Crosstalk between the Mitogen-Activated Protein Kinase Signaling and Protease Pathways in a Model of Glutamate-Induced Oxidative Stress

by

Ruth Anne Stetler

Bachelor of Science, Allegheny College, 1995

Submitted to the Graduate Faculty of

Arts and Sciences in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2005

UNIVERSITY OF PITTSBURGH

Faculty of the Arts and Sciences

This dissertation was presented

by

Ruth Anne Stetler

It was defended on

October 21, 2005

and approved by

Teresa Hastings, Associate Professor, Departments of Neurology and Neuroscience

Ian Reynolds, Professor, Department of Pharmacology

Jun Chen, Associate Professor, Department of Neurology

Xiao-Ming Yin, Associate Professor, Department of Pathology

Edda Thiels, Assistant Professor, Department of Neurobiology

Carol Troy, Associate Professor, Departments of Clinical Pathology and Neurology, Columbia University

Dissertation Advisor: Donald Defranco, Professor, Departments of Pharmacology and Neuroscience

Crosstalk between the Mitogen-Activated Protein Kinase Signaling and Protease Pathways in a Model of Glutamate-Induced Oxidative Stress

Ruth Anne Stetler, PhD

University of Pittsburgh, 2005

Abstract

The molecular mechanisms of alternative forms of cell death are becoming increasingly common, particularly in the case of neurodegenerative diseases. Proteases pathways, such as calpains and caspases, and their interactions with the mitogen-activated protein kinase (MAPK) signaling pathways have not been well-explored, particularly in the context of a pro-toxic role for the extracellular-related kinase (ERK). HT22 cells lack ionotropic glutamate receptors, but are still sensitive to high concentration of extracellular glutamate, which depletes glutathione and causes oxidative toxicity in an ERK-dependent manner. Using a purified clone of HT22 cells which were consistently responsive to glutamate-induced toxicity in an ERK-dependent manner, we have found that caspase-1- and -2-like inhibitors were effective at blocking cell death. Delayed addition of these inhibitors remained protective when added up to 3 hours after glutamate exposure. Both inhibitors decreased ERK phosphorylation at 9 hours following glutamate treatment, a timepoint where the second rise in ERK activation has been found previously. Contrary to previous reports using pharmacological calpain inhibitors, we have found that molecular inhibition of calpains by overexpression of calpastatin, the endogenous calpain inhbitor, was effective at delaying cell toxicity in glutamate treated HT22 cells. Evidence for calpain activation was demonstrated using two separate assay systems. In an *in vitro* enzymatic assay, calpains were found to be activated in the glutamate-induced toxicity of HT22 cells, and that this activation appears to be biphasic in nature, with the early activation MEK-independent and the late activation MEK-dependent. Furthermore, the endogenous calpain target PARP was cleaved to a fragment consistent with calpain-mediated cleavage at late timepoints following glutamate exposure. The appearance of this fragment, consistent with the late MEK-dependent activation, was also dependent on MEK activity. These results indicate for the first time that calpains are indeed activated and involved in cell death execution, that they can be at least in part regulated by MEK activity. Taken together, we have found that glutamate-induced oxidative

stress in HT22 cells represents a form of cell death where caspase-1- or -2-like proteases may function upstream of ERK activation, but that ERK activation is required at least in part for a further, downstream calpain activation.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

Acknowledgements, in my mind, are the most fundamental base to any story. I wish I could explain to each individual how, without them, something would have been lost, and arriving to this point in my life to be able to successfully complete this work would have been more difficult. However, explaining this would require much more than a preface; it would be the whole story itself! So, for all of the people (and animals and mountains and plants and skies) who I have known and with whom I have interacted, I deeply appreciate the lessons that I have learned from each of you. It is what defines me as I am today, and, by extension, what has lead to this work.

However, there are some individuals who I would like to thank because of their more proximal association to this dissertation. My dissertation committee has been absolutely wonderful in their enthusiasm and contribution to this work; I don't know if it is normal for each member to have played such integral roles and be so interactive in committee meetings, but it has been a great experience. Furthermore, my advisors – both past and present – have had profound influence on my growth as a scientist. It goes without saying that this work would not have been possible without the support of my dissertation advisor, Dr. Don Defranco. In particular, Don, your patience and support of my interests has been something that I very much appreciate. Drs. Terri Hastings and Jun Chen, both of you have served as advisors and great sources of support, both personally and professionally. I can in all honesty say that without you, I would not have made it through this process. Dr. Jeff Cross, my advisor at Allegheny College, was the force that switched my career from English literature/Education to Neuroscience, particularly due to a midterm exam question on excitoneurotoxicity. Even a decade later, this question still proves to be something that intrigues me, underscoring the idea that science is a process, not an answer.

In addition, I would like to extend my deep appreciation of the members of the Defranco lab, who have made this time a lot more enjoyable and personable of an atmosphere. Xinjia Wang, Louisa Ho, Yue Luo, Marjet Heitzer, Terry McGuire, Meral Guzey and Bassem Deklabab, you have all been kind with your patience and support, and I appreciate all of the ways that you have helped me out. Thanks!! And Marcia Lewis, I am so happy that you came to our lab; I have enjoyed so much knowing you – your spirit keeps things positive, not to mention your role as a conspirator in chocolate milkshakes definitely has contributed positively to this project!! A special thank you has to be reserved for David Levinthal. David, your support and friendship has meant a lot to me, both through the late hours in the lab (even after you had finished!!!!) and outside the lab as well. Your friendship, which evolved from being labmates, is something that is more valuable than any academic degree. Thanks for being there. Also, without the help of Joan Blaney, the graduate student coordinator, and Jim Kaczynski, the organization of the whole experience in the last several years would have been very difficult. Thank you!

Finally, I would like to thank my family and friends that have had to suffer me on a daily basis outside of the lab. My parents, Betsy and John Howell, Charlie Stetler and Janice Gumbert, my sister, Beth Lichtenwalner, as well as my grandparents, Hal and Mary Beumer and John and Ruth Howell, have given me incredible amounts of unconditional support and had such strong belief in me even when I did not believe in myself. Without the efforts of my mother Betsy Howell, I would have never had the intellectual and educational opportunities which have

culminated in attaining my doctorate. So, thank you, Mother, for having not only believed that I can do anything that I choose to do, but also for your willingness to fight for me when I was not able to do so myself. And Dad, thanks for your Sunday nights at 10; knowing that I will hear your voice once a week encouraging me to get through whatever is going on it has meant a lot. Lastly, Brenna Bogey – you have survived living with me for a significant portion of these years, and remained still a close and dear friend. I cannot imagine not having had you around.

1.0 INTRODUCTION

1.1 PROGRAMMING OF PATHOLOGICAL CELL DEATH

Following pathological stimuli, the process of cell death can occur through a variety of fashions, depending on the cell type, the stimulus, and the molecular machinery. Historically, two traditional models of cell death were proposed: oncosis and apoptosis, originally thought to be diametrically opposed[1]. These models were originally defined by morphological, not biochemical, criteria (Table 1). Only when the biochemical molecules responsible for the morphological characteristics were later further elucidated did the complexities of cell death pathways begin to emerge. Recent studies have suggested that there exists a continuum where most cells die in a process that is neither purely apoptotic nor purely oncotic. Thus, to understand the range of cell death it is necessary to understand the basic morphological as well as biochemical hallmarks.

[1] A note on the terminology needs to be clarified here. There is some argument in the literature about how to refer to the form of cell death involving cellular swelling in order to distinguish it from the shrinkage observed in apoptosis. Many references use the word "necrosis," while others have used "oncosis." In a strictly etymological interpretation, the word "necrosis" is derived from the Greek word "neckros," meaning a corpse or dead body. The addition of the "-is" suffix was a Late Latin derivation which gives the full meaning of the word as "causing to die" or "killing." Apoptosis results in cell death, thus the cell death mechanism of apoptosis that results in killing a cell is, by etymological definition, a necrotic occurrence (i.e., a "causing to die"). The word "oncosis" is derived from the Greek word "onkos," meaning a mass or swelling. The focus on the morphological swelling observed in this form of cell death as diametrically opposed to the cell shrinkage found in apoptotic death is the intention in using this word. Unfortunately, this term is not without confusion, as the Greek implication for swelling was more along the lines of "swelling" in a solid state, and could be applied to the symptomatic appearances of tumours. At any rate, the use of the word "oncosis" in this thesis will be used to imply the form of cell death associated with swelling, rather than shrinkage, of the cell and its components.

Table 1 Distinctive characteristics of cell death models

Morphology	**Apoptosis**	**Oncosis**
Chromatin	200bp fragmentation	Erratic, if present
Nuclear Envelope	Involution	No involution, breaks
Cytosol	Shrinkage	Swelling of contents (mito, ER)
Plasma Membrane	Blebbing	Breakage
Energetic State	ATP loss<80%	ATP loss >80%
Surrounding Tissue	Isolated to cell	Spreads to surrounding cells

1.1.1 Apoptosis

The term “apoptosis” was coined to describe the formation of a nuclear morphology evident in particular forms of cell death, including programmed cell death (PCD) identified in development. This form of cell death was later defined further morphologically by a distinct condensation of chromatin, shrinkage of the cytosol, involution of the nuclear membrane, and the absence of cellular swelling. Additionally, in contrast to oncosis, cells *in vivo* undergoing apoptosis were found to flip a particular phosphatidylserine phospholipid from the inner leaflet of the extracellular membrane to the outer leaflet, to retain integrity of the extracellular membrane, and to maintain ATP levels throughout much of the cell death process.

The mechanisms leading up to the prototypic apoptotic nuclear morphology depend largely on the cell type and stimulus used to induce cell death. Using *C. elegans* as a relatively simplified genetic model, several proteins (*ced* genes) were identified as essential to the appearance of the apoptotic morphology. Later, the mammalian homologues to these genes were discovered and coined “caspases” (cysteinyl-aspartate proteases; see below). The previously morphologically identified chromatin cleavage was found to be a result of the activation of the caspase-activated deoxyribonuclease, CAD, which is released from its inhibitor, ICAD, by caspase-3 cleavage (Chen et al., 2000). For many years, caspase-3 activation alone was used to define apoptosis; however, this currently does not appear to be a legitimate solitary barometer of a specific morphological outcome. For example, caspase activation (including caspase-3) has been found in the absence of the nuclear morphology first attributed to apoptosis (Chen et al., 2000).

1.1.2 Oncosis

Historically, oncotic cells were defined by cellular swelling and subsequent lysis via plasma membrane collapse with few chromatin alterations (Proskuryakov et al., 2003). At the organelle level, extracellular membrane blebbing, mitochondrial swelling and erratic nuclear clumping have been observed prior to the collapse of the plasma membrane and release of intracellular components; oncotic cell death often leads to inflammation in the surrounding tissue due to the release of intracellular material (Liu et al., 2004).

Early plasma membrane permeability was one of the hallmarks of oncosis. Interestingly, there appears to be a sequential permeabilization of the plasma membrane during oncosis. Using renal proximal tubules exposed to anoxic conditions, Chen et al found early uptake of the 633 dalton (Da) fluorescent dye propidium iodide (PI), followed by later uptake of 3 or 70 kDa dextran (Chen et al., 2001). It was noted that cells could be transiently permeable to PI during a noxious stimulus, but if the conditions were altered prior to an unidentified commitment point in death, the cell would no longer exhibit permeability. Therefore, PI uptake, at least in this model, did not represent irreversible cell death, but rather represented cellular toxicity.

The biochemical processes leading to oncotic forms of cell death are not well understood. Originally, oncosis was thought to be a more passive form of death, caused by osmotic swelling of the cytosol, radical loss in ATP production due to swelling of the mitochondria, and subsequent bursting of the plasma membrane. Recently, several molecules and potential pathways have been identified as potential mediators of oncosis, revealing that this form of cell death does not appear to be as passive as once assumed. Increase in intracellular calcium has been correlated with cell death, and in many models, chelation of extracellular or intracellular calcium has afforded protection against toxic stimuli (Choi, 1987; Tan et al., 1998b; Liu et al.,

2004; Artal-Sanz and Tavernarakis, 2005). The compartmentalization of intracellular calcium fluxes also appears to affect the oncotic cell death pathway. Blocking mitochondrial uptake of calcium with FCCP, an inhibitor of the mitochondrial ATPase, effectively blocked cell death in neurons exposed to excitotoxic stimuli (Stout et al., 1998). Signaling molecules, transcriptional activators, inflammatory cytokines, and proteases such as calpains and caspases have been found to be able to alter oncotic cell death (Proskuryakov et al., 2002; Liu et al., 2004; Toledo-Pereyra et al., 2004). Therefore, although still largely undefined, oncotic cell death appears to be at least partly regulated via distinct biochemical pathways.

1.1.3 Continuum between apoptosis and oncosis

Although the morphological states of apoptosis and oncosis appear to be dissimilar, it has become quite clear that the molecular and biochemical machinery typically associated with apoptosis can also play a role in cell death resulting in nuclear morphology more similar to oncosis (Sperandio et al., 2000; Neumar et al., 2003; Proskuryakov et al., 2003; Sperandio et al., 2004). This machinery includes caspases, as well as other proteases, such as calpain and cathespin D, or MAP kinases (Neumar et al., 2003; Sperandio et al., 2004). Additionally, plasma membrane permeabilization can occur during the progression of apoptotic morphology, particularly in cell culture models, where cells are not quickly phagocytosed (Proskuryakov et al., 2003), leading to an inflammation state once thought to be only in oncotic forms of cell death (Jaeschke and Lemasters, 2003). These studies and others have convincingly argued that the strict morphological distinctions made between apoptosis and oncosis do not necessarily translate into biochemical distinctions.

1.2 CELL DEATH MACHINERY

While a series of proteases have been implicated in cell death pathways, the initiation of these pathways under pathological stimuli are often poorly understood, particularly in the cases of neuropathology. Cell death has been studied largely under the contexts of development, inflammation and chemotherapeutic toxicity, and it is from these studies that the generalizations about cell death pathways emanate. However, it should come as no surprise that the mechanisms and conditions under which post-mitotic cells or cells of different phenotypes will vary from the "established" repertoire of cell death mechanisms. It is therefore imperative to have both a solid understanding of the specific conditions in which known cell death programs are activated, as well as an appreciation that alternative programs, diverging at unknown points, exist and can lead to exciting new understandings in the regulation of cell death.

1.2.1 Caspases: General model of caspase pathways

The caspase family of cysteine proteases has been the focus of intense study in the field of cell death over the past decade. Although the activation of upstream apical (or "initiator") caspases (caspase-8, -9, -10, and -2) can vary depending on the cell death stimulus, oligomerization of precursor apical caspases and adaptor proteins has been found to often be critical in inducing activity (Boatright et al., 2003; Boatright and Salvesen, 2003). For example, the formation of the "apoptosome," a large multiprotein complex containing at least caspase-9, APAF-1, and small nucleotides, has been widely acknowledged to be a major point of regulation and initiation of caspase-9 activation (Stefanis, 2005). The upstream signal in caspase-dependent cell death typically converges on common downstream "effector" (or "executioner") caspases

(caspase-3, -6, and -7). The activation of effector caspases often results in an amplification loop to ensure efficient cell death, where effector caspases can activate apical caspases (Fumarola and Guidotti, 2004). This activation of effector caspases culminates in the targeting of cellular substrates. The functional consequence of substrate cleavage is diverse: it can involve activation via disinhibition, production of functional dominant negatives, or translocation. For example, the cleavage of ICAD results in the release and disinhibitionn of CAD (Chen et al., 2000)), poly(ADP-ribose)polymerase (PARP) cleavage leads to the formation of functional dominant negatives that block DNA repair (D'Amours et al., 2001; Decker and Muller, 2002), and cleavage of Bid results in translocation to mitochondria (Gross et al., 1999). In general, the major functions of activated caspases in cell death models are to activate other caspases, and to systematically cleave cellular protein targets, effectively shutting down cellular processes (e.g., DNA repair), ideally while maintaining an intact membrane.

1.2.2 Caspases: General molecular structure

The general structure of the caspase family members includes an N-terminal prodomain of varying length (generally longer in the apical caspases, shorter in the effector caspases), a core catalytic domain, and short C terminal domain (**Fig. 1**). The prodomain on initiator caspases contains either a caspase recruitment domain (CARD) or a death effector domain (DED), both of which promote the oligomerization to itself or with adaptor proteins required for enzymatic activation of the caspase (Chang and Yang, 2000; Chang et al., 2003). The rest of the caspase structure usually is comprised of a large subunit, which contains the actual proteolytic active site, and a small subunit, thought to be essential to induce conformational change that allows full exposure of the active site within the large subunit. To further complicate discussions and

General Domain Structure of the Caspase and Calpain Family

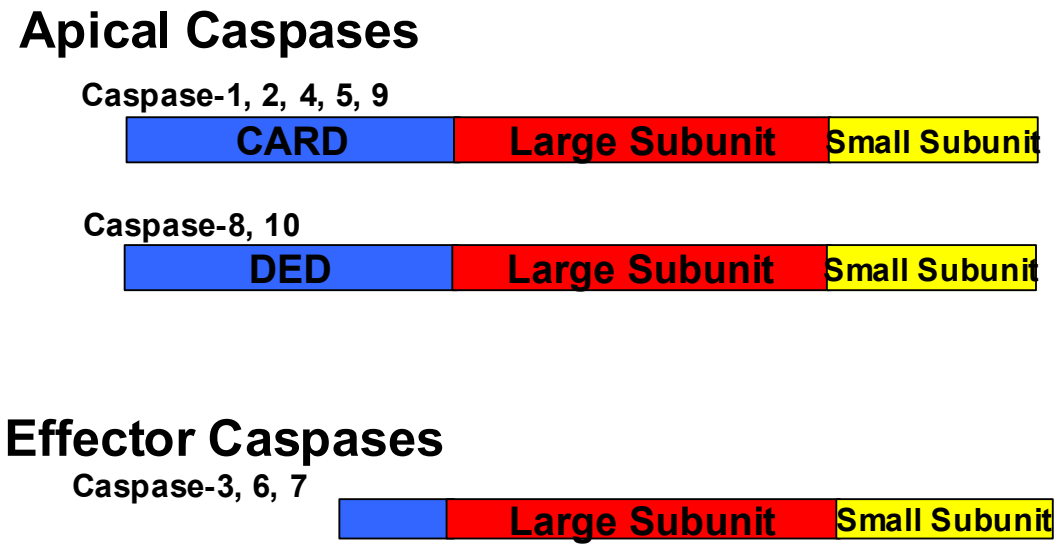


Figure 1 Generalized caspase and calpain protein structures

analyses, the actual activation and cleavage of some apical caspases themselves has revealed a complex network of possibilities or remain relatively obscure (Yamin et al., 1996; Troy and Shelanski, 2003). The full activation of any particular caspase was originally thought to rely on cleavage of both the N- and C-terminals, freeing the catalytic domain. However, this assumption was been called into question by the finding that apical caspases (and caspase-1) have enzymatic activity in the absence of cleavage (Yamin et al., 1996; Stennicke et al., 1999; Boatright et al., 2003), implying that the zymogen itself can be active under certain circumstances. Using processing-site-directed mutants of procaspase-9, the overall caspase-9 activity was reduced, but sufficient caspase-9 activity still supported an apoptotic cascade in the presence of cytosolic factors (Stennicke et al., 1999; Boatright et al., 2003). Furthering these conclusions, Boatright et al demonstrated that dimerization was required in the processing-site-directed mutants of caspase-8 and -9 to induce activity, but that cleavage was neither necessary nor sufficient for apical caspase activation (Boatright et al., 2003). Similarly, caspase-1 was found to contain multiple processing sites, but the zymogen itself was also found to possess enzymatic activity (Yamin et al., 1996) (**Fig. 3**). These apical caspases may operate in normal cells under a tightly controlled, tonic, low activity state that is independent of active cleavage (Launay et al., 2005). The functions of "endogenous" activity under normal physiological conditions remain poorly understood.

1.2.3 Caspase regulation

Caspases exist as zymogens in normal, non-stressed cells. There is now emerging evidence for multiple levels of control systems to regulate caspase activity, when the zymogen is presented in both active and inactive conformations. Inhibitors of apoptosis proteins,

constituting a functional class of proteins named "IAPs", contain a baculoviral IAP repeat (BIR) domain that can bind directly to some, but perhaps not all, caspases (Riedl and Shi, 2004). Caspase-9 recognizes a tetrapeptide in the BIR3 domain of XIAP, and the dimerization of XIAP and caspase-9 via BIR3 blocks an area on caspase-9 required for oligomerization. Hence, activation of caspase-9 is inhibited (Shiozaki et al., 2003; Prunell and Troy, 2004; Riedl and Shi, 2004). Alternately, IAPs can bind via their BIR2 to the catalytic sites of caspases-3 or -7, preventing activity directly (Shi, 2004). IAPs themselves, however, are regulated by another set of proteins (e.g., Smac/Diablo) that act as competitive inhibitors directed to BIR domains in order to sequester IAPs away from caspases, thereby allowing the activation of caspases (Riedl and Shi, 2004). Other regulatory proteins exist to facilitate oligomerization of apical caspases, such as FADD, Apaf-1 or Ipaf-1; these proteins typically contain either the DED sequence (in the case of the caspase-8) or CARD sequence (for caspases-9 and -1), which recognize the analogous sequence on the zymogen prodomain (Budihardjo et al., 1999; Poyet et al., 2001). However, not all DED or CARD containing proteins function to facilitate activation of caspases; some of these proteins are thought to act as dominant negatives, which mimic the function of facilitating proteins by interacting with caspases via the prodomain but prevent oligomerization or association with other critical cytosolic factors necessary for activation (Humke et al., 2000; Thorburn, 2004). For example, in the case of caspase-1, ICEBERG and Pseudo-ICE both serve to displace Rip2, a CARD-containing protein that has been implicated in the formation of the inflammasome (Humke et al., 2000; Druilhe et al., 2001). This displacement has been demonstrated to prevent oligomerization and activation of caspase-1.

Other cellular mechanisms to regulate caspase activation have been proposed, including compartmentalization of caspases and associated cofactors, differentially spliced isoforms, and

phosphorylation. Although the concept of posttranslational modifications, such as phosphorylation, is exceedingly common in regulation of protein activation, the possibility of this form of regulation in caspase activation has only recently been discovered. Human caspase-9 was found to contain phosphorylation sites for both protein kinase B (Akt) and the extracellular-signal-regulated kinase2 (ERK2), although the former site is not conserved across species (Cardone et al., 1998; Fujita et al., 1999; Allan et al., 2003; Johnson and Jarvis, 2004). The ERK2 phosphorylation site on caspase-9 is at Thr-125, part of a conserved MAPK consensus sequence located between the CARD domain and the large subunit of the zymogen (Allan et al., 2003; Johnson and Jarvis, 2004). Okadaic-acid sensitive phosphatases negatively regulate the phosphorylation at Ser-196 (Cardone et al., 1998), but the involvement of phosphatases in regulating phosphorylation at Thr-125 has not been explored. The physiological effect of phosphorylation of caspase-9 appears to be inhibition of activation, since Thr-125 mutants were less able to activate caspase-3 (Allan et al., 2003). A recent study indicated that caspase-2, like caspase-9, may be phosphorylated on its prodomain at Ser-135 (Nutt et al., 2005). However, caspase-9 phosphorylation was mediated by Akt and ERK2 (Cardone et al., 1998; Fujita et al., 1999; Allan et al., 2003); caspase-2 phosphorylation in oocytes appears to occur through the calcium/calmodulin-dependent protein kinase II (CaMKII) (Nutt et al., 2005). Further analysis on potential phosphorylation sequences in the prodomains of apical caspases could yield an interesting link between kinase and protease signaling.

1.2.4 Extrinsic and intrinsic activation pathways

The caspase signaling cascade can be initiated in a variety of manners. Thus far, two pathways have been fairly well detailed and extensively reviewed (Chang and Yang, 2000;

Stefanis, 2005). For the purposes of this discussion, we will outline these two pathways briefly, and then further discuss alternative pathways that are less understood.

The "intrinsic," or mitochondrial, cascade is characterized by the activation of apical caspases, caspase-9 in particular, via internal cellular events, i.e., not due directly to extracellular receptor ligand binding (as is the case in the "extrinsic" pathway, described below). Cellular stresses culminate in an as yet unknown process by acting upon the mitochondria, causing release of cytochrome c. Cytochrome c can be bound by apaf-1, dATP, and caspase-9 to form the apoptosome, resulting in activation of caspase-9. Complexed active caspase-9 can then recruit and activate caspase-3, which subsequently cleaves numerous cellular proteins.

The "extrinsic" cascade begins with the binding of a ligand to a death receptor (e.g. fas ligand binding to the fas receptor). Once bound, the receptors oligomerize, sequestering a variety of molecules in close proximity (Thorburn, 2004). This leads to the recruitment of multiple caspase-8 proteins into the so-called death-inducing signal complex (DISC), analogous to the above described apoptosome. The DISC components oligomerize by virtue of DED domains, rather than the CARD domains in apoptosome-related proteins. The close proximity of a caspase-8 molecule to other caspase-8 molecules can cause autoactivation (Boatright and Salvesen, 2003; Thorburn, 2004). Once caspase-8 is activated, it can then activate downstream caspases either directly or indirectly via cleavage of the proapoptotic protein Bid, which can cause cytochome c release and the formation of the apoptosome (Stefanis, 2005).

1.2.5 Inflammatory caspases

The inflammatory pathway has been described to activate caspases-1, -4, -5, and -11 (Chang and Yang, 2000). The primary function of the inflammatory caspases identified thus far

has been in the role of interleukin processing to a secretable form, inducing inflammation in response to microbial infection (Martinon and Tschopp, 2004). Although ablation of the caspase-1 gene did not show a robust phenotype with the exception of deficiencies in IL-1 processing (Li et al., 1995), caspase-1 knockout mice were resistant to moderate levels of neonatal hypoxia/ischemia (Liu et al., 1999) and focal ischemia (Schielke et al., 1998), implicating caspase-1 in the pathogenesis of neuronal cell death in this model.

The exact mechanism of activation and the downstream targets of the inflammatory caspases are not very well understood, despite the fact that caspase-1 was one of the first mammalian caspases to be cloned. However, the oligomerization of caspase-1 and -11 (the latter is the murine homologue to caspase-4 and -5) has been correlated with an ability to induce cell death by forced overexpression (Yang et al., 1998). This oligomerization is analogous to the apoptosome and DISC formations in that it appears to require multiple cytosolic factors, including proteins containing the CARD molecular domain. However, these proteins are seemingly a distinct subset of CARD-containing proteins than the subset involved in the apoptosome with caspase-9 (Martinon and Tschopp, 2004). The multiprotein complex formed to activate caspase-1 was termed the "inflammasome." Martinon et al found caspase-1 oligomerized with NALP1, Rip2 and Pycard/ASC, all of which contain CARD domains (Martinon et al., 2002). ASC is essential as an adaptor protein for caspase-1, as ASC and caspase-1 have been found to directly interact, resulting in activation of caspase-1 (Martinon et al., 2002; Srinivasula et al., 2002). Perhaps more controversial, but interesting in light of the model our lab deals with, Rip2 has been also found to be associated with the inflammasome. Rip2 is a Raf-1-activated mitogen-activated protein kinase kinase that functions at the same level as but independent of MEK1/2 (Navas et al., 1999). Rip2 directly phosphorylates the

extracellular-regulated kinases-1 and -2 (ERK1/2) on the TEY motif (Thr-183 and Tyr-185; normally associated with MEK1/2 activation of ERK1/2) via a Ras/Raf1 pathway (Navas et al., 1999). Upon structural analysis of Rip2, the serine/threonine kinase domain was located at the N-terminus, whereas a C-terminus CARD domain mediated Rip2 interaction with and potential activation of caspase-1 (McCarthy et al., 1998; Thome et al., 1998; Humke et al., 2000). Following oxygen/glucose deprivation (OGD) in immature cortical neuronal cultures, Rip2 is upregulated and caspase-1 is activated (Zhang et al., 2003). This model has been shown by others to depend on ERK1/2 activation, as U0126, an inhibitor of the upstream kinase MEK1/2, was effective at blocking cell death (Satoh et al., 2000). It is not known if U0126 can inhibit Rip2 activation of ERK1/2. Interestingly, mature cortical cultures were resistant to OGD, did not upregulate Rip2, and did not activate caspase-1, suggesting that immature cultures were incompetent in inflammasome formation. The requirement of Rip2 in the inflammasome, however, may depend on the cellular phenotype and conditions, as Mariathasan et al found that Rip2 deletion did not affect caspase-1 activation in macrophages exposed to pathogens (Mariathasan et al., 2004).

The murine caspase-11 has been thought to be a potential upstream activator of caspase-1, based on the evidence that, although caspase-11 itself cannot process IL-1, the caspase-11 knockout mouse lacked IL-1 processing in a manner similar to the caspase-1 knockout mouse (Wang et al., 1998b). However, the activation of caspase-1 by caspase-11 is not likely a direct event, as caspase-11 could not bind or activate caspase-1 in vitro (Wang et al., 1998b). Interestingly, both the caspase-1 knockout and the caspase-11 knockout are more resistant to neuronal ischemia than wildtypes ((Liu et al., 1999) and unpublished findings in (Wang et al., 1998b)), in particular in neonatal hypoxia/ischemia models.

1.2.6 Caspase-2

Lastly, caspase-2 has been described as the "orphan" caspase (Troy and Shelanski, 2003): On the basis of its sequence, caspase-2 has a long prodomain, and in sequence homology, it resembles closest caspase-9 in the initiator group (Chang and Yang, 2000; Troy and Shelanski, 2003). However, the cleavage recognition sequence on substrates is more similar to the effectors, caspase-3 and -7 (Troy and Shelanski, 2003). Phylogenetically, caspase-2 appears to be the closest homologue to the *C. elegans* gene, *ced-3*. The activation of caspase-2 and the downstream targets are not well defined. Caspase-3 has been shown to be able to cleave caspase-2 in vitro (Li et al., 1997a). Thus, if one assumes that the cleaved form of caspase-2 is the enzymatically active form, then activation of caspase-2 may occur via caspase-3 in some models. However, it has not yet been shown which form of caspase-2 is active (Troy and Shelanski, 2003), and there is evidence that, like the "classical" apical caspase, caspase-2 zymogen is active (Read et al., 2002) (further discussed below). A role for caspase-2 upstream of mitochondria dysfunction and caspase-3 activation has also been demonstrated (Troy and Shelanski, 2003). Purified caspase-2 was also shown in vitro to be able to cleave Bid, which can then translocate to the mitochondria and release cytochrome c (Guo et al., 2002). Caspase-2 was also found to be able to directly induce release of the pro-apoptotic molecules AIF, cytochrome c, and Smac from the mitochondria when added directly to isolated mitochondria in vitro, independent of Bid (Guo et al., 2002). Thus, caspase-2 may have a role in directly and indirectly inducing mitochondrial dysfunction.

Procaspase-2 contains a CARD domain, indicating that perhaps activation could occur via oligomerization (Read et al., 2002; Troy and Shelanski, 2003). Indeed, induced oligomerization of FLAG-tagged caspase-2 in vitro resulted in self-processing (Chang et al.,

2003). A high molecular weight complex isolated from cellular lysates has been found to contain caspase-2; the fractions containing the complex also were enzymatically active with a caspase substrate (Li et al., 1997a), and an CARD-containing adaptor-like protein RAIDD was found to associate with and lead to activation of caspase-2 in some models (Jabado et al., 2004; Wang et al., 2005). Caspase-2 mutants which are incapable of being proteolytically processed retained a limited degree of activity (Baliga et al., 2004). A recent study has now indicated that caspase-2 may be negatively regulated by phosphorylation (Nutt et al., 2005). Caspase-2 containing a S135A mutation was unable to be phosphorylated *in vitro* by CaMKII, and was capable of being proteolytically processed; in the presence of Gp6, an inhibitor of cell death in nutrient-depleted oocytes, expression of this mutant could induce apoptosis in nutrient-depleted oocytes and caspase-3 activation lysates, whereas the wildtype could not (Nutt et al., 2005), indicating that phosphorylation of caspase-2 can block its ability to trigger cell death.

The downstream targets of caspase-2 are even less understood. Lack of a strong phenotype in the caspase-2 knockout resulted in the assumption that caspase-2 was a "redundant" caspase (Troy and Shelanski, 2003). However, deletion of caspase-2 protein by silencing RNA inhibited mitochondrial dysfunction and cell death following DNA damaging toxins exposure (Lassus et al., 2002) and knockout of caspase-2 in mice molecular inhibition of caspase-2 via antisense oligonucleotides effectively blocked beta-amyloid toxicity (Troy et al., 2000).

1.2.7 Calpains

Despite substantial evidence of caspase involvement in various forms of cell death, a number of cell death models have demonstrated alternative, caspase-independent cell death

mechanisms. The use of caspase inhibitors has often resulted in simply a delay of cell death, not longterm protection (Stefanis, 2005), implying that caspase activation may simply be an amplification mechanism for an ongoing cell death program. To further complicate findings, many of the pharmacological protease inhibitors used to implicate specific responses in cell death pathways can have nonspecific effects on other protease systems (Stefanis, 2005). Due to the role of calcium in glutamate induced oxidative stress in HT22 cells, we shall focus the discussion to the calcium-dependent proteases, calpains.

Calpains comprise a family of heterodimeric thiol proteases (Croall and DeMartino, 1991; Markgraf et al., 1998). Currently, 13 known mammalian family members have been found, some of which are expressed in a tissue-specific manner, while others are found ubiquitously (Huang and Wang, 2001). Two of ubiquitously expressed and most characterized calpain isoforms, calpain-1 and calpain-2, require calcium for proteolytic activity, although *in vitro* the necessary Ca^{2+} concentration is much greater for activation of calpain-2 than for calpain-1 (millimolar versus micromolar) (Perrin and Huttenlocher, 2002). While calpains have significant roles in non-injurious cell processes, such as cellular remodeling (Potter et al., 1998), many studies have suggested a role for calpains in the pathogenesis of disease, particularly in ischemia and Alzheimer's disease (Huang and Wang, 2001). Pharmacological calpain inhibition was neuroprotective in focal and global ischemia models (Markgraf et al., 1998; Rami et al., 2000), as well as in a variety of neuronal *in vitro* toxicity studies, including hydrogen peroxide (Ray et al., 2000) and oxygen/glucose deprivation (Newcomb-Fernandez et al., 2001). Cellular calcium buffering mechanisms, such as low-affinity mitochondrial uptake and high-affinity endoplasmic reticular storage, are important regulators of calcium concentrations under physiological conditions (Gunter and Gunter, 2001; Paschen, 2003). Surprisingly, the mitochondrial uptake of

high calcium concentrations is actually associated with inducing cell death, rather than inhibiting cell death. Thus, cells may be capable of surviving with high cytosolic calcium concentrations (Stout et al., 1998), and only initiate cell death programs upon uptake of calcium into the mitochondria. The subcellular localization of calpains is reported to be cytosolic, and translocation to membranes was noted after activation (Perrin and Huttenlocher, 2002). Therefore, it is likely that high cytosolic calcium concentration in various pathophysiological states is not sufficient for calpain activation that would result alone in toxicity.

Calpains exist as 80kDa isoform specific subunits associated with a ubiquitous small 30kDa regulatory subunit. The large subunits contain N-terminal proteolytic domains, followed by an internal domain likely involved in membrane targeting (Perrin and Huttenlocher, 2002). The calcium binding region on the large calpain subunits is thought to be toward the C-terminus, where several calcium-binding EF hand domains were found in some, but not all, isoforms (Huang and Wang, 2001). The mechanisms responsible for calpain activation are not themselves well understood. Like caspases, calpains were originally thought to require proteolysis in order to become fully activated (Michetti et al., 1996). The original model proposed was that calcium binding induced dissociation of the active subunit from the small regulatory subunit, autolysis of the procalpain and translocation to membranes (Liu et al., 2004). However, analogous to the experiments that determined that apical caspases did not need processing to be active, mutations of the processing sites on the large active calpain subunit inhibited autolysis but did not affect activity (Elce et al., 1997). However, differential regulation of calpains via processing may be dependent on different cellular conditions and different calpain isoforms (Liu et al., 2004).

Calpastatin, the endogenously expressed protein inhibitor of calpains, presents another mechanism of calpain regulation. Calpastatin is encoded by a single gene, but contains multiple

promoters and alternative splicings, yielding many different calpastatin proteins with diverse molecular weights (Wendt et al., 2004). Consisting of multiple inhibitory domains stretched along the length of the full protein, calpastatin is capable of reversibly binding and inhibiting several calpain subunits at once (Wendt et al., 2004). Calpastatin binds calpains in the presence of calcium at concentrations lower than that which is required for activation *in vitro* (Otsuka and Goll, 1987), and appear to bind not to the catalytic domains of calpains, but to the calcium binding domains (Ma et al., 1993). Calpastatin itself is not known to bind calcium, therefore it is likely that calpains must first bind calcium in order to be recognized and inhibited by calpastatin (Wendt et al., 2004).

Interestingly, calpastatin has recently been demonstrated to be a target for caspase cleavage both *in vitro* and *in vivo* (Porn-Ares et al., 1998; Wang et al., 1998a; Rami, 2003). The differently translated forms of calpastatin were differentially cleaved by individual caspases *in vitro*, where caspases-3 and -7 could effectively cleave high molecular weight forms of calpastatin, but all forms of calpastatin were more susceptible to cleavage by caspase-1 (Wang et al., 1998a). Caspase-mediated cleavage of calpastatin is thought to release calpastatin from calpains and result in calpain activation, although the possibility exists that caspase-cleaved calpastatin could have alternative functions (Wang et al., 1998a; Rami, 2003), particularly since caspase-1 cleaved calpastatin lost only about 50% of its inhibitory activity (Wang et al., 1998a). However, crosstalk between the calpain and caspase pathways via calpastatin modulation may still prove interesting in the cell death phenotype. Forced overexpression of calpastatin was demonstrated to switch off the more oncotic calpain protease pathway in order to allow an apoptotic phenotype to emerge (Lankiewicz et al., 2000; Neumar et al., 2003). Thus, calpastatin may provide a switch between the oncotic and apoptotic molecular pathways.

There appear to be other possibilities for the regulation of calpain, in addition to inhibition by calpastatin or the presence of calcium. Epidermal growth factor (EGF) stimulation in fibroblasts causes calpain activation that is dependent on the mitogen-activated protein kinase (MAPK)/extracellular regulated kinase (ERK) pathway, and this activation occurs only when the EGF receptor is targeted to the membrane (Glading et al., 2000; Glading et al., 2001; Glading et al., 2004). While calpain does not contain a known ERK consensus sequence, Glading et al found via mutational analysis that Ser50 within the catalytic domain of m-calpain was phosphorlyated by ERK1, and that this phosphorylation served to activate calpain independent of calcium concentration (Glading et al., 2004). The activation of this pathway in pathophysiological conditions is unknown; EGF stimulation results in tail adhesion disassembly and cellular motility, not cell death. However, the possibility remains that calpain activation may not only be dependent on calcium concentrations and an inhibiting protein, but also on phosphorylation signaling pathways.

1.3 MAPK SIGNALING PATHWAY

1.3.1 General MAPK activation

ERK is a member of the mitogen-activated kinase family (MAPK), comprised of sequentially signaling cellular proteins responsive to a variety of stimuli, such as proliferation, differentiation, and cell death. Typically, two of the MAPK family members, c-Jun N-terminal kinase (JNK) and p38, are associated with pro-death signals, whereas ERK activation has been correlated with pro-survival signals (Chang and Karin, 2001). The roles of MAPK family members are diverse and highly complex, and have been reviewed extensively throughout the

literature. Due to the interest in our lab in the role of ERK in the HT22 cell model, we will limit the discussion to this small subsection of a very large signaling pathway network.

The proteins ERK1/2 are effector kinases of the MAPK pathway that phosphorylates specific serine and threonine residues on target substrates located throughout the cell. Although they are two distinct gene products, ERK1 and ERK2 appear to be coupled by the upstream kinases MEK1/2, as inhibitors of MEK1/2 block both ERK1 and ERK2 with equal efficacy. MEK1/2 are mitogen-activated protein kinase kinases that must be dually phosphorylated before having full activity to phosphorylate and activate ERK1/2 by phosphorylation at Thr-183 and Tyr-185 (the TEY motif). MEK1/2 are activated by either Raf1 or B-Raf, which in turn are activated by a small GTP-coupled protein, e.g., Ras, which are coupled to specific cell surface receptors. At virtually all levels of MAPK signaling, phosphatases are thought to negatively regulate kinase activation. Phosphorylation of kinases and substrates alike can result in conformational (and hence functional) changes in protein structure, or recognition by adaptor molecules, as described above for calpain and caspase-9. Between the activation by upstream kinases and inactivation by phosphatases and rapid alterations in protein conformation, as well as traditional methods of regulation by compartmentalization, MAPK signaling appears to be a highly ordered and controlled system of substrate regulation.

1.3.2 ERK1/2 and cell death

The ERK1/2 kinase pathway was historically defined as a cell survival signal involved in the regulation of proliferation and differentiation (Chang and Karin, 2001; Kyosseva, 2004). However, we and others found that ERK1/2 activation can serve to promote cell death induced by glutamate-induced oxidative stress, 6-hydroxydopamine, cerebral ischemia, ceramide,

thapsigargin, brefeldin A, cisplatin, hydrogen peroxide, and potassium withdrawal (Stanciu et al., 2000; Arai et al., 2004; Cheung and Slack, 2004; Subramaniam et al., 2004; Kim et al., 2005; Oh et al., 2005; Park et al., 2005). The exact role for ERK1/2 in promoting cell death is poorly understood. Subramaniam et al found that constitutively active MEK could potentiate plasma membrane permeability and DNA damage following potassium withdrawal in cultured cerebellar granule neurons, whereas U0126 or a dominant negative MEK could reduce the damage (Subramaniam et al., 2004). Similarly, Arai et al observed that U0126 inhibited LDH release (a measure of fairly large plasma membrane permeability) in endoplasmic reticulum-stressed neuroblastoma cells (Arai et al., 2004). In our HT22 cell model, we also observe that MEK inhibition can inhibit cell death and intracellular calcium concentrations (Stanciu et al., 2000). However, more research into the interplay between MAPK and protease pathways may help to elucidate how ERK functions under cellular stresses and cell death.

1.3.3 MAPK and protease pathways

Very little research has been published that investigates the impact of the MAPK signaling pathway on caspase activation. Phosphorylation of upstream bcl-2 family members, such as BAD or bcl-2, have been demonstrated to be dependent on MAPK signaling (Scheid and Duronio, 1998; Blagosklonny et al., 1999), but have starkly different outcomes. The MEK dependent phosphorylation of BAD resulted in a suppression of the proapoptotic functions of BAD and consequently, cell survival (Scheid and Duronio, 1998), whereas the Raf-1 dependent phosphorylation of bcl-2 inactivated the antiapoptotic functions of bcl-2 and promoted cell death (Blagosklonny et al., 1999). However, the bcl-2 family of proteins is quite complex in their

stoichiometry, localization and regulation; thus, interpreting post-translational modifications has proven quite difficult.

Contrary at first glance to ERK1/2 promoting cell death, ERK2 has been demonstrated to directly phosphorylate caspase-9, leading to inhibition of processing and activity (Allan et al., 2003). This inhibition of caspase-9 resulted in inhibition of caspase-3 and suppression of apoptosis. It is also possible that ERK2-directed phosphorylation of caspase-9 (or of BAD) may act be a switch from an apoptotic mode of cell death to an alternative cell death mechanism in different cellular conditions, so that the suppression of apoptosis unmasks activation of other cell death pathways.

Inhibition of MEK1/2 has had differing effects on caspase-3 activation in different models. For example, in the model of potassium withdrawal in cerebellar granule neurons, caspase-3 is activated and cell toxicity was attenuated by MEK1/2 inhibitors (Subramaniam et al., 2004). The caspase activation was unchanged by MEK1/2 inhibition, which suggests that ERK, not caspase-3, triggered DNA damage and plasma membrane permeability. Similar findings were observed in neuroblastoma cells exposed to the endoplasmic reticulum stressor thapsigargin (Arai et al., 2004), where U0126 blocked plasma membrane permeability, but had no effect on the observed caspase-3 or PARP cleavage. Conversely, in a model of cisplatin-induced cell death in renal epithelial cells, MEK1/2 inhibitors not only blocked cell death, but also blocked caspase-3 activation and mitochondrial dysfunction (Kim et al., 2005).

Very little is known about the interactions of MAPK and calpains. Following epidermal growth factor (EGF) stimulation in epithelial cells, m-calpain was found to be directly phosphorylated by activated ERK (Glading et al., 2000; Glading et al., 2001; Glading et al., 2004). This model results in cell migration, not cell death, due to cytoskeletal reorganization.

However, in models of neonatal ischemia (Wang et al., 2003) and of ER stressed neuroblastomas (Arai et al., 2004), calpain activation and ERK activation have been observed together, although the functional significance of this was not pursued, as calpain inhibitors were not effective at inhibiting cell death. Wong et al found that immature cerebellar neurons exposed to estrogens induced ERK1/2 activation, plasma membrane permeability and cell death (Wong et al., 2003). Both the MEK1/2 inhibitor U0126 and the calpain inhibitor PD150606 were effective at blocking the plasma membrane permeability and cell death, suggesting that ERK and calpains may lie in the same pathway in some forms of cell death.

1.4 GLUTAMATE INDUCED OXIDATIVE STRESS

1.4.1 Mechanism

Although oxidative stress and glutamate toxicity appear relevant in many pathological settings, few studies have thoroughly investigated the molecular mechanisms of glutamate-induced oxidative stress. In mature neurons, exposure to glutamate causes toxicity due to activation of ionotropic glutamate receptors and inward flux of calcium (Choi, 1985). Immature neurons and several cell lines lack ionotropic glutamate receptors, but yet are still sensitive to glutamate toxicity (Murphy et al., 1989). Since glutamate toxicity in immature neurons can be mimicked by the removal of cystine from the extracellular medium, it is thus believed that the toxicity to glutamate in neonates is due to the abrogation of the concentration gradient of glutamate, which drives the glutamate/cystine transporter (Murphy et al., 1989). Influx of cystine is necessary for the synthesis of glutathione; thus the proposed mechanism of glutamate toxicity

in cells devoid of ionotropic glutamate receptors is through depletion of intracellular glutathione, resulting in oxidative stress and subsequent cell death (Murphy et al., 1989).

1.4.2 Calcium

Previous studies have found that rises in cytosolic calcium in glutamate treated cells lacking ionotropic glutamate receptors correlate with cell death (Murphy et al., 1989; Tan et al., 1998b; Stanciu et al., 2000). The mechanism responsible for elevations in intracellular calcium under these conditions remains largely undefined. Treatment of glutamate-exposed HT22 cells with U0126, a MEK inhibitor, resulted in full protection against cell death and concurrently decreased cytosolic calcium levels (Stanciu et al., 2000). FCCP was also shown to inhibit both cell toxicity and rises in calcium (Tan et al., 1998b). Ruthenium red, an inhibitor of mitochondrial and endoplasmic reticular calcium uptake, blocked both the formation of reactive oxygen species (measured by DCF), calcium fluxes (measured by Indo-1), and cell toxicity evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Tan et al., 1998b). Furthermore, blockade of extracellular calcium channels with cobalt chloride also inhibited cell toxicity (Tan et al., 1998b). Surprisingly, blocking calpain with pharmacological inhibitors did not inhibit cell toxicity (Tan et al., 1998a). However, the same studies illustrated that these inhibitors can be toxic to HT22 cells at higher doses. Inhibition of calpain can disrupt normal cellular processes, such as cytoskeletal remodelling (Perrin and Huttenlocher, 2002); thus, inhibitor studies may not be an optimal approach to studying calpain activation in cell death models, as they may block normal physiological functions of calpain and lead to a fragile cellular state in the absence of adverse stimuli (Lankiewicz et al., 2000). To date,

there have been no studies investigating the activation of calpain in glutamate-induced oxidative stress independent of pharmacological inhibitors.

1.4.3 Morphology and caspases

The actual molecular mechanism of cell death execution in the glutamate-induced oxidative stress HT22 model also remains unclear. Previous studies have found some evidence of membrane blebbing and perinuclear chromatin condensation, but no clear apoptotic nuclear morphology was observed, even under electron microscopy (Tan et al., 1998a). A role for potential caspase involvement was proposed based on the observation that YVAD.cmk, but not DEVD.cho, could block toxicity as measured by the MTT assay (Tan et al., 1998a; Stanciu et al., 2000). These peptide inhibitors contain short peptide sequences that, at least for caspase-1 (inhibited by YVAD.cmk), are recognized by residues on both the small and large subunits of the tetrameric complex, and bind to the active site cysteine on the large subunit (Wilson et al., 1994). The sequence specificity was based on target sequences of known endogenous substrates, and on molecular screening (Yoshimori et al., 2004). However, these inhibitors are not completely specific for one caspase over another. Furthermore, the comparison between inhibitors containing differing functional groups (e.g., aldehyde (.cho), chloromethylketone (.cmk), or fluoromethylketone (.fmk)) is confounded by the nature of the inhibitors - .cho inhibitors are reversible, while .cmk (and .fmk) inhibitors are irreversible. Additionally, the methylketone groups have been found to be reactive toward cathepsins as well as caspases (Schotte et al., 1999). Thus, the specific role of caspases still needs to be defined through biochemical profiling and targeted inhibition of specific caspases.

1.4.4 Role of ERK in glutamate-induced toxicity in HT22 cells

Interestingly, ERK activation has been found to contribute to glutamate-induced cell death in HT22 cells (Stanciu et al., 2000; Stanciu and DeFranco, 2002). In glutamate-treated HT22 cells, we have found that ERK is strongly activated, and that the noncompetitive MEK inhibitor U0126 completely abrogates cellular toxicity (Stanciu et al., 2000). Several other studies, primarily in the context of neuronal ischemic models, have also found inhibition of MEK and subsequent phosphorylation of ERK to be protective (Namura et al., 2001). Interesting, a link between ERK activation and calpain has been proposed downstream of epidermal growth factor (EGF) signaling via regulation of phosphatases (Cook et al., 1997). Glading et al found that the ERK signal following EGF stimulation resulted in activation of calpain (Glading et al., 2001; Glading et al., 2004). Given the sustained rise in calcium influx associated with the HT22 glutamate model beginning at 4 h following glutamate treatment and continuing throughout the timecourse, it would be interesting to find if ERK activation triggers activation of calpain in HT22 cells.

2.0 THESIS GOALS

The overriding goal of the work presented here was to investigate the interactions between the MAPK signaling pathway at the level of MEK1/2 activation of ERK1/2 and protease activity. Previous work in our lab found that HT22 cells exposed to glutamate died in an ERK-dependent fashion, while others reported that caspase-1-like inhibitors, but not caspase-3-like inhibitors, could effectively block glutamate-induced toxicity. Inhibition of caspase-1-like activity decreased reactive oxygen species (ROS), whereas ERK1/2 inhibition did not decrease ROS, but did decrease calcium. It then becomes reasonable to expect that caspase-1-like activation may occur upstream or independently of ERK1/2 activation, and, if calpains are involved, that calpain activation may occur downstream of ERK1/2 activation.

The first goal of the project was to ensure that the cellular model being employed (glutamate-induced toxicity in HT22 cells) was consistent in the cell death response. Previous reports had found variations in sensitivity of the parent HT22 cell population to glutamate toxicity due to clonal variations and culturing technique (Sagara et al., 1998; Maher, 2001; Dargusch and Schubert, 2002). Due to our observation that the parent HT22 cell population did not always exhibit a robust cell death response, we hypothesized that subclonal analysis would reveal certain populations with less sensitivity than others, and that culture conditions could alter the cell death response. Also, since PI uptake is able to rapidly detect small permeability changes in the plasma membrane, we predicted that PI uptake would be a more sensitive and

quantifiable marker of cellular toxicity than the MTT assay. These results, as well as the confirmation that the chosen subclone responded to glutamate in an ERK-dependent manner, are presented in Chapter 3.

After determining that the model responded consistently to glutamate, we then wished to determine the specific caspases activated following glutamate-induced toxicity in HT22 cells, and to then determine the effects of MEK inhibition on caspase activity as well as molecular caspase inhibition on ERK activation. Because caspase-like inhibition blocked rises in ROS that could not be blocked by a MEK1/2 inhibitor (Tan et al., 1998b; Stanciu et al., 2000), we hypothesized that caspase-like activation occurs upstream of ERK activation. These results are presented in Chapter 4.

While calcium has been implicated in the glutamate-induced toxicity of HT22 cells, a role for calpain in the mechanism of cell death had been ruled out based on the inability of calpain inhibitors to block cell death (Tan et al., 1998a). However, these inhibitors can be toxic to control cells as well. We sought to determine if calpains were activated in HT22 cells exposed to glutamate, if they were involved in the execution of cell death, and if they regulated or were regulated by MAPK. A previous study in our lab had indicated that the MEK1/2 inhibitor U0126 could block intracellular calcium rises following glutamate exposure (Stanciu et al., 2000). On the concept that calpains can be activated by rises in calcium, we predicted that calpains are involved in glutamate-induced oxidative stress, and that they are activated in an ERK-dependent manner. These data are presented in Chapter 5.

3.0 MODEL DEVELOPMENT

3.1 SUMMARY

Although the majority of HT22 cells are sensitive to glutamate toxicity, there exist certain subpopulations of HT22 cells that are resistant to glutamate-induced cell death (Sagara et al., 1998; Dargusch and Schubert, 2002). Therefore, in order to assure that the population of HT22 cells used in these and future studies are homogeneous, the original parent population of HT22 cells were subcloned and assessed for responsiveness to glutamate administered either in fresh or preconditioned media. Toxicity was measured using both MTT assay (as previously described (Stanciu and DeFranco, 2002)) and propidium iodide (PI) uptake. The ERK-dependent aspect of glutamate toxicity in HT22 cells was determined by the efficacy of U0126, a specific MEK inhibitor, in preventing cell death. We found population differences in the parent HT22 cell population, where one of three clones tested had a significantly reduced response to glutamate-induced toxicity. Furthermore, we found that adding glutamate to conditioned media (as opposed to changing to fresh media upon glutamate addition) blunted the cell death response. In the subclone "G", which was selected for all future experiments, we found that glutamate toxicity retained the dependency on the late phase ERK activation, as evidenced by the protective effects of the MEK inhibitor U0126, even when added at late times (i.e., 7 hours) following glutamate exposure.

3.2 INTRODUCTION

HT22 cells are a subclone of HT4 cells, which were originally derived from an SV40 transduced mouse hippocampal tumour (Tan et al., 1998b). Originally isolated and characterized on the basis of their lack of ionotropic glutamate receptors, they have served as an interesting cell model of glutamate-induced toxicity that is independent of excitotoxicity. However, we and others have noted that there exist some inconsistencies in the parent HT22 population in the responsiveness to glutamate or oxidative stress (Sagara et al., 1998; Maher, 2001; Chen et al., 2005a). In one report, subclones of HT22 cells were isolated that were resistant to 10mM glutamate (Sagara et al., 1998). Higher cell densities decreased the sensitivity of HT22 cells to oxidative stress induced by buthionine sulfoximine (Chen et al., 2005a); Maher also claimed in a discussion that cell density had been noted to decrease glutamate-induced toxicity in HT22 cells (Maher, 2001). Cell density can alter many of the culture conditions (Freshney, 2000), such as the formation of cell-cell contacts, or the increased concentration of soluble cellular factors released into the media. To address these discrepancies, we first subcloned our parent HT22 cell population to isolate and characterize individual subclones and their cell death responses to glutamate. Secondly, to address whether the observed attenuated effects of glutamate toxicity could be due to factors in the conditioned media, we treated subclones plated concurrently by either changing to fresh media supplemented with 5mM glutamate, or by adding glutamate to the preexisting media to a final concentration of 5mM glutamate. We further determined whether the new consistently responsive HT22 subclone died following glutamate exposure in an ERK dependent fashion by assessing the protective effects of U0126, a MEK inhibitor, using PI uptake to confirm the results of the previously published MTT results.

We found that 1) statistically significant differences exist between HT22 subclones to glutamate toxicity, 2) conditioned media conferred protection against glutamate toxicity, and 3) delayed treatment of U0126 rescued a glutamate-responsive subclone from glutamate toxicity.

3.3 EXPERIMENTAL PROCEDURES

Cell Culture and Subcloning

HT22 cells were maintained in normal growth media (Dulbecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum (FBS)) between approximately 30 and 70% confluency. To isolate individual cell-derived colonies, 500 cells were plated on a 100 mm dish, and checked within 12 hours to designate well separated single cells by marking the bottom surface of the dish. Media was changed every 2-3 days. Once the designated cells formed a visible colony, the media was removed and a sterile cloning ring (Specialty Media) positioned around the perimeter of the colony. The colony was then trypsinized and replated into an individual well on a 48-well plate. A total of eight colonies were isolated and expanded, and labeled A-H. Once cells grew enough to freeze stocks, three colonies (clones "E", "G", and "H") were randomly chosen to characterize the cell death responsiveness.

Glutamate Treatment

The effects of two methods of glutamate treatment were assessed. In all toxicity experiments, cells were plated at a density of 1.5×10^4 cells/cm^2 in 96 well plates and treated 14-16 h after plating. The three clones, "E", "G" and "H", were plated and treated with the two different methods concurrently. In the first paradigm, the pre-existing media was "spiked" with glutamate to a final concentration of 5mM (0.52 μL of a 1M glutamate solution was added to the

pre-existing 100µl growth media. In order to control for small volume pipetting and mixing, media from at least 6 identical wells were collected together into an eppendorf tube, glutamate was added to a final volume of 5mM, and the media was then equally re-distributed to the wells from whence it came). In the second paradigm, the media was completely changed to fresh media containing 5mM glutamate.

Propidium iodide uptake

Propidium iodide (PI) is a DNA fluorescent dye that intercalates into double stranded nucleic acids. Although impermeable to live cells during short exposures, PI (633 Da) can pass easily into cells with mildly compromised outer membranes, and hence reflect cellular toxicity. At 8, 10, or 12 h following glutamate treatment, PI was added directly to the media to a final concentration of 40µg/mL. After a short incubation period (2-4 minutes at 37ºC), cells were counted using an Olympus inverted microscope with a 600 nm emission filter. Total cells in each field were counted under bright light to give a percent of PI positive cells within each field. Three wells were treated for each condition, and two fields per well were counted. Each experiment was performed over seven different platings (each plating was considered one "n"). Data were analyzed using one-way ANOVA with Tukey's post hoc analysis.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Previous data using HT22 cells had typically used the MTT assay as the primary toxicity assay. MTT is a yellow chemical that contains tetrazolium rings that, when reduced, form formazan crystals visible as dark blue whose absorbance at 570nm can be measured spectrophotometrically. The tetrazolium salt is presumed to be reduced by a mitochondrial succinate-tetrazolium reductase present in metabolically active cells. Loss of this activity has been correlated with loss of cell viability. Cells were plated and treated as described above.

Following 15 h of glutamate exposure, MTT solution was added the cell wells to a final concentration of 0.5mg/mL and incubated for 4 h at 37ºC. Cells were then solubilized overnight in 50% dimethyformamide/20% SDS, pH 4.8 and the absorbance read the following day at 570nm. Three individual wells were read per plating and averaged together; each plating considered one "n". Data were analyzed using one-way ANOVA with Bonferroni's post-hoc test.

3.4 RESULTS

3.4.1 Clonal variations existed within the parent HT22 cell population in responsiveness to glutamate-induced cell toxicity

Three single-cell derived HT22 clones were expanded and assessed for sensitivity to glutamate-induced cell toxicity. Using PI uptake as a measure of cellular toxicity, we found that clones "G" and "H" responded to glutamate-induced toxicity in a timecourse similar to the timecourse previously reported (**Fig. 2A, B**). As early as 8 h following glutamate treatment, both of these clones exhibited a significant cell death response compared to control cells ($p<0.01$ for "G", $p<0.001$ for "H"). The percentage of PI positive cells at all timepoints for clones "G" and "H" were significantly different from controls. Clone "E", however, exhibited a noticeably attenuated response to glutamate. No significant difference between control "E" cells and glutamate treated "E" cells was observed after 8 or 10 h following glutamate treatment. Comparing between subclones at 12 h following glutamate, subclone "E" had significantly fewer PI positive cells than either clone "G" ($p<0.001$) or clone "H" ($p<0.01$) (**Fig. 2B**).

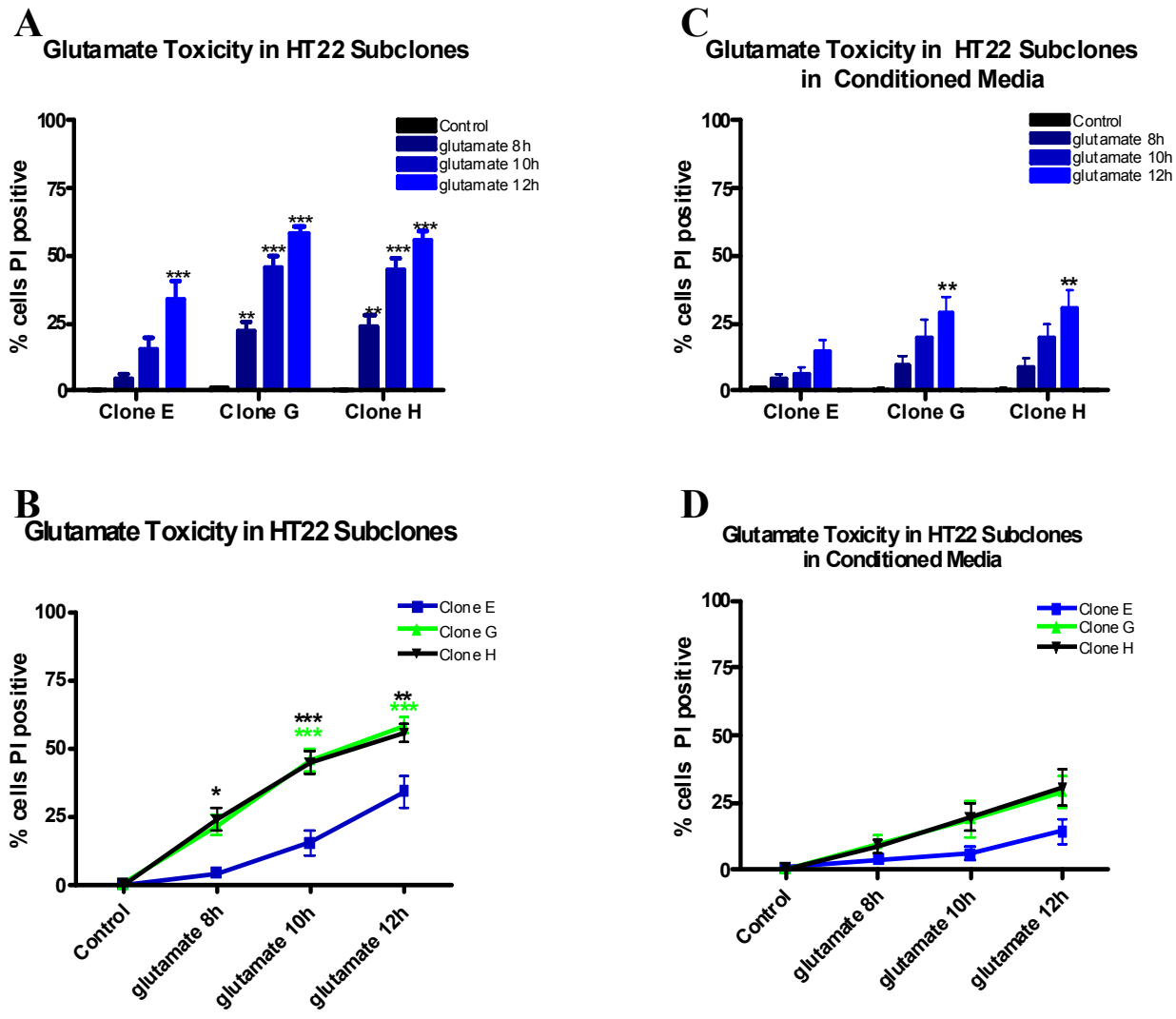


Figure 2 Variations exist among HT22 subclones. Clones E, G, H were plated simultaneously and treated with 5mM glutamate for 8, 10 or 12 hours with either fresh media (A, B) or the pre-existing media (C, D). PI (6 ng/mL) was added to the culture media, incubated for 2-4 minutes and counted under an inverted microscope. Results are expressed as the average percent of PI positive cells ± SEM, n=7. Data on graphs A and B are the same, but represented differentially to describe different statistical relationships, as are the data on graphs C and D the same. Asterisks in graphs A and C represent statistical differences between glutamate treatment and matched controls *within* subclone, whereas asterisks in graphs B and D represent statistical differences between clone E and clones G,H. *p<0.05, **p<0.01, ***p<0.001, according to Tukey's posthoc test of multiple comparisons.

3.4.2 Preconditioned media is protective in all subclonal HT22 cells

In order to assess whether the method of treatment could affect the cell toxicity response to glutamate, we determined whether adding glutamate directly to the pre-existing media (as opposed to a complete fresh media change) could inhibit glutamate-induced toxicity. Cells were plated concurrent to the experiments described above (where glutamate was added with a complete media change), and 0.5µl of a 1M glutamate solution was added to each well. Using PI uptake to measure cell toxicity, there was approximately only half the cell death response when compared to adding glutamate with fresh media in all clones (compare **Fig. 2A with Fig. 2C**). Both clones "G" and "H" reached significance only after 12 h of glutamate exposure compared to control ($p<0.01$) (**Fig. 2C**), whereas clone "E" was not significantly different from control at any of the timepoints tested. Interestingly, no statistical differences between clone "E" compared to clones "G" and "H" was observed in the presence of conditioned media (**Fig. 2D**).

3.4.3 MTT assay reflects a similar trend to PI uptake in measuring glutamate toxicity

Previous studies on HT22 cell toxicity primarily used the MTT assay to quantify cell death. The MTT assay differs from PI uptake in that MTT measures are reflective of the activity of a mitochondrial metabolic enzyme rather than cell membrane integrity. In order to confirm that the variation in clonal lines was not due to difference in assay technique, we performed the MTT assay exactly as described previously (Stanciu and DeFranco, 2002). The differences between subclones were not as remarkable as the results observed using PI uptake (**Fig. 3**). All clones reached the same level of significance ($p<0.01$) following glutamate treatment in fresh media. Conditioned media blunted the effects of glutamate, consistent with the PI uptake data.

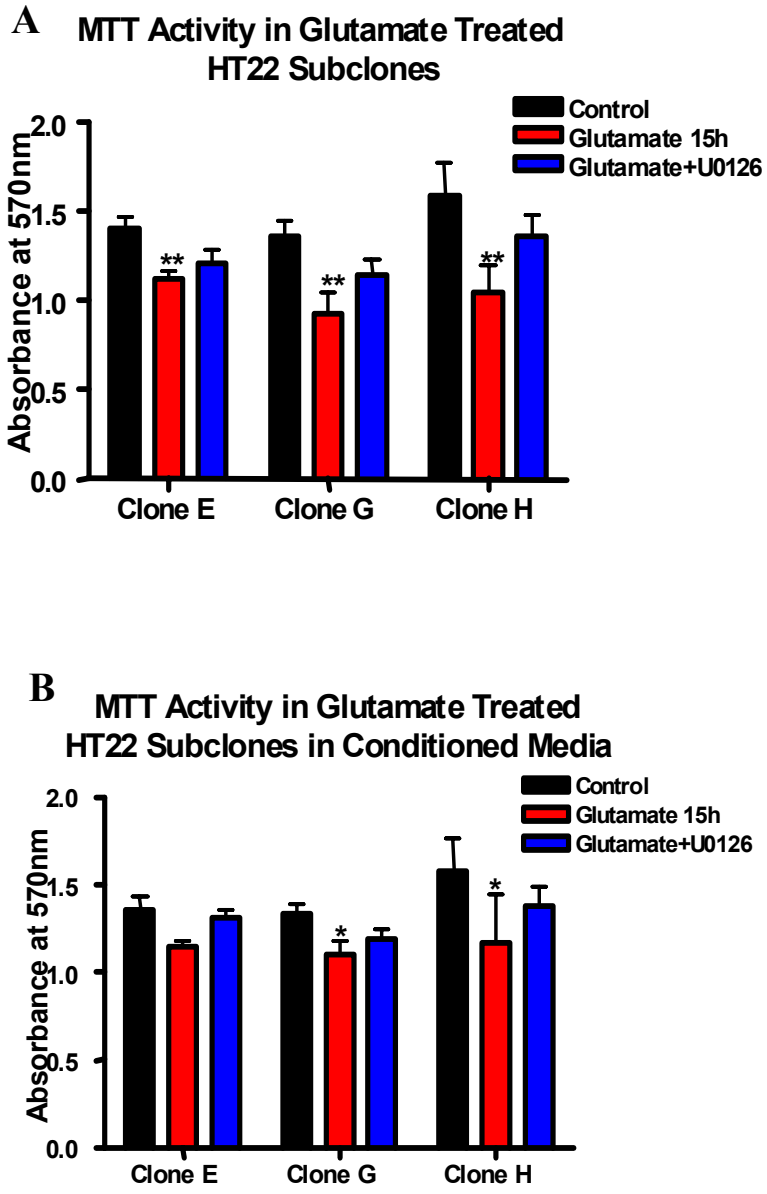


Figure 3 MTT assay reflects similar trend for glutamate-induced toxicity. Clones E, G, H were simultaneously plated and treated with 5mM glutamate in either fresh media (A) or conditioned media (B) for 15 hours. MTT (5mg/mL) was added and incubated 4h at 37ºC. Cells were solubilized overnight and then read at 570nm. Results are expressed as the absorbance at 570nm, ± SEM, n=3. Asterisks represent statistical differences between the glutamate treatment and the subclone control. *$p<0.05$, **$p<0.01$, according to Bonferroni's post hoc multiple comparison test.

In the conditioned media, clone "E" was less responsive to glutamate-induced toxicity, failing to reach significant difference compared to its matched control. Surprisingly, even with the later timepoint (15h of glutamate exposure when the MTT solution was first added, compared to the 12h timepoint using PI uptake), the MTT results did not show a robust cell death response, although by visual observation, the cells were largely rounded and detached from the plate, with rough looking membranes. Therefore, in further toxicity assays after the clonal variations, we relied exclusively on PI uptake as a marker of cellular toxicity, reflecting loss of plasma membrane integrity.

3.4.4 Clone G retains ERK dependency for glutamate-induced cell toxicity

We picked clone "G" to expand and use in all further experiments. Due to the interest in our group on the role of ERK in promoting cell toxicity, we wanted to be certain that clone "G" was still dependent on ERK for glutamate-induced cell toxicity, as had been previously published (Stanciu et al., 2000). ERK1/2 is activated via phosphorylation by the upstream kinase MEK. U0126 is a potent MEK inhibitor, and at low doses exhibits high specificity (Duncia et al., 1998). Treatment of clone "G" HT22 cells with U0126 significantly prevented cell toxicity as measured by PI uptake (**Fig. 4**), even after 24 h of glutamate exposure. Furthermore, U0126 could be added late in the timecourse, where a consistently complete protection was observed even when U0126 was added up to 7 h following glutamate (complete protection indicated by no statistical difference between treatment groups and control). Partial protection was observed in cells treated with U0126 9 h following glutamate exposure ($p<0.001$ when compared to glutamate alone, $p<0.05$ when compared to control). When U0126 was added 11 or 13 h following glutamate, no significant protection was found. Thus, the new subcloned HT22 cells

retain the MEK-dependent aspect of glutamate-induced toxicity, with the critical window for MEK involvement lying within the first 11 h of glutamate toxicity.

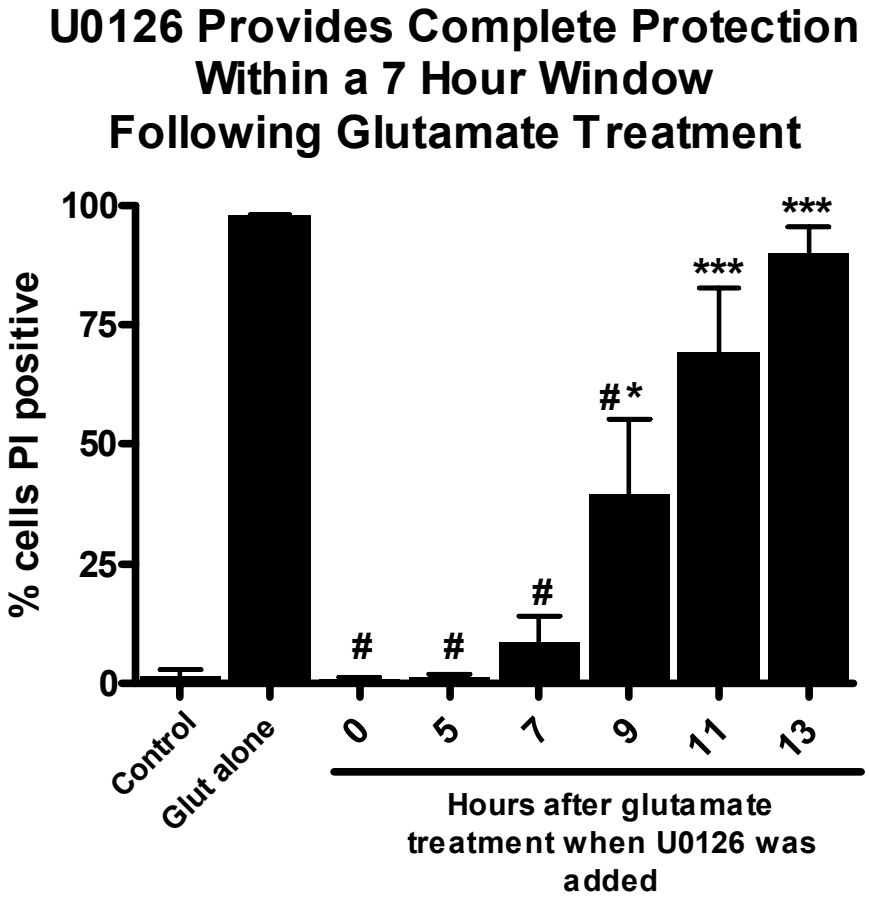


Figure 4 Delayed U0126 protects against glutamate toxicity in HT22 subclone G. Cells were treated with 5mM glutamate (in fresh media) and 10uM U0126 was added after 0, 5, 7, 9, 11, or 13 hours of glutamate exposure. After 24 h from the initial glutamate treatment, PI was added directly to the culture media, incubated 2-4 minutes at 37C, and counted immediately. Data is expressed as the average percent of PI positive cells per field ±SEM, n=3. Asterisks represent statistical differences between the treatment groups and control, where *$p<0.05$ and ***$p<0.001$; hatches represent statistical differences between the treatment groups and glutamate alone, where #$p<0.001$.

3.5 DISCUSSION

Although the benefits of using cell lines to model cell death paradigms is invaluable, caution must be exercised to ensure that the population of cells remains consistent over time and passages. The spontaneous mutation of cell lines in culture over time is a potential source of variability in cellular responsiveness. We have found that in the original parent population of HT22 cells, clonal differences existed in responsiveness to glutamate. Specifically, clones "G" and "H" responded in a consistently robust manner, similar to previously described data (**Fig. 2A**). On the other hand, clone "E" was significantly less responsive to glutamate-induced toxicity (**Fig. 2B**).

Additionally, we have shown that preconditioned media is protective to cells against glutamate-induced toxicity (**Fig. 2C**). Because cells treated with fresh media or preconditioned media were plated concurrently at the same density, we conclude that the protective effects of the preconditioned media are likely to be independent of cell density or cell-cell contacts. This has been confirmed by others in the lab who found that conditioned media from a confluent plate of cells reduced glutamate toxicity when added to a different plate of subconfluent HT22 cells. Many other cell types have been found to release soluble cytoprotective factors into the media (Freshney, 2000), so it is possible that this mechanism may also exist in HT22 cell cultures. A study in HT22 cells exposed to lower (2.5mM) concentrations of glutamate showed that addition of exogenous dopamine, apomorphine or apocodeine could reduce glutamate-induced toxicity mediated by either D4 receptor activation or the capacity of these agents to act as antioxidants (Ishige et al., 2001). By this example, perhaps HT22 cells in culture release neurotransmitters in sufficient quantity over time that could have a cytoprotective effect. However, it is equally possible that a component of the culture media necessary for cell death is depleted. Future

experiments to fractionate preconditioned media components and compare them to fresh media components may address this issue.

Interestingly, we found that the results of the MTT assay were consistent with the results using PI uptake, but were perhaps not as sensitive in detecting cell death. PI uptake occurs in cultured cells undergoing either oncosis or very late stage apoptosis. There appears to be a sequential permeabilization of the plasma membrane during oncosis. Using renal proximal tubules exposed to anoxic conditions, Chen et al found early uptake of the 633 Da fluorescent dye PI, followed by later uptake of 3 or 70 kDa dextran (Chen et al., 2001). This study suggested that PI is a sensitive early marker of cellular toxicity. Interesting to note, the same study found that uptake of PI does not necessarily represent irreversible toxicity, as cells could still be rescued after timepoints where PI uptake was observed. Reduction of MTT to the formazan product, on the other hand, requires active enzymes, predominantly found in mitochondria, although there are data suggesting that cells with compromised mitochondria can still reduce MTT (Mueller et al., 2004). The exact cellular mechanisms responsible for MTT reduction are unclear, as lysosomal enzymes may also be able to reduce MTT (Liu et al., 1997; Liu, 1999). If the reduction of MTT is due to unidentified enzymes, it is difficult then to interpret the relevance of the MTT assay results in terms of cell death. Additionally, many different experimental factors can influence the reduction of MTT (Page et al., 1988). For example, the difference that we observed in the sensitivity of the PI uptake assay and the MTT assay could be confounded by the experimental setup of the MTT assay. The previous reports did not specify any sort of media change or internal control. Because we wished to repeat previous experiments exactly as described, we did not alter the media to phenol red-free, which has been thought to potentially interfere with MTT metabolism. Thus it may be possible to alter

the experimental setup to enhance the sensitivity of this assay. However, given the ambiguity of the nature of the enzymes responsible for reducing MTT to the formazan salt, the MTT assay would still lead to potentially obscure interpretations. Many other cell toxicity assays, such as PI uptake or trypan blue exclusion, shift the focus away from enzymatic activity and instead reflect the integrity of the plasma membrane. Given the lack of enzyme specificity, and the comparison of our PI uptake results compared to the MTT assay results, we chose to rely on PI uptake as a measure of cellular toxicity reflective of loss of plasma membrane integrity.

While the purpose of these experiments was to identify a subclonal population with a consistent response to glutamate, it would be interesting to investigate the less responsive subclones for alterations in ERK signaling. HT22 subclones completely unresponsive to high (10mM) doses of glutamate were examined for differential gene expression patterns, particularly focused on oxidative stress genes, or cross-resistance to other modes of toxicity (Sagara et al., 1998; Dargusch and Schubert, 2002). These subclones expressed higher levels of catalase and had increased activities of enzymes involved in antioxidant pathways, such as γ-glutamylcysteine synthase, GSH reductase and GSH S-transferase (Sagara et al., 1998), and were cross-resistant to amyloid toxicity (Dargusch and Schubert, 2002). ERK1/2 has been implicated in both glutamate toxicity in HT22 cells, and several, but not all, models of amyloid toxicity (Kuperstein and Yavin, 2002). As clone "E" was significantly less responsive to glutamate than clones "G" or "H", it might be interesting to examine whether glutamate-induced ERK activation is also reduced in clone "E". Furthermore, whether the attenuated cell death response in subclone "E" is reflective of resistance to cell death, or simply a delay in the response of cell death, has yet to be determined.

Taken together, we have found that the parent population of HT22 cells consisted of clonal variants that differed in their responsiveness to glutamate-induced toxicity. Conditioned media was significantly protective in all subclones. The MTT assay reflected a similar trend in indicating cell toxicity. Clone "G", which was chosen for all future experiments, was found to retain the previously described ERK-dependent characteristics of glutamate-induced cell death. The results of this study allowed us the use of a purer cell population and a more sensitive cell death assay. The possibility exists for future experiments on the consequences of clonal variations on ERK signaling, as well as the analysis of differential components in conditioned versus fresh media.

4.0 THE ROLE OF CASPASES IN ERK-DEPENDENT CELL DEATH

4.1 SUMMARY

Using the irreversible, semi-selective caspase inhibitors Ac-DEVD.fmk (caspase-3, 6, 7), Ac-IETD.fmk (caspase-8 and granzyme B), Ac-YVAD.fmk (caspase-1, -3, -7) and Ac-VDVAD.fmk (caspase-2), only Ac-YVAD.fmk and Ac-VDVAD.fmk inhibitors were effective at preventing glutamate-induced toxicity in HT22 cells as assessed by PI uptake. Ac-YVAD.fmk could be added as late as 3 h following glutamate treatment and still afford complete protection from glutamate-induced toxicity. We attempted to determine caspase "activation" through immunoblotting for processed forms of individual caspases as well as by *in vitro* caspase activity assays. However, no evidence of caspase-1 or -3 processing or activity was evident by either of these methods, suggesting that the inhibitors may function in a manner independent of the capacity to inhibit caspase-1 activity. Interestingly, we found that the Ac-YVAD.fmk and Ac-VDVAD.fmk inhibitors also inhibited expression of phospho-ERK. Therefore, YVAD.fmk and VDVAD.fmk appear to function upstream of ERK activation following glutamate exposure in HT22 cells, but may possibly prevent toxicity in a caspase-independent manner.

4.2 INTRODUCTION

Caspases comprise a family of proteases that have been implicated in a variety of cell death models. While several "traditional" pathways can be activated in cellular stress models (e.g., the so-called "intrinsic" and "extrinsic" pathways), there exist mechanisms of caspase activation that remain less defined. Although caspase-1 was one of the first mammalian caspases identified, it is still one of the least understood caspases in terms of its mechanism of activation and substrate targeting in models of cellular stress. Caspase-1 was originally isolated from the leukocytic cell line THP.1, and was found to be responsible for the cleavage of interleukin-1β, an inflammatory cytokine that appears to be involved in acute neuronal disease states (Patel et al., 2003). Originally, caspase-1 was thought to be the primary mammalian homologue of the *C. elegans* ced-3 gene, which regulates much of the cell death process in nematodes (Vaux and Strasser, 1996). However, the knockout mouse for caspase-1 did not display a robust phenotype, whereas another ced-3 homologue, caspase-3, was quickly found to be a major executioner cell death protein in many cell death pathways (Kuida et al., 1996). Importantly for our model, one subtle phenotype present in the caspase-1 knockout mouse was a resistance to neonatal hypoxia/ischemia, as well as focal ischemia (Schielke et al., 1998; Liu et al., 1999).

Caspase-2 resembles most closely the C. elegans ced-3 gene in molecular alignment and functional domains, suggesting that it is perhaps the oldest member of the mammalian caspase family (Troy and Shelanski, 2003). Similar to the caspase-1 knockout, the caspase-2 knockout mouse was not grossly abnormal compared to wildtypes (Bergeron et al., 1998). However, *in vitro,* caspase-2 has been implicated in a variety of cell death stimuli by knockdown and antisense techniques, including trophic factor withdrawal (Haviv et al., 1998; Troy et al., 2001) and beta-amyloid (Troy et al., 2000).

HT22 cells die from glutamate exposure with an atypical nuclear morphology which is inconsistent with traditional apoptotic pathways (Tan et al., 1998a). The caspase-3 inhibitor DEVD.cho has been found to be ineffective at preventing glutamate-induced toxicity in HT22 cells, whereas the caspase-1 inhibitor YVAD.cmk was protective (Tan et al., 1998a). The mechanism of inhibition of these two inhibitors differs slightly – the aldehyde (.cho) inhibitors are reversible and are relatively less permeable, whereas the chloromethylketone and fluoromethylketone (.cmk and .fmk) inhibitors are irreversible with increased cell permeability. The endpoint used in these studies was the MTT assay, which measures the ability of cells to convert a tetrazolium salt into a formazan product. This conversion is thought to be based on an enzymatic activity found in the mitochondria and lysosomes (Page et al., 1988; Liu et al., 1997), and has been correlated with cell death. The MTT-reducing enzymatic activity in the mitochondria plays a role in oxidative phosphorylation and ATP synthesis, but the lysosomal-reducing enzymatic activity is not understood. Another problem with interpretation of MTT reduction is that alterations in cell toxicity by pharmacological manipulations downstream or independent of this activity would not be detectable with the MTT assay, thus leading to a possible overestimation of cellular toxicity using this assay.

Previously, the caspase-1 inhibitor YVAD.cmk was found to decrease ROS production in glutamate-exposed HT22 cells (Tan et al., 1998b). In a separate HT22 study, the MEK inhibitor U0126 was found to decrease intracellular calcium levels, but not ROS, following glutamate (Stanciu et al., 2000). Crosstalk between the two signaling pathways had not been directly determined, but these separate results taken together may imply that caspase-1-like activation (rather, activity sensitive to Ac-YVAD.cmk inhibition) lies upstream of MEK activation.

We studied the effects of a panel of semi-selective irreversible (fmk) caspase inhibitors using PI uptake to measure toxicity. We found that only Ac-YVAD.fmk and Ac-VDVAD.fmk were effective at preventing glutamate-induced toxicity. This protection was observed when the inhibitors were added as late as 3 h after glutamate. We were not able to demonstrate caspase-1 activity or processing. Both Ac-YVAD.fmk and Ac-VDVAD.fmk decreased phospho-ERK expression levels at 9 h after glutamate, indicating that the inhibitors act on molecule(s) upstream of ERK activation.

4.3 EXPERIMENTAL PROCEDURES

Materials

Glutamate stock (1M in water) was sterile filtered and stored at -20ºC. Ac-YVAD.fmk, Ac-DEVD.fmk, Ac-IETD.fmk, Ac-VDVAD.fmk, and Ac-VAD.fmk (EMD Biosciences) were each dissolved in DMSO and stored as a 20mM stock at -20ºC. Ac-YVAD.afc was purchased from Biomol and stored away from light as a 10mM stock in DMSO. Propidium iodide (PI) was dissolved in water and stored as a 6mg/mL stock at 4C protected from light. Antibodies used included caspase-3 (Cell Signaling, 1:1000), Santa Cruz caspase-1 (sc-514, 1:200), actin (Santa Cruz, 1:400), phospho-ERK (Cell Signaling, 1:1000), p42/44 MAP kinase (Cell Signaling, 1:2000).

Cell Culture

HT22 cells were maintained in normal growth media (Dulbecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum (FBS)) between approximately 30

and 70% confluency. For all experiments, cells were plated at a density of $1.5x10^4$ cells/cm^2 and treated 14-16 h after plating. For toxicity measurements, PI (40 μg/mL) was added to the culture media, incubated 2-4 minutes at 37ºC, and cells were counted immediately on an inverted microscope. Total cells in each field were counted under bright light to give a percent of PI positive cells within each field. Three wells were treated for each condition, and two fields per well were counted. Each experiment was performed over 2-3 different platings (each plating was considered one "n"). Data was analyzed using one-way ANOVA with Tukey's post hoc analysis.

NIH3T3 cells were grown in DMEM supplemented with 10% FBS and passaged at or before 80% confluency. Staurosoporine was added to fresh media to a concentration of 1μM. Cells were collected as described below under Western blotting.

THP.1 cells were grown in suspension in RPMI 1640 supplemented with 10% FBS and 0.05mM 2-mercaptoethanol. Cultures were maintained in $75mm^2$ flasks, and split every 2-3 days. Cells were collected, resuspended in PBS, split into equal volumes (one for activation, one as "control"), centrifuged for 5 min at 3000 rpm, and resuspended in lysis buffer (50mM Tris pH 7.5, 2mM EDTA, 100mM NaCl, 1% NP-40, 0.1mM $NaVO_4$, 2mM DTT, plus protease inhibitor cocktail). For "activation", the lysate was incubated at 37ºC for 30 min. The lysates were then cleared of the insoluble fraction.

Western Blots

For phospho-ERK westerns, cells were rinsed with ice cold PBS, collected and lysed for 20 minutes on ice in lysis buffer (50mM Tris pH 7.5, 2mM EDTA, 100mM NaCl, 1% NP-40, 0.1mM $NaVO_4$, 2mM DTT, plus protease inhibitor cocktail), spun at 13000 x g for 12 min at 4ºC. The supernatant, containing the soluble protein, was measured for protein concentration

using the Bradford colormetric assay. Sample buffer (350mM Tris pH 6.8, 10.2% SDS, 1.43M 2-mercaptoethanol, 0.17mM bromophenol blue) was added to 100µg of protein, boiled 5 minutes, loaded on a 10% SDS-PAGE gel and transferred using a semi-dry transfer apparatus onto PVDF membrane (0.45µm) at 15V for 20 min.

For caspase-1 westerns, cells were rinsed with ice cold PBS and collected directly into warm sample buffer, boiled 5 min, and loaded on a 15% SDS-PAGE gel. The gel was wet-transferred onto 0.1µm nitrocellulose membrane at 100V for 20 min at 4ºC. Following transfer, the membrane was boiled for 5 min in water.

Membranes were then rinsed quickly in PBST (1X phosphate-buffered saline, 0.1% tween-20), blocked for 1 hour at room temperature in 5% nonfat dry milk/PBST, and incubated overnight at 4ºC in the primary antibody. After several rinses in PBST, secondary IgG-HRP antibodies (Biorad, 1:2000) were incubated for 1.5 hours at room temperature, rinsed several times in PBST, quickly rinsed with chemiluminsence reagents (NEN), and exposed to x-ray film and processed. Densitometry was used to semi-quantify the band intensity, subtracting background and normalizing to loading controls. Data was analyzed using one-way ANOVA with Bonferroni's post hoc test.

Activity assays

Cells were rinsed with ice cold PBS, collected and resuspended in lysis buffer (50mM HEPES, 1mM EDTA, 1mM EGTA, 5mM $MgCl_2$, 0.1% CHAPS) for 20 minutes on ice. 200µg of protein was diluted in reaction buffer (50mM HEPES, 100mM NaCl, 1mM EDTA, 0.1% CHAPS, 10mM DTT, 10% glycerol), and 100µM of the YVAD.afc substrate was added to each well immediately before assay measurements began. The assay plate was maintained in a heated chamber at 37ºC in the fluorimeter, and the reaction was monitored every 10 min at an excitation

wavelength of 400nm and an emission of 505nm. Background values from wells containing substrate but no protein were subtracted from experimental wells. Each lysate was run in duplicate, and the results were compiled from three separate lysates per timepoint. Data were analyzed using one-way ANOVA with Tukey's post hoc test.

4.4 RESULTS

4.4.1 The caspase inhibitors Ac-YVAD.fmk and Ac-VDVAD.fmk, but not Ac-DEVD.fmk or Ac-IETD.fmk, are effective at preventing glutamate induced toxicity.

To determine potential caspase family members that may be involved in glutamate-induced oxidative stress, we used the cell permeable .fmk inhibitors Ac-YVAD.fmk, Ac-VDVAD.fmk, Ac-DEVD.fmk, and Ac-IETD.fmk, all of which are O-methylated to increase cell permeability. Ac-YVAD.fmk and Ac-VDVAD.fmk target caspase-1-like and caspase-2-like proteases respectively, but both are known to also inhibit caspases-3 and -7. Ac-DEVD.fmk inhibits caspases-3, -6, and -7, and Ac-IETD.fmk inhibits caspase-8 and granzyme B. The semi-selective irreversible caspase inhibitors Ac-YVAD.fmk and Ac-VDVAD.fmk were effective at preventing cell death assayed by PI uptake (**Fig. 5**), a cell death measure that is reflective of the integrity of the plasma membrane and is independent of specific enzymatic function. Neither Ac-DEVD.fmk nor Ac-IETD.fmk, irreversible inhibitors of caspase-3-like activity and caspase-8-like activity respectively, were able to inhibit glutamate-induced cell death (**Fig. 5**). Given that Ac-DEVD.fmk was not protective, even at relatively high (100μM) doses, it is probable that the protective effects of Ac-YVAD.fmk and Ac-VDVAD.fmk were not due to inhibition of caspases-3 or -7.

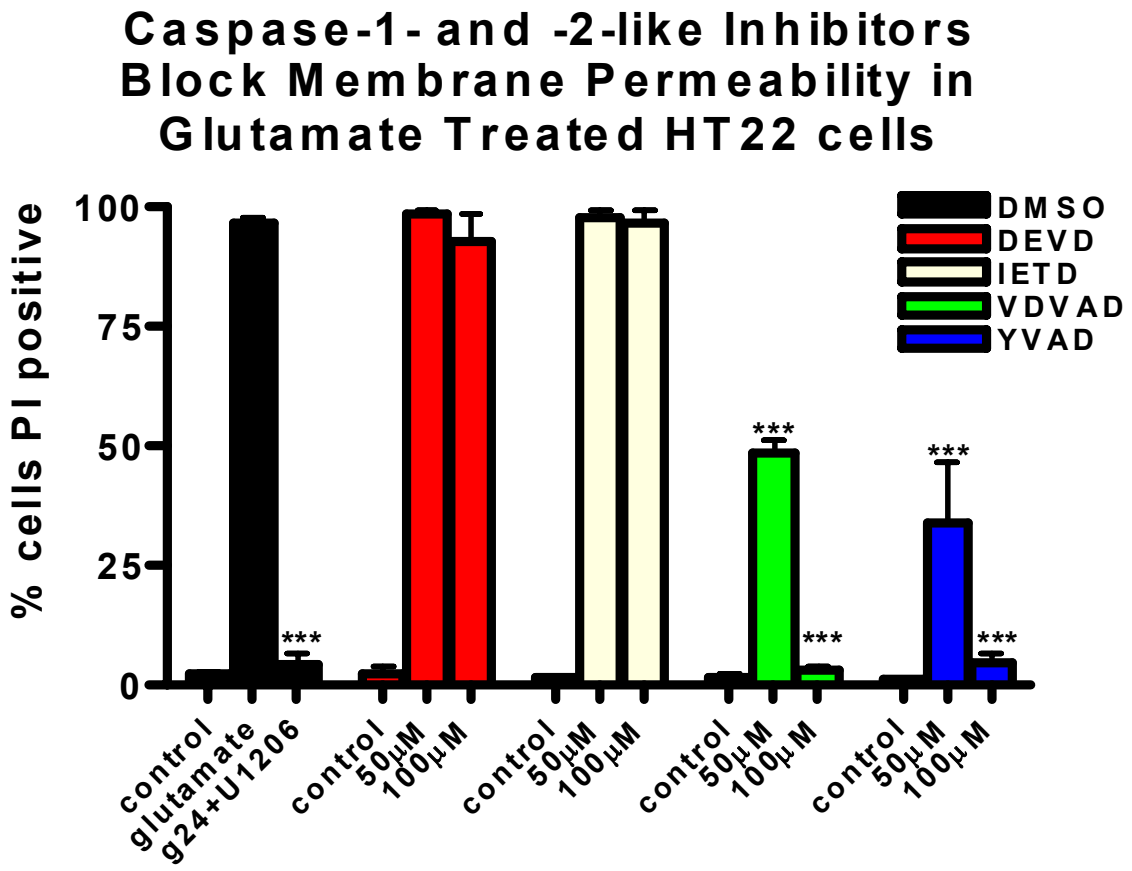


Figure 5 Ac-YVAD.fmk and Ac-VDVAD.fmk, but not Ac-DEVD.fmk and Ac-IETD.fmk, protect against glutamate-induced toxicity in HT22 cells. HT22 cells were treated with glutamate in the presence of 50 or 100μM of the semi-selective irreversible caspase inhibitors Ac-DEVD.fmk (red), Ac-IETD.fmk (beige), Ac-VDVAD.fmk (green) or Ac-YVAD.fmk for 24h, and assessed for glutamate toxicity using PI uptake to measure plasma membrane permeability. All control bars were incubated in the presence of the corresponding inhibitor. Black bars represent untreated HT22 cells or cells treated with glutamate only, or glutamate+10μM U0126. Data are presented as the percent of PI positive cells ±SEM, n=3. Cells were counted from 3 wells (2 fields per well) for each plating, where one plating equals one "n". Data were analyzed using one-way ANOVA and Tukey's post hoc comparisons. ***$p<0.0001$ compared to glutamate treatment alone.

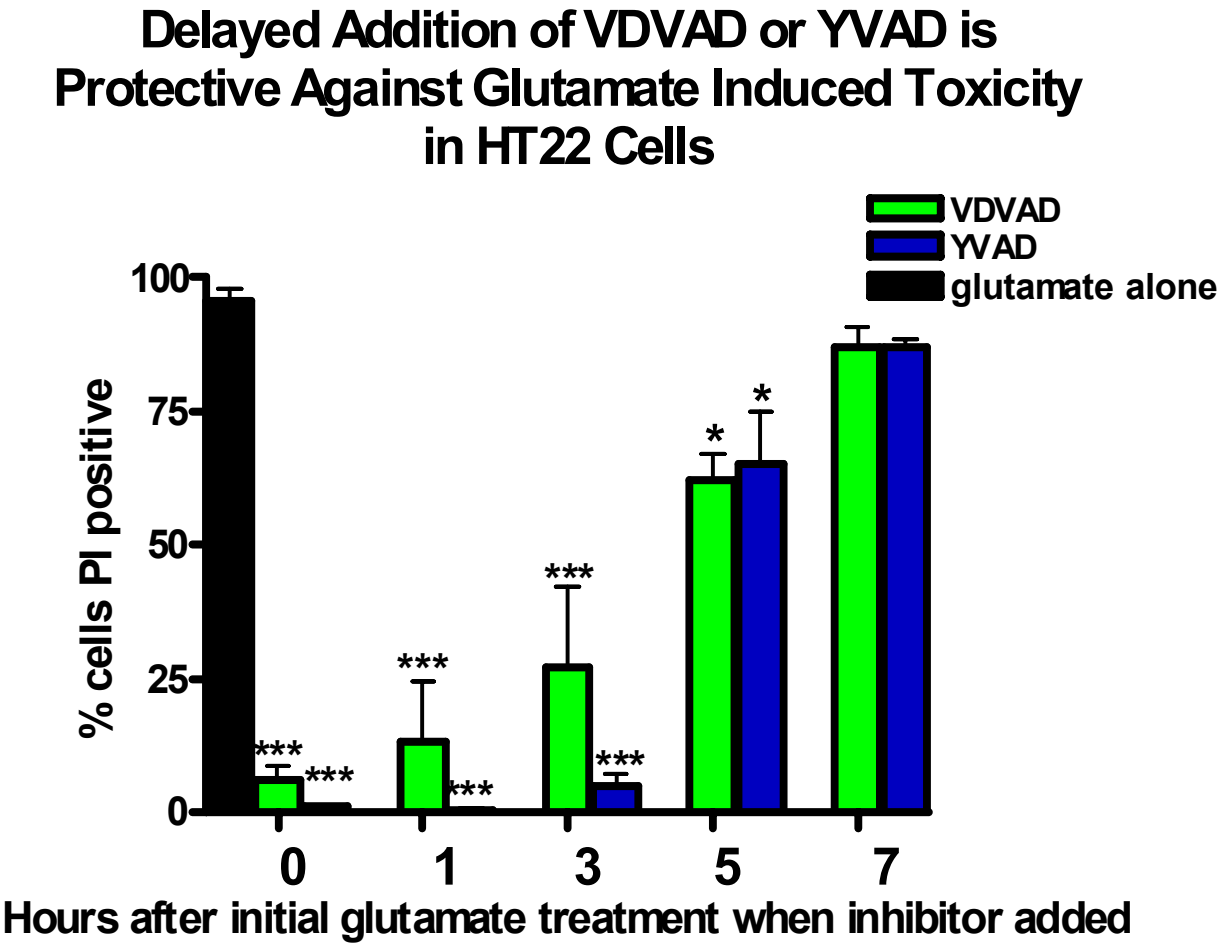


Figure 6 Ac-YVAD.fmk or Ac-VDVAD.fmk could be added as late as 5-7 hours following glutamate treatment and still protect cells from toxicity in HT22 cells. HT22 cells were treated with glutamate for 24h. At various timepoints following glutamate treatment, 100μM of the semi-selective irreversible caspase inhibitors Ac-VDVAD.fmk (green) or Ac-YVAD.fmk was added to the wells. Following 24h of glutamate exposure, cells were assessed with PI uptake to measure plasma membrane permeability. Black bars represent HT22 cells treated with glutamate only. Data is presented as the percent of PI positive cells ±SEM, n=3. Cells were counted from 3 wells (2 fields per well) for each plating, where one plating equals an "n". Data was analyzed using one-way ANOVA and Tukey's post hoc comparisons. ***$p<0.0001$ compared to glutamate only treatment.

Both Ac-YVAD.fmk and Ac-VDVAD.fmk could exert complete protection when added at 3 h after glutamate treatment (F**ig. 6**), but they began to lose their protective effects when added 5 or 7 h following glutamate application. There were no statistical differences in the

effectiveness between Ac-YVAD.fmk and Ac-VDVAD.fmk in blocking glutamate-induced toxicity. Interestingly, both inhibitors exerted a half-maximal effect at 50μM, and complete protection at 100μM (**Fig. 5**). The actual effective inhibitory range of these inhibitors *in vitro* was reported to be in the nanomolar range (Garcia-Calvo et al., 1998). However, use of peptide inhibitors *in vivo* is difficult to compare to K_i values obtained *in vitro*, as these inhibitors are quite difficult to pass the plasma membrane and enter the cell (Ekert et al., 1999). This could suggest that either the intracellular concentration reaches an effective range within these doses, or that off-target molecules can be inhibited with higher drug concentrations.

4.4.2 No evidence of caspase activation or increased activity was detected following glutamate exposure in HT22 cells.

Caspase activation, in particular for executioner caspases, has been associated with the processing of the zymogen into "active" subunits. To confirm that caspase-3 was not involved in the glutamate-induced toxicity model in HT22 cells, Western blots analysis was performed using a caspase-3 antibody that recognizes both the full length zymogen (32kDa) and the large subunit (17kDa). In order to confirm the specificity of the caspase-3 antibody, we ran lysates from 3T3 cells exposed to staurosporine, a classical model of caspase-3 dependent cell death, in addition to lysates from glutamate-treated HT22 cells. Whereas we readily detected the full length caspase-3 protein in both the HT22 and 3T3 lysates, we were not able to detect the cleaved form of caspase-3 in the glutamate-exposed HT22 cells (**Fig. 7C**). The cleaved form of caspase-3 was present in lysates from staurosporine treated NIH3T3 cells. These data corroborate the Ac-DEVD.fmk inhibitor data, suggesting that caspase-3 does not play a role in the toxicity of HT22 cells exposed to glutamate.

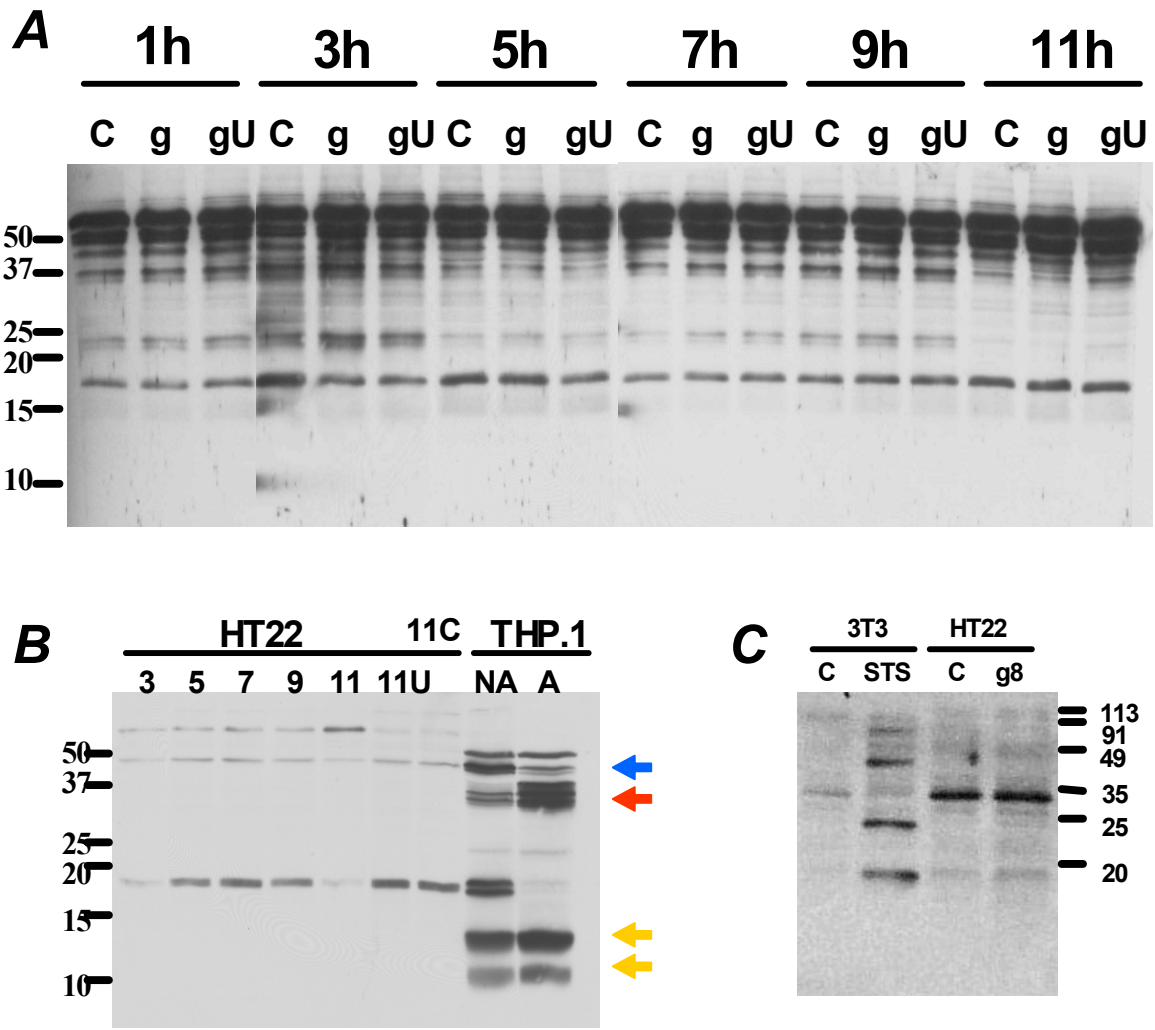


Figure 7 Caspases-1 and -3 are not processed in glutamate-exposed HT22 cells. ***A*** HT22 cells were collected after various timepoints of media change alone ("C"), 5mM glutamate ("g") or glutamate+10μM U0126 immediately into sample buffer and boiled. ***B*** HT22 cells collected following various timepoints (3, 5, 7, 9, 11h) after 5mM glutamate treatment or 11h after glutamate+10μM U0126 (11U) or 11h after media change (11C). Lysates in ***B*** were collected in normal lysis buffer on ice for 10 min and centrifuged at 13000xg. Note the difference in the intensity of the zymogen. Blue arrow indicates the zymogen, red indicates a 35kDa cleavage product, and the yellow arrows indicate the 12 and 10kDa subunit. ***C*** Western blot from lysates of NIH3T3 cells with (STS) or without (C) 8h of staurosporine and lysates of HT22 cells with (g8) or without (C) glutamate for 8h immunoblotted for caspase-3. The 3T3 cells show loss of the zymogen (35kDa) and the appearance of processed product band around 17kDa.

Proteolytic Processing and Isoforms of Caspase-1

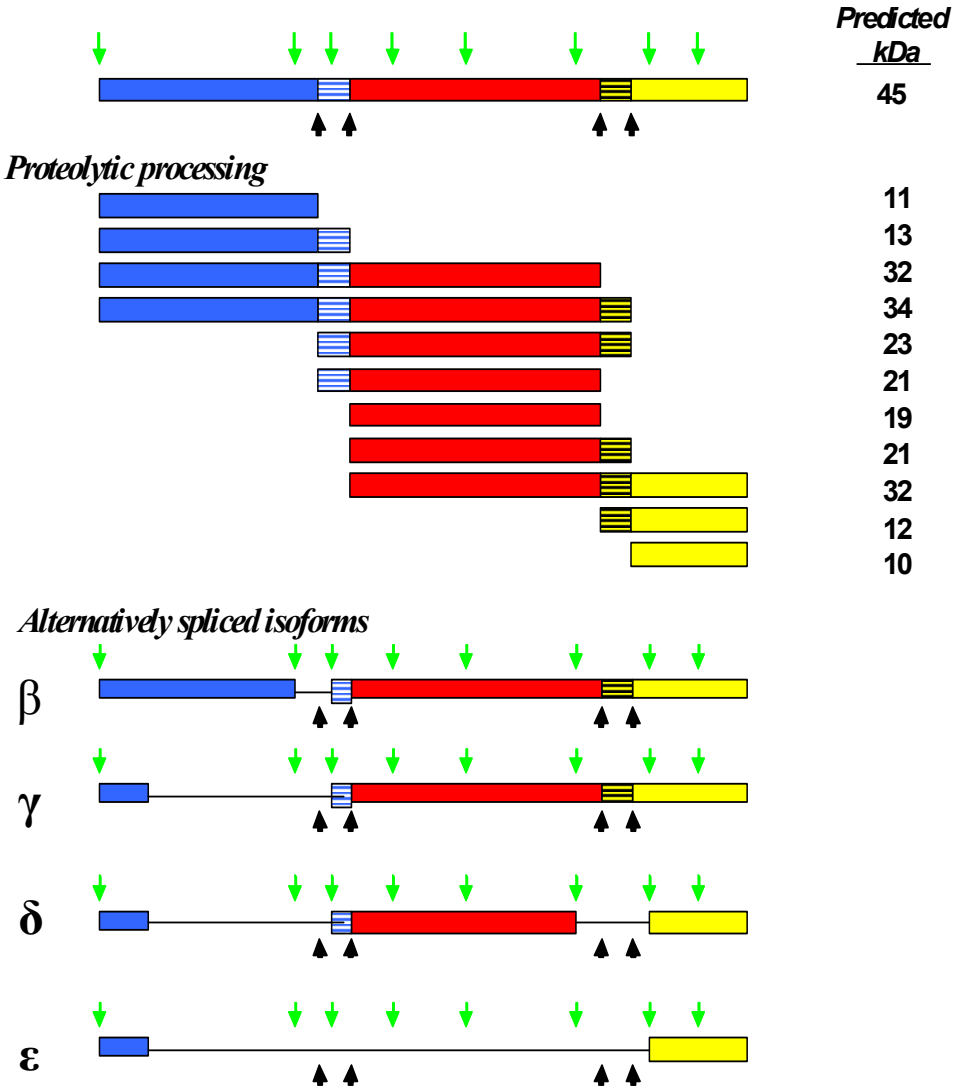


Figure 8 Proteolytic processing and alternative splicing produces many proteolytic fragments. Top, the combination of multiple cleavage sites (black arrows) within the caspase-1 zymogen can produce several known fragments. Bottom, alternative mRNA splice varients cloned from human lysates. Green arrows represent introns. Adapted from Yamin (1996) *JBC* 271 and Alnemri (1995) *JBC 270*

Caspase-1 processing appears to be more complicated than caspase-3. Apical caspases, hallmarked by a long CARD- or DED-containing prodomain, have been found to have enzymatic activity as zymogens (Yamin et al., 1996; Stennicke et al., 1999; Boatright et al., 2003). The caspase-1 zymogen contains at least 5 different processing sites, yielding fragments with predicted sizes of 35kDa, 22kDa, 20kDa, 12kDa or 10kDa, several of which have autoinhibitory function back onto caspase-1 (Yamin et al., 1996) (**Fig. 8**). Using an antibody raised against the C-terminus (within the last 50 amino acids of the murine caspase-1), we have been able to detect the full length caspase-1 in HT22 cells (F**ig. 7A,B)**. However, no cleavage of caspase-1 was detected from lysates of HT22 cells exposed to glutamate. To test the specificity of the bands recognized by the antibody, we also ran activated and non-activated THP.1 cell lysates. The THP.1 lysates showed clear evidence of bands at approximately 12 and 10kDa, as well as 35kDa (**Fig. 7B**). However, given the complicated processing of caspase-1, as well as reports of alternative isoforms found in human (Alnemri et al., 1995; Yamin et al., 1996; Feng et al., 2004) (**Fig. 8**), it would be interesting to determine by cloning analysis which isoform of caspase-1 HT22 cells express.

Due to previous reports that the caspase-1 zymogen possesses enzymatic activity in the absence of processing (Yamin et al., 1996) and our findings that Ac-YVAD.fmk was effective in inhibiting cell toxicity in glutamate exposed HT22 cells, we next sought to measure enzymatic caspase-1 activity using an *in vitro* activity assay with YVAD.afc as a substrate. The substrate should, in theory, bind to the peptide recognition site of activated caspase-1, -3 or -7, resulting in cleavage of the afc moiety and a subsequent shift in fluorescence. Surprisingly, we did not see any statistical difference in caspase-1-like activity between glutamate-treated lysates and controls (**Fig. 9**). However, the positive controls (THP.1 cell lysates) also did not show robust activation,

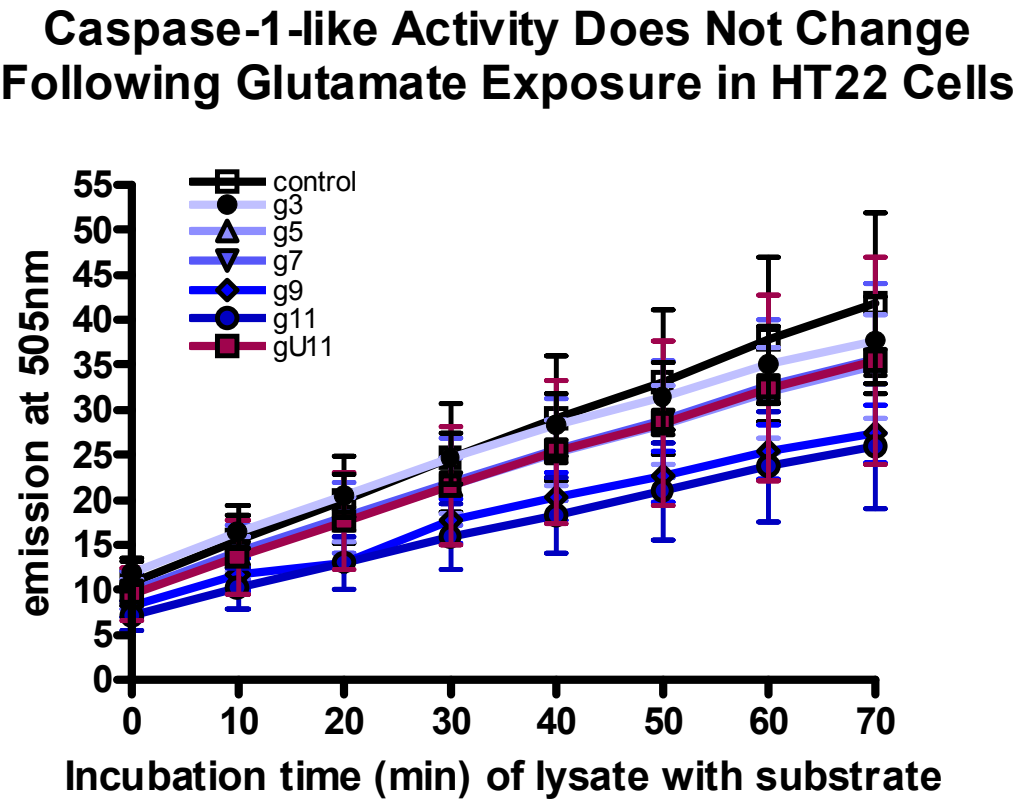
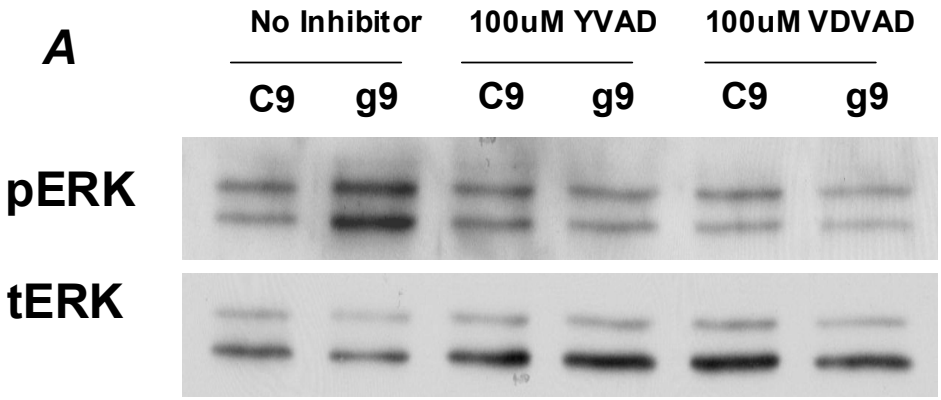


Figure 9 No evidence in alterations of caspase-1-like activity following glutamate exposure in HT22 cells. Lysates were collected at various timepoints (3, 5, 7, 9, 11) from HT22 cells treated with 5mM glutamate or 11h after glutamate+10μM U0126 (11U) or 11h after media change (control), and assessed for enzymatic activity by cleavage of Ac-YVAD.afc. Emissions were recorded every 10min over 1 hour of incubation in a 37C chamber. Data are represented as the average of duplicate wells for each lysate (n=3)±SEM and analyzed using one-way ANOVA.

suggesting that the assay conditions, such as the salts used, may not have been ideal in preserving the protein structure in a form that is enzymatically active (see Discussion below) (Boatright et al., 2003).

4.4.3 YVAD.fmk and VDVAD.fmk block ERK activation at late times following glutamate addition

The interaction of caspase signaling pathways and MAPK pathways has not been extensively explored. We therefore treated HT22 cells with the inhibitors Ac-YVAD.fmk and Ac-VDVAD.fmk (both at 100μM) and assessed ERK1/2 activation 9 h following 5mM glutamate exposure. We found that these caspase inhibitors effectively decreased ERK phosphorylation in the presence of glutamate, which suggests that ERK is downstream of a caspase-like activity (**Fig. 10**). Due to the lack of evidence for significant caspase activity in glutamate-treated HT22 cell lysates, it was not possible to determine the effects of MEK inhibition on caspase activation.



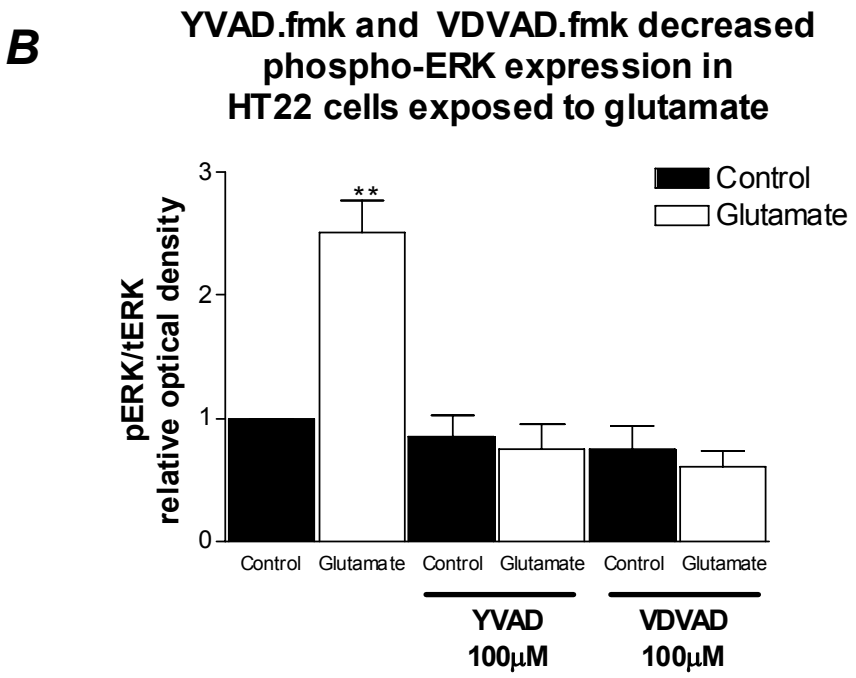


Figure 10 Ac-YVAD.fmk and Ac-VDVAD.fmk inhibit glutamate-induced ERK1/2 activation in HT22 cells. Cells were treated with or without 5mM glutamate in the presence of 100μM YVAD.fmk or VDVAD.fmk for 9h and then collected for Western blots. *A* Western blot of phospho-ERK1/2 (top) and total ERK1/2 (bottom).. *B* Semi-quantification of Western blots (n=3 lysates per group). Data are presented as the optical density (OD) of phosphoERK/OD total ERK, normalized to no drug control±SEM. Analysis was performed using one way ANOVA with Bonferroni's post hoc comparisons.

4.5 DISCUSSION

Previous studies have found that the irreversible caspase-1 inhibitor YVAD.cmk, but not the reversible caspase-3 inhibitor DEVD.cho, was effective at preventing cell toxicity in glutamate-exposed HT22 cells as measured by MTT assay (Tan et al., 1998a). We have corroborated these findings by using .fmk inhibitors and PI uptake as the measure of cell toxicity, which is independent of MTT reductase activity. We found that both the caspase-1 inhibitor Ac-YVAD.fmk and the caspase-2 inhibitor Ac-VDVAD.fmk were equally effective at preventing glutamate toxicity in HT22 cells. Delayed treatment with either of these inhibitors completely prevented toxicity as measured by PI uptake when added to the media up to 3 hours after glutamate, with equal efficacy. The caspase-3 inhibitor Ac-DEVD.fmk and the caspase-9 inhibitor Ac-IETD.fmk were ineffective at preventing glutamate-induced toxicity. The lack of involvement of caspase-3 was further confirmed by Western blot, as no evidence of processing was observed.

Surprisingly, using both Western blot and in vitro activity assays with YVAD.afc as a substrate, we were unable to find any evidence of caspase-1 activity following glutamate exposure in HT22 cells. The inability to detect caspase-1 activity with the *in vitro* peptide substrate could be due to the conditions employed in our experimental setup. In fact, the activities of apical caspases have been proven to be very difficult to detect *in vitro* (Boatright et al., 2003). The addition of kosmotropic salts was reported to be able to stabilize caspase proteins and activity. For example, sodium citrate was effective at the stabilization of caspase-8, and ammonium citrate effective with caspase-9 (Boatright et al., 2003). The sensitivity of active

caspase structures to salt has been observed for caspase-1 as well (Yamin et al., 1996). Future alterations of the lysis buffer and reaction conditions for this assay may enable more reliable measurement of caspase-1 activity. Additionally, with immunoblotting, we observed that our normal lysate preparation procedure (lyse on ice for 20 minutes, centrifuge for 10 minutes) seemed to decrease the detection of even the total zymogen protein. Caspase-1 fragments were found by others to be highly unstable (Miller et al., 1993; Yamin et al., 1996); thus, the lysis and centrifugation that we performed before the activity assay may have resulted in the loss of any observable activity.

In order to address the issues of potential non-specificity of the YVAD peptide substrate, and to avoid problems in the stability of caspase-1 enzymatic activity, a possible alternative caspase-1 assay could involve measuring the secretion of IL-1β using a standard ELISA assay. Provided that HT22 express IL-1, the evidence or absence of cleavage and secretion of IL-1β would be a more reliable and interpretable indicator of caspase-1 activity. If HT22 cells express IL-1β receptors, and given that a recent report identified cytokines (such as IL-1β) as capable of inducing activation of 12-lipoxygenase and subsequent toxicity (Chen et al., 2005b), and that 12-lipoxygenase inhibitors were effective at preventing both ERK activation and glutamate-induced toxicity in HT22 cells (Li et al., 1997b; Stanciu et al., 2000), cytokine processing could be a possible link between caspase-1-like inhibitor actions, ERK and 12-lipoxygenase toxicity in the HT22 cell model.

Caspase-1 has been found to have several intermediary forms in its processing pathway. One report found at least 5 cleavage sites and an alternatively spliced intron, resulting in immunoreactive bands of 42/45kDa, 35kDa, 22kDa, 20kDa, 12kDa and 10kDa (Yamin et al., 1996) (**Fig. 8**). Some of these cleavage forms were less reactive, and were found to be

autoinhibitory to further processing of the proform (Yamin et al., 1996). Additionally, 5 different isoforms of human caspase-1 with varying sizes were found to be produced by alternative splicing variants (Alnemri et al., 1995; Feng et al., 2004) (**Figure 8**). All of these isoforms appear to contain the C-terminus region, against which the antibody used in these studies was raised. The blots from glutamate-treated HT22 lysates indicated several other bands at sizes not normally predicted with the C-terminus antibody; thus, sequence analysis of the caspase-1 expressed form(s) may indicate if these bands are alternative isoforms.

The usage of pharmacological caspase inhibitors has been contested due to the problems with substrate specificity, particularly due to the higher concentrations that prove to be effective in *in vivo* settings (Schotte et al., 1999). With the advent of siRNA techniques, alternatives to pharmacological inhibition of caspases are becoming more widely available. We attempted to knockdown caspase-1 and -2 using siRNA technology, but we were not able to achieve observable downregulation of the protein product in HT22 cells using transient siRNA transfections. This was potentially due to the transfection efficiency, as well as the possible dilution of the siRNA over several cell divisions. Ideally, it would be interesting to see, in the case of caspase-1 or -2 knockdown, if YVAD.fmk and VDVAD.fmk still exert protective effects against glutamate toxicity. Knockdown of caspase-1 or -2 could be protective, leading to the conclusion that the inhibitors are acting in a relatively specific manner. If, however, knockdown of these caspases is not protective, but the inhibitors are still protective in their absence, then the inhibitors are likely acting on an unidentified target. However, for this experiment to be adequately controlled, the possibility of compensatory gene expression must be evaluated. For example, Troy et al found that knockout of caspases-2 altered expression levels of both caspase-9 and the IAP-inhibitor, Diablo (Troy et al., 2001).

Determining the mechanism of action of the YVAD.fmk and VDVAD.fmk inhibitors would prove to be an important insight into glutamate toxicity in the HT22 model. Our data indicate that the effects of these two inhibitors may lie upstream to ERK activation, and provide a novel insight into the mechanism of regulation of ERK function. A role for ERK in cellular toxicity, rather than survival, has only recently been reported in the literature. At present, it is unknown what conditions are necessary for the switch for ERK to promote cellular toxicity rather than survival. If the YVAD.fmk or VDVAD.fmk inhibitors were acting specifically on their caspase substrates, then an interesting hypothesis may lie in the adaptor proteins. Rip2, a protein kinase kinase that functions at the level of MEK1/2 and has been found to directly phosphorylate and activate ERK2 at the same TEY sequence (Navas et al., 1999), was found to associate with caspase-1 (Thome et al., 1998) and when overexpressed can elicit cell death that is dependent on caspase-1 (Zhang et al., 2003). Most of the research focusing on multiprotein complexes involved in activating caspases has examined only the activation of caspases. However, the possibility exists that the so-called "adaptor" proteins themselves undergo alterations as a consequence of oligomerization and become physiologically altered. Hypothetically, caspase-1 associated Rip2 could lead to activation of Rip2 and then phosphorylation of ERK2. Understanding the mechanism of YVAD.fmk- and VDVAD.fmk-based inhibition could lead to further understanding of the signaling pathways and conditions that are necessary for ERK function to be altered.

5.0 THE ROLE OF CALPAINS IN ERK-DEPENDENT CELL DEATH

5.1 SUMMARY

Glutamate-induced toxicity in HT22 cells includes an essential influx of calcium beginning at 4 h following glutamate treatment (Tan et al., 1998b). Calpains are calcium-activated proteases that are sensitive to intracellular calcium concentrations. Calpain inhibitors were found to be ineffective at preventing cell death following glutamate-induced oxidative stress. However, we have found that overexpression of the endogenous calpain inhibitor, calpastatin, can effectively delay glutamate-induced cell death in HT22 cells. This delay of cell death is downstream of MEK1/2-driven activation of ERK1/2, as calpain inhibition did not increase the therapeutic window of U0126, and did not alter the activation of ERK1/2. Using an *in vitro* enzymatic assay, we have found that glutamate causes an increase of calpain-like activity in a biphasic manner. The first increase was observed at early timepoints, whereas the second rise in activity was observed at 11h following glutamate treatment. The activity observed at 11h was inhibited only in part by the MEK inhibitor U0126, but a significant portion of activity was still observed that was MEK-independent. Furthermore, we have found evidence of a PARP fragment consistent with calpain-mediated cleavage, which occurs during glutamate-induced oxidative toxicity. The appearance of this fragment was blocked by U0126. Taken together, these data indicate that 1) calpains are indeed activated following glutamate exposure in HT22

cells, 2) calpain activation is part of an amplification of the cell death signal, and 3) calpain activation is in part dependent on MEK1/2 activity.

5.2 INTRODUCTION

Calpains comprise a family of calcium-activated thiol proteases found to be involved in a multitude of cellular functions including differentiation, proliferation and cell death. The activation and physiological functions of calpains are still poorly understood, partly due to the many tissue-specific isoforms. However, the current concept of calpain activation and regulation has been characterized primarily by the ubiquitously expressed large 80 kDa subunit isoforms μ- and m-calpain (Perrin and Huttenlocher, 2002). Multiple calcium binding domains are present on all known isoforms of calpains, indicating calcium binding as a major regulatory mechanism in the activation of calpains (Huang and Wang, 2001; Liu et al., 2004). The primary difference *in vitro* between the two ubiquitously expressed isoforms lies in their affinities for calcium: μ-calpain (calpain I) is activated by micromolar concentrations of calcium, whereas m-calpain (calpain II) requires millimolar calcium concentrations. While micromolar intracellular concentrations of calcium under normal physiological states are not uncommon, cellular conditions exhibiting millimolar intracellular calcium concentrations are exceedingly rare, and occur primarily only in pathophysiological situations (Liu et al., 2004). Other mechanisms for calpain regulation have been proposed, including autolysis (Michetti et al., 1996) and translocation to the plasma membrane (Fox et al., 1993), but the evidence that these steps result in and are required for activation has been widely debated (Blomgren et al., 1995; Elce et al., 1997; Liu et al., 2004). Interestingly, Glading et al. found that calpain activation can also be

influenced by direct phosphorylation of the large subunits. In particular, cells treated with epidermal growth factor (EGF) were found to activate m-calpain in an ERK-dependent manner, at least in part by direct phosphorylation of m-calpain by ERK, and independent of millimolar calcium concentrations (Glading et al., 2000; Glading et al., 2001; Glading et al., 2004). Calpastatin, the endogenous inhibitor of calpains, can bind and inhibit the catalytic subunit of calpains in the presence of low increases in calcium levels (Otsuka and Goll, 1987; Ma et al., 1993). The full-length calpastatin protein contains multiple binding domains for calpains, and has been shown to be able to bind and inhibit several calpain 80kDa subunits at one time (Wendt et al., 2004).

Following glutamate exposure in HT22 cells, a steady rise in cytosolic calcium concentrations was previously noted, beginning between 4 to 6 h following glutamate exposure, and continuing throughout the timecourse of glutamate toxicity (Tan et al., 1998b). Cell toxicity was attenuated with the inhibition of calcium influx by cobalt chloride (Tan et al., 1998b), which is thought to block calcium uptake into the cell by blocking voltage-dependent calcium channels. Despite the critical involvement of increased calcium influx and cytosolic concentrations in HT22 cells following glutamate exposure, calpain involvement in glutamate-induced oxidative toxicity had previously been ruled out based on the ineffectiveness of pharmacological calpain inhibitors to prevent glutamate-induced toxicity. However, the currently available pharmacological inhibitors lack specificity, potency and permeability (Chatterjee et al., 1997; Mellgren, 1997; Liu et al., 2004). These inhibitors have been found to cause morphological alterations and increased toxicity in control HT22 cells and are ineffective at attenuating glutamate-induced toxicity (**Fig. 11,** (Tan et al., 1998a)). Therefore, we re-examined the role of calpains in glutamate exposed HT22 cells using several methods, including the overexpression of

the endogenous calpain inhibitor calpastatin, an *in vitro* enzymatic calpain activity assay, and the investigation of calpain-mediated breakdown products of known calpain substrates in cellular lysates. Given the critical role of ERK1/2 in mediating glutamate-induced oxidative toxicity, we assessed the effects of calpain inhibition on ERK1/2 signaling and the effects of ERK1/2 inhibition on calpain activity.

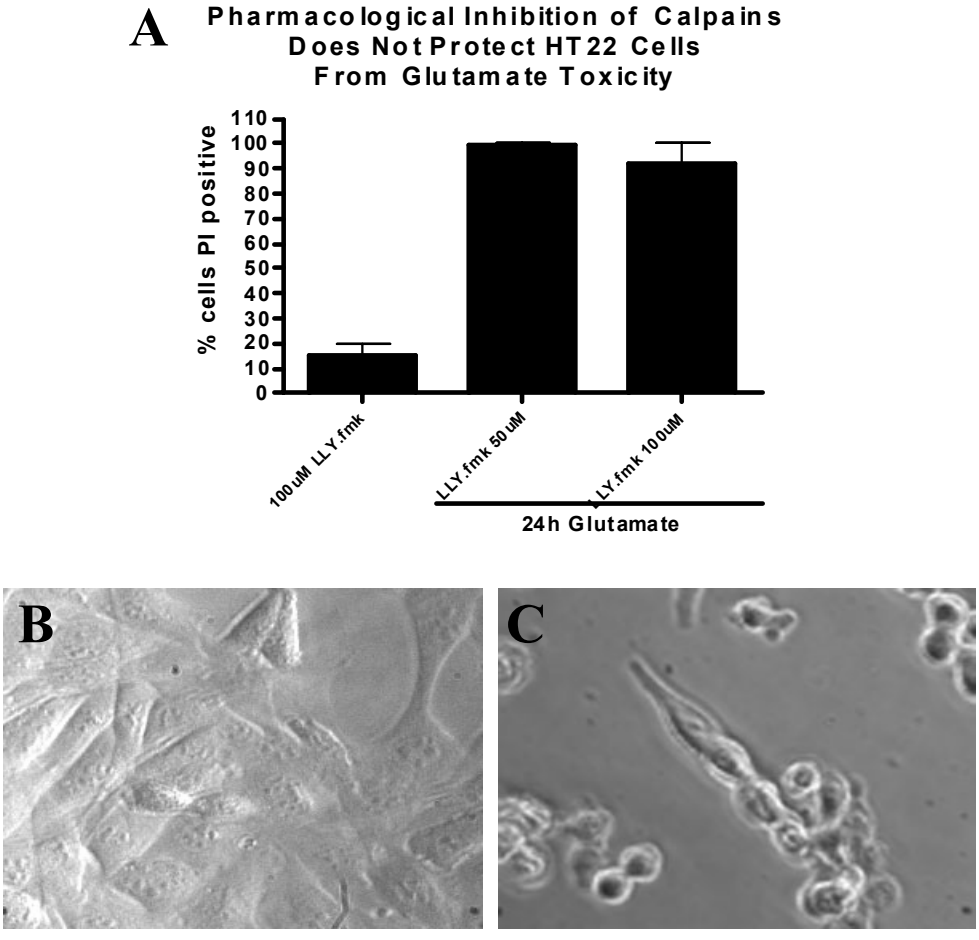


Figure 11 Calpain inhibitors do not protect against glutamate toxicity in HT22 cells. A HT22 cells were treated with 5mM glutamate and either 50 or 100μM LLY.fmk for 24 h. Cells were counted with 40μg/mL propidium iodide added directly into the media. The data are expressed as the percent of PI positive cells ± SEM, taken from 2 fields in each of 3 different wells per plating (n=2 platings). **B, C** Untreated HT22 cells (B) or HT22 cells treated with 100μM LLY.fmk for 24 h (C). LLY.fmk alone induced cell rounding and detachment.

5.3 EXPERIMENTAL PROCEDURES

Materials

The pcDNA3 plasmid containing the rat calpastatin cDNA expressed under control of a CMV promoter was a generous gift from Dr. Jun Chen (Neurology, University of Pittsburgh). The pcDNA3 plasmid containing the mitochondrial localized eYFP cDNA under a CMV promoter was a generous gift from Dr. Ian Reynolds (Pharmacology, University of Pittsburgh). Reagents used included the calpain inhibitor Ac-LLY.fmk (EMD Biosciences), the calpain substrate Suc-LY.amc (Biomol), anti-calpastatin (Santa Cruz, 1:1000 (provided by Dr. Jun Chen)), anti-phosphoERK1/2 (Cell Signaling, 1:1000), anti-ERK1/2 (Cell Signaling, 1:2000), anti-PARP (Cell Signaling, 1:1000), secondary IgGs (Biorad, 1:2000), and chemiluminescence reagents (NEN).

Cell culture

HT22 cells were maintained in normal growth media (Dulbecco's modified essential media (DMEM) supplemented with 10% fetal bovine serum (FBS)) between approximately 30 and 70% confluency. For all experiments, cells were plated at a density of $1.5x10^4$ cells/cm^2 in 96 well plates and treated 14-16 h after plating. For toxicity measurements, PI (40 μg/mL) was added to the culture media, incubated 2-4 minutes at 37ºC, and cells were counted immediately on an inverted microscope. Data was expressed as the percentage of PI positive cells within the fields counted. Each well was counted from two different fields, and three wells per plating were included as one "n". Data was expressed as the averaged percent of PI positive cells ± SEM

derived from 3 platings (i.e., n=3). Statistical analysis was performed using a one-way ANOVA with Tukey's post hoc test.

Transient transfection

HT22 cells were plated at approximately 50-60% confluency on 60mm dish overnight, and transfected the following morning using Lipofectamine 2000. Briefly, 6.67µL lipofectamine was diluted in Optimem, added to 1µl total DNA (for biochemical experiments, 1µL of either pcDNA3/calpastatin or pcDNA3 alone; for cell counts, 0.5µL of eYFP and 0.5µL of pcDNA3/calpastatin or pcDNA3) and incubated at room temperature for 30 min. The cells were rinsed three times with Optimem to remove residual serum, and then the transfection solution (Lipofectamine plus the DNA) was carefully layered onto the cells. The cells were then incubated at 37ºC, 5% CO_2 for 4 h. After incubation, the media was changed to full growth media. The cells were replated at $1.5x10^4$ cells/cm^2 13-15 hours following the start of the transfection, and treated with glutamate 14-16 hours after replating.

***In vitro* enzymatic assay**

Cells were rinsed with ice cold PBS, collected and resuspended in lysis buffer (25mM HEPES, 1mM EGTA, 5mM $MgCl_2$, 0.1% CHAPS). The cells then were lysed for 15 min on ice, centrifuged 10 min at top speed, and the pellet was discarded. Protein concentration of the resulting lysate was determined using the Bradford assay. 100µg of protein was diluted in reaction buffer (20mM Tris-HCl pH 7.5, 50mM NaCl, 0.1% CHAPS, 1mM EDTA, 1mM EGTA, 5mM β-mercaptoethanol, 5mM $CaCl_2$), and 100µM of the fluorogenic substrate Suc-LY.amc (EMD Biosciences) was added to each well immediately before assay measurements began. The assay plate was maintained in a heated chamber at 37ºC in a Perkin Elmer LS55 Luminescence Spectrometer, and the cleavage of the substrate was monitored at 460 nm (excitation at 380nm)

every 10 min. Background values from wells containing substrate and reaction buffer, but no protein, were subtracted from experimental wells. Each lysate was run in duplicate, constituting one "n". The rate of formation of cleaved substrate was derived, and expressed as the average rate (n=4) ± SEM. Statistical analysis was performed using one-way ANOVA with Tukey's post hoc test.

Western blot

Cells were rinsed with ice cold PBS, collected and lysed for 20 minutes on ice in lysis buffer (50mM Tris pH 7.5, 2mM EDTA, 100mM NaCl, 1% NP-40, 0.1mM $NaVO_4$, 2mM DTT, a protease inhibitor cocktail), and centrifuged at 13000 x g in a microtube for 12 min at 4ºC. The supernatant, containing the soluble protein, was measured for protein concentration using the Bradford colormetric assay. Sample buffer (350mM Tris pH 6.8, 10.2% SDS, 1.43M 2-mercaptoethanol, 0.17mM bromophenol blue) was added to 100µg of protein, boiled 5 minutes, and loaded on a 10% SDS-PAGE gel. The gel was transferred using a semi-dry transfer apparatus onto PVDF membrane (0.45µm) at 15V for 20 min. Membranes were then rinsed quickly in PBST (1X phosphate-buffered saline, 0.1% tween-20), blocked for 1 hour at room temperature in 5% nonfat dry milk/PBST, and incubated overnight at 4ºC in the primary antibody (1:1000 for phospho-ERK1/2, calpastatin and PARP, 1:2000 for total ERK1/2, 1:400 for actin). After several rinses in PBST, secondary IgG-HRP antibodies (Biorad, 1:2000) were incubated for 1.5 h at room temperature, rinsed several times in PBST, exposed for 1 min to chemiluminsence reagents (NEN), and exposed to x-ray film and processed. Densitometry was used to semi-quantify the band intensity, subtracting background and normalizing to loading controls. Data were expressed as the averaged normalized data (n=3 platings) ± SEM, and

analyzed using one-way ANOVA with Bonferroni post hoc analysis (for PARP cleavage) or Tukey's post hoc analysis (pERK expression).

5.4 RESULTS

5.4.1 Overexpression of calpastatin, the endogenous calpain inhibitor, delays glutamate-induced cell death in HT22 cells.

As mentioned above, we found that the cell permeable calpain II inhibitor Ac-LLY.fmk was completely ineffective at preventing cell death in glutamate treated HT22 cells (**Fig 11**). However, we also noted gross alterations in control cell morphology, as well as increased cell death in control cells treated with Ac-LLY.fmk in the absence of glutamate. Thus, in order to independently examine if calpains play any role in the initiation or execution of cell death in glutamate treated HT22 cells, we transiently transfected these cells with an expression vector encoding the rat calpastatin cDNA or an empty pcDNA3 vector, along with a separate pcDNA3 expression plasmid encoding a mitochondrial localized eYFP reporter. Each field was counted to determine first, which cells were transfected – all eYFP positive cells were considered to be co-transfected with the calpastatin or empty vector, and all eYFP negative cells were counted as an internal control – and second, which cells were PI positive. After 12 h of glutamate treatment, a significant protection against cell death was observed in calpastatin transfected wells (**Figs. 12, 13**). Twenty-four hours following glutamate treatment, vector-transfected wells were indistinguishable from calpastatin-transfected wells. Thus, the protection observed 12 h following glutamate exposure was only a delay in cell death, not complete protection.

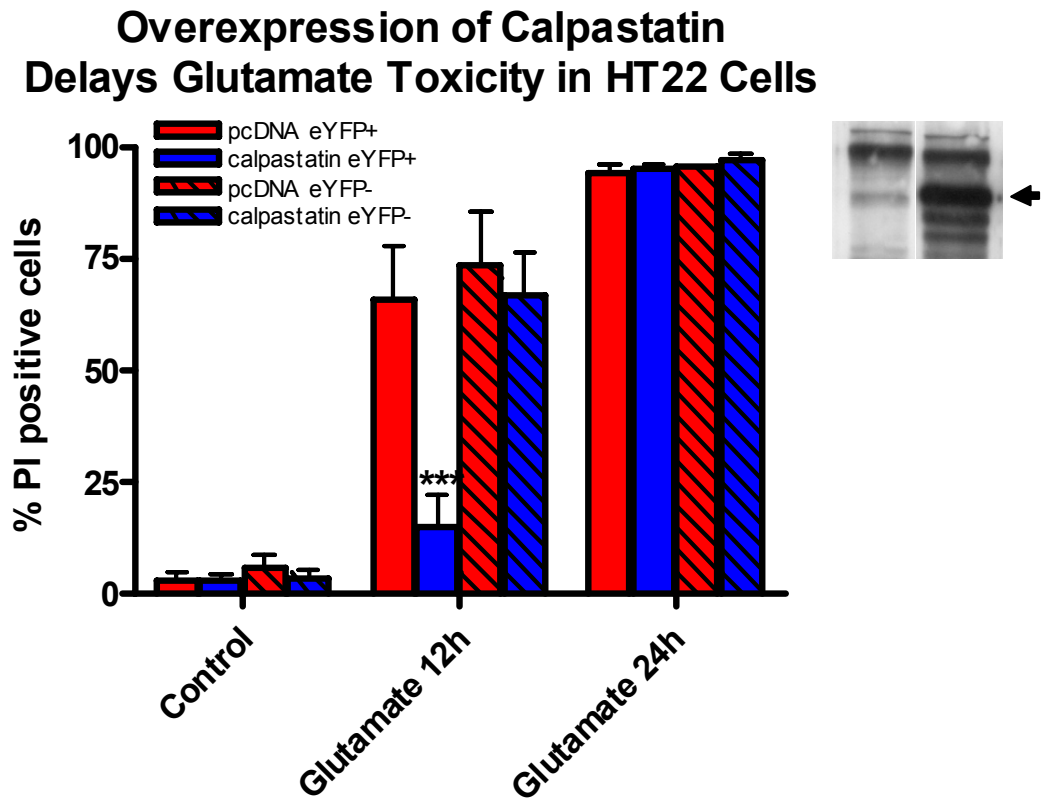


Figure 12 Overexpression of the endogenous calpain inhibitor, calpastatin, protects HT22 cells from glutamate-induced toxicity at 12 h, but not 24 h, following glutamate treatment. HT22 cells were transfected with either pcDNA3 or pcDNA3 containing the calpastatin cDNA, and co-transfected with a mitochondrial targeted eYFP reporter plasmid (pcDNA). Cells were then replated and treated with 5mM glutamate for 12 or 24 h, at which point they were counted in the presence of PI. Solid bars indicate cells that were eYFP positive, hatched bars were cells that were eYFP negative within the transfected wells. Red bars indicate cells that were in wells transfected with pcDNA, blue bars indicate cells that were in wells transfected with calpastatin. Data are presented as mean±SEM. ***$p<0.001$, according to one-way ANOVA with Tukey's post hoc analysis. Inset: Western blot image is of cells overexpressing calpastatin (right lane) or transfected with vector only (left lane). Arrow indicates the band at approximately 90kDa, the predicted size for the calpastatin protein.

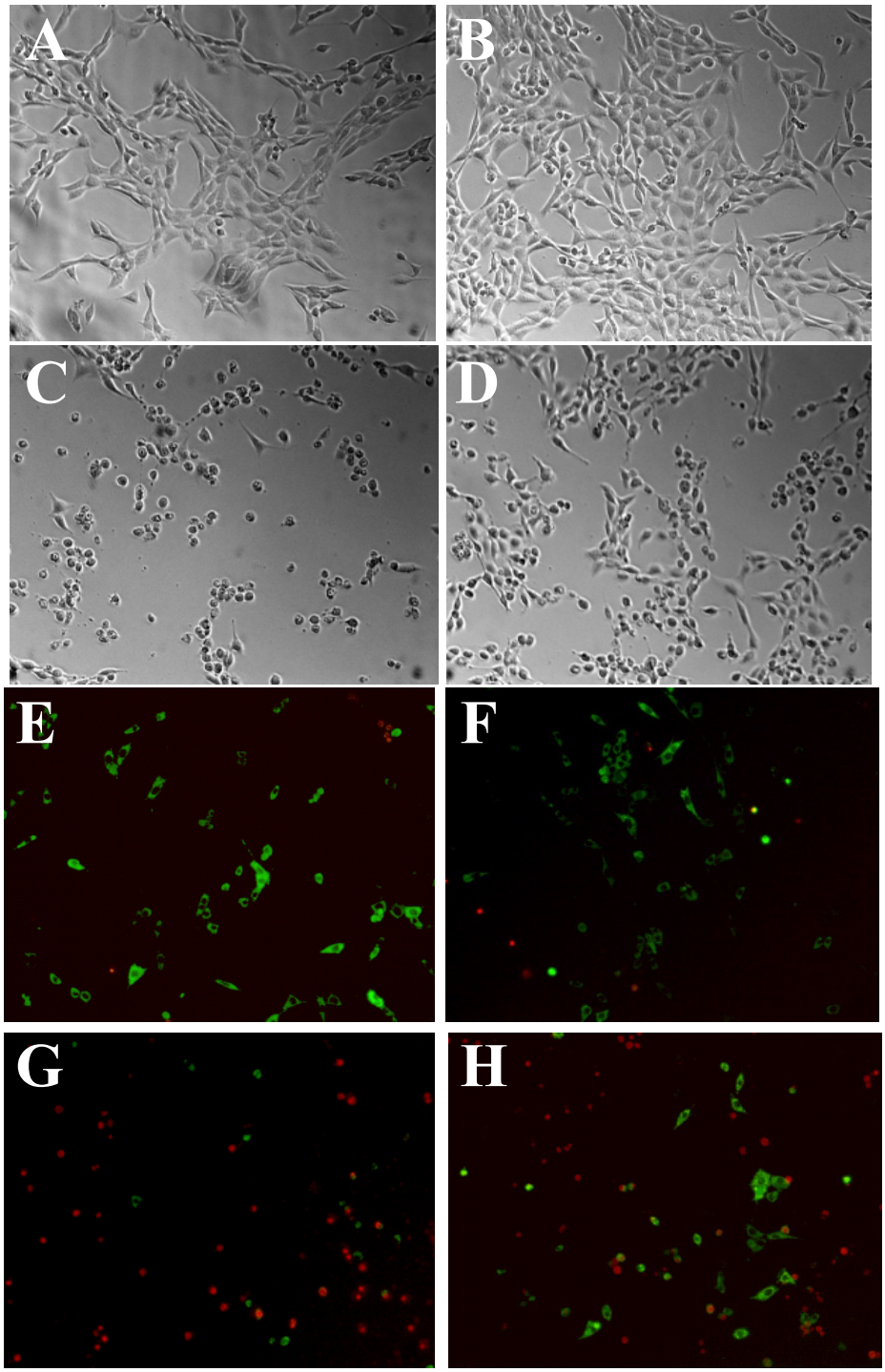


Figure 13 Representative photomicrographs of calpastatin or vector only transfected cells. Cells were transfected with empty vector (A, C, E, G) or a vector encoding calpastatin (B, D, F. H) plus a mitochondrial encoded eYFP reporter construct. A, B, E, F are representative fields from control cells, whereas C, D, G, H are fields taken from wells treated with 5mM glutamate for 12 h. Wells in E-H were treated with 40µg/mL PI for 2-4 min at 37ºC. Green cells represent transfected (eYFP positive) cells, red cells represent dying (PI positive) cells. Note the morphology of the green cells in G versus H.

We next investigated whether transient calpastatin overexpression could alter ERK activation. We found no significant differences between empty vector and calpastatin transfected samples (**Fig. 14**). However, based on the cell counts of populations co-transfected with the eYFP reporter plasmid, the transfection efficiency in HT22 cells tends to be around 30%, so any subtle effects may be difficult to assess biochemically in a transiently transfected population.

In order to see whether the delay in cell death due to calpastatin could prolong the posttreatment window for delayed U0126 protective effects, we transiently transfected cells with eYFP/calpastatin or eYFP/vector alone and added U0126 either 0, 5, 7, 9, 11, or 13 h following glutamate treatment. Cells were counted 24 h following the initial glutamate treatment in the manner described above. No significant difference existed between vector transfected and calpastatin transfected cells, suggesting that calpastatin-mediated delay is unaffected by MEK activity (**Fig, 15**). It is worthwhile to note that the transfection itself had a slightly protective effect only when in combination with delayed U0126 treatment (**compare Fig. 4 and Fig. 15**). This is surprising, because the transfection of the empty vector did not affect the response to glutamate alone compared to non-transfected cells. It is possible that, perhaps via the transcriptional machinery, transfected cells are less acutely ERK1/2-dependent in the timing of death, resulting in an increase in the therapeutic window for U0126, but not complete protection.

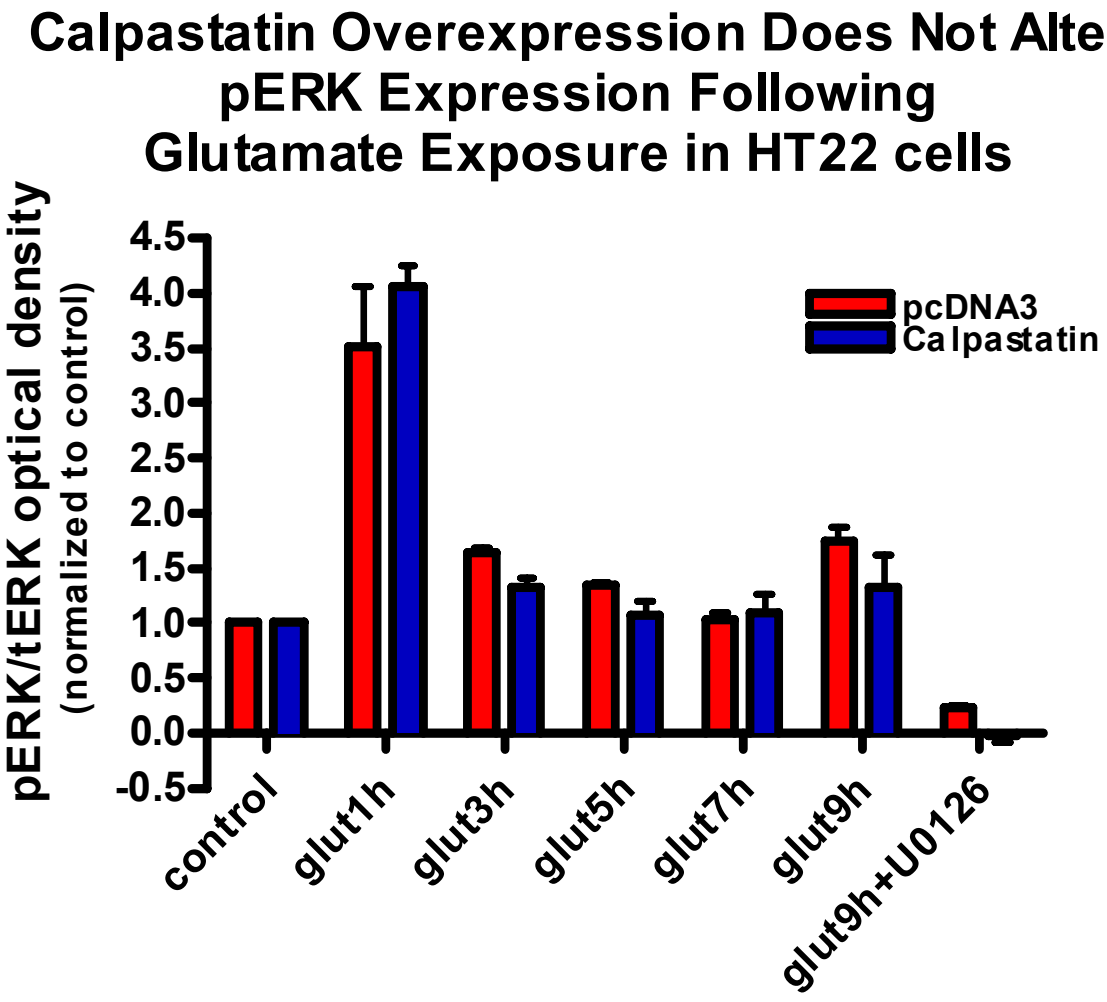


Figure 14 No significant differences in ERK phosphorylation between calpastatin- and vector-transfected cells. HT22 cells were transfected with 10µg of either pcDNA or pcDNA encoding calpastatin. The cells were then replated, collected and lysed, run on SDS-PAGE, and immunoblotted for phospho-ERK, stripped and then reblotted for total ERK. Blots were semi-quantified using optical density imaging analysis software, with the background subtracted. The data are expressed as the average pERK/tERK from three individual platings per timepoint, run on separate immunoblots. Bars are the mean±SEM. No statistical differences were observed between vector transfected lysates (red) and calpastatin transfected lysates (blue) using one-way ANOVA with Tukey's post hoc analysis.

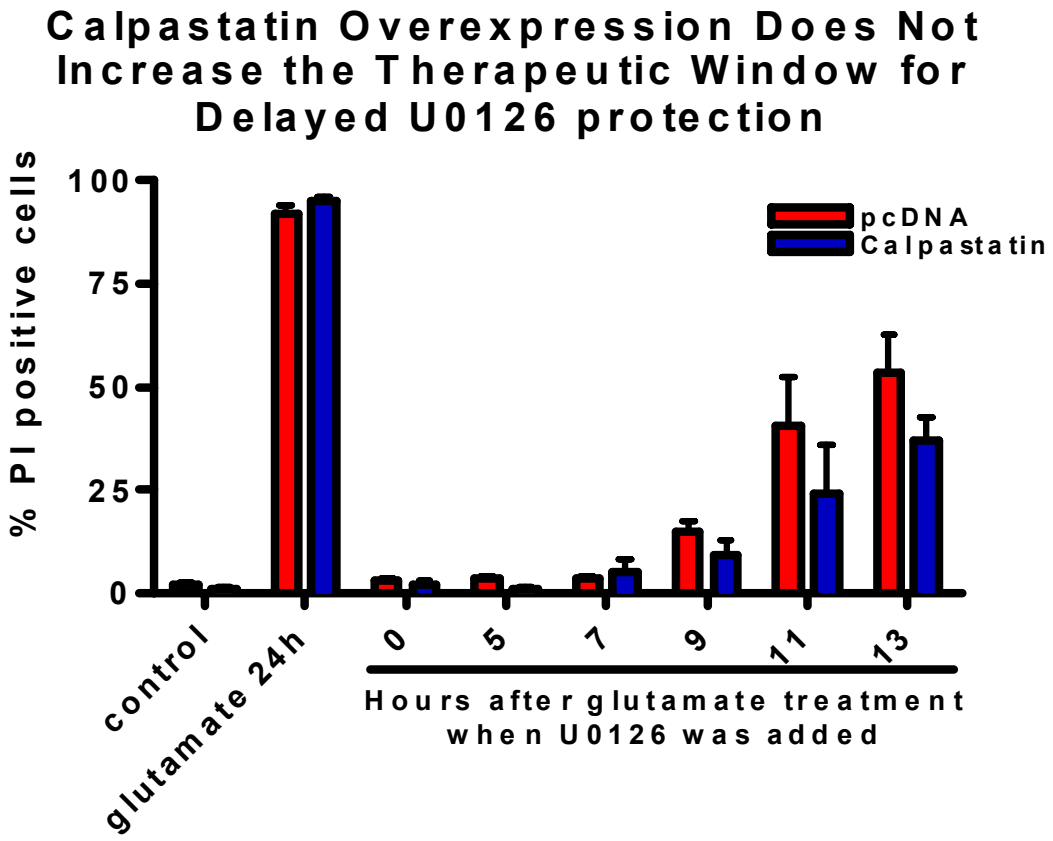


Figure 15 The therapeutic window for delayed U0126 is not affected by calpastatin overexpression. HT22 cells were transfected and plated as described in Fig. 12, and then treated with 5mM glutamate. U0126 was added (10μM) at 0, 5, 7, 9, 11, or 13 h following glutamate. The cells were counted after 24h of glutamate treatment. Only transfected (eYFP positive) cells are shown above. Data are presented as the mean±SEM taken from 4 individual platings (n=4), with 3 wells per plating counted. There were no statistical differences between pcDNA transfected cells and calpastatin transfected cells using one-way ANOVA with Tukey's post hoc test.

5.4.2 Calpain-like activity occurs in two phases following glutamate exposure in HT22 cells, one of which is MEK dependent, the other is MEK independent.

Calpain activity can be measured *in vitro* using a peptidyl substrate (Ac-LY.amc), which, upon cleavage, emits a fluorogenic signal. This peptide sequence is recognized by both μ- and m-calpains, and in the experimental paradigm, we used a high calcium reaction buffer in order to detect general calpain activity. The resulting background-subtracted data were graphed as the individual assay points (every 10 minutes) during the hour-long incubation of lysate and substrate, and analyzed by deriving the rate of substrate cleavage over one hour of incubation. We found that glutamate significantly increased calpain-like activity in all samples compared to control (**Fig. 16**). Statistical analyses using Tukey's post test indicated that the latest glutamate timepoint – 11 hours – was significantly increased above the earlier glutamate timepoints. There were no differences within the earlier timepoints.

Interestingly, while the MEK inihibitor U0126 significantly decreased calpain activity at 11h post-glutamate treatment compared to glutamate alone, it did not completely ablate activity down to control levels. In fact, calpain activity in the samples from HT22 cells treated with U0126 plus glutamate for 11 h remained significantly increased above the activity measured in control samples ($p<0.01$). Thus, these data suggest that at least part of the calpain activity induced by glutamate treatment is MEK-independent, while a significant portion is MEK-dependent.

5.4.3 Glutamate exposure leads to the evidence of ERK-dependent, calpain-sized PARP breakdown products.

Given that the peptide substrate used in the *in vitro* activity assay may also be reflective of the 20S proteasome activity, we sought evidence of calpain activity using a method not dependent upon pharmacological substrates. PARP is a known intracellular target of both caspases and calpains, but is cleaved differentially by these different protease families. Caspases cleave full length PARP (116kDa) into 89 and 24kDa fragments, whereas calpains cleave PARP into 70 and 40kDa fragments. Eleven hours following glutamate exposure, we found a significant loss of the full length PARP protein, and a corresponding increase in a band at approximately 40kDa, consistent with the calpain-mediated cleavage product. At earlier timepoints, neither the loss of full length PARP nor the occurrence of a band around 40kDa was observed.

Consistent with the *in vitro* activity data suggesting a distinct calpain activity 11 h following glutamate exposure that is MEK dependent, we found that addition of U0126 to the cells blocked the formation of a 40kDa PARP band 11 h following glutamate exposure, along with the loss of the 116kDa full length protein (**Fig. 17**). Thus, these data provide *in vivo* evidence for calpain activity during glutamate-induced oxidative stress, which was blocked by MEK inhibition.

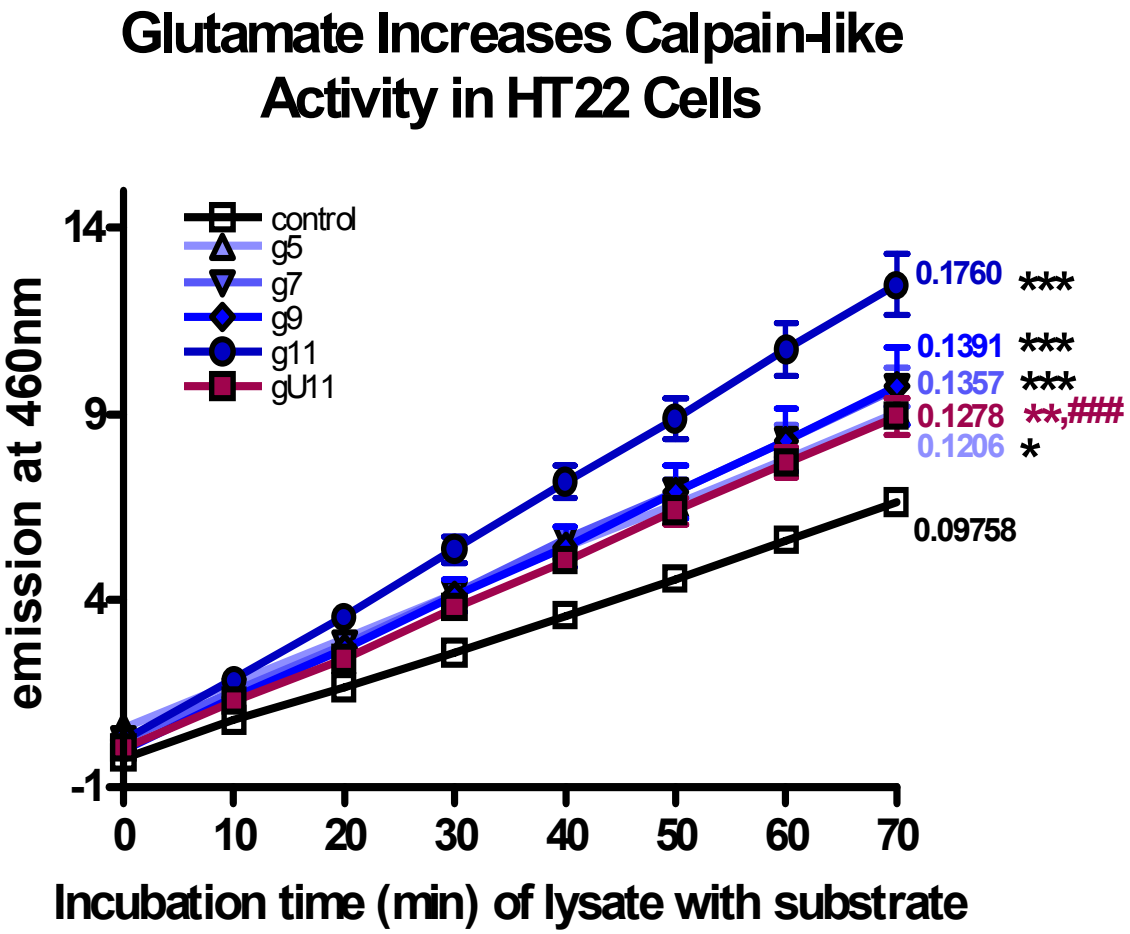


Figure 16 Calpain activity is increased in all lysates from glutamate treated HT22 cells, with a second phase of activity present 11h following glutamate treatment. HT22 cells were plated on 60mm dishes as described and treated with 5mM glutamate for either 5, 7, 9, or 11 h, or 5mM glutamate+10μM U0126 for 11h. Cells were lysed and 100μg of protein was added to reaction buffer containing 100μM Suc-LY.amc and immediately read on a fluorimeter set at an excitation wavelength of 360nm and an emissions wavelength of 460nm in a heated (37ºC) chamber. The emissions wavelength was recorded every 10 min for 1 h. Duplicate wells were averaged, and background values (obtained from wells with substrate but no protein) were subtracted. Data are graphed as the averaged emission over time from 4 different cell lysates, and the numbers listed at the right are the derived slope of the curve for each glutamate timepoint. Error bars represent the SEM, and statistical significance was determined from the averaged rate using one way ANOVA with Tukey's post hoc analysis. *p<0.05, **p<0.01, ***p<0.001 compared to control; ##p<0.001 compared to 11h glutamate.

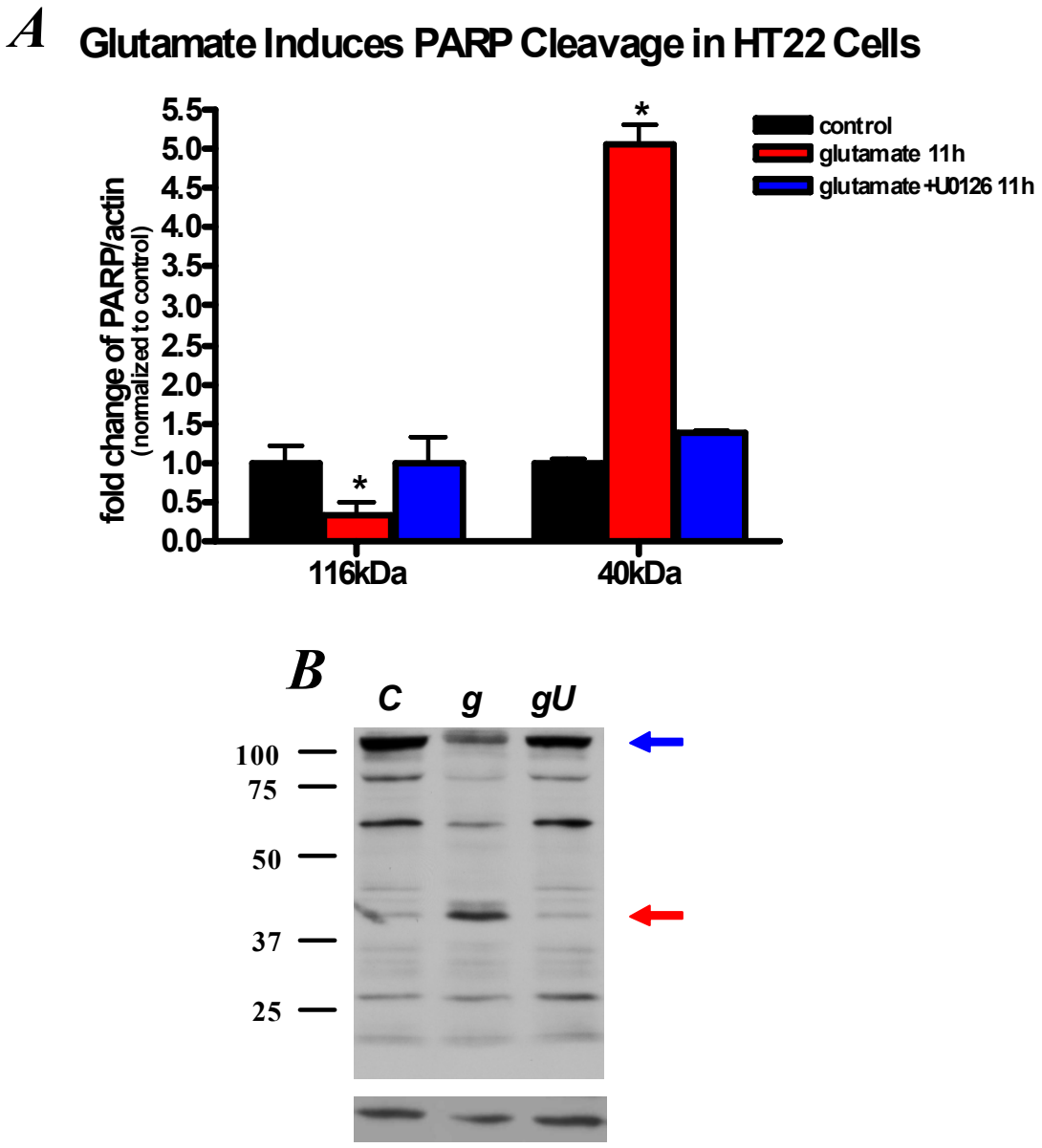


Figure 17 Evidence of a calpain-sized breakdown product of PARP. Cells were plated in 60mm dishes as described, treated with 5mM glutamate alone or with 10µM U0126 for 11h, collected, lysed and immunoblotted for PARP, an endogenous substrate of calpains *in vivo*. Data was obtained by semi-quantification using densitometry to determine the optical density (OD) of the PARP band divided by the OD of actin, obtained by stripping and reblotting the same membrane. A: Data are expressed as the averaged fold change normalized to control taken from 3 different lysates ± SEM, *$p<0.05$ using one-way ANOVA with Bonferroni's post hoc analysis. B shows a representative PARP immunoblot (top), which was stripped and reblotted for actin (bottom). "C" control lysates, "g" glutamate 11h, "gU" glutamate+10µM U0126 11h. The blue arrow indicates the 116kDa PARP; the red arrow indicates the 40kDa calpain-mediated PARP fragment

5.5 DISCUSSION

Despite multiple reports indicating that pharmacological calpain inhibitors are ineffective at preventing glutamate-induced oxidative toxicity (**Fig. 11,** (Tan et al., 1998a; van Leyen et al., 2005)), we have found evidence that calpains do indeed play a role in the execution of cell death in HT22 cells. Molecular inhibition of calpains by overexpression of the endogenous calpain inhibitor, calpastatin, was effective in delaying cell toxicity 12 h following glutamate treatment. Evidence of increased calpain activity was found with both an *in vitro* enzymatic assay and evidence of calpain sized fragments of an endogenous substrate, PARP.

Overexpression of the endogenous calpain inhibitor, calpastatin, has been regarded as the most reliable and specific method of calpain inhibition currently available (Neumar et al., 2003). However, as calpastatin itself can be a substrate for protease cleavage (Porn-Ares et al., 1998; Wang et al., 1998a; Neumar et al., 2003), the inhibition afforded by calpastatin overexpression could, theoretically, be overcome by cellular mechanisms in which calpastatin is degraded by proteases. Glutamate-treated HT22 cells overexpressing calpastatin exhibit only a delay in membrane permeability, indicating that either alternative mechanisms are in place that lead to membrane permeability, or that the delay in toxicity might be reflective of cellular compensation to counteract the overexpression of calpastatin.

In assessing toxicity in glutamate-induced oxidative stress, we used the approach of counting the number of "dying" cells with uptake of the small fluorescent dye, PI. This dye measures early membrane permeability, typically found in dead or dying cells. Calpains have been found to cleave many cytoskeletal and adhesion proteins both *in vitro* and *in vivo*. Cleavage of these proteins, such as paxillin or talin, can lead to cell detachment, rounding and disassembly of focal adhesions (Carragher et al., 1999). The calpain-dependent hydrolysis of

paxillin, talin and viniculin were demonstrated to occur concurrently with plasma membrane permeability in pathological settings (Liu and Schnellmann, 2003). Inhibition of calpain blocked both the hydrolysis of these proteins and the membrane compromise, indicating that calpain may mediate plasma membrane permeability in oncotic states via hydrolysis of cytoskeletal proteins (Liu and Schnellmann, 2003; Liu et al., 2004). The particular cytoskeletal changes that may occur following glutamate-induced oxidative stress are currently unknown. However, cell rounding and detachment as well as membrane permeability occur in these cells in response to glutamate (**Fig. 13**), indicating that cytoskeletal alterations are hallmarks of glutamate-induced toxicity. It is possible that the cleavage of cytoskeletal proteins may lead to an excitotoxic spread to surrounding cells, accounting for the progressive nature of the occurrence of PI positive cells following glutamate exposure in HT22 cells. Inhibition of cytoskeletal alterations may delay this wave of toxicity, consistent to the observation that calpastatin overexpression delays cell toxicity as measured by PI uptake (**Fig. 12**). In cerebellar granule neurons exposed to low potassium and immature cerebellar neurons exposed to estrogens, inhibitors of MEK1/2 were found to prevent membrane permeability, although the mechanism was not understood. If calpain induces plasma membrane permeability, then our data demonstrating an ERK-dependent rise in late calpain activation may indicate a mechanism for ERK triggering membrane permeability via calpain.

In addition to cytoskeletal proteins, a variety of potential targets exist for activated calpains, including components of the cell death machinery. Calpains have been shown to cleave many proteins in *in vitro* studies, but confirming these proteins as bona fide calpain targets in cellular conditions has yielded fewer candidates. We have identified PARP as a probable target of calpain in the HT22 glutamate toxicity model; however, other targets likely exist. While

originally thought to enhance degradation of targeted proteins, calpain activity can instead modify protein function, e.g. by cleaving at interdomain boundaries, producing functional sub-fragments. Furthermore, calpain generated protein sub-fragments may act to integrate into and alter structural assemblies (as in the case of focal adhesions), act as naturally produced dominant negatives (as in the case of PARP, (D'Amours et al., 2001)), or alter subcellular localization (as in the case of AIF (Polster et al., 2005)).

On the basis of the *in vitro* activity, there appears to be calpain-like activity following glutamate exposure in HT22 cells. However, the small peptide calpain substrate, Suc-LY.amc, has been found to be not only a substrate for calpains themselves, but also for proteinase activity by the 20S proteasome at higher concentrations (Mellgren, 1997). A recent study found that inhibition of the proteasome with the compound lactacystin was protective against glutamate-induced toxicity in HT22 cells (van Leyen et al., 2005), indicating that proteasome activity may be involved in this model. However, according to the data derived from an alternative activity assay specific for proteasome activity, glutamate treatment alone does not increase proteasome activity above control levels (van Leyen et al., 2005), whereas using the *in vitro* calpain activity assay, a significant increase in calpain-like activity was observed following glutamate exposure. Thus, it is likely that the increase observed using the Suc-LY.amc substrate is not reflective of proteasome activity, although other proteases than calpains, such as cathepsins, may also cleave Suc-LY.amc. The involvement of these alternate proteases in HT22 cells would warrant further investigation.

If both phases of Suc-LY.amc cleavage can be attributed to calpains, then perhaps one of the more interesting observations from the data presented here is that calpain activity seems to be in part dependent on MEK (as PARP cleavage and the higher calpain activity 11 h following

glutamate treatment was blocked by U0126), but also has a MEK-independent component as well. Calpain-like activity was increased significantly above control in the presence of U0126+glutamate and calpastatin overexpression did not significantly increase the therapeutic window for delayed U0126 addition (**Figs 16 and 15**), arguing that calpains and MEK-mediated protection may in part function independently. It would be interesting to investigate if calpains feedback onto ERK signaling by cleavage of protein phosphatases, leading to a decreased role for MEK activity at later timepoints of glutamate exposure. Calpains have been found to be able to cleave phosphatases such as calcineurin/PP2B, a PSTP, leading to increased *activation* of the phosphatase in ischemia (Wu et al., 2004). Conversely, calpains have also been demonstrated to cleave oxidized (reversibly inactivated) protein tyrosine phosphatases (PTPs), leading to the irreversible *inactivation* of the phosphatase (Gulati et al., 2004). This discrepancy may explain how calpain may play a protective role in early phases of cell death, and a toxic role in later phases (Lankiewicz et al., 2000). For example, Lankiewicz et al found that calpain inhibitors added to hippocampal cultures 1 hour before or 2 hours after NMDA exposure was ineffective at preventing cell death, whereas the same inhibitors added either concurrently with or 1 hour after NMDA exposure was effective at blocking cell death(Lankiewicz et al., 2000). This presents a possibility that particular phases of calpain activity present in the cells may have opposing effects on cellular survival. Although the second rise in ERK activation was not significantly decreased 9h following glutamate exposure in lysates transiently transfected with calpastatin (**Fig. 14**), the pattern of ERK signaling and phosphatase activity are not known at later timepoints concurrent with the late-phase calpain activity (11 h glutamate treatment). The issue of a potential dual nature of calpain activity could also explain why pharmacological calpain inhibitors do not exert protection in the HT22 glutamate model. In our hands and in other

reports, calpain inhibitors added concurrently with glutamate were not protective. Given the possible biphasic nature of calpain activity, it might be interesting to determine whether delayed addition of calpain inhibitors at early timepoints following glutamate treatment exerts protective effects in HT22 cells.

The upstream regulation of calpain activity appears to be multifaceted and complex. Glading et al. found that m-calpain can be activated by direct phosphorylation by ERK following EGF stimulation, mediating cell detachment and disassembly of focal adhesions (Glading et al., 2000; Glading et al., 2001; Glading et al., 2004). Given that the second phase of calpain activity was ERK dependent in HT22 cells (**Fig. 16, 17**), it is possible that the second burst of calpain activity is due to direct phosphorylation of m-calpain by ERK. However, calpains can also be regulated by the endogenous inhibitor of calpain, calpastatin, and by calcium levels. Calpastatin can be cleaved by caspases (caspase-1 in particular), resulting in the activation of calpains (Porn-Ares et al., 1998; Wang et al., 1998a; Neumar et al., 2003; Rami, 2003). In HT22 cells, the early increase in calpain-like activity does not appear to be toxic by itself, as this level of activity was not statistically different from calpain activity in the presence of U0126 (**Fig. 16**), which affords complete protection (F**ig. 4**). Addition of the caspase inhibitors Ac-YVAD.fmk and Ac-VDVAD.fmk at early timepoints is also protective in this model, and caspase-like inhibition decreased the expression of phospho-ERK (**Fig. 6**), suggesting that the earlier caspase-like activity is MEK independent. It would be interesting to investigate early calpain-like activity in the presence of caspase inhibitors, as caspase-like activity might be responsible for the initial activation of calpains. Alternatively, the timepoints that we assayed were consistent with previous reports indicating that cytosolic calcium levels increase in glutamate treated HT22 cells between 4 and 6 hours following glutamate exposure, and continue to rise throughout the

timecourse (Tan et al., 1998b). Thus, the early phase of calpain-like activity may be reflective of the commencement of rises in cytosolic calcium.

While the *in vitro* activity assay by itself cannot confirm specific calpain activation in HT22 cells, the detection of a PARP fragment, consistent with the 40kDa calpain-mediated cleavage product, at late timepoints following glutamate exposure suggests that calpains are activated in HT22 cells undergoing glutamate-induced oxidative stress. The calpain involvement appears to be during an amplification of an upstream ERK-mediated cell death signal, due to the observation that inhibition of calpain results only in a delay of cell death and that *in vivo* evidence of calpain activity (PARP cleavage) occurs at late timepoints. Understanding the exact role(s) of calpain activity could lead to further insights and possible approaches to the prevention of cellular toxicity or the extension of therapeutic windows.

6.0 CONCLUDING REMARKS

The role of the caspase and calpain protease families in cell death has been studied extensively in a variety of experimental models, but yet the linkage between proteases and stress signaling pathways remains less understood. The MAPK family primarily consists of ERK, JNK and p38; the latter two were historically associated with toxicity pathways, whereas ERK was thought to be involved in cell survival. However, glutamate-induced oxidative toxicity in HT22 cells presents an interesting paradigm in cell death pathways as ERK has been found to promote cell death, rather than survival (Stanciu et al., 2000). This novel role for ERK activation in promoting cell death has been recently identified in several other cell death models, including toxicity induced by ischemia, 6-OHDA, MPP+, cisplatin, ceramide, thapsigargin and amyloid beta (Chu et al., 2004). Many of these models also undergo obscure cell death pathways. This raises the possibility that exploring the relationship between protease families and MAPK signaling in the HT22 glutamate toxicity model might provide important clues into both the mechanism of death, and the conditions under which ERK may function as a toxic rather than protective factor. Upon finding evidence for calpain activation (Chapter 4), we determined that calpain activity is, at least in part, MEK-dependent and that the semi-selective caspase inhibitors Ac-YVAD.fmk and Ac-VDVAD.fmk decreased ERK activation (Chapter 3). MEK and caspase inhibitors conferred complete protection against glutamate-induced toxicity, whereas overexpression of the endogenous calpain inhibitor, calpastatin, only delayed cell death. Thus,

we propose that HT22 cells exposed to glutamate activate a caspase-1- or -2-like protease, which in turn leads to ERK activation, leading to both calpain activation and cell death.

6.1 VARIATIONS IN GLUTAMATE SENSITIVITY EXISTS IN THE HT22 CELL MODEL

Cellular model systems, such as clonal cell lines, provide an excellent experimental tool for dissecting molecular pathways, and can often lead to an increased understanding in the possible mechanisms associated with *in vivo* disease settings. However, cells grown and maintained in culture conditions are not subject to the controls of the natural environment, and can rapidly undergo molecular and morphological changes (Freshney, 2000; Freshney, 2001; Stacey and MacDonald, 2001). HT22 cells themselves were originally derived from a parent population of hippocampal cells (HT-4). These cells were isolated from a mouse with a spontaneous hippocampal tumour that was a result of transformation with an SV-40 virus. The HT-4 cells were then subcloned and assessed for sensitivity to glutamate-induced toxicity. From these subclones, the HT22 cell line displayed robust sensitivity and were then further characterized and used to model glutamate-induced oxidative stress. However, we and others have found inconsistencies in the cell death response in the HT22 cell line. For example, Sagara et al found and subcloned a line of HT22 cells resistant to extremely high glutamate concentrations (Sagara et al., 1998). Within this subclone, alterations in expression levels of oxidative stress genes, such as catalase, and increased enzymatic activities of the glutathione antioxidant pathways were observed, leading to the conclusion that this subclone had altered

capacities for coping with oxidative stress. Cell culturing conditions can also affect cell sensitivity to toxins. Cell density, cell-cell contacts and soluble components in the extracellular media have been suggested to affect toxicity models (Freshney, 2001). In order to both obtain a homogeneous population of HT22 cells and normalize the cell culturing conditions, we subcloned and characterized three lines derived from the parent HT22 population. Of these three subclones, we found that one clone was significantly less responsive to glutamate in all conditions and assays tested, whereas the other two clones responded consistently to glutamate-induced oxidative toxicity. Furthermore, we found that in all subclones, glutamate treatment using conditioned media fails to induce toxicity, while glutamate treatment in fresh media leads to reproducible cell death. The subclone of HT22 cells that was chosen for all future experiments in the lab was confirmed to have retained the ERK-dependent aspect of glutamate toxicity, where addition of the MEK inhibitor U0126 as late as 7 h following glutamate treatment provided complete protection.

6.2 CASPASE-1 AND -2-LIKE INHIBITORS ARE PROTECTIVE AGAINST GLUTAMATE TOXICITY, AND INHIBIT ERK PHOSPHORYLATION.

The specificity of pharmacological caspase inhibitors has been questioned in many cases (Ekert et al., 1999; Schotte et al., 1999), as these inhibitors cannot readily target one particular individual caspase over other caspases, especially within the same subfamily and at the high concentrations sometimes required for *in vivo* studies. However, when used as a panel, inferences about specificity may be made based on the overlapping activities of these inhibitors. If Ac-YVAD.fmk demonstrates effective protection against cell death, and Ac-DEVD.fmk

cannot protect at all, then a stronger rationale for investigating caspase-1 over caspase-3 or -7 would exist. Furthermore, non-specific effects on cathepsin inhibition may also be argued, since both of these inhibitors should equally target cathepsins (Schotte et al., 1999). Such is the case in glutamate-induced toxicity in HT22 cells. We have found that both the caspase inhibitors Ac-YVAD.fmk and Ac-VDVAD.fmk (caspase-2) were equally effective at protecting HT22 cells against glutamate-induced toxicity. Both Ac-DEVD.fmk and Ac-IETD.fmk (caspase-8 and granzyme B) were completely ineffective at preventing glutamate-induced toxicity, even at high doses. The possibility that Ac-YVAD.fmk or Ac-VDVAD.fmk acts on an unknown substrate could still be an issue. To this end, we attempted to observe activation of caspase-1, in particular. No evidence of caspase-1, -2, -3, or -12 processing was observed in HT22 cells, nor did we find evidence of activity based on an *in vitro* activity assay using YVAD.afc as a substrate. However, this negative result cannot conclusively rule out caspase-1 or -2 involvement in glutamate-induced oxidative toxicity. Neither of these two enzymes requires processing to be catalytically active (Yamin et al., 1996; Troy and Shelanski, 2003), and *in vitro* activity assays of apical caspases have been reported to be technically challenging (Boatright et al., 2003). A further study examining processing of the cytokine IL-1 may prove to be more insightful in determining a potential role for caspase-1 in this model. IL-1 is an endogenous substrate for active caspase-1, resulting in the cleavage and release of IL-1β. If IL-1β is formed and released following glutamate treatment in HT22 cells, then the caspase-1 activation profile could be further studied. Additionally, IL-1β has been shown in other models to stimulate 12-LOX production. Given that 12-LOX inhibitors were protective in HT22 cells exposed to glutamate and that glutamate activated 12-LOX in these cells (Li et al., 1997b; Stanciu et al., 2000), additional studies could examine if 12-LOX-dependent toxicity lies downstream of

caspase activation and IL-1β release. If, on the other hand, IL-1β is not formed, then there exists a stronger probability that caspase-1 is not involved in this model, and that 12-LOX formation is independent of cytokine production.

Perhaps the strongest technique to determine a role for caspases in glutamate-induced HT22 toxicity would be siRNA targeting of caspases-1 or -2. We have attempted to use this technique, but significant knockdown was not attained, potentially due to the dilution from multiple cell passages following transfection. However, if stable inducible siRNA cell lines were able to knockdown these caspases, careful control would need to be taken to observe any potential changes in gene expression, such as other caspase family members or regulators, such as the IAPs or Diablo, as this has been reported in the literature following caspase knockdown (Troy et al., 2001). Perhaps less technically difficult, an analogous approach to the calpastatin experiments described in Chapter 4 would be to overexpress endogenous inhibitors of caspase-1 activation, e.g., ICEBERG or Pseudo-ICE. If caspase-1 is involved, then we would expect to find that overexpression of ICEBERG or Pseudo-ICE might alter glutamate-induced toxicity.

We have not pursued a possible role of caspase-2 or -11 rather than caspase-1 in this model, other than the finding via immunoblotting that no changes in the caspase-2 zymogen expression throughout the glutamate timecourse. The issues that are present in detecting the involvement of caspase-1 are also present for caspase-2, i.e., cleavage of the apical caspase is neither sufficient nor necessary for activation, the parameters of the *in vitro* assay are not well defined, and downstream targets are widely unknown (Troy and Shelanski, 2003). One potential marker of *in vivo* activation could be to detect cleavage of golgin-160 (Mancini et al., 2000). Golgin-160 can be cleaved by caspases-2, -3 and -7 (Troy and Shelanski, 2003), but as the caspase-3- and -7-like inhibitors were completely ineffective at blocking cell death, then by

elimination, if golgin-160 is cleaved it would likely be a result of caspase-2 activity. However, golgin-160 cleavage may not necessarily be present in all forms of caspase-2 mediated cell death. Again, siRNA would be a useful tool if it becomes technically feasible in these cells. Unfortunately, specific endogenous inhibitors for caspase-2 are currently unknown, as IAPs do not seem to be able to inhibit either caspases-1 or -2 (Deveraux et al., 1997; Roy et al., 1997; Ho et al., 2005).

However, using the caspase inhibitors effective at preventing glutamate-induced toxicity (i.e., Ac-YVAD.fmk and Ac-VDVAD.fmk), we have found that these two inhibitors were effective at blocking late ERK activation. Although we cannot yet directly attribute this result to caspase-1 or -2 activation, it is exciting nonetheless because it places a potential protease activity upstream of or modulating ERK-mediated toxicity. Interestingly, Zhang et al. found that transfection of Rip2 (a mitogen-activated protein kinase kinase that is capable of activating ERK2) can induce cell death in wildtype immature mouse primary cortical neurons (Zhang et al., 2003). However, when Rip2 was transfected into caspase-1 knockout cultures, the cell death response was greatly attenuated (Zhang et al., 2003). These findings present evidence that a mitogen-activated kinase kinase in the ERK2 pathway may act to promote cell death and function in a caspase-1-dependent manner.

If caspase-1 or -2 can be demonstrated to directly regulate HT22 glutamate toxicity, then the question may be posed as to how caspase-3 is not then activated? The sequence of events between caspase-1 or -2 activation and caspase-3 processing are not well understood. Caspase-2 has been demonstrated in some models to be upstream of the mitochondrial gateway, and can eventually lead to caspase-3 activation via caspase-9. As an interesting point of regulation, several studies suggest that caspase-9 activation can be inhibited by phosphorylation of the

zymogen by ERK2 (Allan et al., 2003). Additionally, Allan et al found that the inhibition of caspase-9 activation could be attenuated by okadaic acid, a serine/threonine directed phosphatase inhibitor, in cytosolic extracts. ERK2 is activated in the HT22 glutamate model to promote cell death, and its activity is regulated at least in part by ERK-directed, okadaic acid-sensitive phosphatases. Thus, ERK may also function to silence the activation of the intrinsic caspase pathway, pushing the cell death pathway towards a non-apoptotic mechanism. This is likely not the sole function of ERK in the glutamate toxicity model, as inhibition of ERK activation with the MEK inhibitor U0126 completely protects HT22 cells from toxicity, and does not simply alter the cell death phenotype. However, the downstream targets of ERK activation have remained elusive in the HT22 toxicity model. Furthermore exploring targets in the intrinsic caspase cascade would provide an interesting integration of ERK activity with the non-traditional form of cell death exhibited by HT22 cells.

6.3 CALPAINS ARE ACTIVATED FOLLOWING GLUTAMATE-INDUCED TOXICITY IN HT22 CELLS

Previous reports indicated that calcium influx into HT22 cells is critical for glutamate-induced toxicity (Tan et al., 1998b). However, surprisingly, pharmacological calpain inhibitors were completely ineffective at preventing cell death ((Tan et al., 1998a) and **Fig. 11**). At higher doses, these inhibitors also displayed toxicity in control cells. Using non-pharmacological approaches, we found clear evidence that calpains are indeed activated following glutamate exposure in HT22 cells, and that they appear to play a role in the execution of cell death. Although our *in vitro* calpain activity assay using Suc-LY.amc is sensitive to 20S proteasome

activity as well as calpain activity, a recent study by van Leyen et al indicated that HT22 cells exposed to glutamate did not exhibit increased 20S proteasome activity, measured by the z-LLL.amc substrate (van Leyen et al., 2005). The potential for a biphasic activation of calpains could be an interesting line of research to pursue, as a portion of late calpain activity was clearly MEK-dependent whereas a significant portion remained that was MEK-independent. The MEK-dependent phase could be a result of direct phosphorylation and activation of m-calpain by ERK1/2, as has been found to be possible in *in vitro* settings (Glading et al., 2004). Analyses to determine if calpains are phosphorylated, and if that phosphorylation is ERK-dependent, might give insight into the downstream targets of ERK activation in this model.

Additionally, further experiments on the nature of the potential biphasic calpain activation might lead to further insights into the response of HT22 cells to glutamate exposure. Lankiewicz et al noted that pretreatment of neurons with calpain inhibitors was ineffective at blocking NMDA-induced excitotoxicity, while the same inhibitors were effective at reducing NMDA toxicity when added within the first hour of exposure (Lankiewicz et al., 2000). We and others have demonstrated that calpain inhibitors added at the same time as glutamate were ineffective at blocking cell death in HT22 cells, but it is currently not know if delayed treatment with calpain inhibitors, added closer to the second phase of activation, could potentially block glutamate-induced cell death.

6.4 GLUTAMATE-INDUCED TOXICITY IN HT22 CELLS APPEARS TO ACTIVATE CASPASE-1- OR -2-LIKE PROTEASES UPSTREAM OF ERK ACTIVATION, WHICH IN TURN INDUCES A SECOND PHASE OF CALPAIN ACTIVATION.

The understanding of multiple layers of cell death activation is critical for translational research targeting therapeutics that can be differentially administered during the progession of a disease state. We present here evidence that caspase-like proteases may function to increase activation of ERK. ERK activation may then directly or indirectly promote cell death by the activation of calpains (**Fig. 18**). At different points during this mechanism there likely exist feedback loops furthering crosstalk between these and other pathways. For example, ERK has been demonstrated to be able to directly phosphorylate and activate calpains, which have in turn been demonstrated to be able to cleave some, but not all, phosphatases (Gulati et al., 2004). It would be interesting to determine if calpains cleave ERK-directed phosphatases, leading to enhanced ERK activation and an amplification of calpain activity.

The interplay between caspases and calpains was not examined in the data presented here. The delicate balance in crosstalk between the caspase and calpain protease pathways has recently come to light in many different models of cell death. Caspases have been found to be able to cleave calpastatin, the endogenous inhibitor of calpain, resulting in the release and subsequent activation of calpain. An example of the effects of crosstalk between caspases and calpains can be inferred from the observations of caspase-1 knockout phenotypes. Caspase-1 knockout mice are resistant to neonatal hypoxia/ischemia, and display, as expected, reduced formation of IL-1β. Surprisingly, these mice also demonstrated reduction in the levels of IL-1α. IL-1α, another byproduct of IL-1 cleavage, is similar to IL-1β in that it is formed by cleavage of IL-1. However, in the case of IL-1α, this cleavage is mediated not by caspase-1, but rather by

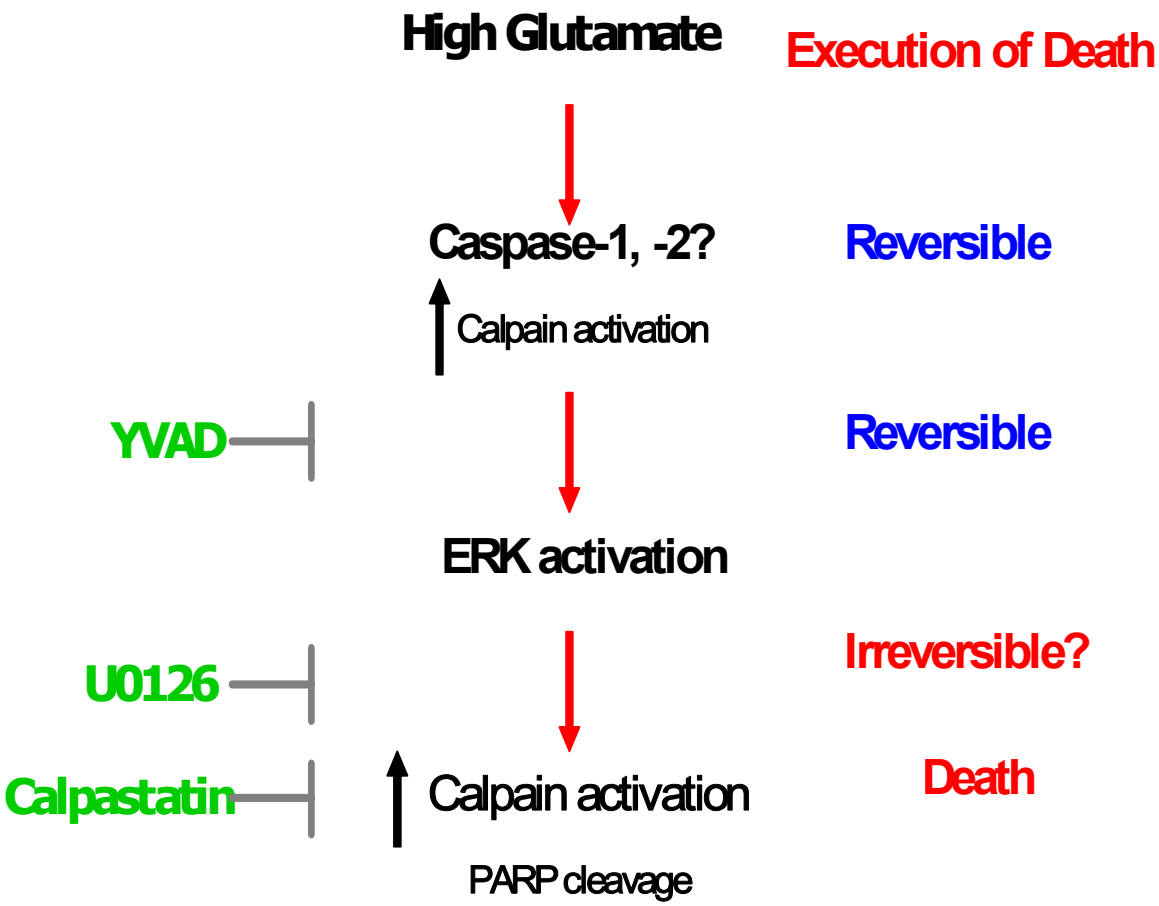


Figure 18 Proposed model of glutamate-induced cell death.

calpains. Because the caspase-1 knockout mouse exhibits reduction in both processed forms, it may be the case that ablation of caspase-1 results in decreased calpain activation due to the absence of calpastatin cleavage (Wang et al., 1998a). Interestingly, calpains may also function to activate caspases, either directly, as has been shown using purified caspase-12 and m-calpain, or indirectly. For example, Bcl-xl has been identified as a substrate for calpain cleavage (Nakagawa and Yuan, 2000). Calpain was found to cleave the loop domain in bcl-xl, a region that has also been demonstrated to contain caspase-1 and -3 cleavage sites (Clem et al., 1998; Fujita et al., 1998). Cleavage at this loop domain produces a functional shift in the role of bcl-x in cell death, where bcl-xl has a protective function against cell death, and the cleaved form has a proapoptotic role. However, in the HT22 glutamate toxicity model, we have not been able to conclusively demonstrate caspase activation, either due to the lack of caspase involvement in this model of cell death, or due to the limitations of the experimental setup. Despite this, armed with caspase inhibitors that exert a protective effect in this model, it would be interesting to explore if these inhibitors block the observed calpain activity.

The data presented in these studies provide strong evidence for the ERK-dependent involvement of calpain in executing glutamate-induced toxicity in a purified clone of glutamate responsive HT22 cells. Furthermore, caspase-1- and -2-like inhibitors were found to inhibit ERK signaling, suggesting a role for proteases upstream of ERK activation. Given the recently defined role for ERK in the promotion of cell death, the interplay between known cell death protease systems and ERK signaling may yield important new insights in the mechanisms of non-traditional cell death. Interestingly, many of the models exhibiting ERK-dependent toxicity involve neuronal cells, and it has long been established that neurodegeneration involves cell death mechanisms that deviate substantially from mechanisms found in other cellular models.

Neurodegeneration has the distinction of typically evading detection until late morphological stages, well past the onset of the actual molecular mechanisms initiating toxicity. Therefore, in order to obtain an arsenal of therapeutics that can be differentially administered throughout the degeneration cycle, it is critical to understand the progression of the cell death signal, even at late timepoints. The interplay between proteases and signaling kinases can further fine tune therapeutics to approach treatments in a multipronged manner, where the dream of a 'silver bullet' is exchanged for the sequential targeting substrates in a progressive and logical manner.

7.0 BIBLIOGRAPHY

Allan LA, Morrice N, Brady S, Magee G, Pathak S, Clarke PR (2003) Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. Nat Cell Biol 5:647-654.

Alnemri ES, Fernandes-Alnemri T, Litwack G (1995) Cloning and expression of four novel isoforms of human interleukin-1 beta converting enzyme with different apoptotic activities. J Biol Chem 270:4312-4317.

Arai K, Lee SR, van Leyen K, Kurose H, Lo EH (2004) Involvement of ERK MAP kinase in endoplasmic reticulum stress in SH-SY5Y human neuroblastoma cells. J Neurochem 89:232-239.

Artal-Sanz M, Tavernarakis N (2005) Proteolytic mechanisms in necrotic cell death and neurodegeneration. FEBS Lett 579:3287-3296.

Baliga BC, Read SH, Kumar S (2004) The biochemical mechanism of caspase-2 activation. Cell Death Differ 11:1234-1241.

Bergeron L, Perez GI, Macdonald G, Shi L, Sun Y, Jurisicova A, Varmuza S, Latham KE, Flaws JA, Salter JC, Hara H, Moskowitz MA, Li E, Greenberg A, Tilly JL, Yuan J (1998) Defects in regulation of apoptosis in caspase-2-deficient mice. Genes Dev 12:1304-1314.

Blagosklonny MV, Chuman Y, Bergan RC, Fojo T (1999) Mitogen-activated protein kinase pathway is dispensable for microtubule-active drug-induced Raf-1/Bcl-2 phosphorylation and apoptosis in leukemia cells. Leukemia 13:1028-1036.

Blomgren K, Kawashima S, Saido TC, Karlsson JO, Elmered A, Hagberg H (1995) Fodrin degradation and subcellular distribution of calpains after neonatal rat cerebral hypoxic-ischemia. Brain Res 684:143-149.

Boatright KM, Salvesen GS (2003) Mechanisms of caspase activation. Curr Opin Cell Biol 15:725-731.

Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, Ricci JE, Edris WA, Sutherlin DP, Green DR, Salvesen GS (2003) A unified model for apical caspase activation. Mol Cell 11:529-541.

Budihardjo I, Oliver H, Lutter M, Luo X, Wang X (1999) Biochemical pathways of caspase activation during apoptosis. Annu Rev Cell Dev Biol 15:269-290.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC (1998) Regulation of cell death protease caspase-9 by phosphorylation. Science 282:1318-1321.

Carragher NO, Levkau B, Ross R, Raines EW (1999) Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. J Cell Biol 147:619-630.

Chang DW, Ditsworth D, Liu H, Srinivasula SM, Alnemri ES, Yang X (2003) Oligomerization is a general mechanism for the activation of apoptosis initiator and inflammatory procaspases. J Biol Chem 278:16466-16469.

Chang HY, Yang X (2000) Proteases for cell suicide: functions and regulation of caspases. Microbiol Mol Biol Rev 64:821-846.

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410:37-40.

Chatterjee S, Ator MA, Bozyczko-Coyne D, Josef K, Wells G, Tripathy R, Iqbal M, Bihovsky R, Senadhi SE, Mallya S, O'Kane TM, McKenna BA, Siman R, Mallamo JP (1997) Synthesis and biological activity of a series of potent fluoromethyl ketone inhibitors of recombinant human calpain I. J Med Chem 40:3820-3828.

Chen D, Stetler RA, Cao G, Pei W, O'Horo C, Yin XM, Chen J (2000) Characterization of the rat DNA fragmentation factor 35/Inhibitor of caspase-activated DNase (Short form). The endogenous inhibitor of caspase-dependent DNA fragmentation in neuronal apoptosis. J Biol Chem 275:38508-38517.

Chen J, Liu X, Mandel LJ, Schnellmann RG (2001) Progressive disruption of the plasma membrane during renal proximal tubule cellular injury. Toxicol Appl Pharmacol 171:1-11.

Chen J, Small-Howard A, Yin A, Berry MJ (2005a) The responses of Ht22 cells to oxidative stress induced by buthionine sulfoximine (BSO). BMC Neurosci 6:10.

Chen M, Yang ZD, Smith KM, Carter JD, Nadler JL (2005b) Activation of 12-lipoxygenase in proinflammatory cytokine-mediated beta cell toxicity. Diabetologia 48:486-495.

Cheung EC, Slack RS (2004) Emerging role for ERK as a key regulator of neuronal apoptosis. Sci STKE 2004:PE45.

Choi DW (1985) Glutamate neurotoxicity in cortical cell culture is calcium dependent. Neurosci Lett 58:293-297.

Choi DW (1987) Ionic dependence of glutamate neurotoxicity. J Neurosci 7:369-379.

Chu CT, Levinthal DJ, Kulich SM, Chalovich EM, DeFranco DB (2004) Oxidative neuronal injury. The dark side of ERK1/2. Eur J Biochem 271:2060-2066.

Clem RJ, Cheng EH, Karp CL, Kirsch DG, Ueno K, Takahashi A, Kastan MB, Griffin DE, Earnshaw WC, Veliuona MA, Hardwick JM (1998) Modulation of cell death by Bcl-XL through caspase interaction. Proc Natl Acad Sci U S A 95:554-559.

Cook SJ, Beltman J, Cadwallader KA, McMahon M, McCormick F (1997) Regulation of mitogen-activated protein kinase phosphatase-1 expression by extracellular signal-related kinase-dependent and Ca2+-dependent signal pathways in Rat-1 cells. J Biol Chem 272:13309-13319.

Croall DE, DeMartino GN (1991) Calcium-activated neutral protease (calpain) system: structure, function, and regulation. Physiol Rev 71:813-847.

D'Amours D, Sallmann FR, Dixit VM, Poirier GG (2001) Gain-of-function of poly(ADP-ribose) polymerase-1 upon cleavage by apoptotic proteases: implications for apoptosis. J Cell Sci 114:3771-3778.

Dargusch R, Schubert D (2002) Specificity of resistance to oxidative stress. J Neurochem 81:1394-1400.

Decker P, Muller S (2002) Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. Curr Pharm Biotechnol 3:275-283.

Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) X-linked IAP is a direct inhibitor of cell-death proteases. Nature 388:300-304.

Druilhe A, Srinivasula SM, Razmara M, Ahmad M, Alnemri ES (2001) Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins. Cell Death Differ 8:649-657.

Duncia JV, Santella JB, 3rd, Higley CA, Pitts WJ, Wityak J, Frietze WE, Rankin FW, Sun JH, Earl RA, Tabaka AC, Teleha CA, Blom KF, Favata MF, Manos EJ, Daulerio AJ, Stradley DA, Horiuchi K, Copeland RA, Scherle PA, Trzaskos JM, Magolda RL, Trainor GL, Wexler RR, Hobbs FW, Olson RE (1998) MEK inhibitors: the chemistry and biological activity of U0126, its analogs, and cyclization products. Bioorg Med Chem Lett 8:2839-2844.

Ekert PG, Silke J, Vaux DL (1999) Caspase inhibitors. Cell Death Differ 6:1081-1086.

Elce JS, Hegadorn C, Arthur JS (1997) Autolysis, Ca2+ requirement, and heterodimer stability in m-calpain. J Biol Chem 272:11268-11275.

Feng Q, Li P, Leung PC, Auersperg N (2004) Caspase-1zeta, a new splice variant of the caspase-1 gene. Genomics 84:587-591.

Fox JE, Taylor RG, Taffarel M, Boyles JK, Goll DE (1993) Evidence that activation of platelet calpain is induced as a consequence of binding of adhesive ligand to the integrin, glycoprotein IIb-IIIa. J Cell Biol 120:1501-1507.

Freshney I (2001) Application of cell cultures to toxicology. Cell Biol Toxicol 17:213-230.

Freshney RI (2000) Culture of animal cells: A manual of basic techniques, 4th Edition: Wiley-Liss.

Fujita E, Jinbo A, Matuzaki H, Konishi H, Kikkawa U, Momoi T (1999) Akt phosphorylation site found in human caspase-9 is absent in mouse caspase-9. Biochem Biophys Res Commun 264:550-555.

Fujita N, Nagahashi A, Nagashima K, Rokudai S, Tsuruo T (1998) Acceleration of apoptotic cell death after the cleavage of Bcl-XL protein by caspase-3-like proteases. Oncogene 17:1295-1304.

Fumarola C, Guidotti GG (2004) Stress-induced apoptosis: toward a symmetry with receptor-mediated cell death. Apoptosis 9:77-82.

Garcia-Calvo M, Peterson EP, Leiting B, Ruel R, Nicholson DW, Thornberry NA (1998) Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem 273:32608-32613.

Glading A, Chang P, Lauffenburger DA, Wells A (2000) Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. J Biol Chem 275:2390-2398.

Glading A, Uberall F, Keyse SM, Lauffenburger DA, Wells A (2001) Membrane proximal ERK signaling is required for M-calpain activation downstream of epidermal growth factor receptor signaling. J Biol Chem 276:23341-23348.

Glading A, Bodnar RJ, Reynolds IJ, Shiraha H, Satish L, Potter DA, Blair HC, Wells A (2004) Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. Mol Cell Biol 24:2499-2512.

Gross A, Yin XM, Wang K, Wei MC, Jockel J, Milliman C, Erdjument-Bromage H, Tempst P, Korsmeyer SJ (1999) Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 274:1156-1163.

Gulati P, Markova B, Gottlicher M, Bohmer FD, Herrlich PA (2004) UVA inactivates protein tyrosine phosphatases by calpain-mediated degradation. EMBO Rep 5:812-817.

Gunter TE, Gunter KK (2001) Uptake of calcium by mitochondria: transport and possible function. IUBMB Life 52:197-204.

Guo Y, Srinivasula SM, Druilhe A, Fernandes-Alnemri T, Alnemri ES (2002) Caspase-2 induces apoptosis by releasing proapoptotic proteins from mitochondria. J Biol Chem 277:13430-13437.

Haviv R, Lindenboim L, Yuan J, Stein R (1998) Need for caspase-2 in apoptosis of growth-factor-deprived PC12 cells. J Neurosci Res 52:491-497.

Ho PK, Jabbour AM, Ekert PG, Hawkins CJ (2005) Caspase-2 is resistant to inhibition by inhibitor of apoptosis proteins (IAPs) and can activate caspase-7. Febs J 272:1401-1414.

Huang Y, Wang KK (2001) The calpain family and human disease. Trends Mol Med 7:355-362.

Humke EW, Shriver SK, Starovasnik MA, Fairbrother WJ, Dixit VM (2000) ICEBERG: a novel inhibitor of interleukin-1beta generation. Cell 103:99-111.

Ishige K, Chen Q, Sagara Y, Schubert D (2001) The activation of dopamine D4 receptors inhibits oxidative stress-induced nerve cell death. J Neurosci 21:6069-6076.

Jabado O, Wang Q, Rideout HJ, Yeasmin M, Guo KX, Vekrellis K, Papantonis S, Angelastro JM, Troy CM, Stefanis L (2004) RAIDD aggregation facilitates apoptotic death of PC12 cells and sympathetic neurons. Cell Death Differ 11:618-630.

Jaeschke H, Lemasters JJ (2003) Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. Gastroenterology 125:1246-1257.

Johnson CR, Jarvis WD (2004) Caspase-9 regulation: an update. Apoptosis 9:423-427.

Kim YK, Kim HJ, Kwon CH, Kim JH, Woo JS, Jung JS, Kim JM (2005) Role of ERK activation in cisplatin-induced apoptosis in OK renal epithelial cells. J Appl Toxicol 25:374-382.

Kuida K, Zheng TS, Na S, Kuan C, Yang D, Karasuyama H, Rakic P, Flavell RA (1996) Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384:368-372.

Kuperstein F, Yavin E (2002) ERK activation and nuclear translocation in amyloid-beta peptide- and iron-stressed neuronal cell cultures. Eur J Neurosci 16:44-54.

Kyosseva SV (2004) Mitogen-activated protein kinase signaling. Int Rev Neurobiol 59:201-220.

Lankiewicz S, Marc Luetjens C, Truc Bui N, Krohn AJ, Poppe M, Cole GM, Saido TC, Prehn JH (2000) Activation of calpain I converts excitotoxic neuron death into a caspase-independent cell death. J Biol Chem 275:17064-17071.

Lassus P, Opitz-Araya X, Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. Science 297:1352-1354.

Launay S, Hermine O, Fontenay M, Kroemer G, Solary E, Garrido C (2005) Vital functions for lethal caspases. Oncogene 24:5137-5148.

Li H, Bergeron L, Cryns V, Pasternack MS, Zhu H, Shi L, Greenberg A, Yuan J (1997a) Activation of caspase-2 in apoptosis. J Biol Chem 272:21010-21017.

Li P, Allen H, Banerjee S, Franklin S, Herzog L, Johnston C, McDowell J, Paskind M, Rodman L, Salfeld J, et al. (1995) Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock. Cell 80:401-411.

Li Y, Maher P, Schubert D (1997b) A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron 19:453-463.

Liu X, Schnellmann RG (2003) Calpain mediates progressive plasma membrane permeability and proteolysis of cytoskeleton-associated paxillin, talin, and vinculin during renal cell death. J Pharmacol Exp Ther 304:63-70.

Liu X, Van Vleet T, Schnellmann RG (2004) The role of calpain in oncotic cell death. Annu Rev Pharmacol Toxicol 44:349-370.

Liu XH, Kwon D, Schielke GP, Yang GY, Silverstein FS, Barks JD (1999) Mice deficient in interleukin-1 converting enzyme are resistant to neonatal hypoxic-ischemic brain damage. J Cereb Blood Flow Metab 19:1099-1108.

Liu Y (1999) Understanding the biological activity of amyloid proteins in vitro: from inhibited cellular MTT reduction to altered cellular cholesterol homeostatis. Prog Neuropsychopharmacol Biol Psychiatry 23:377-395.

Liu Y, Peterson DA, Kimura H, Schubert D (1997) Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. J Neurochem 69:581-593.

Ma H, Yang HQ, Takano E, Lee WJ, Hatanaka M, Maki M (1993) Requirement of different subdomains of calpastatin for calpain inhibition and for binding to calmodulin-like domains. J Biochem (Tokyo) 113:591-599.

Maher P (2001) How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. J Neurosci 21:2929-2938.

Mancini M, Machamer CE, Roy S, Nicholson DW, Thornberry NA, Casciola-Rosen LA, Rosen A (2000) Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. J Cell Biol 149:603-612.

Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, Roose-Girma M, Erickson S, Dixit VM (2004) Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. Nature 430:213-218.

Markgraf CG, Velayo NL, Johnson MP, McCarty DR, Medhi S, Koehl JR, Chmielewski PA, Linnik MD (1998) Six-hour window of opportunity for calpain inhibition in focal cerebral ischemia in rats. Stroke 29:152-158.

Martinon F, Tschopp J (2004) Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. Cell 117:561-574.

Martinon F, Burns K, Tschopp J (2002) The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10:417-426.

McCarthy JV, Ni J, Dixit VM (1998) RIP2 is a novel NF-kappaB-activating and cell death-inducing kinase. J Biol Chem 273:16968-16975.

Mellgren RL (1997) Specificities of cell permeant peptidyl inhibitors for the proteinase activities of mu-calpain and the 20 S proteasome. J Biol Chem 272:29899-29903.

Michetti M, Salamino F, Tedesco I, Averna M, Minafra R, Melloni E, Pontremoli S (1996) Autolysis of human erythrocyte calpain produces two active enzyme forms with different cell localization. FEBS Lett 392:11-15.

Miller DK, Ayala JM, Egger LA, Raju SM, Yamin TT, Ding GJ, Gaffney EP, Howard AD, Palyha OC, Rolando AM, et al. (1993) Purification and characterization of active human interleukin-1 beta-converting enzyme from THP.1 monocytic cells. J Biol Chem 268:18062-18069.

Mueller H, Kassack MU, Wiese M (2004) Comparison of the usefulness of the MTT, ATP, and calcein assays to predict the potency of cytotoxic agents in various human cancer cell lines. J Biomol Screen 9:506-515.

Murphy TH, Miyamoto M, Sastre A, Schnaar RL, Coyle JT (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2:1547-1558.

Nakagawa T, Yuan J (2000) Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. J Cell Biol 150:887-894.

Namura S, Iihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, Moskowitz MA, Bonventre JV, Alessandrini A (2001) Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. Proc Natl Acad Sci U S A 98:11569-11574.

Navas TA, Baldwin DT, Stewart TA (1999) RIP2 is a Raf1-activated mitogen-activated protein kinase kinase. J Biol Chem 274:33684-33690.

Neumar RW, Xu YA, Gada H, Guttmann RP, Siman R (2003) Cross-talk between calpain and caspase proteolytic systems during neuronal apoptosis. J Biol Chem 278:14162-14167.

Newcomb-Fernandez JK, Zhao X, Pike BR, Wang KK, Kampfl A, Beer R, DeFord SM, Hayes RL (2001) Concurrent assessment of calpain and caspase-3 activation after oxygen-glucose deprivation in primary septo-hippocampal cultures. J Cereb Blood Flow Metab 21:1281-1294.

Nutt LK, Margolis SS, Jensen M, Herman CE, Dunphy WG, Rathmell JC, Kornbluth S (2005) Metabolic Regulation of Oocyte Cell Death through the CaMKII-Mediated Phosphorylation of Caspase-2. Cell 123:89.

Oh HL, Seok JY, Kwon CH, Kang SK, Kim YK (2005) Role of MAPK in ceramide-induced cell death in primary cultured astrocytes from mouse embryonic brain. Neurotoxicology (in press).

Otsuka Y, Goll DE (1987) Purification of the Ca2+-dependent proteinase inhibitor from bovine cardiac muscle and its interaction with the millimolar Ca2+-dependent proteinase. J Biol Chem 262:5839-5851.

Page M, Bejaoui N, Cinq-Mars B, Lemieux P (1988) Optimization of the tetrazolium-based colorimetric assay for the measurement of cell number and cytotoxicity. Int J Immunopharmacol 10:785-793.

Park BG, Yoo CI, Kim HT, Kwon CH, Kim YK (2005) Role of mitogen-activated protein kinases in hydrogen peroxide-induced cell death in osteoblastic cells. Toxicology 215:115-125.

Paschen W (2003) Endoplasmic reticulum: a primary target in various acute disorders and degenerative diseases of the brain. Cell Calcium 34:365-383.

Patel HC, Boutin H, Allan SM (2003) Interleukin-1 in the brain: mechanisms of action in acute neurodegeneration. Ann N Y Acad Sci 992:39-47.

Perrin BJ, Huttenlocher A (2002) Calpain. Int J Biochem Cell Biol 34:722-725.

Polster BM, Basanez G, Etxebarria A, Hardwick JM, Nicholls DG (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280:6447-6454.

Porn-Ares MI, Samali A, Orrenius S (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. Cell Death Differ 5:1028-1033.

Potter DA, Tirnauer JS, Janssen R, Croall DE, Hughes CN, Fiacco KA, Mier JW, Maki M, Herman IM (1998) Calpain regulates actin remodeling during cell spreading. J Cell Biol 141:647-662.

Poyet JL, Srinivasula SM, Tnani M, Razmara M, Fernandes-Alnemri T, Alnemri ES (2001) Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1. J Biol Chem 276:28309-28313.

Proskuryakov SY, Gabai VL, Konoplyannikov AG (2002) Necrosis is an active and controlled form of programmed cell death. Biochemistry (Mosc) 67:387-408.

Proskuryakov SY, Konoplyannikov AG, Gabai VL (2003) Necrosis: a specific form of programmed cell death? Exp Cell Res 283:1-16.

Prunell GF, Troy CM (2004) Balancing neuronal death. J Neurosci Res 78:1-6.

Rami A (2003) Ischemic neuronal death in the rat hippocampus: the calpain-calpastatin-caspase hypothesis. Neurobiol Dis 13:75-88.

Rami A, Agarwal R, Botez G, Winckler J (2000) mu-Calpain activation, DNA fragmentation, and synergistic effects of caspase and calpain inhibitors in protecting hippocampal neurons from ischemic damage. Brain Res 866:299-312.

Ray SK, Fidan M, Nowak MW, Wilford GG, Hogan EL, Banik NL (2000) Oxidative stress and Ca2+ influx upregulate calpain and induce apoptosis in PC12 cells. Brain Res 852:326-334.

Read SH, Baliga BC, Ekert PG, Vaux DL, Kumar S (2002) A novel Apaf-1-independent putative caspase-2 activation complex. J Cell Biol 159:739-745.

Riedl SJ, Shi Y (2004) Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 5:897-907.

Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. Embo J 16:6914-6925.

Sagara Y, Dargusch R, Chambers D, Davis J, Schubert D, Maher P (1998) Cellular mechanisms of resistance to chronic oxidative stress. Free Radic Biol Med 24:1375-1389.

Satoh T, Nakatsuka D, Watanabe Y, Nagata I, Kikuchi H, Namura S (2000) Neuroprotection by MAPK/ERK kinase inhibition with U0126 against oxidative stress in a mouse neuronal cell line and rat primary cultured cortical neurons. Neurosci Lett 288:163-166.

Scheid MP, Duronio V (1998) Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation. Proc Natl Acad Sci U S A 95:7439-7444.

Schielke GP, Yang GY, Shivers BD, Betz AL (1998) Reduced ischemic brain injury in interleukin-1 beta converting enzyme-deficient mice. J Cereb Blood Flow Metab 18:180-185.

Schotte P, Declercq W, Van Huffel S, Vandenabeele P, Beyaert R (1999) Non-specific effects of methyl ketone peptide inhibitors of caspases. FEBS Lett 442:117-121.

Shi Y (2004) Caspase activation, inhibition, and reactivation: a mechanistic view. Protein Sci 13:1979-1987.

Shiozaki EN, Chai J, Rigotti DJ, Riedl SJ, Li P, Srinivasula SM, Alnemri ES, Fairman R, Shi Y (2003) Mechanism of XIAP-mediated inhibition of caspase-9. Mol Cell 11:519-527.

Sperandio S, de Belle I, Bredesen DE (2000) An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci U S A 97:14376-14381.

Sperandio S, Poksay K, de Belle I, Lafuente MJ, Liu B, Nasir J, Bredesen DE (2004) Paraptosis: mediation by MAP kinases and inhibition by AIP-1/Alix. Cell Death Differ 11:1066-1075.

Srinivasula SM, Poyet JL, Razmara M, Datta P, Zhang Z, Alnemri ES (2002) The PYRIN-CARD protein ASC is an activating adaptor for caspase-1. J Biol Chem 277:21119-21122.

Stacey G, MacDonald C (2001) Immortalisation of primary cells. Cell Biol Toxicol 17:231-246.

Stanciu M, DeFranco DB (2002) Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010-4017.

Stanciu M, Wang Y, Kentor R, Burke N, Watkins S, Kress G, Reynolds I, Klann E, Angiolieri MR, Johnson JW, DeFranco DB (2000) Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures. J Biol Chem 275:12200-12206.

Stefanis L (2005) Caspase-dependent and -independent neuronal death: two distinct pathways to neuronal injury. Neuroscientist 11:50-62.

Stennicke HR, Deveraux QL, Humke EW, Reed JC, Dixit VM, Salvesen GS (1999) Caspase-9 can be activated without proteolytic processing. J Biol Chem 274:8359-8362.

Stout AK, Raphael HM, Kanterewicz BI, Klann E, Reynolds IJ (1998) Glutamate-induced neuron death requires mitochondrial calcium uptake. Nat Neurosci 1:366-373.

Subramaniam S, Zirrgiebel U, von Bohlen Und Halbach O, Strelau J, Laliberte C, Kaplan DR, Unsicker K (2004) ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. J Cell Biol 165:357-369.

Tan S, Wood M, Maher P (1998a) Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem 71:95-105.

Tan S, Sagara Y, Liu Y, Maher P, Schubert D (1998b) The regulation of reactive oxygen species production during programmed cell death. J Cell Biol 141:1423-1432.

Thome M, Hofmann K, Burns K, Martinon F, Bodmer JL, Mattmann C, Tschopp J (1998) Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. Curr Biol 8:885-888.

Thorburn A (2004) Death receptor-induced cell killing. Cell Signal 16:139-144.

Toledo-Pereyra LH, Toledo AH, Walsh J, Lopez-Neblina F (2004) Molecular signaling pathways in ischemia/reperfusion. Exp Clin Transplant 2:174-177.

Troy CM, Shelanski ML (2003) Caspase-2 redux. Cell Death Differ 10:101-107.

Troy CM, Rabacchi SA, Friedman WJ, Frappier TF, Brown K, Shelanski ML (2000) Caspase-2 mediates neuronal cell death induced by beta-amyloid. J Neurosci 20:1386-1392.

Troy CM, Rabacchi SA, Hohl JB, Angelastro JM, Greene LA, Shelanski ML (2001) Death in the balance: alternative participation of the caspase-2 and -9 pathways in neuronal death induced by nerve growth factor deprivation. J Neurosci 21:5007-5016.

van Leyen K, Siddiq A, Ratan RR, Lo EH (2005) Proteasome inhibition protects HT22 neuronal cells from oxidative glutamate toxicity. J Neurochem 92:824-830.

Vaux DL, Strasser A (1996) The molecular biology of apoptosis. Proc Natl Acad Sci U S A 93:2239-2244.

Wang KK, Posmantur R, Nadimpalli R, Nath R, Mohan P, Nixon RA, Talanian RV, Keegan M, Herzog L, Allen H (1998a) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. Arch Biochem Biophys 356:187-196.

Wang Q, Maniati M, Jabado O, Pavlaki M, Troy CM, Greene LA, Stefanis L (2005) RAIDD is required for apoptosis of PC12 cells and sympathetic neurons induced by trophic factor withdrawal. Cell Death Differ.

Wang S, Miura M, Jung YK, Zhu H, Li E, Yuan J (1998b) Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE. Cell 92:501-509.

Wang X, Zhu C, Qiu L, Hagberg H, Sandberg M, Blomgren K (2003) Activation of ERK1/2 after neonatal rat cerebral hypoxia-ischaemia. J Neurochem 86:351-362.

Wendt A, Thompson VF, Goll DE (2004) Interaction of calpastatin with calpain: a review. Biol Chem 385:465-472.

Wilson KP, Black JA, Thomson JA, Kim EE, Griffith JP, Navia MA, Murcko MA, Chambers SP, Aldape RA, Raybuck SA, et al. (1994) Structure and mechanism of interleukin-1 beta converting enzyme. Nature 370:270-275.

Wong JK, Le HH, Zsarnovszky A, Belcher SM (2003) Estrogens and ICI182,780 (Faslodex) modulate mitosis and cell death in immature cerebellar neurons via rapid activation of p44/p42 mitogen-activated protein kinase. J Neurosci 23:4984-4995.

Wu HY, Tomizawa K, Oda Y, Wei FY, Lu YF, Matsushita M, Li ST, Moriwaki A, Matsui H (2004) Critical role of calpain-mediated cleavage of calcineurin in excitotoxic neurodegeneration. J Biol Chem 279:4929-4940.

Yamin TT, Ayala JM, Miller DK (1996) Activation of the native 45-kDa precursor form of interleukin-1-converting enzyme. J Biol Chem 271:13273-13282.

Yang X, Chang HY, Baltimore D (1998) Autoproteolytic activation of pro-caspases by oligomerization. Mol Cell 1:319-325.

Yoshimori A, Takasawa R, Tanuma S (2004) A novel method for evaluation and screening of caspase inhibitory peptides by the amino acid positional fitness score. BMC Pharmacol 4:7.

Zhang WH, Wang X, Narayanan M, Zhang Y, Huo C, Reed JC, Friedlander RM (2003) Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. Proc Natl Acad Sci U S A 100:16012-16017.