Divergent roles for the ERK1/2 signaling pathway in neuronal oxidative stress

by

Yue Luo

MD, Capital University of Medical Sciences, 1997

MSc, National University of Singapore, 2001

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

2006

UNIVERSITY OF PITTSBURGH

School of Arts and Sciences

This dissertation was presented

By

Yue Luo

It was defended on

July 13, 2006

and approved by

Teresa Hastings, Associate Professor, Department of Neurology and Neuroscience

Charleen Chu, Associate Professor, Department of Pathology and Neuroscience

Yu Jiang, Assistant Professor, Department of Pharmacology

Stephen Meriney, Associate Professor, Department of Neuroscience

Nina Schor, Professor, Department of Pediatrics, Neurology and Neuroscience

Henry Jay Forman, Professor, School of Natural Sciences, University of California, Merced

Dissertation Advisor: Donald DeFranco, Professor, Department of Pharmacology and

Neuroscience

Divergent roles for the ERK1/2 signaling pathway in neuronal oxidative stress

Yue Luo, PhD

University of Pittsburgh, 2006

In the mouse HT22 hippocampal cell line and immature primary cortical neurons, excessive glutamate treatment results in intracellular cysteine depletion, subsequent glutathione loss and the steady accumulation of reactive oxygen species (ROS). This form of oxidative stress ultimately leads to cell death. Previous data from our laboratory had shown that delayed and persistent activation of extracellular signal-regulated kinases-1/2 (ERK1/2) is associated with glutamate induced oxidative toxicity in HT22 cells and immature primary neurons. In addition, U0126, a specific inhibitor of the ERK-activating kinase, MEK-1/2, inhibits ERK activation and prevents cells death induced by glutamate. However the mechanisms responsible for this chronic activation of ERK during oxidative stress have not been well characterized.

Results from this thesis demonstrated that overexpression of a dominant negative mutant of MEK1 blocked glutamate toxicity in transfected HT22 cells. These data confirmed previous results and illustrated that ERK1/2 activation is necessary for oxidative toxicity. However overexpression of a constitutively active MEK1ERK chimera (LA-MEK1ERK2) that induced robust ERK activation and translocation into nucleus did not trigger toxicity in HT22 cells. Thus, ERK1/2 phosphorylation and activation is not sufficient for glutamate induced cell oxidative toxicity. Activation of ERK1/2 in HT22 cells has a distinct kinetic profile with an initial peak occurring between 30 minutes and 1 hour of glutamate treatment and a second peak typically emerging after 6 hours. I demonstrate here that the initial phase of ERK1/2 induction is due to

activation of metabotropic glutamate receptor type I (mGluRI). ERK1/2 activation by mGluRI contributes to an HT22 cell adaptive response to oxidative stress as glutamate induced toxicity is enhanced upon pharmacological inhibition of mGluRI. The protective effect of ERK1/2 activation at early times after glutamate treatment is mediated by a restoration of glutathione (GSH) levels that are reduced due to depletion of intracellular cysteine pools. Additional results suggest that mGluRI may be involved in regulating mRNA and protein levels of glutamate-cysteine ligase (GCL), which would lead to enhanced glutathione synthesis. Thus, ERK1/2 activation by mGluRI protects HT22 cells from oxidative toxicity through upregulation of GCL transcription and translation, and subsequent enhancement of GSH levels.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

This thesis and any success in my graduate study could not have been accomplished without the kind help, encouragement and guidance of many people. I would like to express my sincere appreciation to them here.

First of all, I would like to thank my supervisor, Dr. Donald DeFranco. Don is not only a great scientist, but also an outstanding mentor, editor and friend. I could never have imagined my graduate study without him, the type of dream supervisor that every graduate student desires. His encouragement and advice accompanied me through all the frustration and hard times. Especially, as a non-native English speaker, I would thank him for the patient training in scientific writing.

I also want to thank all the members of my committee for providing me with precious suggestion and guidance both in my study and career. They are Dr. Teresa Hastings, Dr. Charleen Chu, Dr. Yu Jiang, Dr. Stephen Meriney, Dr. Nina Schor and Dr. Henry Forman. I also obtained generous help from Dr. Elias Aizenman, Dr. Jane Cavanaugh, Dr. Terrance Kavanagh, Dr. Melanie Cobb, and Dr. Ian Reynolds for providing their labs' experimental reagents, plasmids, and antibodies.

I want to express my thanks to all the members of the DeFranco Lab, past and present, who have given me much help in my experiments and great memories to cherish. Thanks to Louisa Ho, Xinjia Wang, John Caltagarone, Marjet Heizer, Gary Kohanbash, Natalia Kubicki,

1.0   INTRODUCTION

1.1   NEURONAL CELL DEATH

Neuronal cell death not only plays an important role in the normal development of the nervous system but also is involved in many neurodegenerative diseases such as Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), retinitis pigmentosa, several forms of cerebellar degeneration, spinal muscular atrophy, and Alzheimer's disease (AD) (234). The pathogenesis of many neurological diseases is characterized by the gradual loss of neurons in certain regions, which result in defects of CNS function such as movement and cognition (205).

Two distinct mechanisms of cell death have been characterized, i.e. apoptosis and necrosis (109). Apoptosis is a carefully controlled cell death process, which keeps the content of the dying cell intracellular. It is initiated by both physiological and pathological stimuli. However, necrosis is a relatively uncontrolled cell death mode and is generally correlated with injury (205). Table 1 shows a comparison of essential features and mechanisms of apoptosis and necrosis.

Recently, increasing studies have shown that necrosis and apoptosis represent morphological expressions of a shared biochemical network through caspase (cysteinyl-aspartate protease) -dependent or –independent machinery. Mounting evidence is emerging to support an apoptosis-necrosis cell death continuum (149, 264). In this continuum, coexisting apoptotic and

necrotic mechanisms induce neuronal death; thus, some of the boundaries between apoptosis and

necrosis are becoming less well defined.

Table 1. Comparison of Necrosis vs Apoptosis.

Adapted from (62)

	Apoptosis	Necrosis
Pathological Features		
Pattern of death	Individual cells	Whole groups of cells
Cell shape changes	Cell shrinkage	Cell swelling and lysis
Plasma membrane changes	Membrane preservation, cell surface blebbing	Early membrane breakdown
Organelle changes	Involution, contraction, "apoptotic bodies"	Organelle swelling and disruption
Nuclear changes	Chromatin condensation and fragmentation	Karyolysis, pyknosis (or karyorrhexis)
DNA breakdown	Internucleosomal DNA fragmentation, free 3'-ends	Diffuse and random DNA degradation
Cell degradation	Phagocytosis without cell infiltration or inflammation	Marked inflammation, with macrophage invasion
Mechanisms		
Causes	Developmental/programmed Degenerative changes Growth factor deprivation Mild ischemia, radiation, etc.	Toxins Massive ischemia Radiation (high dose)
Cellular processes	Programmed events Δ membrane phospholipid asymmetry Organelles preserved/shrunk Energy (ATP) dependence Requires protein synthesis Requires new RNA transcription	Noncoordinated events Cell membrane rupture Mitochondrial swelling Energy independence No protein synthesis requirement No RNA synthesis requirement
Molecular Events	Mitochondrial permeability transition Mitochondrial cytochrome c release Caspase activations Internucleosomal endonucleases Transglutaminase activation Poly(ADP-ribose) polymerase cleavage	ATP depletion Enzymatic digestion Protein denaturation Diffuse DNA digestion

1.1.1   Apoptosis

The term apoptosis (from Greek: falling off) was not introduced to describe cell death until 1971 (108). In 1972, Kerr et al., illustrated and compared necrosis with apoptosis in liver cells (109). They defined apoptosis as cell death that was not only morphologically distinct from necrosis but also possessed distinct biochemical and molecular features. Apoptosis plays an important role in early development and growth of normal adult tissues. It is regulated not only by physiological stimuli but also induced by various disease conditions (205).

Apoptosis is also referred to as programmed cell death (PCD) or active cell death (ACD), since it requires controlled and pre-programmed gene expression. Also, PCD can occur at a precise time during development. Apoptosis has distinctive morphological and biochemical characteristics such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation. The earliest indications of apoptotic cell death are morphological alterations within the cell such as chromatin condensation, disappearance of the nucleolus, and alterations of the cell surface, characterized by the occurrence of blebs. These signs are followed by a margination of the chromatin at the inner surface of the nuclear membrane. Eventually the activation of a variety of nucleases leads to the fragmentation of DNA. DNA degradation during apoptosis generally occurs at two levels: initially high molecular weight fragments are generated while at later stages nucleosome-sized DNA fragments appear. This requires a number of specific DNases, which are activated specifically during apoptosis. The specific pattern of internucleosomal fragmentation of DNA can be used to identify an apoptotic cell. On agarose gel electrophoresis, a DNA "ladder," could be seen, which is a large series of electrophoretic bands differing from their neighbors in molecular weight of about 180 base pairs. There is no marked inflammatory reaction as cytoplasmic condensation is observed in apoptotic cells before they are

4

sequestered by phagocytes or surrounding cells. Apoptotic cells do not release damaging material to extracellular space and therefore limit death to the apoptotic cell.

A multi-step pathway is involved in apoptosis. Proteins such as caspases play an essential role in apoptosis. Caspases are a group of enzymes with a crucial cysteine residue that can cleave other proteins after an aspartic acid residue, a specificity which is unusual among proteases. They are apoptosis-specific proteases. There are more than ten members of the caspase family that form an apoptotic cascade to execute apoptosis in response to death signal. They are divided into two categories: upstream initiator caspases and downstream effector caspases. Initiator caspases cleave inactive pro-forms of effector caspases, thereby activating them. For example, caspase-9, an initiator, can be activated by the death signals and then cleave procaspase 3 after a specific aspartic acid residue into a short form that is the active form of caspase 3 (2). Effector caspases such as caspase-3 and caspase-7 in turn cleave other protein substrates within various subcellular compartment resulting in the apoptotic process. Caspase-3 (also known as CPP32, apopain, YAMA) has been identified as a key mediator of apoptosis in mammalian cells. Activated caspase 3 may translocate into the nucleus and cleave substrates such as lamin A and DNA fragmentation factor (DFF) 40 and 45 (172). The active form of DFF have DNAse activity. DFF 40 and 45 act on DNA and cause DNA fragmentation and chromatin condensation which are the hallmarks of apoptosis (172).

The importance of caspase 3 during morphogenetic cell death in nervous system is also demonstrated through genetic studies. Caspase 3 knockout mice exhibted profound dysfunction and aberrant development in the brain such as hyperplasia and disorganized cellular deployment (120).

Another important family of proteins involved in neuronal apoptosis is the Bcl-2 family proteins. Bcl-2 and Bcl-XL are anti-apoptotic proteins; however, Bak, Bim, Bad and Bax are pro-apoptotic (172). They modulate apoptosis by influencing cytochrome c release from mitochondria. Releasing of cytochrome c into the cytosol activates pro-caspase 9 and then initiates unapoptotic cascade. Bcl-2 is a negative regulator of cytochrome c release and therefore is anti-apoptotic. Transgenic mice that overexpress Bcl-2 have larger brains resulting from decreased apoptosis (150, 263). On the contrary, Bax is a pro-apoptotic Bcl-2 family protein. Bax knock-out mice display significantly decreased apoptosis of synapse-bearing neurons whose survival are neurotropic factor-dependent (43).

1.1.2   Necrosis

Scientific papers mentioning necrosis could be found as early as 1930s. The process of necrosis is uncontrolled and passive and does not require energy. Necrosis generally occurs in response to acute injury such as that generated by toxic substances and trauma. Necrotic cells swell and rupture, releasing their cellular components to the extracellular space and causing inflammation to neighboring tissues. The damaging effects of necrosis are even worse in neuronal cells given the release of neurotransmitters such as glutamate, which can cause excitotoxic injury to their neighbors (131). Morphologically, necrosis is characterized by a disruption of cellular membranes and a swelling of the cytoplasm and mitochondria, ending in the complete disintegration of organelles. The process terminates with total cell lysis. Biochemical features of necrosis include loss of regulation of ion homeostasis, random digestion of DNA and DNA fragmentation after lysis. In necrosis, DNA breakdown is more diffuse and random than in apoptosis, leading to a "smear" on gel electrophoresis rather than a DNA "ladder".

1.1.3 Cell death and acute and chronic neurological diseases

Neuronal cell death occurs in neuropathological conditions, both in response to acute injury such as stroke and during chronic neurodegenerative diseases. Necrosis and apoptosis may be present simultaneously within a damaged region of the brain, and be related to the magnitude of the toxic stimuli (91).

Acute insults such as hypoxia, stroke, trauma, and infection cause harsh, usually focal injuries to the central nervous system. In general, such severe injuries to the brain result in rapid necrosis in the core regions, although in most cases apoptosis is also observed (91). In addition, delayed "secondary" injury occurs in a "penumbra" zone which is the area bordering the most severely damaged core of ischemia (61). Blood flow in the penumbra zone is gradually reduced to below a functional level but still adequate to sustain morphological integrity for a certain period of time, which depends on the extent of residual perfusion (86, 87). Clinically, this penumbra zone is usually considered as the most promising target for acute stroke therapy because the therapeutic window is extended for several hours (13). Part of cell death in these penumbra areas appears to be caspase-mediated and has apoptotic characteristics (174, 189, 213). Thus, a variety of acute neurological diseases may, in part, also involve apoptotic cell death.

Many inherited or sporadic neurodegenerative diseases can be characterized by progressive nervous system dysfunction. These disorders are often associated with atrophy of the affected central or peripheral nervous system structures. Defects in these neurons cause them to function abnormally, eventually bringing about their death and resulting in cognitive and/or motor defects. These disorders have classically been categorized by their clinical symptoms and therefore reflect their neuropathologic features.

One category of these diseases is known for mainly targeting motor systems, and may rarely affect cognition function; and only in later stages of the disease. Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease (HD), spinobulbar atrophy, and the spinocerebellar ataxias are included in this class. The pathoetiology of these disorders is predicated upon involvement of spinal motor neurons, corticospinal, striatal, or cerebellar neurons (91).

Another group of diseases is known for predominantly having effects on cognition and only partially on motor function. Some common neurodegenerative disorders such as Alzheimer's disease (AD), and certain related disorders, such as Pick's disease, are in this category (91).

The major neurodegenerative diseases and their affected neurons are summarized in Table 2.

**Table 2. Selective neuron losses happen in a variety of neurodegenerative diseases.
          Adapted from (91)**

Neurodegenerative Disorders	Severely Affected Cells
Amyotrophic lateral sclerosis/ Lou Gehrig's disease	Spinal motor neurons, corticospinal (layer 5) Betz cells
Parkinson's disease	Substantia nigra pars compacta, locus ceruleus, vagus dorsal motor nucleus, sympathetic ganglia
Huntington's disease	Caudo-putamen medium spiny interneurons, cortical layers 3, 5, and 6 neurons
Olivopontocerebellar atrophy/ spinocerebellar ataxia type 1 (SCA-1)	Cerebellar Purkinje cells, dentate nucleus, inferior olive
Machado-Joseph disease/ spinocerebellar ataxia type 3 (MJD1/SCA-3)	Dentate nucleus, red nucleus, substantia nigra pars compacta, Purkinje cells, brainstem motor nuclei
Alzheimer's disease	Entorhinal neurons, hippocampal neurons, cortical neurons (late in disease)

1.2 OXIDATIVE STRESS AND NEURONAL CELL DEATH

1.2.1 Oxidative stress

Oxidative stress is defined as conditions under which prooxidants (e.g. free radicals, reactive oxygen and reactive nitrogen species) exceed the ability of antioxidant systems to neutralize them.

1.2.2 Oxidative stress and neuronal cell death

An excess of free radical groups in the body can create a potentially unstable cellular environment associated with tissue damage. For instance, oxidative stress can trigger neuronal cell death and has been implicated in both acute lesions and chronic neurodegenerative diseases (217, 226). Growing evidence from experimental models and human patients studies have indicated that oxidative stress plays an important role in ischemia (204), trauma (35), ALS (218), PD (74) and AD (165). Investigations of intracellular signal transduction pathway affected by oxidative stress is crucial not only for clarification of the pathological basis for cell death in these disorders, but also for providing potential therapy strategies (184).

Superoxide dismutases (SOD) are among the most important cellular defense mechanisms fighting against oxidative stress. In normal conditions, cytosolic copper zinc superoxide dismutase (Cu/Zn SOD), mitochondrial manganese superoxide dismutase (Mn SOD) and iron-containing superoxide dismutase (Fe SOD) are responsible for maintaining low levels of intracellular O_2^- by catalyzing its conversion to oxygen and H_2O_2 (155). In the nervous system, normally, endogenous Cu/Zn SOD is ubiquitously expressed (173). Mutations in the Cu/Zn

SOD1 which inhibit its dimerization and enzymatic activity (45) are associated with the development of the familial form of ALS (199). Consistent with this, over-expression of Cu/Zn SOD in adult transgenic rats show marked neuroprotection from acute and chronic damage such as traumatic brain injury (160). Oxidative stress is also involved in PD animal models induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropypridine (MPTP) administration. Transgenic mice overexpressing cystolic Cu/Zn SOD1 are more resistant to MPTP-induced dopaminergic neurotoxicity than wild type mice (188). In addition, selective neuronal NO synthase (nNOS) inhibitors prevent neurons against MPTP-induced neurtoxicity and mutant mice that lack the nNOS gene (nNOS-/-) are notably more resistant to MPTP-induced neurtoxicity compared with the controls (78, 153, 187).

The role of oxidative stress in the pathology of acute neuronal injury, such as stroke, is also well established. A robust increase in ROS in selective regions of the brain is observed shortly after reperfusion of ischemic regions, which leads to neuronal cell death that occurs over a short period of time (within days) (111, 156). As mentioned previously, this form of delayed neuronal cell death could be found in the penumbral regions (105). Similarly, acute neuronal death induced by potent neurotoxins can involve oxidative stress. For example, MDMA administration in mice induces striatal neuronal cell death that is dependent upon free radical formation in affected neurons (23). In addition, a commonly used industrial and household biocide, methylisothiozolinone, causes acute cell death in cortical neurons cultures via the production of ROS (57).

Therefore, ROS production and neuronal toxicity are intimately associated with a range of chronic and acute neurological disorders.

1.2.3   ROS and its metabolism

Oxidative stress can be generated by the increased production of free radicals and/or decreased elimination of free radical molecules. This imbalance leads to the accumulation of oxidative molecules that can damage susceptible cells.

Oxygen containing free radicals such as superoxide (O_2^-), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) are referred to as reactive oxygen species (ROS) which has been reported to contribute to both necrosis and apoptosis through damaging lipids, proteins and DNA (217). ROS can interact with nitrogen containing molecules. For example, O_2^- reacts with nitric oxide ($NO^.$) forming peroxynitrite ($ONOO^-$), a member of the family of reactive nitrogen species (RNS) (217). A growing body of evidence has shown that ROS could act as important signaling molecules in nearly all cells in the body via oxidation-dependent and reversible changes in cellular protein function. For example, hydrogen peroxide (H2O2) is regarded as a specific diffusible signaling molecule functioning in processes of learning and memory (106). Collectively, ROS and RNS molecules are necessary for important functions in the cell (44), but the accumulation of these molecules in excess is harmful to normal cellular physiological function. If this damage is irreversible, then it may cause cellular injury, mutation, cancer, and eventually cell demise (223).

Under normal physiological conditions, ROS are produced as a byproduct of metabolic reactions. ROS are generated as a result of energy production in mitochondria (from the electron transport chain), as part of an antimicrobial (246) or antiviral (76) response, as well as from detoxification reactions performed by the cytochrome P-450 system (27, 208). Environmental agents such as ultraviolet light, ionizing radiation, redox chemicals and smoking also lead to ROS production. As the principal organelles of oxygen consumption, mitochondria are a

significant source of ROS production in the cell. During oxidative phosphorylation, ATP is formed as a result of the transfer of electrons from NADH or $FADH_2$ to O_2 by a series of electron carriers. Ultimately, O_2 is reduced to H_2O. This process takes place in mitochondria of eukaryotic cells. Although the majority of O_2 is reduced to H_2O during this procedure, a partial reduction of oxygen results in the creation of unstable oxygen radicals that interact with molecular oxygen or water to generate ROS. Cells in cardiac tissue and the nervous system, i.e. cardiomyocytes and neurons respectively, have greater energy requirements and a higher metabolic rate. Because of increased demands on mitochondrial oxidative phosphorylation, these cells consume more oxygen than other cells.

The production of ROS can be compensated by antioxidants, such as small molecular weight dietary supplements, including vitamin E and vitamin C; small molecular weight peptides and cofactors, including glutathione (GSH) and pyruvate; and enzymes, including superoxide dismutase (SOD), glutathione peroxidase and catalase (223). When cells are in an imbalanced state, in which the levels of oxidants exceed the capacity of antioxidants, oxidative damage occurs in nuclear and mitochondrial DNA, proteins, and lipids.

1.3   FUNCTION, METABOLISM AND REGULATION OF GLUTATHIONE

1.3.1   Function of glutathione

GSH, an important antioxidant, is a tripeptide comprised of glutamate, cysteine, and glycine. The primary biological function of glutathione is to act as a non-enzymatic reducing agent to maintain protein cysteine thiol side chains in a reduced state. GSH is also used to prevent

oxidative stress in most cells and helps to remove free radicals that can damage normal function of DNA, lipid and protein etc. As individuals grow older, GSH levels drop, and the ability to detoxify free radicals decreases (195). GSH is found ubiquitously in human body. For example, it is found in the liver where it detoxifies harmful substances. GSH is also present in the nervous system, blood, lungs and intestinal tract. This powerful antioxidant protects the body from the damaging effects of free radicals that may lead to neurodegenerative disease.

In addition to its powerful antioxidant function, GSH acts as a strong anti-toxin, allowing the body to eliminate numerous xenobiotic and carcinogenic chemicals (55). It is essential for immune cell-mediated immune reactions (20) and cell proliferation (185). It is critical to the maintenance of the integrity of red blood cells (18). In addition, GSH is a storage and transport form of cysteine in cells (55). It is widely recognized that deficiencies in the GSH system lead to significant apoptosis and ultimately, morbidity (80).

1.3.2 Synthesis and metabolism of GSH

GSH is synthesized in cell by two continuous steps and catalyzed by two enzymes. First, glutamate-cysteine ligase (GCL) also known as γ-Glutamylcysteine (γGluCys) synthetase (GCS) uses glutamate and cysteine as substrates to generate the dipeptide γGluCys, which is then combined with glycine to form GSH by glutathione synthetase (GS) (157) (Fig 1). Both of the two reactions are ATP-dependent. The intracellular GSH level is regulated via a negative feedback of GCL by the end product GSH (197).
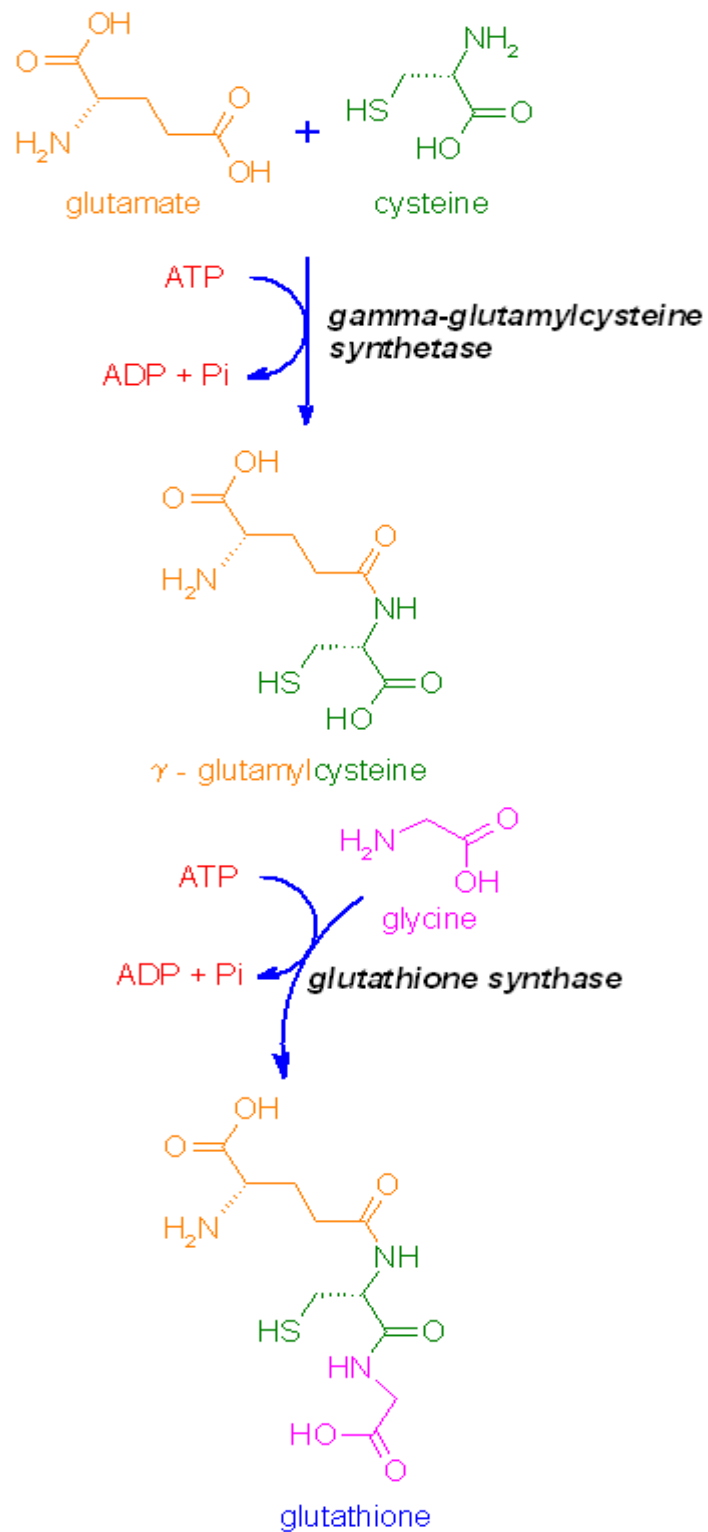
Figure 1. The synthesis of glutathione (GSH).

Glutathione exists in both reduced form and an oxidized dimer – glutathione disulfide (GSSG) form in cells. GSSG typically accounts for only 1-2% of total cellular glutathione. GSH and GSSG are reversibly converted to one another by the action of two enzymes. GSH peroxidase (GPx) and glutathione reductase (GR) (Fig 2).

$$2\,H_2O \quad\nwarrow\nearrow\quad GSSG \quad\nwarrow\nearrow\quad 2NADPH$$
$$GPx \qquad\qquad GR$$
$$H_2O_2 \quad\nearrow\nwarrow\quad 2GS\,H \quad\nwarrow\nearrow\quad NADP^+$$
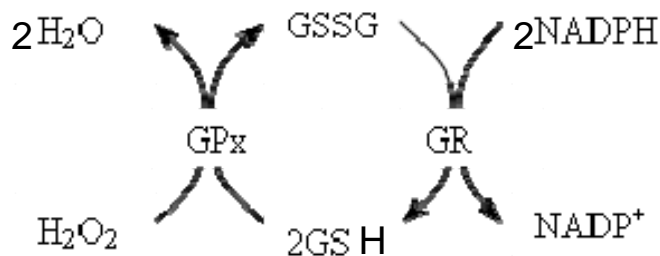
Figure 2.   Switch between reduced GSH and oxidized (GSSG) glutathione.

During the reactions catalyzed by GPx and GR, glutathione is not consumed but recycled (Fig 2). On the other hand, the level of the total intracellular GSH is depleted when the glutathione-S-conjugates are formed by glutathione-S-transferases (GST) or when glutathione is released outside of cells (55). Glutathione consumed by these processes will be replaced by newly synthesized GSH under normal conditions. The reactions catalyzed by GST and γ-glutamyl transpeptidase (γGT) are generally considered as cell defense systems against oxidative stress and xenobiotics. γGT uses extracellular GSH and glutathione conjugates as substrates. With this enzyme, the γ-glutamyl moiety from GSH or a glutathione conjugate are transferred onto an acceptor molecule, thereby forming the dipeptide CysGly or the CysGly conjugate, respectively (158, 233). Ectopeptidases can hydrolyze CysGly to cysteine and glycine, which are consequently taken up by cells and serve as precursors for cellular GSH synthesis again (55).

1.3.3   Regulation of intracellular GSH

As mentioned previously, two enzymes catalyze GSH synthesis, i.e. GCL and GS. GCL, the rate-limiting enzyme in GSH synthesis, is a heterodimer composed of two subunits, GCLM and GCLC. GCLC is the catalytic domain, with a molecular weight of 73kDa and is the target for GSH feedback inhibition (49). GCLM subunit, with a molecular weight of 31kDa, has modulatory or regulatory effects on GCLC. GCLM alone does not exhibit catalytic function (49), but enhances the enzyme activity of GCLC by lowering the K(m) for glutamate and increasing the K(i) to GSH inhibition (41): i.e. it decreases negative feedback inhibition of GCLC by GSH.

A variety of chemicals have been suggested to induce glutathione biosynthesis through increased transcription of GCL. These compounds include those generating ROS and those forming glutathione-conjugates (249). In addition, a large number of conditions are shown to regulate intracellular GSH concentration by increasing its synthesis rate. These conditions include exposure to heavy metal mercury (252), hyperglycemia (240), heat shock (117), ROS such as H_2O_2 (190), nitric oxide (161) or ROS producing compounds (248). Since GCLC is feedback inhibited by GSH, depletion of GSH can lead to a short-term upregulation in GSH synthesis. With some limit, a decrease in GSH will result in a temporary rise in the activity of pre-existing GCL by lessening the feedback inhibition by GSH, inducing a short-term enhancement in GSH synthesis (197). However, increased synthesis of GCL subunits via a combination of increased transcription and mRNA stability is the main reason for increased de novo synthesis of GSH (139, 260).

1.4 GLUTATHIONE AND NEUROLOGICAL DISORDERS

1.4.1 Glutathione in brain

Compared with other organs the brain is particularly sensitive to the generation of ROS and very reliant on detoxification of ROS, since the loss of neurons in adult brain can't generally be compensated by generation of new neurons.

First of all, the brain uses 20% of the oxygen consumed by the body but constitutes only 2% of the body weight (55). Thus, during oxidative phosphorylation in brain, there is a potential to generate a large quantity of ROS. Secondly, some brain areas contain a high content of iron, which can assist the generation of ROS (72). Third, the brain is abundant in lipids with unsaturated fatty acids, which are targets for lipid peroxidation (81). Lastly, the brain has only low to moderate activities of SOD, catalase and GPx compared to other organs such as liver or kidney.

The concentration of GSH in the brain varies from 1 to 3 mM with astrocytes containing higher GSH concentrations than neurons (196). The precursors of GSH can cross blood-brain barrier and therefore GSH precursors in the blood can be used for new GSH synthesis in brain (58). In addition, some precursors for GSH synthesis in neurons, i.e. CysGly and glutamate, are released from astrocytes whose important role in protection against neuronal oxidative stress has been demonstrated (15).

1.4.2   Glutathione and neurological disorders

GSH plays multiple roles in the nervous system such as a free radical scavenger, redox modulator of iontropic receptor activity, and possible neurotransmitter (55). GSH loss can exaggerate oxidative stress and may also increase the levels of excitotoxic molecules. Furthermore, GSH depletion is well known to contribute to neurological damage. Decreased glutathione levels have been implicated in the pathogenesis of PD (17), AD (251), HD (21), ALS (38), cerebral ischemia-reperfusion injury (141) and schizophrenia (219).

The majority (90%) of total cellular glutathione accumulates within the cytoplasm, with the remainder localizes predominantly in mitochondria (194). The mitochondrial pool of GSH functions to maintain normal mitochondrial function by keeping protein thiols unoxidized. In many cells, glutathione in the mitochondria is much more important than the larger cytoplasmic pool in maintaining cell viability and limiting damage to various potentially toxic treatments (254). Following the complete loss of both the mitochondrial and cytoplasmic glutathione, cerebellar granule neurons in culture exhibit marked dysfunction and neurotoxicity (254). Therefore, neurons have a similar dependence on mitochondrial glutathione as other cell types. Loss of GSH in neurons may induce mitochondrial damage and neurological dysfunction such as is seen in PD, AD, multiple sclerosis, stroke and ALS (84, 85).

1.5 NRF2 AND GLUTATHIONE SYNTHESIS REGULATION

1.5.1 Phase II detoxification enzymes and antioxidants

Environmental toxicants and metabolites of xenobiotic chemicals can result in neurodegenerative diseases such as PD and AD (171). To defend cells against oxidative insults, various phase II detoxification enzymes and antioxidants work together to reduce damage caused by oxidative stress (127).

Biotransforming enzymes are categorized as Phase I and Phase II enzymes, which indicate functionalization and conjugation respectively (124, 250). The Phase I enzymes, composed mainly of the cytochrome P450 supergene family of enzymes, are called 'activators'. By adding single nitrogen or oxygen molecules, they modify their target molecules to facilitate their removal as reactive molecules. Conjugation reactions by Phase II enzymes, 'excretors', then follow. Phase II reactions generally result in a xenobiotic chemical being converted into a water-soluble compound that can be removed through the urine or bile (136).

Many reports indicate that phase II detoxification enzymes and antioxidants genes are regulated by an antioxidant responsive element (ARE), which is located within the promoter regions of these genes (99). The genes driven by ARE include GST A1, GST P1, NQ01, Glutamate-cysteine ligase (GCL), ferritin-L, metalloththionein-1, and UDP-glucuronyl transferase (UGT).

Many factors have been suggested to regulate ARE activity. NF-E2-related factor2 (Nrf2), belonging to the basic leucine zipper family of proteins, serves as one of the most important ones. Nrf2, a transcriptional factor, was first identified in 1994 (162) and then illustrated to regulate ARE activation in NQ01 in 1996 (243). Interestingly, Nrf2 is also found to

bind the ARE of GCL (163) and transcriptionally upregulate the basal and inducible GCL subunit genes (99, 101, 247).

1.5.2   Roles of Nrf2 in defense against oxidative stress

A variety of antioxidant and detoxification enzymes are positively regulated by the ARE sequence, including Nrf2 which serves as a regulator of the ARE-driven cellular protection system against ROS and electrophiles. Consistent with this, numerous studies have shown that Nrf2 protects many cell types and organ systems from a broad spectrum of toxic insults through both known ARE-driven genes and novel cell type-specific genes (128). Specifically, Nrf2 plays an important role in protecting cells against various toxins in lung (191), liver (28), GI tract (59) and nervous system (48).

A large body of evidence indicates that oxidative stress is a prominent pathological feature in many neurodegenerative diseases, including ALS, AD, and PD. More recently, the Nrf2-ARE signaling pathway is considered as a promising drug target to combat oxidative stress in neurodegenerative disorders (242).

1.5.3   Signaling pathways regulating Nrf2 activation

The molecular mechanism and signaling pathways that regulate the activation of Nrf2 have been the subject of considerable investigation for many years. Kelch repeat family proteins, such as Keap1 in mouse, sequester Nrf2 within cytoplasm (100) and affect its turnover (115, 210). Nrf2 turns over rapidly via the ubiquitin-26S proteasome protein degradation system (210) with a short half-life from 15 min (170) to 3 h (227). Keap1 is known as a repressor protein for Nrf2

since Keap1 directly binds to Nrf2 and prevents its nuclear accumulation. Oxidative stress and electrophilic agents antagonize Keap1 inhibition of Nrf2 activity in vivo so that Nrf2 accumulates within the nucleus and regulates the transcription of genes encoding detoxification and antioxidant enzymes by binding to their AREs (100). Interestingly, several groups have in parallel indicated that Keap1 functions as an adaptor that bridges Nrf2 to Cullin 3 (cul3)-based E3 ligase (39, 68, 114, 227, 265). Thus, Keap1 negatively regulates Nrf2 stability in part by targeting Nrf2 for ubiquitination by the cul3 and subsequent degradation by the proteasome pathway. Under oxidative stress and electrophiles, the Keap1-mediated Nrf2 degradation pathway is impaired, so that the de novo synthesized Nrf2 does not accumulate within the nucleus (115).

1.5.4   Regulation of Nrf2 activation

Nrf2 activation is regulated by several factors such as transcription, intracellular localization and protein stability. For example, CBP and p300 were shown to mediate Nrf2 transactivation (107, 266). This thesis will focus on one important mechanism of Nrf2 regulation, protein stability.

Some inducers of ARE-dependent genes do not affect the mRNA levels of Nrf2 while leading to an overall increase of Nrf2 protein levels (98, 170, 227, 247). These data indicate that posttranscriptional mechanisms play a crucial role in regulating Nrf2 activity. Further experiments also showed that the half-life of Nrf2 was prolonged in cells exposed to the same inducers, which supported the conclusion that increases in the stability of Nrf2 results in its accumulation within the nucleus. In addition, oxidative stress has been found to activate Nrf2 through effects on its protein stability (170, 227). Specifically, proteasome inhibitors were used

to show a decreased degradation of Nrf2 by the ubiquitin-dependent proteasome pathway. Collectively, stabilization of Nrf2 is sufficient to induce its nuclear accumulation (171).

Protein phosphorylation has also been believed to regulate the stability of Nrf2 protein. The protein phosphatase inhibitor okadaic acid results in accumulation of Nrf2 in HepG2 cells treated with tBHQ (an ARE inducer) (170). It has been reported that the mitogen activated protein kinases (MAPK) signaling pathway may also be involved in the regulation of the ARE response (261, 262). The MAPK family members and pathway will be defined and discussed in next section. For example, the extracellular signal-regulated kinase (ERK) pathway inhibitor, PD98059 or U0126 attenuates the inducing effects of tBHQ on Nrf2 protein levels (170). In addition, inhibitors of the ERK signaling pathway abolished Nrf2 phosphorylation under hypoxic stimuli (179). ERK-directed phosphorylation is required for Nrf2 nuclear translocation during PDTC induced GCLM gene expression (267). In general, phosphorylation by the MAPK/ERK pathway increases Nrf2 stability (170) which may contribute to the upregulation of GCLC and GCLM (267).

A model has been proposed in which xenobiotics (BHA, tBHQ, GTP, EGCG, PEITC, sulforaphane) activate the MAPK pathway via an electrophilic-mediated stress response, leading to the transcriptional activation of Nrf2/Maf heterodimers on ARE/EpRE enhancers. The subsequent induction of cellular defense/detoxifying genes including Phase II enzymes, may then protect cells against toxic insults and thereby enhance cell survival (118).

PKC is the only kinase that has been shown to directly phosphorylate Nrf2 in vitro (95). Although MAPK-dependent phosphorylation may be required for Nrf2 nuclear translocation, results from several groups suggest that it is unlikely that Nrf2 itself is a direct target for MAPK phosphorylation (214, 267). Mutation of the conserved MAPK consensus phosphorylation sites

in Nrf2 did not alter Nrf2 transactivation of GCLM gene expression or Nrf2 interaction with the

Keap1 (267). The activation of Nrf2 pathways have been summarized in Fig 3.
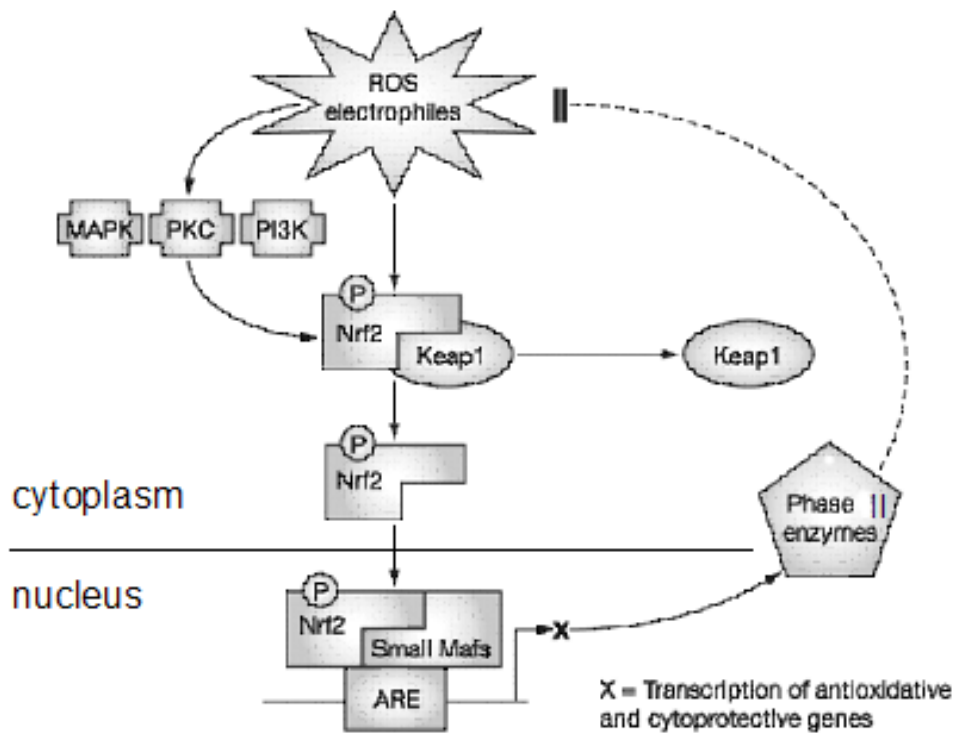
Figure 3.   The simplified mechanisms that can activate Nrf2.
Adapted from (224)

1.6   MITOGEN ACTIVATED PROTEIN KINASES (MAPKS)

1.6.1   MAPKs members

The MAPK family members include extracellular signal-regulated kinase (ERK), stress-activated protein kinase/N-terminal Jun Kinase (SAPK/JNK) and p38 MAPK. All of them have been involved in neuronal injury and disorders (14, 31, 258).

MAPKs are activated upon dual phosphorylation at specific tyrosine and threonine residues by upstream MAPK kinases, which in turn are phosphorylated and activated by MAPK kinase kinases such as Raf. In particular, ERK1 and ERK2, which are activated by the MAPK/ERK kinase-1/2 (MEK1/2), are emerging as important regulators of neuronal responses to both functional (learning and memory) (229) and pathologic stimuli (176).

MAPKs are multi-functional signal transducing kinases that are involved in many facets of cellular regulation including gene expression, cell proliferation, cell survival and cell death (182). MAPKs are activated not only by growth factors but also by stresses such as oxidative stress, heat shock, genotoxins, UV irradiation and inflammatory cytokines (123).

1.6.2   MAPKs and neuronal toxicity

The functions of individual MAPK family members in the brain are diverse. Traditionally, specific MAPK family members such as JNK and p38 MAPK have been associated with neuronal cell death while ERK has been linked to neuronal cell survival. The balance of activation between p38 / JNK and ERK oppose each other to determine whether cells die or survive (255).

However, individual MAPKs do not function exclusively as pro-death or pro-survival factors as a variety of recent papers have demonstrated a role for ERK1/2 in neuronal cell death. Furthermore, recent reports have illustrated a crucial protective role of both p38 (125) and JNK (7) in a variety of models. However, the activation of ERK1/2 contributes to neuronal survival in serum-starved neuronal cultures (255), in an *in vivo* animal model of hypoxia-ischemia (82) and in the brain-derived neurotrophic factor (BDNF) -mediated protection of camptothecin-induced cortical neuronal toxicity (90). Model systems such as glutamate-induced oxidative stress (133, 207, 226), zinc toxicity (212), Fe / ß-amyloid toxicity (122), focal ischemia (4, 175), brain trauma (166), seizure-induced neurotoxicity (168), 6-hydroxydopamine toxicity (121), methylisothiazolinone insults (57), and okadaic acid (OA) toxicity (202) have all exhibited ERK dependent neuronal toxicity. ERK activation not only promotes neuronal toxicity but also cell death in other cell types such as astrocytes, oligodendroglia, vascular smooth muscle, fibroblasts and renal epithelial cells (34).

1.7   ERK AND NEURONAL OXIDATIVE TOXICITY

ERK-1 and ERK-2 (ERK1/2) are two closely related isoforms of the MAPK family. Various reports indicate that ERK signaling, in addition to its classical involvement in proliferation, differentiation (209), learning and memory (256) and cell survival (46), can also promote neuronal cell death and be involved in the pathogenesis of neurodegenerative diseases such as AD and PD (34). For example, oxidative toxicity could be blocked upon inhibition of ERK1/2 activation in a neuronal cell line and primary immature neuron cultures (207, 226). Furthermore, in animal models of stroke, a block of ERK1/2 activation by intravenous injection of U0126, an

inhibitor of the ERK1/2 activator MEK-1, protects against neuronal cell damage after forebrain ischemia and focal cerebral ischemia (169).

It is interesting to note that the role of ERK1/2 can be different even within the same cell type, since activation of ERK1/2 protects HT22 cells from serum withdrawal, but not from glutamate-induced oxidative toxicity (200, 226). The divergent roles of ERK1/2 in various neurotoxicity models are likely because of the heterogeneity in the temporal and spatial pattern of ERK1/2 activation. Increasing evidence suggests that kinetics, intensity and duration of ERK1/2 activation, as well as its subcellular localization, may determine whether downstream targets will trigger beneficial or detrimental effects on neuronal cells (34, 36). Specifically, it has been suggested that persistent nuclear retention of active phosphorylated-ERK1/2 (p-ERK1/2) is associated with glutamate-induced oxidative toxicity (225). In PC12 cells, prolonged nerve growth factor (NGF) -induced differentiation is characterized by a more prolonged, persistent accumulation of ERK within nuclei; however, rapid and transient epidermal growth factor (EGF)-induced proliferation causes a brief activation of ERK without translocation into nuclei (148).

ERK1 (44 kDa) and ERK2 (42 kDa) appear to be similarly regulated in most conditions. It is difficult to distinguish the functions of ERK1 from ERK2 with pharmacological methods, because of the absence of specific ERK1 or ERK2 inhibitors (e.g. MEK1/2 inhibitors such as PD98059 and U0126 inhibit phosphorylation of ERK1 and 2 to comparable levels). Nevertheless, gene knockout mice exhibit obviously different phenotypes. ERK1-deficient mice are viable, fertile, and normal in size. However, ERK1 is implicated with a specific role in the development of thymocytes, as the number of mature thymocytes was decreased to half in the ERK1-deficient mice (178). Furthermore, ERK1-deficient mice show enhanced synaptic

plasticity and learning in striatal-mediated tasks (154), but not in those that are hippocampal- or amydgala-dependent (211). On the contrary, ERK2-deficient mice are embryonic lethal, indicating that ERK2 is more vital for embryonic development and that ERK1 could not compensate for the absence of ERK2 during development (154).

The clearly identified nuclear targets of ERK1/2 have remained fairly limited. Most identified targets are transcription factors, such as CREB, Elk-1, and the AP-1 complex, emphasizing the importance of active ERK1/2 translocation into the nucleus. In addition, ERK may affect transcription indirectly via the activation of cytoplasmic targets, such as p90 ribosomal S6 kinase (RSK), that translocate into the nucleus and activate transcription factors such as estrogen receptor, c-Fos, and CREB (65).

1.8   GLUTAMATE-INDUCED HT22 CELL TOXICITY

1.8.1   Experimental model

Extracellular glutamate acts on neurons via several pathways that include binding to ionotropic receptors (i.e. kainate, AMPA, and NMDA receptors), metabotropic receptors (mGluRs), high affinity glutamate uptake transporters, and a chloride-dependent antiporter (X_c^-).

The X_c^- is ubiquitously expressed in mammalian cells. Extracellular cystine is imported via X_c^- in exchange for intracellular glutamate (206). Cystine is rapidly converted to cysteine in the cytoplasm and used as precursor for protein and GSH synthesis as described above. Intracellular glutamate concentrations are higher inside than outside the cell. Thus, there is a steady and strong concentration gradient that drives this exchange. Due to the low solubility of

cystine (the lowest of all common amino acids) and its propensity to undergo thiol-disulfide exchange reaction with proteins, the intracellular concentration of cystine is very low (97). Intracellular cysteine concentrations are dependent upon the activity of the X_c^- exchanger, and free cysteine is an important precursor for glutathione synthesis. Therefore the activity of the X_c^- exchanger directly regulates glutathione levels in the cell, and inhibition of the exchanger, by increasing extracellular glutamate, leads to the depletion of intracellular glutathione (167). While both neurons and astrocytes express and utilize the X_c^- system, there are no reported differences in the efficiency of this system between the two cell types (5).

In this project, glutamate-induced HT22 cell toxicity is used as experimental model. HT22 cells, derived from mouse hippocampus, lack ionotropic glutamate receptors. Therefore glutamate treatment of these cells leads to cell death through oxidative stress, but not excitotoxity (230). Oxidative glutamate toxicity is induced by inhibition of cystine uptake into cells, leading to depletion of intracellular cystine and the reduction of GSH. With the decreasing supply of GSH, there is a buildup of excessive amounts of ROS in cells (230).

HT22 cells have been used as a neuronal cell line for the study of glutamate-induced oxidative toxicity for more than ten years, but the signaling pathway that causes toxicity in these cells remains controversial. 5mM glutamate can induce HT22 cell death in several hours. Following glutamate treatment in HT22 cells, activation of 12-lipoxygenase (12-LOX), influx of Ca^{2+} into the cells and subsequent ROS accumulation have been demonstrated (135). The characteristics of the Ca^{2+} channels that are involved in Ca^{2+} influx are not clear. Several other studies have revealed that glutamate-induced oxidative toxicity is dependent upon the activation of the phospholipase C (PLC) isoform phosphatidylcholine-specific PLC (PS-PLC) and protein kinase C (PKC) (134, 143). In addition, it has been found that multiple MAPK members are

involved in toxicity and that ERK may play a protective role (143). However, data from our group suggests that the establishment of glutamate-induced oxidative toxicity depends specifically upon the persistent activation of ERK1/2 (226). The pro-death role of ERK has been replicated in both HT22 cells and in immature primary cortical cultures (133, 207, 225, 226). Thus MEK inhibitors such as U0126 protect both HT22 cells and primary cortical neurons from oxidative toxicity and ERK translocation into nuclei. The exact downstream targets of ERK1/2 activation that are required for oxidative toxicity have not been clarified.

1.8.2   Oxytosis

Extensive research studies have suggested that glutamate-induced cell death pathway in HT22 cells or primary cortical culture is clearly distinct from classical apoptosis or necrosis. The unique form of programmed neuronal cell death resulted from oxidative stress and ROS accumulation is therefore named oxytosis (231).

Morphologically, oxidative glutamate toxicity has some of the features of apoptosis such as the requirement for macromolecular synthesis, but it lacks the hallmarks of apoptosis, such as DNA fragmentation and chromatin condensation (231). At the ultrastructural level, in cells under oxytosis, nuclei remain intact with minimal swelling or chromatin condensation. More ultrastructural changes are found in cytoplasm. For example, both inner and outer mitochondria membranes are swollen and cristae are lost. Golgi bodies and endoplasmic reticulum (ER) also appear swollen (232). While in apoptosis cells, intracellular organelles shrink.

Although glutamate-induced neuronal toxicity has some necrotic morphological features, biochemically it resembles some features of apoptosis. For example, macromolecule synthesis is required and selective caspase/protease inhibitors such as Ac-YVAD-CMK, TLCK and TPCK

protect cells from death (232). However, caspase 3 is not activated and involved in glutamate induced neuronal toxicity whereas it is a common feature for apoptosis (232).

Oxytosis may occur by a series of sequential steps: a loss in GSH levels, an increase in the production of ROS and a vast influx of Ca^{++} which occurs shortly before cell death. Other factors such as macromoleular synthesis, lipoxygenase activation, soluble guanylyl cyclase (sGC) activation and cGMP production may also be involved.

1.9   METABOTROPIC GLUTAMATE RECEPTORS AND NEURONAL CELL TOXICITY

1.9.1   Members of the mGluRs

Glutamate receptors are composed of two major superfamilies: the ligand-gated ion-channel ionotropic receptors, and the metabotropic glutamate receptors (mGluRs). mGluRs, with seven transmembrane domains, are coupled to effector systems through GTP binding proteins (G-Protein). Based on sequence similarity, signal transduction, and pharmacology, the mGluRs are further subdivided into three groups, i.e. Group I, II and III mGluRs (37). The mGluRs belong to a family of G protein-coupled receptors (GPCRs).

Group I (mGluRI) consists of mGluRs 1 and 5, and is positively coupled to phosphoinositide hydrolysis via Gq/G11 (60). The first mGluR, which is known as mGluR1a, was cloned and expressed (93, 152) in 1991. Group II, consisting of mGluRs 2 and 3, and group III, composed of mGluRs 4, 6, 7 and 8 are both negatively coupled via Gi to adenylate cyclase (AC) activity. DmGluRA has been cloned from *Drosophila melanogaster*. This metabotropic

glutamate receptor shows a high homology with the mammalian group II mGluRs, and therefore is considered in group II. The existence of multiple isoforms resulting from the alternative splicing of the mGluR genes has been demonstrated during the process of molecular cloning.

mGluRs are widely distributed in mammalian central nervous systems including in the hippocampus, basal ganglia (201), Purkinje cells in cerebellum (113) and even in the visual system (71). Astrocytes are also reported to express certain subtypes of mGluRs (9). In this project, we study HT22 cells which express mGluR1 and 5, but not 2 or 3 (203).

1.9.2   Structure of mGluRs

mGluRs have a N-terminal extracellular domain, seven transmembrane domains (TMDs) separated by short intra- and extracellular loops, and a cytoplasmic C-terminal domain. Their N-terminal extracellular domains are relatively large compared to other GPCRs.

Several amino acids i.e. Arg78, Arg106, Ser165 and Thr188 in the N-terminus are responsible for glutamate reorganization. This region is predicted to form a two-globular binding pocket for glutamate. There is a cysteine rich domain between thre N-terminal loop and TMDs, whose function is not fully resolved. However, this cysteine rich domain may be involved in maintaining three dimensional structure or intramolecular transduction (37, 88). Cys140 and possibly with Cys67 and Cys109 are important for receptor dimerization (239). Certain residues on the intracellular loop of TMDs are critical for the G protein binding. The intracellular C-terminal domain has the phosphorylation sites and regions that interact with other proteins (37, 88). Growing evidence also supports that different regions of the mGluR1 are involved in the functional activation of distinct effector responses (88).

1.9.3   Signal transduction pathway of mGluRs

The signal transduction pathway of mGluRs has not been fully clarified as of yet. Here we take mGluRI as an example. Functional activation of the phospholipase C (PLC)/ inositol 1,4,5-trisphosphate (IP3) /Ca^{++} pathway or PLC/ protein kinase C (PKC) is always detected after mGluRIs activation. Additional coupling to other transduction pathways is dependent on the system in which the mGluRIs have been characterized. Stimulation of cAMP formation through AC has also been reported for mGluR1 in several cell types such as CHO cells (8), BHK (baby hamster kidney) cells (235), and human embryonic kidney 293 cells (69, 180). A similar effect of mGluR5 has also been reported in LLC-PK1 cells (103) but not in CHO cells (1). Further studies have also shown that mGluRI mediated stimulation of AC and PLC are regulated independently rather than at different stages on the same pathway (88). In addition to PLC and AC pathways, mGluRIs are also believed to affect certain cation channels. For example, in cultured primary striatal neurons, mGluRI up-regulates CREB phosphorylation via the intracellular Ca^{2+} release-induced activation of L-type Ca^{2+} channels (147). In cerebellar granule cells, activation of mGluRIs inhibits TASK and TREK type K^{+} channels (29). Moreover, the potential for alternative coupling with other intracellular signaling cascades such as ERK in MAPK (19, 77), PLA2 (8) and PLD (112) has been widely documented. The signal transduction pathway of mGluRI has been illustrated in Fig 4.
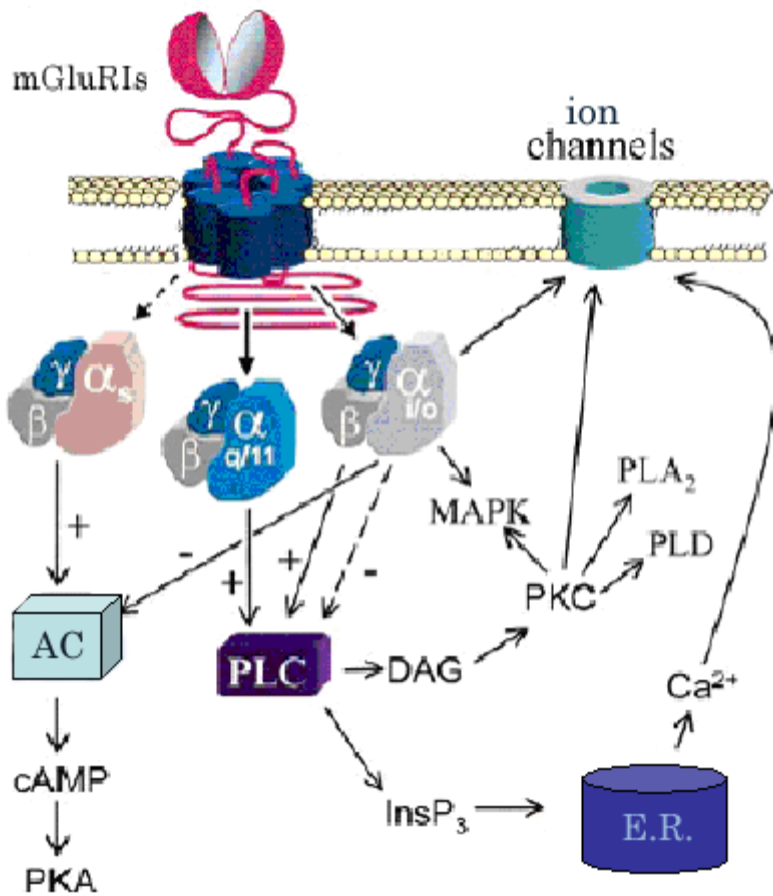
Figure 4.   Signal transduction pathways activated by the mGluR1a through coupling to different G-proteins.
   Adapted from **(88)**

ER: endoplasmic reticulum

PLA_2: phospholipase A_2

PLD: phospholipase D

DAG: diacylglycerol

PKA: cAMP-dependent protein kinase

1.9.4   mGluRs and neuronal cell death

Due to the ubiquitous distribution of glutamatergic synapses, mGluRs have the potential to participate in a wide range of function of CNS. mGluRs have been implicated in a variety of physiological functions, including neurotransmission, long-term potentiation (LTP), and reciprocal interactions with the ionotropic glutamate receptors (37).

mGluRs-mediated neuroprotection against a variety of insults is well established in the literature, and each of the subgroups has been documented to provide neuroprotection (62). They have been shown to be involved in neurotoxic insults, spinal cord injury, ischemia, epilepsy, multiple sclerosis, amyotrophic lateral sclerosis and more recently oxidative stress and diabetes (222).

For instance, due to mGluRs' role in the motor circuit, their ligands may contribute to alleviation of some motor dysfunctions in ALS, HD, PD and cerebellar ataxia (37). As certain mGluRs in thalamic and spinal cord neurons are responsible for noxious stimuli, selective antagonists could be used for the treatment of acute and chronic pain (37). mGluRs also contribute to synaptic plasticity, induction of hippocampal LTP and enhancement cognitive function (70). mGluRIs dysfunction has been suggested to be involved in the pathogenesis of cognitive impairment and dementia in AD (3). Agonists of these receptors may have the potential to act as cognitive enhancing agents in patients with AD or other memory impairments.

The mechanism of action of mGluR likely involves activation of the phosphatidylinositol 3-kinase (PI3K) and MAPK signaling pathways, which in turn are likely lead to decreased ROS concentration and ultimately a decrease in oxidative stress and programmed cell death (54, 96). Additional evidence suggests that mGluRs regulate ROS production and oxidative stress albeit by different receptor groups depending upon the neurotoxic insult and neuronal type. Activation

of mGluRII by mGluR3 agonist APDC prevents the formation of ROS in the mitochondria and blocks caspase activation in DRG neurons in response to elevated glucose levels (222). More interestingly, Sagara & Schubert showed that mGluRI activation prevents oxidative stress-induced cell death in HT22 cells, most likely due to buffering of ROS by GSH (230).

2.0   THESIS GOALS

The goal of work presented in this thesis is to examine the mechanism of ERK1/2 activation under both pro- and anti-apoptotic conditions in a single cell type and investigate the relationship between these contrary actions of ERK1/2. This will be accomplished through the following two specific aims:

Aim 1: To determine whether ERK1/2 activation is necessary and sufficient for glutamate induced cell toxicity.

Aim1: Rationale: Previous reports from our lab have established that ERK1/2 activation is biphasic with peak activation initially occurring within 30 min to 1 hr of 5mM glutamate treatment followed by a second peak of activation that initiates within 6 hr of glutamate exposure. U0126 treatment not only blocks ERK1/2 activation but also cell toxicity induced by glutamate. The second peak of ERK1/2 activation may be relevant for toxicity since delayed U0126 addition (2 hr following glutamate) is protective. However, the role of ERK1/2 in neuronal cell death is controversial. In contrast to the results found by our lab, studies from other groups have indicated that phosphorylation of ERK1/2 is essential to protect HT22 cells from death triggered by a variety of stimuli such as withdrawal of serum (200). Our previous studies were mainly based on a pharmacological strategy where U0126

was used to block ERK1/2 activation. More selective molecular tools, such as overexpression of a dominant negative mutant of the ERK1/2 activating kinase, MEK-1, would be useful to confirm the role of ERK1/2 in glutamate-induced oxidative toxicity. The results from these studies are presented in Chapter 3.

Aim 2: To examine the impact and mechanism of mGluR activation on oxidative toxicity and ERK1/2 activation.

Aim2: Rationale. HT22 cells express metabotropic glutamate receptors (mGluRs) that are also activated in response to glutamate treatment. mGluR-mediated neuroprotection against a variety of insults is well established in the literature, and each of the subgroups has been documented to provide neuroprotection. For example, in HT22 cells and primary cortical neurons specific agonist and antagonist of mGluR type I (mGluRI) have anti- and proapoptotic roles, respectively (203). The mechanism of the neuroprotective action of mGluRI in these cells is not clear although it is likely to involve activation of the phosphatidylinositol 3-kinase (PI3K) and ERK1/2 signaling pathways. Thus, ERK1/2 activation may serve opposing roles in neuronal oxidative toxicity acting through mGluRs to promote survival while affecting distinct targets to induce cell death.

The results of these studies shown in Chapter 3 provided insights into the link between mGluR activation, ERK1/2 activation and glutathione depletion in glutamate-induced oxidative toxicity in HT22 cells. Specifically, I examined whether the early phase of ERK1/2 activation would provide a protective function against glutamate toxicity in HT22 cells through limiting glutathione depletion. In order to identify the mechanism of ERK1/2 effects on GSH

metabolism, I examined potential targets of ERK1/2 during the early and late phase of induction that may act to limit glutathione depletion. Glutathione synthesis, as discussed above is controlled by the first enzyme (rate limiting enzyme) in its synthetic pathway, GCL, which generates γ-L-glutamyl-L-cysteine (GC) from glutamate and cysteine. Therefore, I hypothesize that ERK1/2 may modulate GSH through regulating GCS, which is possibly dependent on the transcrition factors such as Nrf-2 or AP1. The results from these studies are presented in Chapter 4.

3.0 DISTINCT MECHANISMS OF ERK1/2 ACTION AT EARLY VERSUS LATE PHASES OF OXIDATIVE STRESS

3.1 SUMMARY

Glutamate-induced oxidative toxicity is mediated by glutathione depletion in the HT22 mouse hippocampal cell line. Previous results with pharmacological agents implicated the extracellular signal-regulated kinases-1/2 (ERK1/2) in glutamate toxicity in HT22 cells and immature embryonic rat cortical neurons. In this report, we definitively establish a role for ERK1/2 in oxidative toxicity using dominant negative MEK1 expression in transiently transfected HT22 cells to block glutamate-induced cell death. In contrast, chronic activation of ERK (i.e. brought about by transfection of constitutively active ERK2 chimera) is not sufficient to trigger HT22 cell death demonstrating that ERK1/2 activation is not sufficient for toxicity. Activation of ERK1/2 in HT22 cells has a distinct kinetic profile with an initial peak occurring between 30 minutes and 1 hour of glutamate treatment and a second peak typically emerging after 6 hours. We demonstrate here that the initial phase of ERK1/2 induction is due to activation of metabotropic glutamate receptor type I (mGluRI). ERK1/2 activation by mGluRI contributes to an HT22 cell adaptive response to oxidative stress as glutamate induced toxicity is enhanced upon pharmacological inhibition of mGluRI. The protective effect of ERK1/2 activation at early times after glutamate treatment is mediated by a restoration of glutathione (GSH) levels that are

reduced due to depletion of intracellular cysteine pools. Thus, ERK1/2 appears to play dual roles in HT22 cells acting as part of a cellular adaptive response during the initial phases of glutamate-induced oxidative stress and contributing to toxicity during later stages of stress.

3.2   INTRODUCTION

Oxidative stress can contribute to neuronal toxicity and has been implicated in both acute injury and chronic neuropathological conditions (183, 217). Many in vitro models have been used to examine the mechanistic basis for neuronal cell death induced by oxidative stress. For example, oxidative toxicity can be induced by glutamate treatment in the HT22 mouse hippocampal cell line (42, 134, 226) and immature primary embryonic rat cortical neuron cultures (133, 134, 193). In these models, glutamate treatment leads to glutathione (GSH) depletion and subsequent accumulation of reactive oxygen species (ROS) (230). Many intracellular second messenger pathways are required for oxidative toxicity in HT22 cells including, arachidonic acid metabolites, cyclic GMP (cGMP) and calcium (230). In addition, signaling kinases, such as mitogen-activated protein kinases (MAPK), are activated upon glutamate-induced oxidative stress in HT22 cells and primary neurons and are likely to affect targets that either limit or promote oxidative toxicity.

Extracellular signal-regulated kinases-1/2 (ERK1/2) has been implicated in glutamate-induced neuronal oxidative toxicity based upon the neuroprotective effects of U0126, a specific inhibitor of the ERK1/2-activating kinase, MEK-1/2 (226). U0126 is also effective at reducing brain injury following focal ischemia in rodents suggesting that ERK1/2 may also promote neuronal cell death resulting from acute injury *in vivo* (169). However, the role of ERK1/2 in

41

neuronal cell death remains controversial (26, 34, 89). In a number of studies, ERK1/2 has been found to promote neuronal survival and reduce cell death induced by various insults (36, 75, 221). Furthermore, even in HT22 cells, ERK1/2 may limit toxicity in response to specific insults such as serum withdrawal (200).

Distinct kinetic profiles of ERK1/2 activation are observed in response to different extracellular signals and also can be associated with differential compartmentalization of active ERK1/2 within the cell. For example, in PC12 cells, prolonged, NGF-induced activation of ERK1/2 leads to its persistent accumulation within nuclei; however, rapid and transient EGF-induced activation of ERK1/2 is unable to trigger its efficient nuclear translocation (36, 148). Interestingly, increasing evidence suggests that the kinetics and duration of ERK1/2 activation, as well as its subcellular localization, may direct ERK1/2 towards downstream targets that will either promote or limit neuronal survival (34, 36, 121).

In HT22 cells and primary neurons, glutamate induces a biphasic activation of ERK1/2 (226). Persistent activation of ERK1/2 in these cells is mediated primarily through the oxidative inhibition of select ERK phosphatases (132). However, the mechanisms responsible for the initial rapid activation of ERK1/2 by glutamate in HT22 cells and primary neurons are not known. Furthermore, the impact of the first wave of ERK1/2 activation on the response of HT22 cells and primary neurons to oxidative stress has not been established.

In this report, we show that rapid activation of ERK1/2 by glutamate in HT22 cells is driven by type I metabotropic glutamate receptors (mGluRIs). Furthermore, mGluRIs activation of ERK1/2 represents a cellular defense response that attempts to limit glutathione depletion resulting from glutamate induced cysteine depletion. This protective response mediated by ERK1/2 is however unable to overcome an overwhelming and chronic oxidative stress that

utilizes ERK1/2 in its final stages to promote neuronal cell death. Thus, ERK1/2 activation may serve opposing roles in neuronal oxidative toxicity acting initially through effects on glutathione metabolism to limit oxidative stress, but serving as a necessary signal to trigger cell death when cellular defense against oxidative stress is exhausted.

3.3   MATERIALS AND METHODS

Plasmids.

The expression plasmids for LAERK2-MEK1 and ERK2-MEK1 were kind gifts from Dr. Melanie Cobb (198). pMCL-MEK1 Lys-97$\rightarrow$Met (K97M), an HA-tagged dominant negative (DN) mutant of MEK1 was provided by Dr. Jane Cavanaugh (146). The mitochondrial-targeted enhanced yellow fluorescent protein (eYFP) expression plasmid was a gift from Dr. Ian Reynolds, while the expression plasmid for the ELK-1/GAL4 fusion protein, the luciferase reporter, and the constitutive renilla reporter plasmids were obtained from Strategene (La Jolla, CA).

Cell culture.

HT22 cells, a mouse hippocampal cell line, were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), 100 units of penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO_2.

Cell viability assay.

HT22 cells grown in 24 well plates were incubated for 10 min with 1 μl (1:1000 dilution) of a 6.25 mg/ml solution of propidium iodide (PI) to visualize dead or dying PI-positive cells (133). Cells were observed under an inverted fluorescence microscope equipped with phase-contrast optics (Nikon Eclipse TE200). Three random fields were counted for each condition in at least three separate cultures. For experiments that did not involve transfection, the fraction of PI-positive cells was scored relative to total cells per field visualized by phase contrast microscopy. In transfection experiments, the extent of PI positive staining was scored in eYFP-positive transfected cells.

Transfection and Western blotting.

Cells were transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA) using conditions recommended by the supplier. During transfection, cells were maintained in serum- and antibiotic-free medium. Following 4 h of exposure to DNA-lipofectamine mixture, cells were refed with medium containing 10% FBS.

On the following day, cells were washed, scraped and collected into ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.4), pelleted at $2-3 \times 10^3$ x g for 5 min, and disrupted in Lysis Buffer (50 mM Tris-Cl, pH 7.5, 2 mM EDTA, 100 mM NaCl, 1% Nonidet P-40, 100 μM NaVO4, 100 μM NaF, 2 mM dithiothreitol) supplemented with 5 μl/ml of a protease inhibitor cocktail (Sigma Chemical Corporation, St. Louis, MO). The lysates were then centrifuged at 14,000 x g for 10 min at 4°C. Equivalent amounts of total protein, 30 μg, were separated by SDS-PAGE on 10% polyacrylamide gels and then transferred to polyvinylidene fluoride membranes (Millipore,

Bedford, MA). Membranes were blocked with 5% dry milk in PBS, 0.1% Tween 20. Membranes were then incubated with primary antibodies (anti- phospho- ERK and anti-total ERK both from Cell Signaling, Beverly, MA) overnight at 4°C with 3% dry milk, washed 3 x 10 min with PBS, 0.1% v/v Tween 20, and then exposed to the appropriate horseradish-peroxidase conjugated secondary antibody for 1 h at room temperature. Membranes were again washed 3 x 10 min with PBS, 0.1% Tween 20, and immunoreactive bands were detected by enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) using standard x-ray film (Kodak, Rochester, NY). Several different exposure times were used for each blot to ensure linearity of band intensities. Densitometry was performed using a Personal Densitometer SI (Molecular Dynamics, Amersham Biosciences) linked to the ImageQuant 5.2 software (Molecular Dynamics).

Luciferase assay.

Components of the Path Detect In Vivo Signal Transduction Pathway trans-Reporting System (Stratagene, La Jolla, CA) were used in order to monitor ELK-1-dependent transcription in HT22 cells (132). Cells were transfected with an expression plasmid coding for an ELK-1/GAL4 fusion protein, a reporter plasmid containing the luciferase gene linked to a synthetic promoter containing 5 tandem GAL4 binding sites, a renilla expression plasmid, and various MEK1 expression plasmids (i.e. DN-MEK1, LA-MEK1ERK2). In this system, luciferase activity is a measure of the extent of ELK-1 activated transcription. Both luciferase activity and renilla activity were examined with the Dual-Glo Luciferase Assay System (Promega, Madison, WI) using a luminometer (Wallac Victor3, Perkin Elmer Biosciences, Boston, MA) according to

the manufacturer's specifications. Results shown were from at least three separate experiments. All luciferase values were normalized to the internal control, renilla, within each sample.

Glutathione concentration measurement.

A glutathione assay kit from Cayman Chemical Company (Ann Arbor, MI) was used to measure glutathione concentrations. Cells from two 60 mm plates were collected and sonicated in 50 μl ice-cold lysis buffer (i.e. 50 mM MES buffer, pH6-7, containing 1 mM EDTA). After centrifuging at 10,000 x g for 15 min at 4°C, the supernatant was collected for deproteination with the exception of a small amount of cell lysate that was used to determine lysate protein concentration. Deproteination was necessary to avoid interference in the assay due to particulates and protein sulfhydryl groups. An MPA reagent (10% metaphosphoric acid in water; Sigma) and 4 M TEAM reagent (triethanolamine from Sigma) were used to deproteinize lysates. An equal volume of ice-cold MPA was added to ice-cold supernatant and left on ice for 10 min. Samples were then centrifuged at 14,000 x g in a microfuge for 5 min at 4°C. 50 μl of TEAM reagent per ml of supernatant was added to supernatant. 50 µl of sample or standard provided in the kit was applied to each sample well. An Assay Cocktail mix was prepared with the following reagents: MES Buffer (11.25 ml), reconstituted Cofactor Mixture (0.45 ml), reconstituted Enzyme Mixture (2.1 ml), water (2.3 ml), and reconstituted DTNB (0.45 ml). 150 µl of the freshly prepared Assay Cocktail was added to each of the wells containing standards and samples. The plate was incubated in the dark on an orbital shaker at room temperature. Absorbance was measured at 405 or 414 nm using a plate reader 25 min later. An end point method was used to calculate and determine the sample glutathione concentration according to the instructions provided by the supplier. All glutathione concentration values were normalized to the protein concentration within each sample.

Statistical analysis.

Comparisons of multiple mean values were accomplished by analysis of variance (ANOVA) with Bonferroni's post-hoc tests for significance. Comparisons of two means were performed using a paired t-test. P values less than 0.05 were taken to be significant, and all data was analyzed using Graph Pad Prism version 3.0 for Windows (GraphPad Software, San Diego California USA).

3.4   RESULTS

3.4.1   ERK1/2 activation is necessary for glutamate-induced oxidative toxicity in HT22 cells

ERK1/2 has been implicated in both neuronal cell survival and cell death although the basis for its disparate effects is not known. In fact, even in HT22 cells, opposing roles for ERK1/2 have been proposed (143, 200, 226). Since part of the discrepancy for ERK1/2's role in neuronal cell survival may be due to secondary effects of pharmacological agents used to block ERK1/2 activation, we used a molecular approach to definitively assess the role of ERK1/2 in HT22 cell response to oxidative stress.

HT22 cells were therefore cotransfected with dominant negative MEK1 (DN-MEK1) and mitochondrial-targeted enhanced yellow fluorescent protein (mt-eYFP) expression plasmids. Since the cotransfected plasmids enter cells with equal probability, eYFP positive cells were likely to contain DN-MEK1 DNA. DN-MEK1 contains a point mutation at position 97 (i.e. lysine to methionine) that inactivates its kinase activity (146). As a result, the DN-MEK1

interferes with activation of wild type MEK1. Cell death was measured in transfected cells 7 h after 5 mM glutamate treatment using a PI staining assay (133). As shown in Figure 5A, DN-MEK1 overexpression blocked glutamate induced oxidative toxicity in HT22 cells. These data confirm our previous results with a pharmacological inhibitor of MEK1 (i.e. U0126) and demonstrate that ERK1/2 activation is necessary for glutamate toxicity.

To confirm the inhibitory effect of DN-MEK1 on ERK1/2 activation, we used a sensitive assay to detect functional ERK1/2 in nuclei. Specifically, HT22 cells were transfected with the DN-MEK1 expression plasmid along with an expression vector for an ELK-1-GAL4 fusion protein and a luciferase reporter gene under the control of a promoter containing 5 tandem GAL4 DNA binding sites i.e. GAL4 UAS-luciferase (UAS: upstream activator sequences). Given that ELK-1 is an established nuclear target of ERK1/2, enhanced transactivation activity resulting from its phosphorylation by nuclear localized ERK1/2 is easily monitored through the activity of the luciferase reporter (132).

As shown in Figure 5B, 10 μM U0126 treatment decreased luciferase activity from GAL4UAS-reporter significantly in both untreated and glutamate treatment HT22 cells. Luciferase activity from the Gal4UAS-luciferase reporter was induced nearly two-fold following a 7 h treatment with 5 mM glutamate (Fig 5B) consistent with previous results of ERK1/2 activation using this assay (132). The expression of DN-MEK1 significantly reduced luciferase activity from the GAL4UAS-reporter in glutamate treated cells (Fig 5C). The reduced effectiveness of DN-MEK1 to block ELK-1 activated transcription relative to U0126 treatment most likely results from variable expression of the DN-MEK1 within individual transfected cells. Nonetheless, these results confirm the expected inhibition of ERK1/2 activity in the nucleus of HT22 cells upon overexpression of DN-MEK1.

3.4.2 Prolonged ERK activation is not sufficient to induce toxicity in HT22 cells

In order to assess whether chronic activation of ERK was sufficient to induce toxicity in HT22 cells, we used constitutively active (CA) ERK2 chimeric proteins. MEK1ERK2 is a fusion protein with constitutive ERK2 activity that localizes in the cytoplasm (198). In the CA ERK2 fusion LAMEK1ERK2, four leucines in MEK1 that are crucial for its nuclear export have been mutated to alanines (67). As a result, the LAMEK1ERK2 fusion protein is localized in the nucleus (198). Furthermore, by virtue of its retention within nucleus, LAMEK1ERK2 exhibits a 5-10 fold higher ERK2 activity than MEK1ERK2 (198). To verify the activation of ERK signaling by LAMEK1ERK2, the Path Detect in Vivo Signal Transduction Pathway trans-Reporting System was used to access ELK-1-dependent gene expression. MEK1ERK2/LAMEK1ERK2 expression plasmids along with an expression vector for an ELK-1/GAL4 fusion protein and the GAL4UAS-luciferase reporter gene were transfected into HT22 cells. Cell lysates were collected and luciferase activity was measured 18 h after transfection. As shown in Figure 6A, LAMEK1ERK2 overexpression can induce robust ERK1/2 activation compared to that of MEK1ERK2. The enhanced activation of the GAL4UAS luciferase reporter in LAMEK1ERK2 transfected cells likely results from its more efficient localization within nuclei.

We then accessed the effects of LAMEK1ERK2 overexpression on HT22 cell viability using the PI staining method. As shown in Figure 6B, both LAMEK1ERK2 and MEK1ERK2 transfected cells showed no enhanced toxicity as compared to empty vector transfected HT22 cells. Robust ERK activation upon overexpression of LAMEK1ERK2 chimera also did not unleash toxicity in HT22 cells exposed to a low dose of glutamate (1.5 mM). As will be shown below, 1.5 mM glutamate can trigger cell death under conditions of altered mGluRIs activity.

Thus, although necessary, prolonged ERK activation is not sufficient to induce toxicity in HT22 cells nor does it predispose cells to toxicity under conditions of minimal glutamate exposure.

3.4.3   Metabotropic glutamate receptors group I (mGluRIs) are involved in glutamate-induced oxidative toxicity

HT22 cells express both type 1 and type 5 metabotropic glutamate receptors (mGluR1 and mGluR5) (203). These receptors are likely to be activated during glutamate exposure that triggers oxidative stress. Since mGluRIs have been suggested to play a protective role in glutamate toxicity (203), we sought to examine their role in promoting or attenuating ERK1/2 dependent toxicity in HT22 cells. HT22 cells were therefore pretreated with a mGluRI antagonist (*R,S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) or mGluRI agonist (*R,S*)-3,5-dihydroxyphenylglycine (DHPG) at 500 or 1000 μM respectively. Glutamate was added 30 min later at 5 mM (maximally toxic dose) or at 1.5 mM, a dose that exhibits minimal cytotoxic effects. Seven hours after 5 mM glutamate and 22 h after 1.5 mM glutamate treatment, cell viability was measured by PI staining. 1.5 mM glutamate treatment of HT22 cells for 22 h only results in a 17% reduction in cell viability as assessed by PI staining (Fig 7A). However, a 30 min pretreatment with AIDA potentiated toxicity in the presence of 1.5 mM glutamate resulting in 66% cell death after 22 h (Fig 7A). In contrast, a 30 min pretreatment of HT22 cells with DHPG significantly reduced the minimal toxicity induced by 1.5 mM glutamate treatment to 5% (Fig 7A). By blocking ERK1/2, the toxicity observed in HT22 cells treated with glutamate and AIDA or DHPG was reduced. In HT22 cells treated with 5 mM glutamate, AIDA and DHPG have similar effects as those observed in 1.5 mM glutamate treated cell (Fig 7B). These results confirm that mGluRIs play a role in limiting oxidative toxicity induced by glutamate treatment

(203) and establish that limiting mGluRIs activation promotes ERK1/2 dependent toxicity at sub-threshold levels of glutamate.

3.4.4   Effects of mGluRIs activation on the ERK1/2 activation in glutamate-induced oxidative toxicity

Given the protective effects of mGluRIs activation on ERK1/2 dependent oxidative toxicity in HT22 cells, we set out to investigate whether mGluRIs impacts ERK1/2 activation. Western blots were therefore performed with lysates prepared from HT22 cells treated with 1.5 mM glutamate and AIDA or DHPG and probed to detect phosphorylated ERK1/2. In order to assess the kinetics of ERK1/2 activation under the various conditions, protein samples were then collected at different time points (i.e. 30 min, 1 h, 6 h, 10 h and 22 h) following glutamate addition. In all cases, the extent of ERK1/2 activation was measured relative to total ERK1/2 quantified from stripped blots (e.g. see Fig 9).

As shown in Figure 8A, treatment of HT22 cells with 1.5 mM glutamate alone induced a 3 fold activation of ERK1/2 that peaked within 30 min. ERK1/2 activation did not persist and eventually was reduced to levels approximately 40% below baseline following 10 h of treatment with 1.5 mM glutamate (Fig. 8A). Continued exposure to 1.5 mM glutamate alone (i.e. 22 h total) generated a second peak of ERK1/2 activation (Figs. 8A and 9B).

A role for mGluRIs in rapid transient activation of ERK1/2 by 1.5 mM glutamate was supported by the observed prevention of this activation by a 30 min pretreatment with AIDA (Fig 8B). Specifically, the early phase of ERK1/2 activation (i.e. within 30 min of glutamate treatment) was reduced 34% by AIDA (Fig 9A). The addition of DHPG along with 1.5 mM glutamate did not lead to a significant effect on the initial phase of ERK1/2 activation (Fig 9A).

Base line levels of activated ERK1/2 during the nadir between early and late phases of activation were often lower than that observed in unstimulated cells. Therefore, we compared the extent of ERK1/2 activation at 22 h relative to 10 h of glutamate treatment to more clearly illustrate the late phase of activation (Fig 9B).

Using this approach, we observed that AIDA enhanced the second phase of ERK/2 activation nearly 4 fold (Fig 9B). However, continuous exposure of HT22 cells to 1.5 mM glutamate and DHPG did not generate a second peak of ERK1/2 activation (Fig 9B). These results identify a dual role for mGluRIs in regulating ERK1/2 activation. Activation of mGluRIs by glutamate triggers activation of ERK1/2 that may function to limit toxicity. However, in the absence of mGluRIs activation, a delayed activation of ERK1/2 is enhanced that functions to promote cell death.

3.4.5 The role of ERK1/2 in glutathione depletion

Glutamate induced oxidative toxicity is triggered by glutathione depletion (230). A role for ERK1/2 in this component of glutamate toxicity has not been established. We therefore measured intracellular glutathione concentrations at various times after glutamate treatment in the presence or absence of U0126. Consistent with previous studies, a 5 mM glutamate treatment induced prolonged glutathione depletion in HT22 cells (Fig 10A). GSH levels dropped to 84% in 2 h and 33% in 6-8 h relative to basal line levels. Although U0126 protects HT22 cells from glutamate toxicity, it does not trigger the restoration of GSH levels in glutamate treated HT22 cells (Fig 10B). In this experiment, U0126 was added coincident with glutamate and therefore blocked both early and late phases of ERK1/2 activation. However, when U0126 was added 2 h following 5 mM glutamate treatment to allow for the early but not late phase of ERK1/2

activation, GSH levels returned to about 82% of basal line levels within 22 h (Fig 10C). This delay in U0126 treatment protects HT22 cells from glutamate toxicity (226). Thus, ERK1/2 activation via mGluRIs signaling within the first few hours of glutamate treatment is required for HT22 cells to restore GSH levels that were reduced due to cysteine depletion.

As illustrated above, a suboptimal dose (i.e. 1.5 mM) of glutamate generates minimal toxicity in HT22 cells that is only apparent following prolonged exposure (22 h). Therefore, we investigated the kinetics of GSH depletion in response to a low dose of glutamate. Under these conditions, ERK1/2 activation is observed only at early times after glutamate addition. 1.5 mM glutamate induced an early depletion of GSH in 4-8 h to about 27% of basal line levels, which is comparable to the extent of GSH depletion in 5 mM glutamate treated cells. However, a rebound in GSH levels occurred between 16-22 h which restores GSH to basal line levels (Fig 10D). Interestingly, inhibition of mGluRIs activation by a 30 min pretreatment with AIDA abolished the rebound in GSH levels that occurs in response to a 1.5 mM glutamate treatment. GSH levels remained low (i.e. 18-46% of basal line levels) from 4-22 h (Fig 10E). Under these conditions, only the late phase of ERK1/2 activation is observed. Thus, in the absence of mGluRIs activation, a compensatory mechanism that acts to restore GSH levels is not operating and cell death can result in HT22 cells from even a minimal exposure to glutamate.
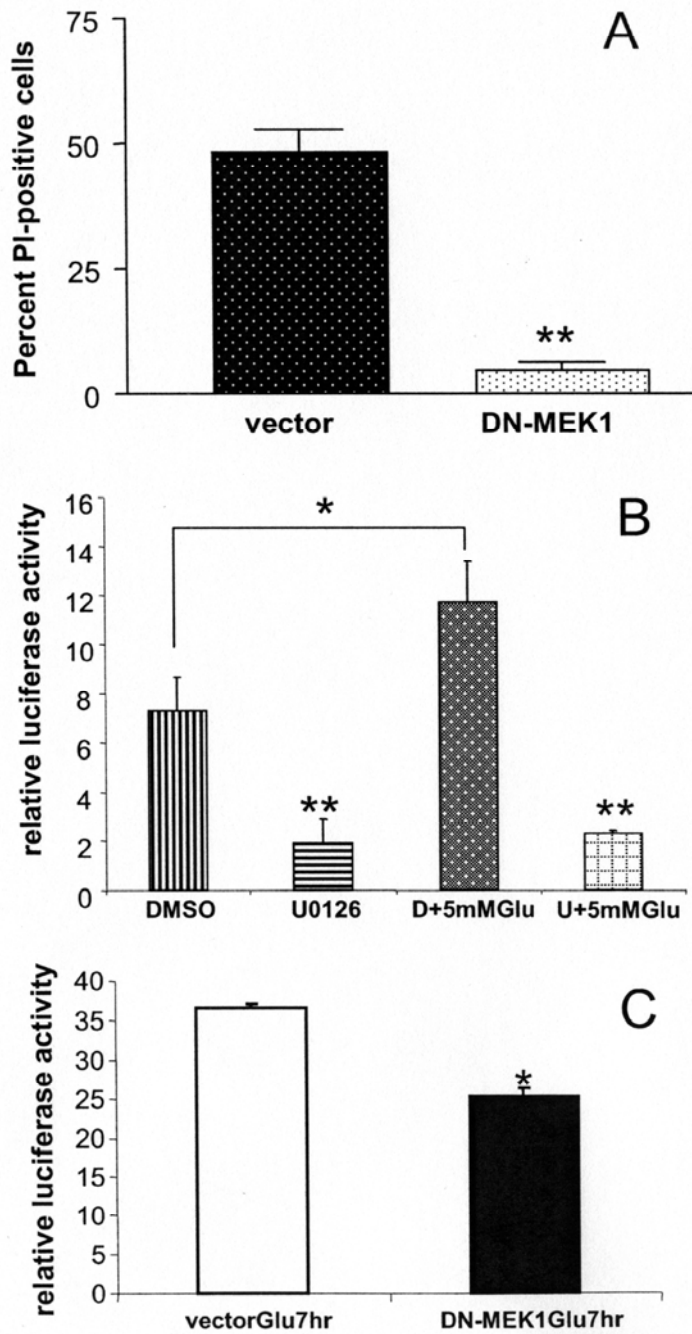
Figure 5. DN-MEK1 reduces ERK1/2 activition and blocks glutamate-induced toxicity in HT22 cells.

(A) HT22 cells were cotransfected with either an empty plasmid vector or DN-MEK1 and mt-eYFP. The following day, cell death was induced by a 7 h treatment with 5 mM glutamate. Cell death in transfected cells was measured using a PI staining assay and was

expressed as the percentage of PI and mt-eYFP double positive cells relative to the total mt-eYFP positive cells. The percentage of cell death was decreased from 48% to 5% by expression of DN-MEK1. Data shown are from 3 separate experiments (** P<0.01).

(B) HT22 cells were transfected with an empty plasmid vector and then treated with either DMSO, 10 μM U0126, 5 mM glutamate or glutamate plus U0126. Luciferase activity was measured from a cotransfected ELK-1 reporter plasmid. Luciferase activity from the Gal4UAS-luciferase reporter was induced nearly two-fold upon a 7 h 5 mM glutamate treatment. However, U0126 inhibited luciferase activity in both DMSO and glutamate treated groups (C) DN-MEK1 expression decreased ELK-1 driven luciferase activity in cells treated for 7 h with 5 mM glutamate. (*P<0.05, **P<0.01).
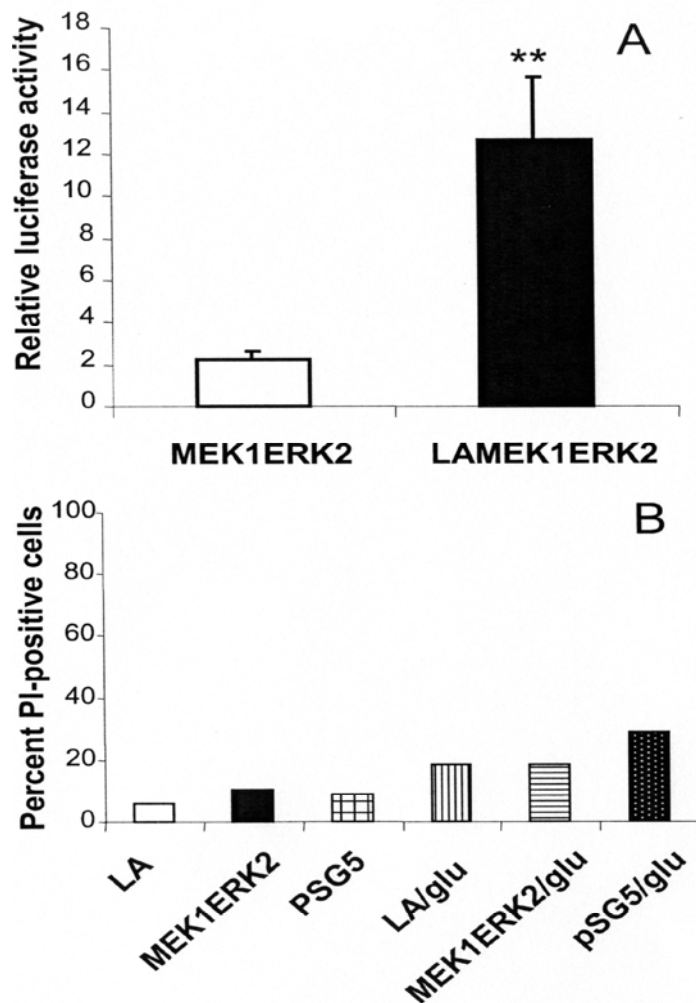
Figure 6. Chronic activation of ERK1/2 is not sufficient to induce HT22 cell death.

(A) A constitutively active (CA) LAMEK1-ERK2 fusion plasmid was transfected into HT22 cells and luciferase activity measured from an ELK-1driven luciferase reporter plasmid. Expression of LAMEK1ERK2 plasmid induced a robust increase in luciferase activity compared to transfections with a MEK1ERK2 plasmid (**P<0.01).

(B) HT22 cells were transfected with LAMEK1ERK2 (LA), MEK1ERK2 fusion plasmid or empty vector PSG5 (PSG5). Cell toxicity was detected using the PI staining assay in the presence or absence of a 22 h 1.5 mM glutamate treatment (Glu). Activation of ERK1/2 in LA transfected cells did not trigger cell death (*P<0.05).
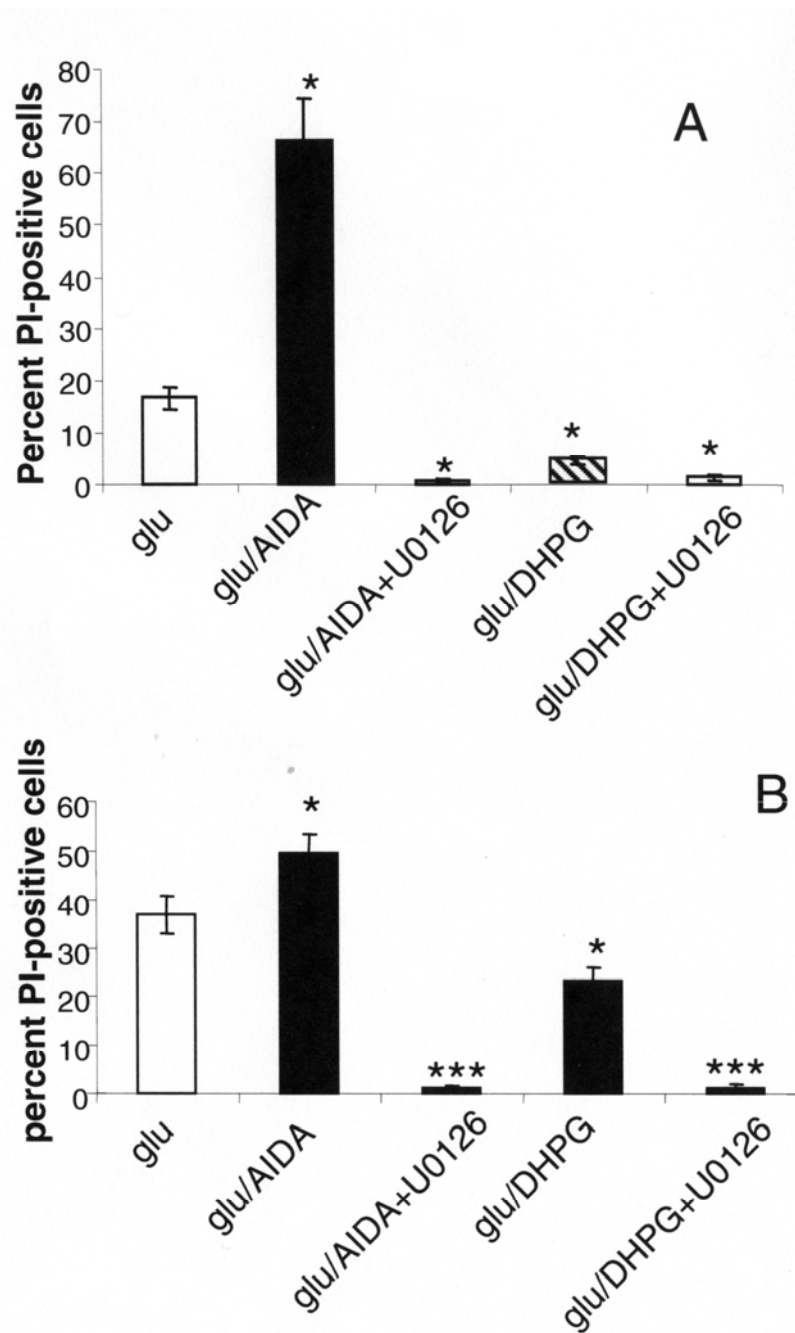
Figure 7. Effects of mGluRI activation or inactivation on glutamate toxicity in HT22 cells.

The percentage of cell death was measured using a PI staining assay as described above. The mGluRI antagonist AIDA enhanced glutamate (Glu) toxicity while the mGluRI agonist DHPG decreased glutamate toxicity at both 1.5 mM (A) and 5 mM glutamate (B). In both groups, U0126 prevented cell death ($*P<0.05$, $***P<0.001$).
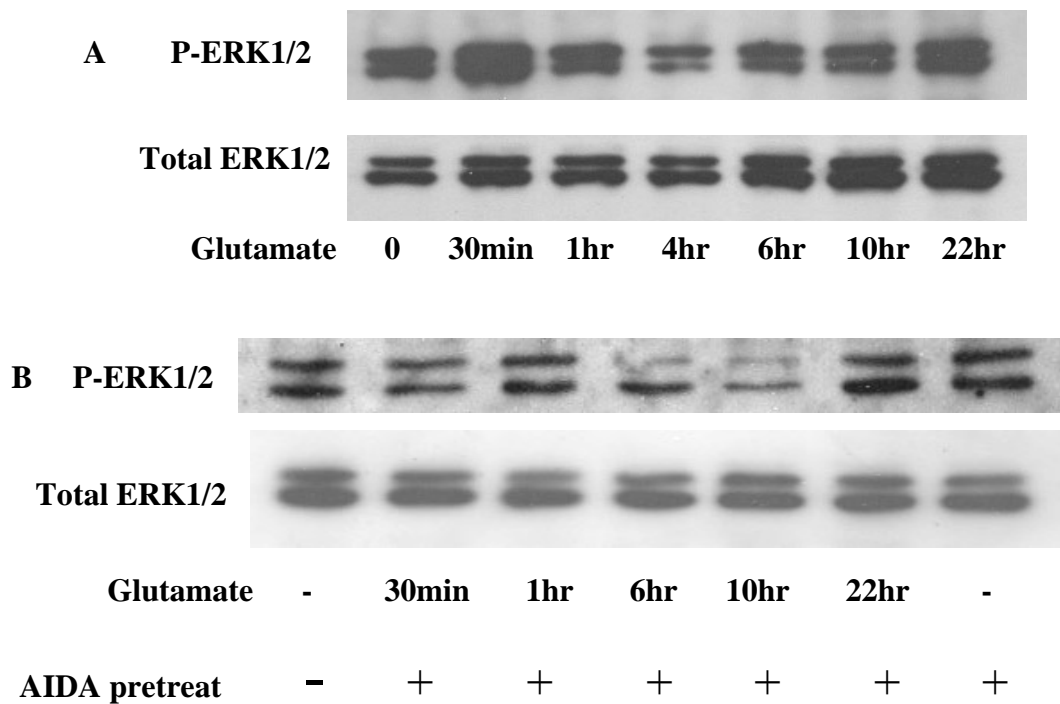
Figure 8. Kinetics of ERK1/2 activation following glutamate treatment.

Western blot analysis in A, B and C show p-ERK1/2 and total ERK1/2 levels in lysates prepared from HT22 cells treated for various lengths of time with 1.5 mM glutamate. (A) Treatment of HT22 cells with 1.5 mM glutamate alone induced a transient 3 fold activation of ERK1/2 (n=3) within 30-60 min. ERK1/2 activation did not persist and returned to basal line levels within 10 h. Upon continuous exposure to 1.5 mM glutamate, activated ERK1/2 levels gradually increased above its nadir attained between 4-6 h following glutamate addition. (B) Pretreatment of HT22 cells with AIDA blocked the transient activation of ERK1/2 while enhancing the delayed secondary rise in ERK1/2 activation.
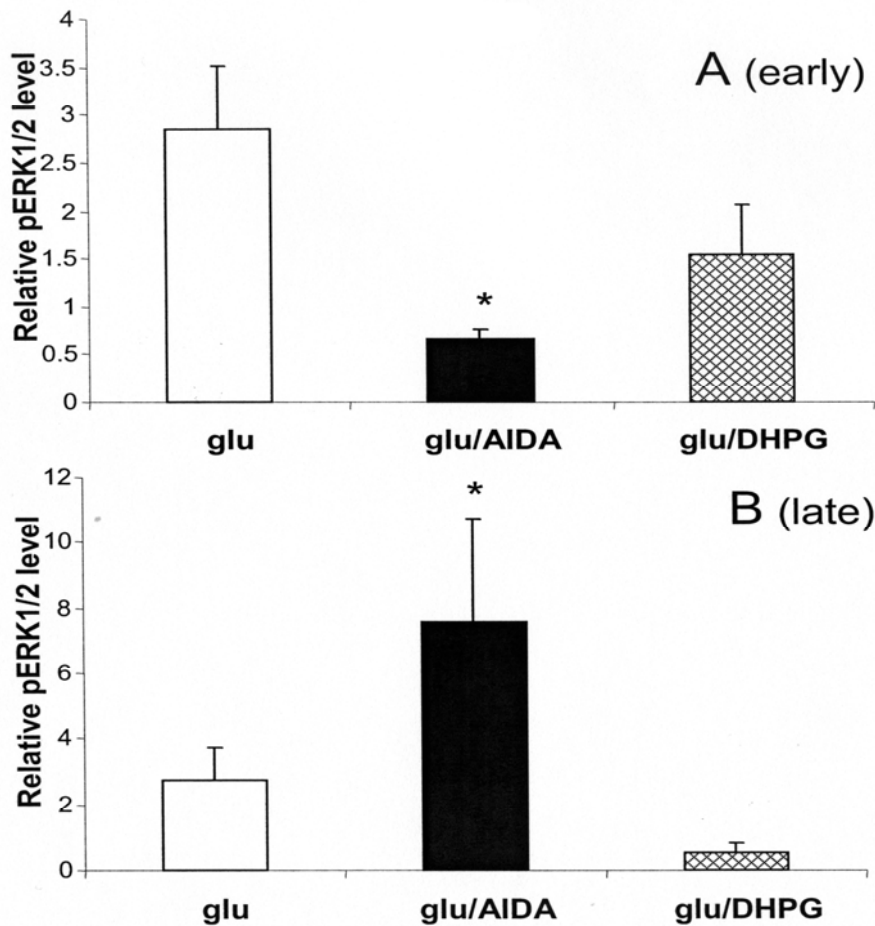
Figure 9. Quantitative analysis of ERK1/2 activation in glutamate treated HT22 cells.

Autoradiograms from Western blots shown in Figure 8 and replicates were subjected to densitometric analysis and relative pERK1/2 levels (normalized to total ERK1/2) were quantified using NIH Image (n>=3). As shown in (A), AIDA inhibited by 4 fold the activation of ERK1/2 that occurs within 30 min of 1.5 mM glutamate treatment (*P<0.05). DHPG did not significantly affect ERK1/2 activation within 30 min of glutamate treatment. (B) The second phase of ERK1/2 activation is illustrated by comparisons of ERK1/2 phosphorylation at 22 versus 10 h of 1.5 mM glutamate treatment. An AIDA pretreatment enhanced this late phase of ERK1/2 activation approximately 3 fold while DHPG inhibited the late phase of ERK1/2 activation 5 fold (*P<0.05).

59
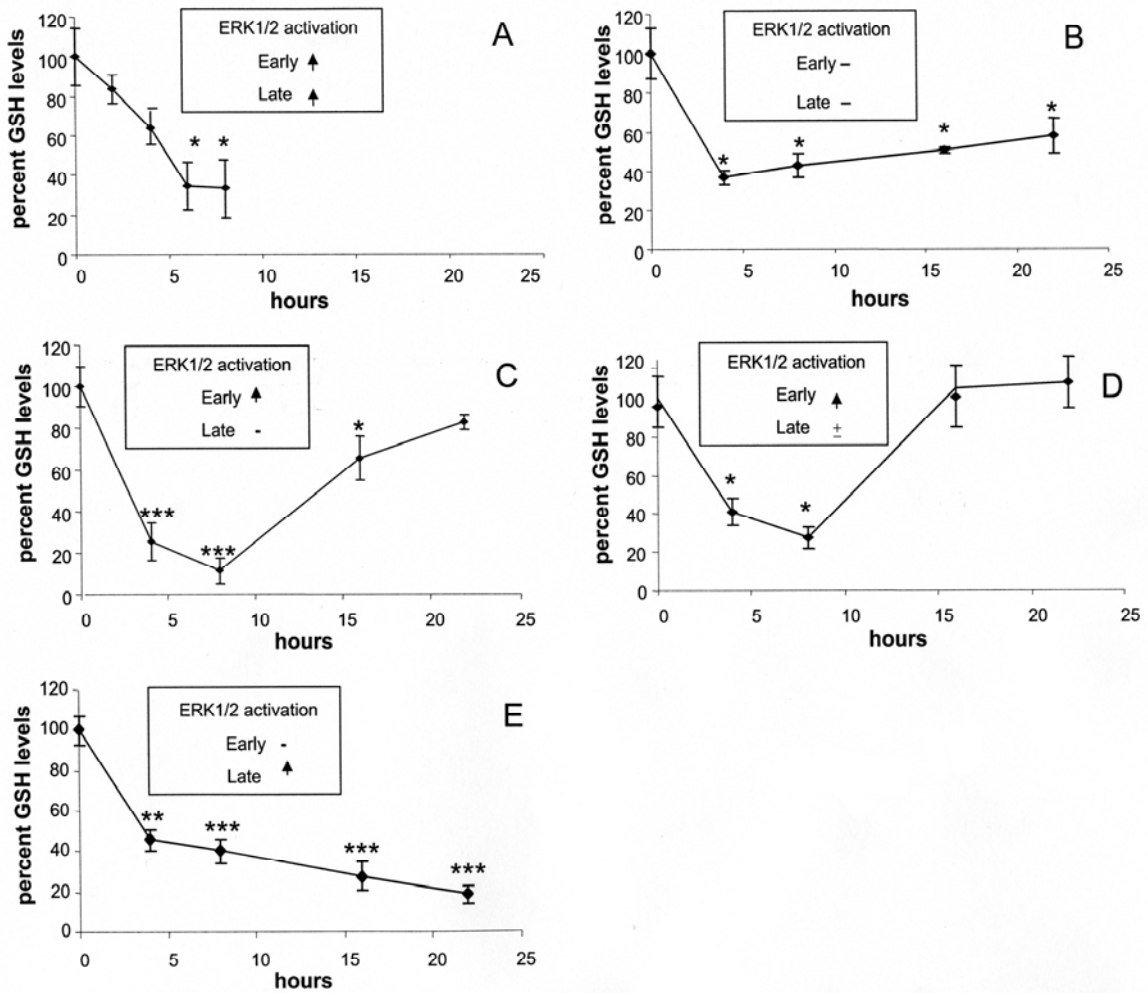
Figure 10. Effects of ERK1/2 on glutathione depletion.

(A) HT22 cells were treated with 5 mM glutamate and GSH levels measured at 2, 4, 6 and 8 h afterwards. A significant reduction of GSH levels to 30% of basal line levels was detected by 6-8 h. (B) HT22 cells were treated with 5 mM glutamate and 10 μM U0126 simultaneously and GSH levels examined 4, 8, 16 and 22 h afterwards. U0126 did not affect GSH depletion during the time course although it blocks glutamate toxicity. (C) HT22 cells were exposed to 10 μM U0126 2 h after the initiation of a 5 mM glutamate treatment and GSH levels examined 4, 8, 16 and 22 h after glutamate addition. GSH levels decreased to 25% and 11% after 4 and 8 h respectively. A rebound of GSH levels to 82% was observed within 22 h of glutamate

treatment. (D) HT22 cells were treated with 1.5 mM glutamate and GSH levels assayed at 4, 8, 16 and 22 h afterwards. 1.5 mM glutamate induced a significant decrease of GSH in 4-8 h which rebounded to basal line GSH levels within 16-22 h. (E) HT22 cells were pretreated with AIDA for 30 min then exposed to 1.5 mM glutamate. GSH levels were assayed at 4, 8, 16 and 22 h afterwards. AIDA treatment induced a prolonged and significant GSH depletion.

Data shown above are representative of 4 separate experiments. The GSH reading was normalized to protein concentration. HT22 cells' GSH level was set as 100% (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

3.5   DISCUSSION

3.5.1   ERK1/2 and Neuronal Cell Death

It is now well recognized that ERK1/2 plays disparate roles in neurons, acting in some cases to promote cell survival (46, 221) while also participating in neuronal cell death and the pathogenesis of neurodegeneration (34). While a mechanistic basis for diverse effects of ERK1/2 in neurons is beginning to emerge, it is often difficult to make meaningful comparisons of results obtained in different cell lines or neuronal cell types. In this study, we have uncovered both prosurvival and cell-death promoting activity of ERK1/2 in one neuronal cell line exposed to a single toxic stimulus. We (226) and others (73) had previously shown that ERK1/2 is required for glutamate-induced oxidative toxicity relying principally on results obtained with pharmacological inhibitors of ERK1/2 activation such as the MEK1 inhibitor U0126. In the present study, we provide more definite support for this conclusion by showing a protective effect of DN-MEK1 on glutamate-induced oxidative toxicity in HT22 cells. Interestingly, we also showed that chronic ERK1/2 activation itself is not sufficient to induce cellular toxicity. Thus, ERK1/2 must cooperate with other pathways or cellular components affected by oxidative stress to contribute to toxicity.

Our initial studies of the kinetics of ERK1/2 activation in response to glutamate treatment revealed a biphasic pattern with an early peak of activation occurring within 30-60 minutes and a second peak emerging within approximately 6-8 hours (226). While recent results from our laboratory established a role for oxidative inhibition of ERK1/2 phosphatases in the second peak of ERK1/2 activation in glutamate-treated HT22 cells and primary cortical neurons (133), the signaling pathway that triggers the initial phase of ERK1/2 activation in these models remained

undefined. In this report, we show that the initial phase of ERK1/2 activation is due to the activation of mGluRI. The transient nature of this rapid activation of ERK1/2 is likely due to desensitization of mGluRI which occurs upon continuous exposure of HT22 cells to glutamate (203). Importantly, our results demonstrate that ERK1/2 can indeed function as it does in many other neuronal and non-neuronal cells, as a pro-survival kinase. Specifically, in early phases of glutamate-induced oxidative stress, ERK1/2 is a component of a cellular defense pathway that seeks to overcome oxidative stress by restoring depleted glutathione levels. However, as oxidative stress continues to develop, this cellular defense pathway is overwhelmed, perhaps due in part to desensitization of mGluRI, and ERK1/2 becomes a necessary player as oxidatively-stressed cells enter the final stages of a unique cell death pathway (230). If the initial phase of ERK1/2 activation is blocked (i.e. by a mGluRI antagonist), HT22 cells become hypersensitive to a subthreshold dose of glutamate. The toxicity that results in HT22 cells from low doses of glutamate in the absence of mGluRI action remains ERK1/2 dependent.

3.5.2   Mechanism of ERK1/2 activation by mGluRIs

mGluRs are G protein-coupled receptors (GPCR) with seven transmembrane domains. These receptors have been implicated in a variety of physiological functions, including neurotransmission, long-term potentiation, and reciprocal interactions with the ionotropic glutamate receptors (37). mGluR-mediated neuroprotection against a variety of insults is well established, and each of the subgroups has been shown to provide neuroprotection in various contexts. Furthermore, activation of mGluRs has been shown to be protective against a variety of neurotoxic insults in vivo including spinal cord injury, ischemia, epilepsy, multiple sclerosis, amyotrophic lateral sclerosis and more recently oxidative stress and diabetes (222).

HT22 cells and primary embryonic rat cortical neurons lack ionotropic glutamate receptors (144) and do not express mGluRII (203). Therefore, HT22 cells serve a good model to illustrate the role of mGluRI in neuronal cell death. In fact, specific agonists and antagonists of mGluRIs have been shown to have anti- and proapoptotic roles, respectively in HT22 cells and primary cortical neurons (203). Our results not only establish ERK1/2 as a mediator of mGluRI effect on oxidative toxicity but identify the downstream target of ERK1/2 (i.e. glutathione metabolism, see below) that is responsible for its initial neuroprotective effects.

The pro-survival effects of mGluR have previously been shown to be due to activation of the phosphatidylinositol 3-kinase (PI3K) and MAPK signaling pathways, which in turn may lead to decreased ROS accumulation (54, 96). Additional evidence suggests that mGluRs regulate ROS production and oxidative stress albeit by distinct subtypes (e.g. group I, II and III) depending upon the neurotoxic insult and neuronal type. For example, activation of mGluRII prevents ROS generation in the mitochondria of dorsal root ganglia neurons in response to elevated glucose levels (222). The mechanism of the neuroprotective action of mGluRI in these cells was not clear although it was suggested to involve activation of PI3K and ERK1/2 signaling pathways (54, 96). Our results confirm the neuroprotective effects of mGluRI in HT22 cells and establish a role for ERK1/2 activation in mGluRI action to limit toxicity. Unfortunately, this protective response of HT22 cells driven by active ERK1/2 is not sufficient to overcome the overwhelming oxidative stress that ensues following prolonged exposure to a high concentration of glutamate.

3.5.3   ERK1/2 activation and glutathione depletion

Glutathione is the predominant non-protein thiol antioxidant in mammalian and plant cells (64). Glutamate, cysteine and glycine are the three precursors of GSH, which plays an important role in many cellular processes such as metabolism of xenobiotic (110) and endogenous oxidants, cell proliferation (52), and regulation of gene transcription (10). Among those, the most well-known function of GSH is antioxidant defense by acting as a coenzyme in glutathione peroxidase or GST-catalyzed reactions (49, 63, 253). GSH depletion has been implicated in the pathogenesis of many neurological diseases such as Parkinson's disease (102). Although some cells can take up GSH directly from their surroundings, adequate GSH levels are maintained by de novo synthesis. GSH concentrations in cells are determined by the balance of its synthesis and consumption. As discussed and evaluated by Hansen (2004), GSH synthesis is controlled by the first enzyme (rate limiting enzyme) in the synthetic pathway, glutamate-cysteine ligase (GCL), which converts glutamate to cysteine (83). Some environmental factors can alter intracellular GSH levels by influencing this reaction (52). For example, H_2O_2 (190) and some reactive species generated during exposure to toxins could upregulate GSH production (215). Unfortunately, this increase is limited and short-lived (49).

GSH depletion and elevations in ROS levels do not represent the terminal, irreversible phase of cell death in oxidatively stressed HT22 cells. The protective effects of U0126 are not associated with a restoration of GSH levels (this report) or reduction in elevated ROS (226). Therefore, active ERK1/2 is more proximal to the irreversible step in HT22 cell oxidative toxicity and is downstream of ROS activation. Although there is not a link between GSH metabolism and the death promoting action of the late phase of ERK1/2 activation, the initial pro-survival effects of active ERK1/2 (i.e. driven by mGluRI activation) appear to be due to its

impact on GSH metabolism. Specifically, in HT22 cells that are triggered to activate ERK1/2 only at early times after glutamate addition (i.e. delayed U0126 addition), a rebound occurs that restores GSH to basal line levels. Without the initial activation of ERK1/2, this restoration of GSH levels in glutamate treated HT22 cells does not occur.

Previous studies in HNE treated L2 cells (rat lung epithelial) have shown that inhibition of ERK1/2 by PD98059 blocked GCLc (catalytic subunit of GCL) mRNA but not GCLm (modulator subunit of GCL) mRNA (49). Furthermore, ERK1/2 was found to upregulate GCLc/GCLm mRNA through two possible pathways. One is through the increase in AP-1 DNA binding and the other through an increase in binding of the Nrf2 transcription factor to ARE binding sites (97). Future studies will reveal whether any of these mechanisms apply to the effect of ERK1/2 on GSH metabolism in HT22 cells.

3.6   CONCLUSIONS

In summary, the dual roles of ERK1/2 as a prosurvival and death promoting kinase can be observed within a single neuronal cell type (i.e. HT22 cells) exposed to a single toxin (i.e. glutamate). Specifically, at early stages of glutathione depletion-induced oxidative stress, ERK1/2 influences GSH metabolism and facilitates the recovery of GSH levels while at later stages, ERK1/2 becomes a necessary component of oxidative toxicity. Thus, any therapeutic intervention directed at the ERK1/2 pathway must take into account the divergent effects of this signaling pathway particularly within cells and tissues where oxidative stress is a contributing factor in cell death.

4.0   POSSIBLE MECHANISM OF ERK1/2 REGULATION TO GLUTATHIONE THROUGH GLUTAMATE-CYSTEINE LIGASE (GCL) FOLLOWING GLUTAMATE-INDUCED OXIDATIVE TOXICITY IN HT22 CELLS

4.1   INTRODUCTION

ERK1/2 activation exerts different effects on GSH depletion during the early and late phases of glutamate treatment but the mechanism responsible for ERK1/2 effects on GSH metabolism are unknown. Distinct targets of ERK1/2 differentially affected during the early and late phases of induction that may act to limit glutathione depletion were examined.

GSH biosynthesis is regulated by the activity of the biosynthetic enzymes and the availability of cysteine (97). Glutathione synthesis, as discussed in the Introduction, is controlled by the first enzyme (rate limiting enzyme) in the synthetic pathway, glutamate-cysteine ligase (GCL), which creates γ-L-glutamyl-L-cysteine (GC). The second enzyme required for *de novo* synthesis of glutathione is glutathione synthase (GS), formerly called glutathione synthetase. The GCL holoenzyme is a heterodimer of ~104 kDa. It can be separated under non-denaturing conditions to yield two subunits, a catalytic (GCLC; 73 kDa) and a modulatory (GCLM; 31 kDa) subunit (66). The GCLM has a modulatory function affecting the affinity of the GCLC for glutamate and GSH (94). The GCLM protein contributes to the decreased feedback inhibition of

GSH to GCLC in transgenic mice expressing the HIV transactivator Tat in liver (33) and aged rats brain tissue (137).

As GCL activity is feedback-inhibited by GSH, rapid depletion of intracellular GSH by conjugation reactions or other reasons can increase GCL activity. Therefore rapid GSH increases are thought to occur via increased activity of existing GCL as an adaptive response (32). However, as the rate-limiting enzyme during GSH synthesis, increased GCL gene expression has also been reported to act to maintain intracellular glutathione homeostasis during oxidative stress (52, 220).

A large number of conditions have been shown to regulate intracellular GSH concentrations through increased GCL activity. These conditions include exposure to quinones (140), heavy metal mercury (252), ionizing radiation (164), ROS such as H_2O_2 (177, 190), nitric oxide (161) or ROS producing compounds (248). It has been observed that the effect of stimuli on GCL and GSH levels may be agonist, model (in vivo vs in vitro), species, and even cell type specific.

Recent studies have shown that nuclear factor erythroid2-related factor 2 (Nrf2) governs basal and inducible GCL subunits expression by means of an antioxidant response element (ARE) contained within the GCL promoter (228). Also, inhibition of ERK1/2 or p38 kinases by PD98059 or SB202190, respectively, results in a 50% reduction in GCL gene induction, while simultaneous inhibition completely eliminates induction in HepG2 cells. Induction of GCL expression is associated with an increase in the binding of transcription factors such as Nrf2 and AP1 to the GCL ARE (268).

Therefore, I hypothesize that ERK1/2 may modulate GSH through regulating GCL gene transcription. I detected changes in GCL protein and mRNA levels at different times after

glutamate treatment, which is consistent with effects of ERK1/2 on GSH levels. The combination of glutamate with AIDA pretreatment was used to examine whether the change of GCLC and GCLM expression is due to glutamate activation of mGluRIs.

4.2   MATERIAL AND METHODS

Cell culture

HT22 cells, a mouse hippocampal cell line, were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Atlanta Biologicals, Norcross, GA), 100 units of penicillin, and 100 μg/ml streptomycin at 37°C and 5% CO2.

Cell viability assay

HT22 cells grown in 24 well plates were incubated for 10 min with 1 μl (1:1000 dilution) of a 6.25 mg/ml solution of propidium iodide (PI) to visualize dead or dying PI-positive cells (133). Cells were observed under an inverted fluorescence microscope equipped with phase-contrast optics (Nikon Eclipse TE200). Three random fields were counted for each condition in at least three separate cultures. The fraction of PI-positive cells was scored relative to total cells per field visualized by phase contrast microscopy.

Chemicals

2(3)-*tert*-butyl-4-hydroxyanisole (BHA) (Cat# B1253, from Sigma) was dissolved in absolute ethanol. The stock of 200mM BHA is saved in -20°C.

mGluRI antagonist (*R,S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) (Cat# 0904, from Tocris Bioscience, Ellisville, Missouri) was dissolved in 1.1eq. NaOH. The stock of 100 mM is saved at -80 °C. mGluRI agonist (*R,S*)-3,5-Dihydroxyphenylglycine (DHPG) (Cat# 0342, from Tocris Bioscience, Ellisville, Missouri) was dissolved in 1 eq. NaOH. The stock of 100 mM is saved at -80 °C.

L-glutamic acid (Cat# G-5889, from Sigma) was dissolved in water to 1M, filtered then stocked in -20 °C. MEK1 inhibitor, 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio) butadiene (U0126) (Cat# 662005, from Calbiochem, San Diego, California) was dissolved in DMSO. The stock of 10 mM is saved at -20 °C.

RT-PCR (reverse transcription-polymerase chain reaction)

Total RNA was extracted from HT22 cells using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the instructions provided by manufacturer. To reverse transcript, 1 μg of RNA was incubated with 100 μl of reaction mix containing 25 mM $MgCl_2$, 25 mM deoxynucleotide triphosphates (PerkinElmer), 10x PCR II Buffer (Life Technologies), 40 U/μl RNAsin RNase inhibitor (Promega), 45 μM random hexamers (Integrated DNA Technologies, Coralville, IA), 200 U/μl Superscript reverse transcriptase (Life Technologies), and nuclease-free water (Ambion). Control experiments were performed without reverse transcriptase to control for the contamination of DNA. The samples were incubated at 25°C for 10 min, at 48°C for 30 min, and at 95°C for 5 min followed by 4°C for 5 min to inactivate the reverse transcriptase.

For amplification, a PCR containing a cDNA aliquot along with AmpliTaq Gold DNA polymerase in a volume of 25 μl was used according to the manufacturer's instructions (Applied

Biosystems, Foster City, CA). The sequences of the primers and probes are as follows (all sequences 5' to 3'): *Gclc* sense primer, ATGTGGACACCCGATGCAGTATT; *Gclc* antisense primer, TGTCTTGCTTGTAGTCAGGATGGTTT; *Gclm* sense primer, GCCACCAGATTTGACTGCCTTT; *Gclm* antisense primer, CAGGGATGCTTTCTTGAAGAGCTT; GAPDH: sense primer, 5'-CATCACCATCTTCCAGGAGCGAGA-3'; and antisense primer, 5'-GTCTTCTGGGTGGCAGTGATGG-3'. Expression levels were derived from comparison with serial dilutions of a known reference standard and values are normalized to GAPDH. Thermocycling conditions involved an initial denaturation step at 94°C for 12 min followed by 23 cycles at 94°C for 30 sec and 56°C for 30 sec and 72°C for 30 sec. Specific PCR amplification products were separated on a 12% PAGE and detected by Ethidium Bromide (EtBr) staining. Experiments were performed with at least triplicates for each data point.

Western blotting

Cells were collected and prepared as described in Chapter 2. The protein samples were separated by SDS-PAGE on 10-12% polyacrylamide gels and then transferred to polyvinylidene fluoride membranes. After blocking with 5% dry milk in PBS, 0.1% Tween 20, membranes were incubated with primary antibodies/antisera (anti-GCLC and anti-GCLM both provided by Dr. Terrance Kavanagh; anti-Nrf2 (Sc-722) from Santa Cruz biotechnology Inc, California; anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, CSA-335) from Stressgen, Victoria, British Columbia, Canada) overnight at 4°C with 3% dry milk, washed 3 x 10 min with PBS, 0.1% v/v Tween 20, and then exposed to the appropriate horseradish-peroxidase conjugated

secondary antibody for 1 h at room temperature. See the details of the following development and analysis steps in Chapter 3.

Statistical analysis

Comparisons of multiple mean values were accomplished by analysis of variance (ANOVA) with Bonferroni's post-hoc tests for significance. Comparisons of two means were performed using a paired t-test. P values less than 0.05 were taken to be significant, and all data was analyzed using Graph Pad Prism version 3.0 for Windows (GraphPad Software, San Diego California USA).

4.3   RESULTS

4.3.1   Alterations in GCL mRNA levels following glutamate treatment in HT22 cells

The mRNA levels of GCLC and GCLM were examined in HT22 cells treated with 5mM glutamate and 1.5mM glutamate in the presence or absence of a pretreatment with the mGluRI antagonist AIDA. RNA samples were extracted from HT22 cells and then RT-PCR experiments were performed with corresponding primers. All of the RT-PCR results were repeated more than 3 times and normalized to mRNA levels of the housekeeping GAPDH gene.

The results showed that 5mM glutamate induced a slight increase in both GCLC and GCLM mRNA levels that appeared to peak around 2 hours (Fig 11A). The strongest induction was obtained after a 2 hr glutamate treatment as shown in Fig 11, with a 1.7 and 2 fold induction in GCLC (Fig 11B) and GCLM (Fig 11C) respectively compared to that of control HT22 cells. However, there is no significant statistical difference compared with that of control cells at any time point and only a trend toward significance.

In contrast, 1.5mM glutamate treatment enhanced the mRNA levels of GCLC in HT22 cells gradually with a peak of induction observed after 22 hours with P value of 0.02. A 30 minute pretreatment with AIDA inhibited the rise of GCLC mRNA induced by 22 hr 1.5 mM glutamate with P value of 0.03. In fact the level of GCLC mRNA in AIDA treated cells after 22 hr 1.5mM glutamate was significantly below basal levels (i.e. 70%, Fig 12 A & B).

The data from GCLM showed a trend of enhanced mRNA levels in HT22 cells treated with 1.5mM glutamate after 22 hours without significant difference. There is a significant inhibition when we compare the GCLM mRNA level of 1.5 mM glutamate treatment after 22 hr to that with a 30 minute pretreatment with AIDA (with a P value of 0.04) (Fig 12 C). AIDA alone did not affect the mRNA levels of GCLC and GCLM following 22 hr (D). These results provide a potential explanation for the effects of mGluRI activation on the restoration glutathione levels in HT22 cells treated with 1.5 mM glutamate.

4.3.2   Effect of glutamate treatment on GCL protein levels in HT22 cells

At the protein level, 5mM glutamate triggered an increase in GCLC levels that peaks at 1-2 hr, as shown by Western blot analysis (Fig 13A). However, GCLM was not affected by glutamate

treatment (Fig 13A). Quantitative analysis of GCLC activation in glutamate treated HT22 cells from three separate experiments is shown in Fig 13B. A statistically significant two fold of activation of GCLC was obtained following a 2 hr 5mM glutamate treatment with P value of 0.04. The levels of GCLC decreased slightly upon longer exposures to glutamate.

Consistent with the results from RT-PCR assays, 1.5mM alone induced a gradual rise in GCLC protein with a peak observed following 16-22 hr (Fig 14A). Quantitative analysis of GCLC activation in glutamate treated HT22 cells from three separate experiments is shown in Fig 14B. A 2.8 fold of activation of GCLC was observed following 16-22 hr 1.5mM glutamate treatment that was statistically significant with P value of 0.03.

A 30min pretreatment with AIDA abolished the induction of GCLC protein by 1.5mM glutamate and even led to a reduction of GCLC protein levels to below basal line at 22 hr after glutamate (Fig 15A). Quantitative analysis of GCLC activation in glutamate treated HT22 cells from three separate experiments is shown in Fig 15B. GCLC expression was reduced to approximately 30% of basal levels following 22 hr of 1.5mM glutamate treatment with AIDA (Fig 15B) with P value less than 0.01. However, GCLM protein levels did not show a similar response with glutamate and AIDA (Fig 15A).

4.3.3 Effects of glutamate treatment on Nrf2 expression in HT22 cells

A 2-8 hr treatment of HT22 cells with 5mM glutamate led to an enhancement of Nrf2 levels as revealed by Western blot analysis. However, as shown in Fig 16, the pattern of Nrf2 expression was not very consistent (Fig 16). Similarly, Nrf2 protein expression after 10-22 hr 1.5mM glutamate with/without AIDA 30 pretreatment shows some variability. It can not be concluded

74

from our results whether the early activation of ERK1/2 contributes to a rebound of glutathione levels in glutamate treated HT22 cells through an enhanced stabilization of Nrf2.

At this point, our analysis of Nrf2 expression is not sensitive enough to prove whether Nrf2 is the regulator of GCL transcription in glutamate treated HT22 cells. The inconsistency of the results from Nrf2 can not exclude the possible role of ERK1/2 and mGluRI regulation of GCL via transcription factors such as Nrf2 or AP1. Further and more sensitive experiments such as chromatin-immunoprecipitation (CHIP) assays or electrophoretic mobility shift assay (EMSA) may be helpful to verify Nrf2's role in glutamate-induction of GCL transcription. Specifically, CHIP assay may visualize factors bound to the GCL promoter in native chromatin in cells and EMSA can quantify the DNA binding activity of GCL promoter factor in extracts. The inconsistency of Nrf2 western blot results may be due to rapid degradation and instability of the Nrf2 protein.

A GCLC

GCLM

GAPDH

5mM Glu   _   0.5   2   4   6   8   (hr)

B

C
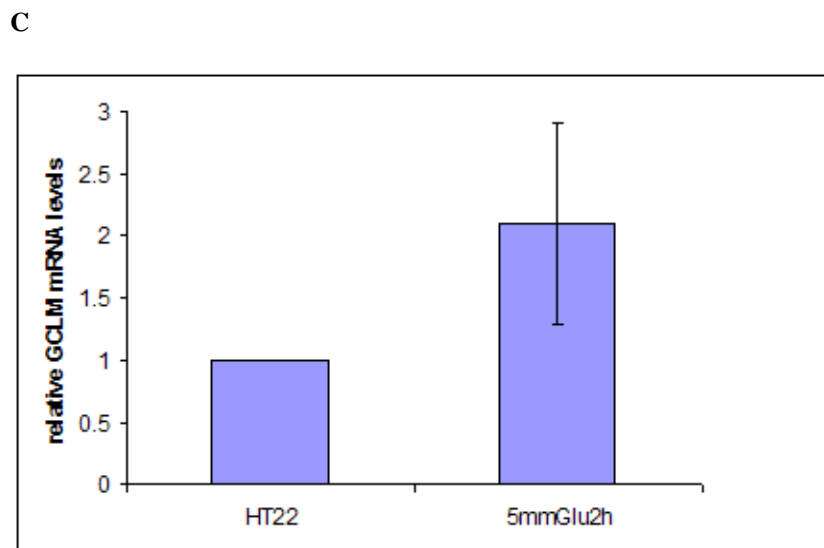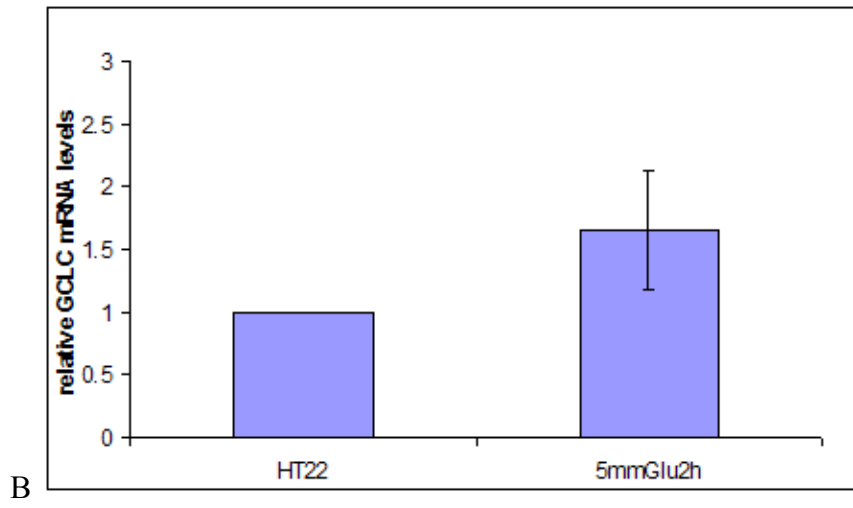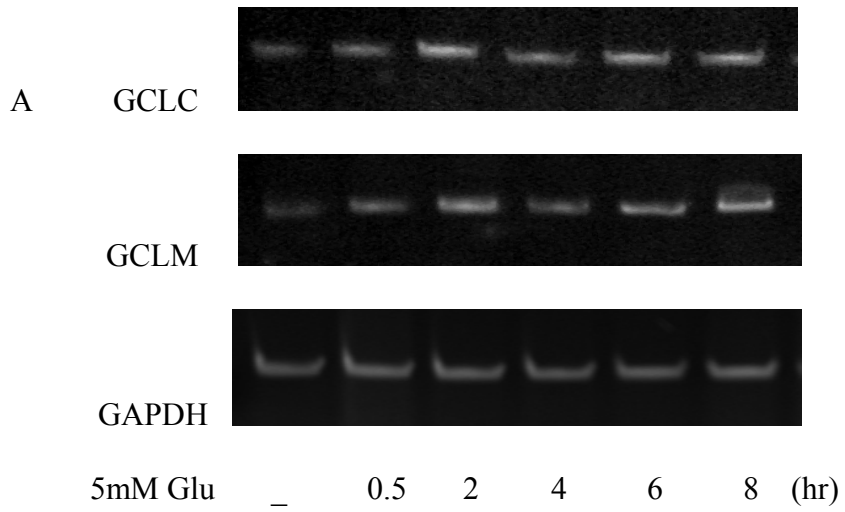
Figure 11. The GCLC and GCLM mRNA levels in glutamate treated HT22 cells.

The mRNA levels of GCLC and GCLM slightly increased in HT22 cells treated with 5mM glutamate with a peak around 2hr. There may be a trend towards glutamate induction of GCLC and GCLM mRNA.

GCLC

GCLM

GAPDH

HT222 1.5mM Glu22hr AIDA/1.5mM Glu22hr

A

B
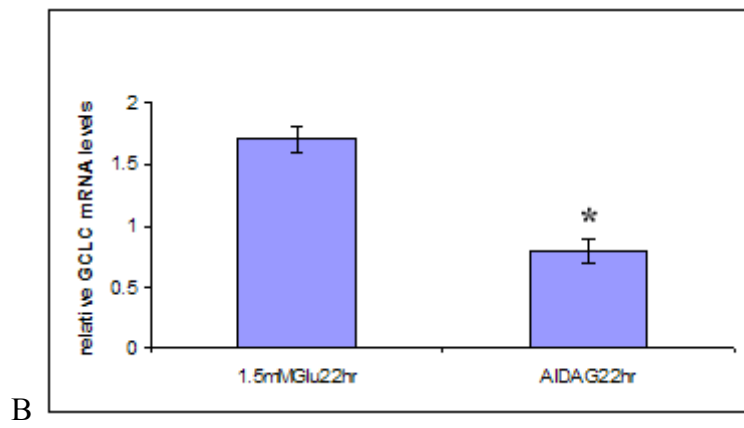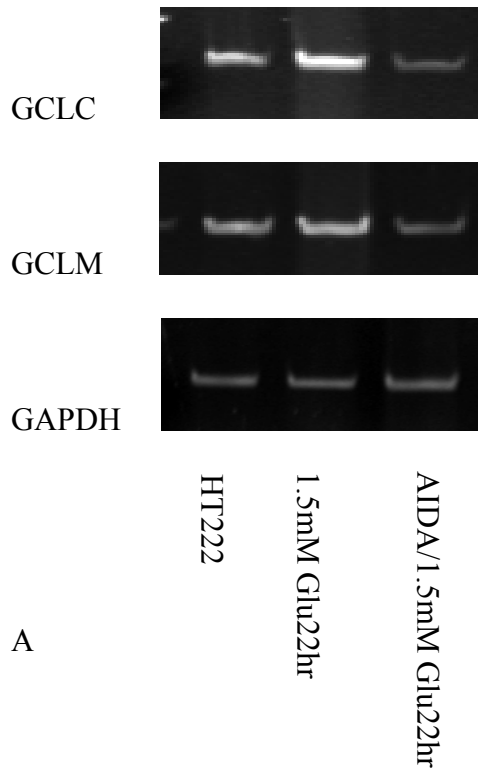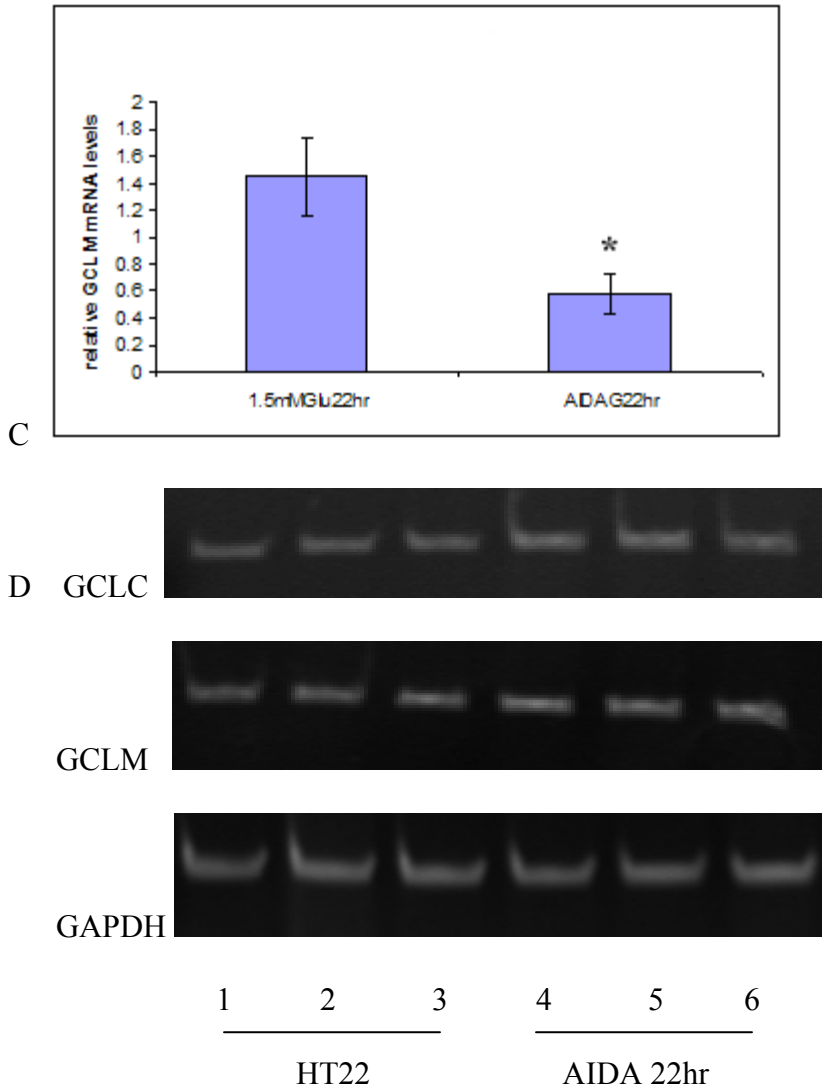
Figure 12. Effects of AIDA on glutamate induction of GCLC and GCLM mRNA levels in HT22 cells.

A 30 minute pretreatment with AIDA significantly inhibited the rise of GCLC (A, B) and GCLM (A, C) mRNA induced by 1.5 mM glutamate to levels below the basal expression level of HT22 cells (*P<0.05). As a control, AIDA alone did not affect the mRNA levels of GCLC and GCLM after 22 hr (D).

A

GCLC

GCLM

GAPDH

5mM glutamate   --   0.5   1   2   4   6   8 (hr)

B
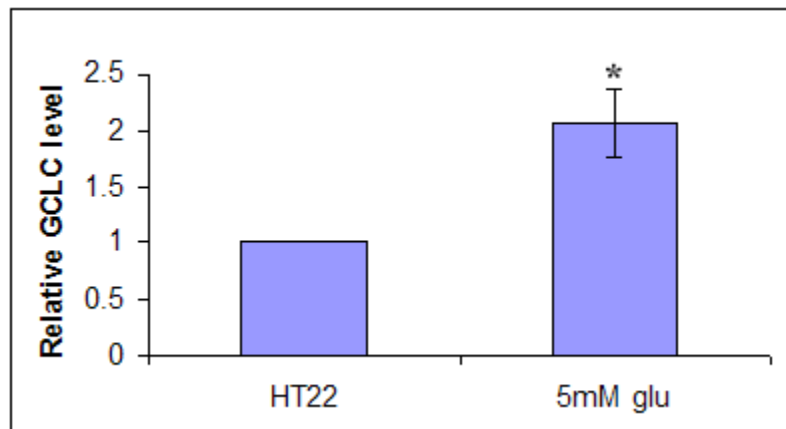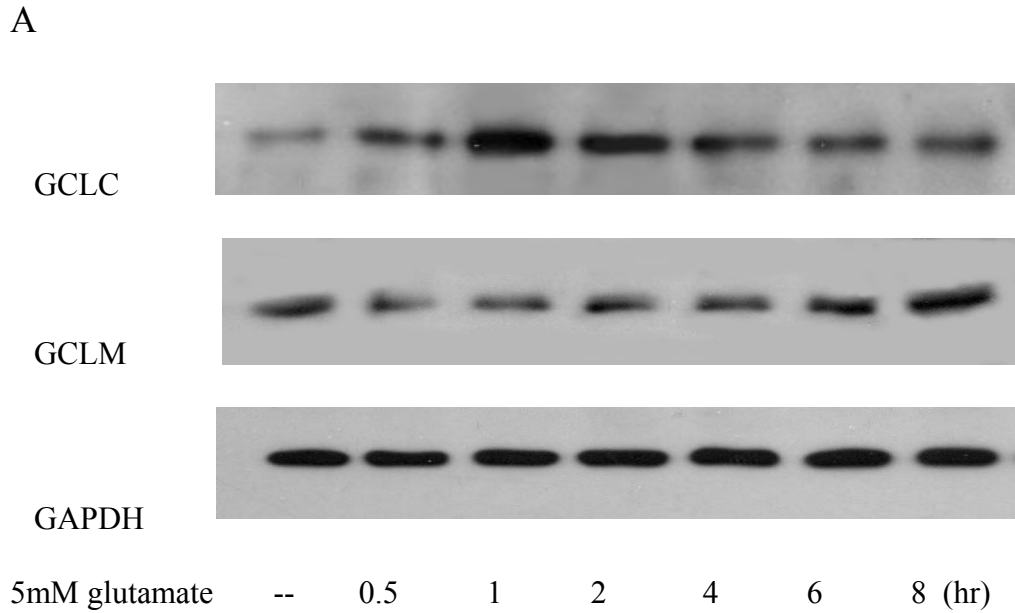
Figure 13. The GCLC and GCLM protein levels in 5mM glutamate treated HT22 cells.

GCLC protein levels are upregulated following a 5mM glutamate treatment of HT22 cells with a peak around 1-2 hours (A). Autoradiograms from Western blots shown in A and replicates were subjected to densitometric analysis and relative GCLC levels were quantified using NIH Image (n>=3) (B). About 2 fold of induction was obtained following 2 hr 5mM glutamate treatment (*P<0.05). However, GCLM is not modulated by glutamate (A).

A GCLC

GCLM

GAPDH

1.5mM glutamate --   0.5   1   4   6   10   16   22 (hr)
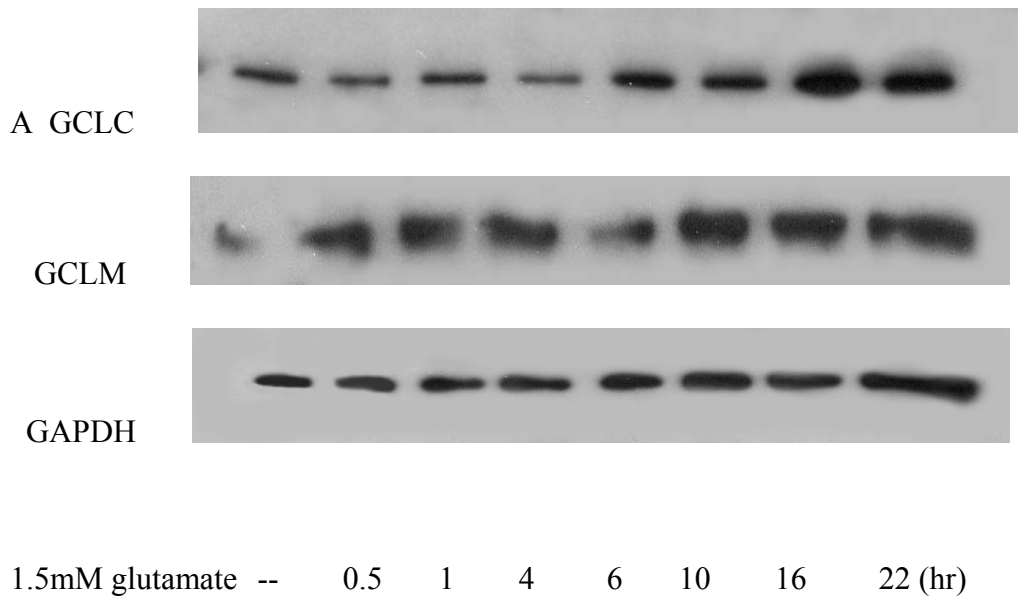
B
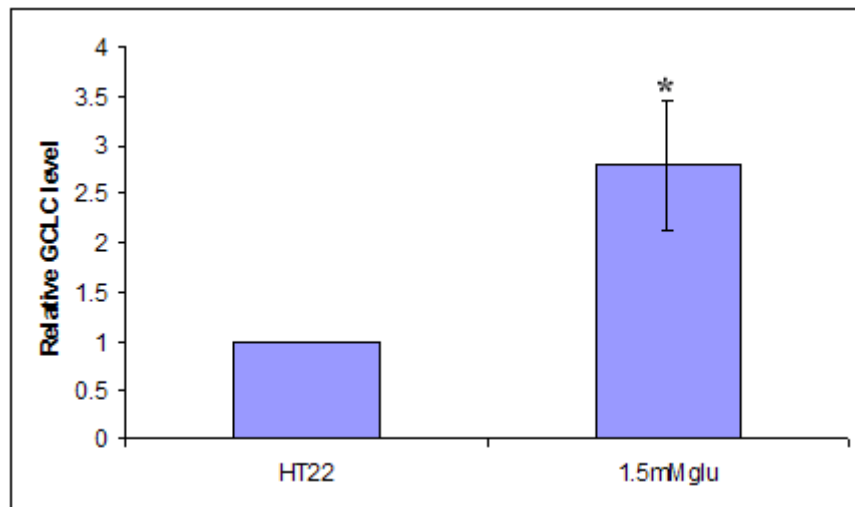
Figure 14. The GCLC and GCLM protein levels in 1.5mM glutamate treated HT22 cells.

GCLC and GCLM protein levels in HT22 cells treated with 1.5mM glutamate for the indicated times were visualized by Western blot analysis (A). Autoradiograms from Western blots shown in A and replicates were subjected to densitometric analysis and relative GCLC levels were quantified using NIH Image (n>=3) (B). About 2.8 fold of induction was obtained following 16-22 hr 1.5mM glutamate treatment (*P<0.05). However, GCLM is not modulated by glutamate (A).

81

A

GCLC

GCLM

GAPDH

| 1.5mM glutamate | -- | 0.5 | 1 | 4 | 6 | 10 | 16 | 22 (hr) |
| AIDA | -- | + | + | + | + | + | + | + |
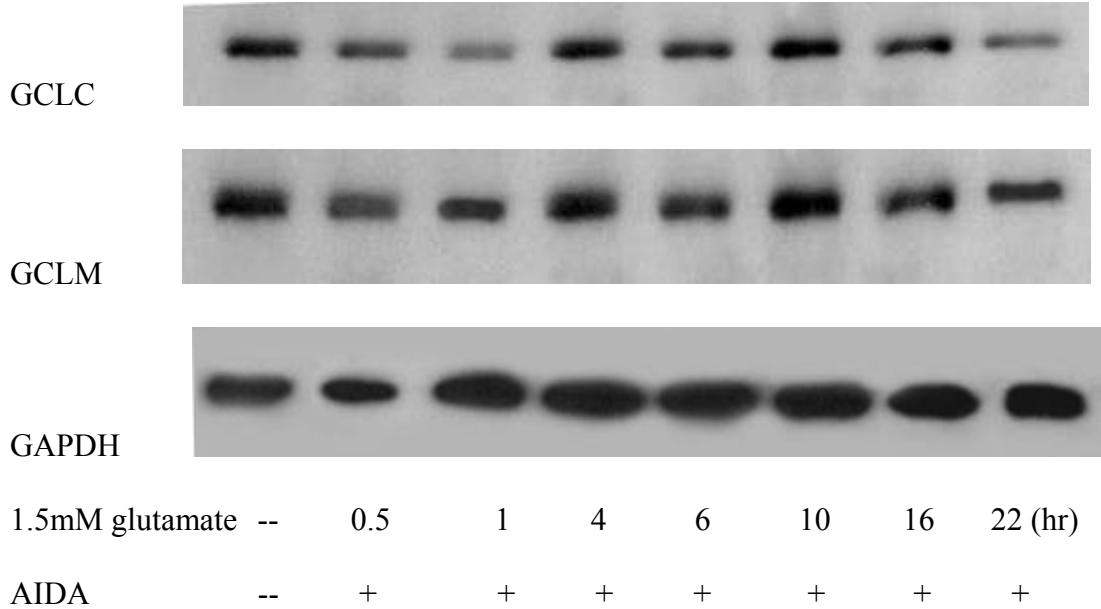
B
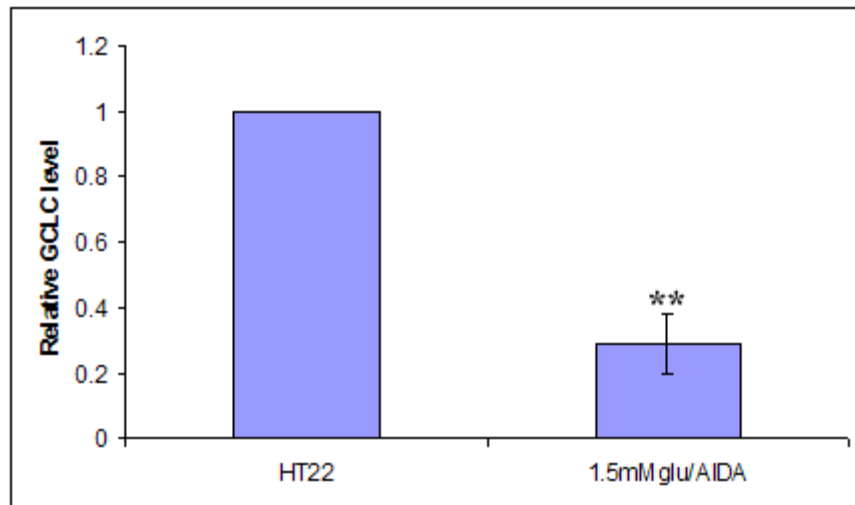
Figure 15. Effects of AIDA on glutamate induction of GCLC and GCLM protein levels in HT22 cells.

GCLC, GCLM and GAPDH protein levels in HT22 cells treated with 1.5mM glutamate in the presence or absence of AIDA for the indicated times were visualized by Western blot analysis (A). Autoradiograms from Western blots shown in A and replicates were subjected to

densitometric analysis and relative GCLC levels were quantified using NIH Image (n>=3) (B). GCLC expression was reduced to 30% of basal line following 22 hr of 1.5mM glutamate treatment in the presence of AIDA pretreatment (**P<0.01) (B). However, GCLM is not modulated by glutamate and AIDA.

A

Nrf2

GAPDH

5mMglutamate --   0.5   1   2   4   6   8 (hr)

B

Nrf2

GAPDH

5mMglutamate --   0.5   2   4   6   8 (hr)
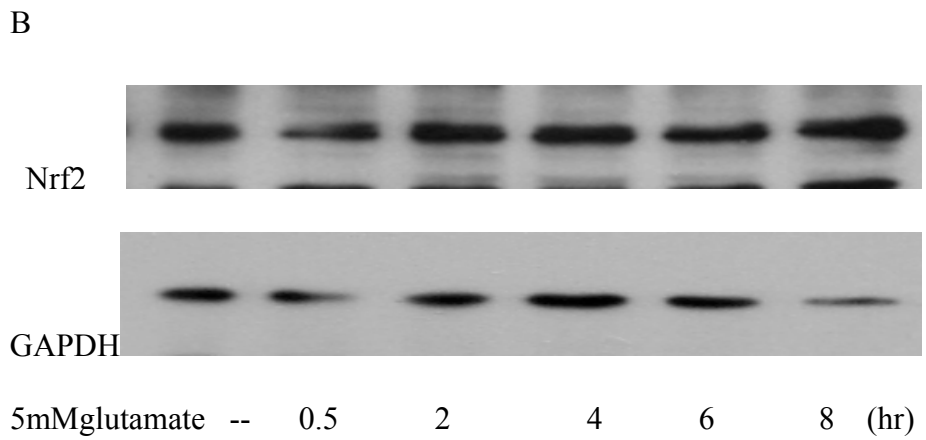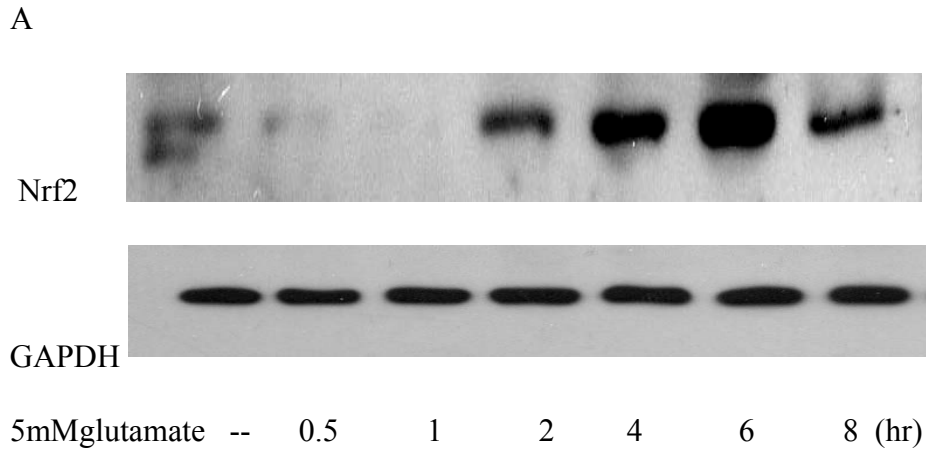
Figure 16. Nrf2 protein levels are not consistently affected by glutamate.

In one experiment, Nrf2 protein expression was induced following a 2-8 hr 5mM glutamate treatment (A). In a separate experiment, Nrf2 protein expression was similar at different times i.e. 0.5, 2, 4, 6 and 8 hr after 5mM glutamate treatment (B).

4.4   DISCUSSION

4.4.1   GCL change following glutamate-induced oxidative toxicity

Intracellular de novo GSH synthesis is determined by two important factors, GSH precursors and the rate-limiting enzyme in GSH synthesis, GCL. In glutamate-induced oxidative toxicity in HT22 cells, the low content of intracellular cystine has been well verified. This thesis explored whether glutamate affects GCL expression via mGluRI.

The de novo synthesis of GSH is regulated at several levels in the cell. In general, the consumption of GSH by conjugation or reduction reactions leads to an increased production of GSH. In part, the increased GSH production is because of the upregulated activity of pre-existing GCL as a result of weakened GSH feedback inhibition (197). In addition, the synthesis of new GCL subunits through increased transcription and/or mRNA stability has also been suggested to result in the increased production of GSH (139). Comparing the two mechanisms to regulate intracellular GSH, the inhibition in GSH feedback inhibition is transient and increased GCL synthesis is prolonged. It is widely believed that the increase in GSH levels is an adaptive response for the cell by which the cell prepares to defend itself against coming stresses (119).

The time course studies have provided insights into the kinetics of GCL induction. For the catalytic subunit GCLC, 5mM glutamate upregulation occurs at an early phase after glutamate exposure, i.e. within 1-2 hr. This occurs at both mRNA and protein level. This result confirmed the notion addressed above that the early peak of ERK1/2 activation is protective. This neuronal protection works through upregulation of GCLC, increased GSH de novo synthesis and subsequent maintenance of base line GSH level.

Although both GCL mRNA and protein exhibit a small increase at the early phases of glutamate treatment, it didn't reverse the GSH depletion in HT22 cells caused by 5mM glutamate. Oxidative stress generated by high glutamate treatment may be too overwhelming to cells. However, at the protein level, 5mM glutamate did not induce a parallel change of GCLM and GCLC.

Some previous reports from other groups have illustrated that in response to oxidative stress, alterations in cellular GSH are correlated with GCLC to a greater extent than GCLM (119, 126). For example, effects of oxidative stress were examined in HBE1 cells, immortalized human bronchial epithelial cells from a normal individual, by treatments with 10 μM 4-hydroxy-2-nonenal (4HNE), 5 μM 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), and 25 μM *tertiary*-butylhydroquinone (tBHQ) (119). All three compounds increased the steady-state mRNA levels of both *Gclc* and *Gclm* but resulted in different induction of GCLC and GCLM proteins. Specifically, 4HNE and DMNQ induced changes in GCLM protein at levels that bordered on statistical significance. tBHQ had no effect on GCLM protein levels under any conditions. However, all three chemicals induced significant induction in GCLC protein levels. In addition, all of the three oxidative stress inducers can increase the GCLC:GCLM molar ratio in HBE1 cells (119).

Therefore, although oxidative stress increases the steady-state levels of GCLC and GCLM mRNA, the magnitude of induction of GCL protein varies between the two subunits, which may implicate some form of translational regulation. Together with results from molar ratio of GCLC:GCLM studies, it has been suggested that de novo GSH biosynthesis does not require equal increases in GCL subunit protein (126). Our results reinforce this idea in glutamate induced oxidative toxicity in a neuronal cell line. More interestingly, more recently, study from

HepG2/C3A (from human hepatoma) cells has demonstrated the differential up-regulation of GCL subunits in response to cysteine deprivation (126).

1.5 mM glutamate induced a gradual increase in GCL mRNA and protein levels with a peak observed at 22 hr. A pretreatment with AIDA, a mGluRI antagonist, inhibited induction of GCL. mRNA and protein results are consistent with those from intracellular GSH measurements. Therefore, AIDA may promote toxicity through its inhibition of GCL mRNA and protein induction and subsequent GSH depletion. Results illustrated here confirmed the neuroprotective role of mGluRIs in glutamate-induced oxidative toxicity and provided some possible mechanisms.

4.4.2   The mechanism of ERK1/2 effect to GCL regulation

The upregulation of GCL under oxidative stress has been well documented. However, the signaling pathways responsible are still being characterized. MAPK is one of the targets being investigated for a role in oxidative stress induced GCL expression. The role of MAPK in GCL activation is cell type and model dependent. For example, the MEK1/2 inhibitor PD98059 completely inhibited the activation of GCLC in primary cultured rat alveolar epithelial type II (AT2) cells treated with HNE (138). p38 and JNK pathway inhibitors had no effect on the transcription on GCL in the same model (241). However, in human bronchial epithelial HBE1 cells, PD98059 is ineffective against HNE-mediated induction of GCL (50), which seems to be JNK pathway dependent (50).

Nrf2, a transcription factor, binds to the ARE within the GCL gene and could lead to increased GCLC transcription. As summarized by Iles and Liu, upregulation of GCLC/GCLM might work through ERK phosphorylation, increased Nrf2 activity and translocation into nuclei,

or more ARE binding pathway in oxidative stress induced by HNE (97). So far, there is no direct evidence for phosphorylation of Nrf2 by ERK. A recent paper showed that ERK pathway contributes to Nrf2 protein stabilization and subsequent transactivation activity in HepG2 cells (170). This finding has provided us a reasonable explanation for the effect of protective role of ERK1/2, Nrf2 and GCL upregulation in glutamate-induced oxidative stress.

Other possible factor could not be excluded in regulating GCL activity. For example, activated ERK1/2 could upregulate AP-1 binding via its downstream target c-fos. Both human GCL subunits contain consensus binding sites for many transcription factors including ARE and activator protein 1 (AP-1). AP-1 binding is required for basal and inducible expression of GCLC, GCLM and GS under models of tert-Butylhydroquinone (TBH) (259), curcumin (51) and HNE treatment (50). Further studies examining AP-1 binding need to be performed to clarify if it is also involved in glutamate-induced oxidative toxicity.

5.0   SUMMARY AND CONCLUSIONS

5.1   ROLES OF ERK1/2 IN GLUTAMATE-INDUCED OXIDATIVE TOXICITY

ERK1/2 has been well documented to play disparate roles in neurons. In some cases, ERK1/2 promotes cell survival, while it also can participate in neuronal cell death and the pathogenesis of neurodegenerative diseases. In this thesis, we have demonstrated both prosurvival and pro-death activities of ERK1/2 in one neuronal cell line exposed to a single toxic stimulus, i.e. glutamate-induced oxidative toxicity in the mouse hippocampal cell line, HT22 cells.

In this thesis, I showed that rapid activation of ERK1/2 by glutamate in HT22 cells is driven by type I metabotropic glutamate receptors (mGluRIs). Furthermore, mGluRI activation of ERK1/2 represents a cellular adaptive response and defense system that attempts to control glutathione loss resulting from glutamate induced cysteine depletion. This protective response mediated by ERK1/2 is however unable to overcome an overwhelming and prolonged oxidative stress that utilizes ERK1/2 in its final stages to promote neuronal cell death. Thus, ERK1/2 activation may serve opposing roles in neuronal oxidative toxicity acting initially through effects on glutathione metabolism to limit oxidative stress, but serving as a necessary signal to trigger cell death when cellular defense against oxidative stress is exhausted.

5.2 LOCATION, DURATION AND INTENSITY OF ERK1/2 ACTIVATION AFFECTS ITS ROLE IN CELL TOXICITY

This thesis also supports the hypothesis that the intensity and duration of ERK1/2 activation is crucial in determining whether it serves a beneficial or detrimental role in neuronal oxidative stress.

Neuronal cell function is also dramatically influenced by the subcellular localization of activated ERK1/2. It has been reported that sustained nuclear localization of ERK1/2 is critical for long-term potentiation (181), differentiation (36) and cell injury after hypoxia and ischemia (245) in neurons. In addition, it is noteworthy that the subcellular localization of ERK1/2 is also critical to trigger glutamate-induced oxidative toxicity in HT22 cells. Because ERK1/2 substrates are found in various subcellular compartments (75), the biological outcome of ERK1/2 activation will depend in part upon the localization of ERK1/2 and its accessibility to potential targets within that compartment (34, 186).

Previous results from our groups have established that ERK1/2 translocation into nuclei is necessary for glutamate toxicity in both HT22 cells and primary cortical neurons (132, 225). Specifically, indirect immunofluorescence staining showed nuclear staining of HT22 cells following 5mM glutamate addition (225). Results presented in this thesis also confirmed this hypothesis using a sensitive, quantifiable assay of ERK1/2 function in the nucleus. Given that ELK-1 is an established nuclear target of ERK1/2, enhanced transactivation activity resulting from its phosphorylation by nuclear localized ERK1/2 is easily monitored through the activity of a luciferase reporter. In this system, luciferase expression is controlled by the activation of an Elk-1-GAL4 fusion by active-ERK1/2 in the nucleus. The results showed that GAL-4UAS-luciferase activity was induced nearly 2 fold with a 7h 5mM glutamate treatment.

Furthermore, the neuroprotective effect of the mutant ERK1/2 phosphatase, MKP3 C293S mutant transfection, which enhances ERK1/2 phosphorylation but blocks its nuclear translocation, demonstrates the necessity for active ERK1/2 nuclear retention for oxidative toxicity in neurons (132). MKP3 C293S, a catalytically inactive mutant of MKP3, retains the ability to bind to ERK1/2 and therefore functions as a dominant negative to limit the access of endogenous ERK1/2 to phosphatases (132). Previous work from our lab has established the ability of MKP3 C293S to restrict nuclear translocation of active ERK1/2 in HT22 cells, which is consistent with results from a fibroblast cell line (22). MKP3 C293S restricts ERK1/2 localization to the cytoplasm through its NH_2-terminal domain, which can selectively interact with ERK1/2 with high affinity. Overexpression of MKP3 C293S can protect both HT22 cells and primary cortical neurons from glutamate-induced oxidative toxicity (132). Collectively, these results confirmed that glutamate-induced oxidative toxicity in HT22 cells is accompanied by the translocation of functional ERK1/2 into the nucleus.

Therefore, ERK1/2 nuclear translocation and the action of its possible nuclear targets are vital to bring about oxidative toxicity in neurons. A variety of ERK1/2 nuclear targets have been identified. Some of them are transcription factors such as CREB. Therefore, new transcription or translation may be required for the glutamate-induced oxidative toxicity. Some studies using transcription/translation inhibitors support this hypothesis. For example, Actinomycin D, a transcription inhibitor, prevents the increase in ROS and protects the HT22 cells from glutamate-induced oxidative toxicity (230). The translation inhibitor, cycloheximide, is also able to protect HT22 cells and keeps ROS levels low during glutamate exposure (230). Since in our model, the secondary peak of ERK1/2 activation occurs at a time when cells begin to die, there may not be enough time for transcription and translation to contribute newly synthesized proteins to cell

death pathway. Further studies are needed to clarify the role of ERK1/2 regulated transcription in HT22 cell toxicity.

Results from Chapter 2 also demonstrated that ERK1/2 activation is necessary but not sufficient to induce toxicity in HT22 cells. First, overexpression of a dominant negative MEK1 protein in transfected cells confirmed that ERK1/2 activation is necessary for glutamate-induced neuronal toxicity. This molecular tool confirmed results obtained with a pharmacological inhibitor of MEK1/2, U0126. Next, we set up a system which induced prolonged ERK1/2 activation in HT22 cells. Although a 6-fold ERK1/2 activation was generated in HT22 cells by overexpression of the MEK-ERK fusions, no cell toxicity was observed. Thus, some other factors induced by oxidative stress in HT22 cells must be combined with ERK1/2 activation to trigger neuronal toxicity.

5.3   THE MECHANISM OF DELAYED ERK1/2 ACTIVATION

The work presented in this thesis demonstrated the opposing roles of ERK1/2 in early and late phases following glutamate-induced oxidative toxicity and identified the signal pathway responsible for the first peak of ERK1/2 activation, i.e. mGluRIs. The mechanisms of delayed secondary peak of ERK1/2 activation are still not fully clear, although ERK1/2-directed phosphatase inactivation may play a role in this process (132). In primary immature cortical cultures, ERK1/2-directed phosphatase activity is specifically inhibited during the late phase (14 hr) of glutamate-induced oxidative toxicity, but not in the early phase (1 or 6 hr) (132).

These results together with data from Chapter 2 suggest a possible dynamic mechanism resulting in biphasic ERK1/2 activation. Under glutamate-induced oxidative stress, mGluRIs is

initially activated; rapid ERK1/2 phosphorylation and glutathione restoration follow as a cell adaptive and self defensive system. Unfortunately, if the oxidative stress is too potent and prolonged, such as in the late phase of 5mM glutamate treatment, the mGluRIs are inactivated, probably via desensitization. In the meanwhile, selective ERK1/2-directed phosphatase may be also inhibited which leads to a late phase of ERK1/2 activation.

5.4   MGLURIS RAPID DESENSITIZATION

Desensitization is defined as the tendency of a receptor response to decrease with time after exposure to an agonist or ligand (203). It has previously been shown that mGluRI's protection from glutamate toxicity is rapidly desensitized in HT22 cells and primary cultures. For example, HT22 cells pretreated with a low dosage (100 µM) of glutamate are much more sensitive to a second toxic dose of glutamate that ranges from 10-500 µM) (203). An earlier report has even demonstrated that in cerebellar neurons cultures mGluRs desensitization involves, at least in its rapid component, activation of PKC (25).

The desensitization studies also provide the explanation of the biphasic ERK1/2 activation with glutamate treatment. Loss of ERK1/2 activation following an initial peak may be due to desensitization and the late peak of activation may be due to a different mechanism excluding mGluR involvement. This explanation is consistent with our previous finding that U0126 added after first peak of ERK1/2 activation blocked the late phase of activation and was protective.

5.5   IMPACT OF GLIA ON NEURON RESPONSE IN OXIDATIVE STRESS

The majority of the work in this thesis has been performed in the HT22 cell line. Other factors in central nervous system, however, need to be taken into consideration if the results presented here are to be applied to mechanisms of neurodegenerative diseases.

For example, growing reports have demonstrated that dynamic and complex interactions between astrocytes and neurons secure brain function and survival under both physiological and pathological conditions (237).

As effective sensors of the brain microenvironment, astrocytes react quickly at the genomic (e.g., production of beneficial factors) and non-genomic level (e.g., regulation of water homeostasis and blood-brain barrier during brain edema and ischemia) (237).

The mechanism underlying astrocytes' protection is complex and includes the production of various substrates for neurotransmitters (79), transferring energy substrates such as lactate and pyruvate to neurons (6), providing metabolites (53) and ions (130) and modulating inflammation after ischemia (6). In addition, many factors synthesized and secreted by astrocytes contribute to neuronal survival in neurological disorders such as ischemia. These factors are composed of heat shock protein (Hsp32) (116), metallothionein1/2 (MT-1/2) (238), thioredoxin (TRX) (92), metalloproteinases (MMPs) (40), nerve growth factor (NGF) (129), brain-derived neurotrophic factor (BDNF) (236), glial cell-derived neurotrophic factor (GDNF) (257), basic fibroblast growth factor (bFGF) (104), neutrophins 3, 4, 5 (NTs) (151) and ciliary neurotrophic factor (CNTF) (11) etc. Among them TRX and MTI/II are scavengers of ROS (237).

A critical role of astrocytes in protecting neuron toxicity induced by oxidative stress has been described (216). Astrocytes have stronger potential antioxidative ability than neurons (142, 145, 192). It is well documented that astrocytes can protect neurons from damage caused by

oxidative stress induced by various compounds such as dopamine, H_2O_2, and 6-hydroxydopamine and nitric oxide (30, 47, 159).

The mechanism underlying the antioxidant properties of astrocytes involves their delivery of GSH and/or GSH precursors such as cysteine, cystine, and the CysGly dipeptide from glia to neurons for GSH synthesis (56). It appears that the Nrf-2-dependent increase in glial GSH synthesis is both necessary and sufficient for the protection of neurons.

Collectively, future studies need to be performed to verify the mechanism shown in this thesis in vivo or in neuron-glia co-culture, for example, in primary cortical mixed cultures.

5.6   NEUROPROTECTION OF MGLURIS IN OXIDATIVE STRESS

In studies published by Schubert and colleagues (143), glutamate toxicity in HT22 cells was found not to be ERK1/2-dependent but driven by JNK and/or p38 MAPK activation. However, results from our laboratory and other groups (207) suggest that glutamate toxicity in HT22 cells is ERK1/2-dependent. Furthermore, ERK1/2 dependent oxidative toxicity has also been confirmed in primary neuron cultures (24) and linked to ischemic cell death in rodent models of transient focal ischemia (169).

In fact, specific agonists and antagonists of mGluRIs have been shown to have anti- and pro-apoptotic roles, respectively in HT22 cells and primary cortical neurons (203). As mentioned above, contrary to our results, the same group suggested that glutamate induced HT22 cell death is not ERK1/2 dependent. In addition, for the past 4 years we have been working with a subclone of HT22 cells isolated from a screen of single cell-derived clones with reproducible sensitivity to glutamate toxicity. Therefore, it is necessary for us to evaluate the impact of mGluRIs agonists

and antagonists on glutamate toxicity in HT22 cells prior to biochemical analysis of role of mGluRIs activation on ERK1/2 phosphorylation.

mGluR-mediated neuroprotection against a variety of toxic stimuli such as ischemia (12), nitric oxide treatment (244) and neuropathy in diabetes (16) has been well established. Activation of each of the three subgroups can provide protection in the area they express (222). MAPK is one of the most common pathways mGluRs function through to display its neuroprotective role (222).

The data presented here provide insight into the mechanisms by which a specific cellular signaling pathway, the ERK1/2-MAPK pathway, is recruited during oxidative stress-induced neuronal cell death.

The work in this thesis not only confirmed the neuroprotective role of mGluRIs activation in glutamate-induced oxidative stress but also give insights into some mechanisms of toxicity. With mGluRI agonist and antagonist, DHPG and AIDA respectively, it has been clearly demonstrated that mGluRIs protect cells from oxidative toxicity induced by 5mM and 1.5mM glutamate. ERK1/2 phosphorylation at the early phase is one consequence of mGluRI activation in our model. Glutathione measurements provided potent evidence that first peak of ERK1/2 activation protects HT22 cells from oxidative toxicity by upregulation of inreacellular GSH. Results from Chapter 3 further illustrated the GCL link between ERK1/2 activation and GSH.

As discussed in the Introduction, intracellular GSH de novo synthesis is controlled by the rate-limiting enzyme GCL. The results presented in Chapter 3 demonstrated the dynamic change of GCLC and GCLM in mRNA level (by RT-PCR) as well as protein (by Western blot) in glutamate-induced oxidative toxicity. It has been suggested that the restoration of GSH by the early phase of ERK1/2 activation is through GCLC modulation. Therefore, effects on precursors

and biosynthesis enzymes (GCL) both contribute to alteration in GSH concentration following glutamate treatment in HT22 cells.

Transactivation of the GCL gene may be regulated by transcription factors such as Nrf2 and/or AP1. Preliminary experiments presented in this thesis are not sensitive enough to prove if Nrf2 is the major regulator of GCL transcription in HT22 cells. More sensitive assays such as a chromatin-immunoprecipitation (CHIP) assay or electrophoretic mobility shift assays (EMSA) may be helpful to verify Nrf2's role in glutamate-induced oxidative toxicity. For example, the CHIP assay can visualize transcription factors bound to promoter in native chromatin in cells, while the EMSA measures DNA binding in extracts. These experiments may help to prove if binding of Nrf2 to ARE of GCL is upregulated following glutamate induced oxidative stress and explore other potential transcription factors binding to ARE of GCL.

In conclusion, the work presented in this thesis illustrated the opposing roles of ERK1/2, i.e. pro-survival and pro-death, in a single cell type. In addition, the mechanism of the neuroprotective role of ERK1/2 was revealed. Early phase of ERK1/2 activation, induced by mGluRI, aids in protecting the cells against oxidative stress by upregulating intracellular GSH level. Furthermore, modulating GCL subunit at transcriptional and translational level has been shown to be part of the mechanism of restoration of GSH. However, when the intracellular oxidative stress is too overwhelming and prolonged, the late phase of ERK1/2 activation is triggered and used as a factor contributing to cell death.

Studies from our lab have shown that the increased accumulation Zn^{2+} in neuronal cells under oxidative stressed is responsible for the selective inhibition of ERK-phosphatases and ensuing ERK1/2 activation and cell death. Interestingly, the late phase of ERK1/2 activation also functions to maintain elevated intracellular levels of Zn^{2+}. Thus the elevation of intracellular

97

Zn^{2+} within damaged neurons can trigger a robust positive feedback operating through activated ERK1/2 that rapidly induce Zn^{2+} dependent cell death. ROS has been considered as an important factor triggers the Zn^{2+} accumulation and following cell toxicity which can explain our previous results in Chapter 3 (Fig 6B). Specifically, constitutive ERK1/2 activation alone does not promote cell death because ERK1/2 activation itself might not affect Zn^{2+} release.

The mechanism of glutamate-induced oxidative toxicity in HT22 cells and the protective ERK1/2 signal transduction pathway have been summarized in Fig 17.
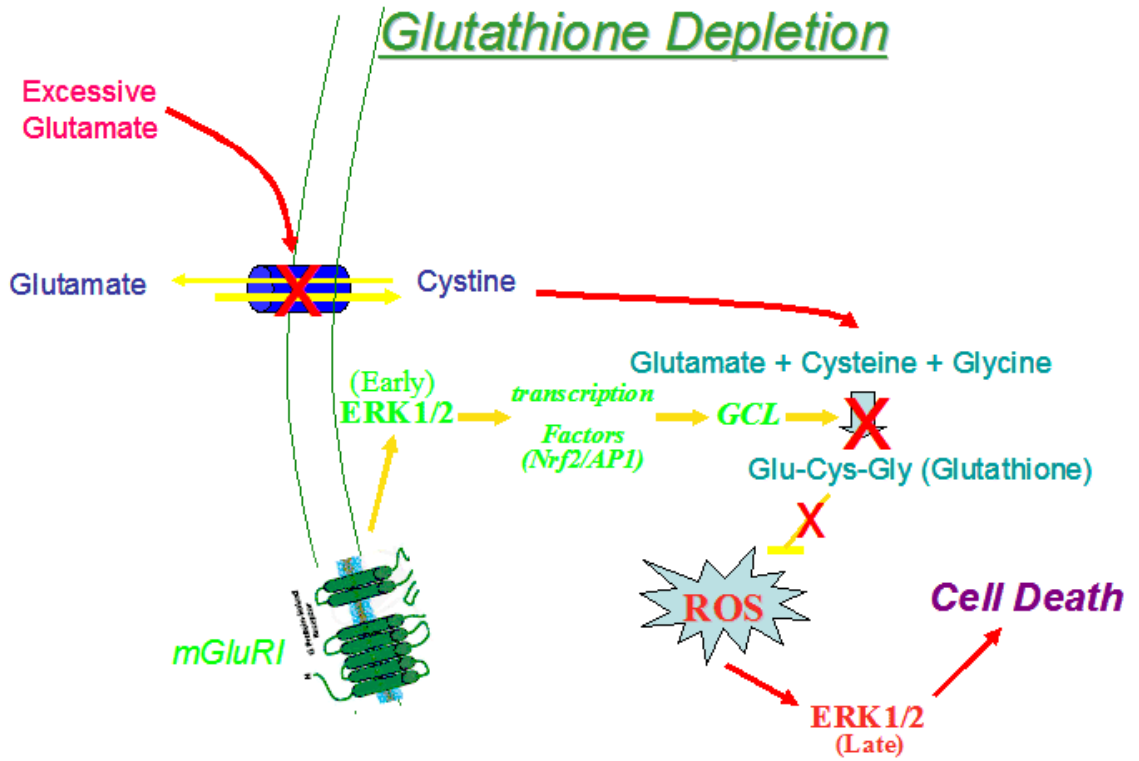
Figure 17. Summary of glutamate-induced oxidative toxicity and mechanism of ERK1/2's neuroprotective effect.

BIBLIOGRAPHY

1. **Abe, T., H. Sugihara, H. Nawa, R. Shigemoto, N. Mizuno, and S. Nakanishi.** 1992. Molecular characterization of a novel metabotropic glutamate receptor mGluR5 coupled to inositol phosphate/Ca2+ signal transduction. J Biol Chem **267:**13361-8.
2. **Adams, J. M., and S. Cory.** 2002. Apoptosomes: engines for caspase activation. Curr Opin Cell Biol **14:**715-20.
3. **Albasanz, J. L., E. Dalfo, I. Ferrer, and M. Martin.** 2005. Impaired metabotropic glutamate receptor/phospholipase C signaling pathway in the cerebral cortex in Alzheimer's disease and dementia with Lewy bodies correlates with stage of Alzheimer's-disease-related changes. Neurobiol Dis **20:**685-93.
4. **Alessandrini, A., S. Namura, M. A. Moskowitz, and J. V. Bonventre.** 1999. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci U S A **96:**12866-9.
5. **Allen, J. W., G. Shanker, K. H. Tan, and M. Aschner.** 2002. The consequences of methylmercury exposure on interactive functions between astrocytes and neurons. Neurotoxicology **23:**755-9.
6. **Anderson, M. F., F. Blomstrand, C. Blomstrand, P. S. Eriksson, and M. Nilsson.** 2003. Astrocytes and stroke: networking for survival? Neurochem Res **28:**293-305.
7. **Andreka, P., J. Zang, C. Dougherty, T. I. Slepak, K. A. Webster, and N. H. Bishopric.** 2001. Cytoprotection by Jun kinase during nitric oxide-induced cardiac myocyte apoptosis. Circ Res **88:**305-12.
8. **Aramori, I., and S. Nakanishi.** 1992. Signal transduction and pharmacological characteristics of a metabotropic glutamate receptor, mGluR1, in transfected CHO cells. Neuron **8:**757-65.
9. **Aronica, E., E. A. van Vliet, O. A. Mayboroda, D. Troost, F. H. da Silva, and J. A. Gorter.** 2000. Upregulation of metabotropic glutamate receptor subtype mGluR3 and mGluR5 in reactive astrocytes in a rat model of mesial temporal lobe epilepsy. Eur J Neurosci **12:**2333-44.
10. **Arrigo, A. P.** 1999. Gene expression and the thiol redox state. Free Radic Biol Med **27:**936-44.
11. **Asada, H., N. Y. Ip, L. Pan, N. Razack, M. M. Parfitt, and R. J. Plunkett.** 1995. Time course of ciliary neurotrophic factor mRNA expression is coincident with the presence of protoplasmic astrocytes in traumatized rat striatum. J Neurosci Res **40:**22-30.
12. **Bao, W. L., A. J. Williams, A. I. Faden, and F. C. Tortella.** 2001. Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia. Brain Res **922:**173-9.

13. **Baron, J. C., R. von Kummer, and G. J. del Zoppo.** 1995. Treatment of acute ischemic stroke. Challenging the concept of a rigid and universal time window. Stroke **26:**2219-21.

14. **Barone, F. C., E. A. Irving, A. M. Ray, J. C. Lee, S. Kassis, S. Kumar, A. M. Badger, J. J. Legos, J. A. Erhardt, E. H. Ohlstein, A. J. Hunter, D. C. Harrison, K. Philpott, B. R. Smith, J. L. Adams, and A. A. Parsons.** 2001. Inhibition of p38 mitogen-activated protein kinase provides neuroprotection in cerebral focal ischemia. Med Res Rev **21:**129-45.

15. **Benarroch, E. E.** 2005. Neuron-astrocyte interactions: partnership for normal function and disease in the central nervous system. Mayo Clin Proc **80:**1326-38.

16. **Berent-Spillson, A., A. M. Robinson, D. Golovoy, B. Slusher, C. Rojas, and J. W. Russell.** 2004. Protection against glucose-induced neuronal death by NAAG and GCP II inhibition is regulated by mGluR3. J Neurochem **89:**90-9.

17. **Bharath, S., M. Hsu, D. Kaur, S. Rajagopalan, and J. K. Andersen.** 2002. Glutathione, iron and Parkinson's disease. Biochem Pharmacol **64:**1037-48.

18. **Bhattacharya, J., and S. Swarup-Mitra.** 1987. Reduction in erythrocytic GSH level and stability in Plasmodium vivax malaria. Trans R Soc Trop Med Hyg **81:**64-6.

19. **Boulware, M. I., J. P. Weick, B. R. Becklund, S. P. Kuo, R. D. Groth, and P. G. Mermelstein.** 2005. Estradiol activates group I and II metabotropic glutamate receptor signaling, leading to opposing influences on cAMP response element-binding protein. J Neurosci **25:**5066-78.

20. **Bounous, G., and J. H. Molson.** 2003. The antioxidant system. Anticancer Res **23:**1411-5.

21. **Browne, S. E., R. J. Ferrante, and M. F. Beal.** 1999. Oxidative stress in Huntington's disease. Brain Pathol **9:**147-63.

22. **Brunet, A., D. Roux, P. Lenormand, S. Dowd, S. Keyse, and J. Pouyssegur.** 1999. Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. Embo J **18:**664-74.

23. **Camarero, J., V. Sanchez, E. O'Shea, A. R. Green, and M. I. Colado.** 2002. Studies, using in vivo microdialysis, on the effect of the dopamine uptake inhibitor GBR 12909 on 3,4-methylenedioxymethamphetamine ('ecstasy')-induced dopamine release and free radical formation in the mouse striatum. J Neurochem **81:**961-72.

24. **Canals, S., M. J. Casarejos, S. de Bernardo, R. M. Solano, and M. A. Mena.** 2003. Selective and persistent activation of extracellular signal-regulated protein kinase by nitric oxide in glial cells induces neuronal degeneration in glutathione-depleted midbrain cultures. Mol Cell Neurosci **24:**1012-26.

25. **Catania, M. V., E. Aronica, M. A. Sortino, P. L. Canonico, and F. Nicoletti.** 1991. Desensitization of metabotropic glutamate receptors in neuronal cultures. J Neurochem **56:**1329-35.

26. **Cavanaugh, J. E.** 2004. Role of extracellular signal regulated kinase 5 in neuronal survival. Eur J Biochem **271:**2056-9.

27. **Cederbaum, A. I.** 1989. Oxygen radical generation by microsomes: role of iron and implications for alcohol metabolism and toxicity. Free Radic Biol Med **7:**559-67.

28. **Chan, K., X. D. Han, and Y. W. Kan.** 2001. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proc Natl Acad Sci U S A **98:**4611-6.

29. **Chemin, J., C. Girard, F. Duprat, F. Lesage, G. Romey, and M. Lazdunski.** 2003. Mechanisms underlying excitatory effects of group I metabotropic glutamate receptors via inhibition of 2P domain K+ channels. Embo J **22:**5403-11.

30. **Chen, Y., N. E. Vartiainen, W. Ying, P. H. Chan, J. Koistinaho, and R. A. Swanson.** 2001. Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. J Neurochem **77:**1601-10.

31. **Cheung, E. C., and R. S. Slack.** 2004. Emerging Role for ERK as a Key Regulator of Neuronal Apoptosis. Sci STKE **2004:**PE45.

32. **Choi, J., R. M. Liu, and H. J. Forman.** 1997. Adaptation to oxidative stress: quinone-mediated protection of signaling in rat lung epithelial L2 cells. Biochem Pharmacol **53:**987-93.

33. **Choi, J., R. M. Liu, R. K. Kundu, F. Sangiorgi, W. Wu, R. Maxson, and H. J. Forman.** 2000. Molecular mechanism of decreased glutathione content in human immunodeficiency virus type 1 Tat-transgenic mice. J Biol Chem **275:**3693-8.

34. **Chu, C. T., D. J. Levinthal, S. M. Kulich, E. M. Chalovich, and D. B. DeFranco.** 2004. Oxidative neuronal injury. The dark side of ERK1/2. Eur J Biochem **271:**2060-6.

35. **Cole, K., and J. R. Perez-Polo.** 2004. Neuronal trauma model: in search of Thanatos. Int J Dev Neurosci **22:**485-96.

36. **Colucci-D'Amato, L., C. Perrone-Capano, and U. di Porzio.** 2003. Chronic activation of ERK and neurodegenerative diseases. Bioessays **25:**1085-95.

37. **Conn, P. J., and J. P. Pin.** 1997. Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol **37:**205-37.

38. **Cookson, M. R., and P. J. Shaw.** 1999. Oxidative stress and motor neurone disease. Brain Pathol **9:**165-86.

39. **Cullinan, S. B., J. D. Gordan, J. Jin, J. W. Harper, and J. A. Diehl.** 2004. The Keap1-BTB protein is an adaptor that bridges Nrf2 to a Cul3-based E3 ligase: oxidative stress sensing by a Cul3-Keap1 ligase. Mol Cell Biol **24:**8477-86.

40. **Cunningham, L. A., M. Wetzel, and G. A. Rosenberg.** 2005. Multiple roles for MMPs and TIMPs in cerebral ischemia. Glia **50:**329-39.

41. **Dalton, T. P., M. Z. Dieter, Y. Yang, H. G. Shertzer, and D. W. Nebert.** 2000. Knockout of the mouse glutamate cysteine ligase catalytic subunit (Gclc) gene: embryonic lethal when homozygous, and proposed model for moderate glutathione deficiency when heterozygous. Biochem Biophys Res Commun **279:**324-9.

42. **Davis, J. B., and P. Maher.** 1994. Protein kinase C activation inhibits glutamate-induced cytotoxicity in a neuronal cell line. Brain Res **652:**169-73.

43. **Deckwerth, T. L., J. L. Elliott, C. M. Knudson, E. M. Johnson, Jr., W. D. Snider, and S. J. Korsmeyer.** 1996. BAX is required for neuronal death after trophic factor deprivation and during development. Neuron **17:**401-11.

44. **Decoursey, T. E., and E. Ligeti.** 2005. Regulation and termination of NADPH oxidase activity. Cell Mol Life Sci **62:**2173-93.

45. **Deng, H. X., A. Hentati, J. A. Tainer, Z. Iqbal, A. Cayabyab, W. Y. Hung, E. D. Getzoff, P. Hu, B. Herzfeldt, R. P. Roos, and et al.** 1993. Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. Science **261:**1047-51.

46. **Dent, P., D. B. Reardon, J. S. Park, G. Bowers, C. Logsdon, K. Valerie, and R. Schmidt-Ullrich.** 1999. Radiation-induced release of transforming growth factor alpha activates the epidermal growth factor receptor and mitogen-activated protein kinase

pathway in carcinoma cells, leading to increased proliferation and protection from radiation-induced cell death. Mol Biol Cell **10:**2493-506.

47. **Desagher, S., J. Glowinski, and J. Premont.** 1996. Astrocytes protect neurons from hydrogen peroxide toxicity. J Neurosci **16:**2553-62.

48. **Dhakshinamoorthy, S., and A. G. Porter.** 2004. Nitric oxide-induced transcriptional up-regulation of protective genes by Nrf2 via the antioxidant response element counteracts apoptosis of neuroblastoma cells. J Biol Chem **279:**20096-107.

49. **Dickinson, D. A., and H. J. Forman.** 2002. Cellular glutathione and thiols metabolism. Biochem Pharmacol **64:**1019-26.

50. **Dickinson, D. A., K. E. Iles, N. Watanabe, T. Iwamoto, H. Zhang, D. M. Krzywanski, and H. J. Forman.** 2002. 4-hydroxynonenal induces glutamate cysteine ligase through JNK in HBE1 cells. Free Radic Biol Med **33:**974.

51. **Dickinson, D. A., K. E. Iles, H. Zhang, V. Blank, and H. J. Forman.** 2003. Curcumin alters EpRE and AP-1 binding complexes and elevates glutamate-cysteine ligase gene expression. Faseb J **17:**473-5.

52. **Dickinson, D. A., D. R. Moellering, K. E. Iles, R. P. Patel, A. L. Levonen, A. Wigley, V. M. Darley-Usmar, and H. J. Forman.** 2003. Cytoprotection against oxidative stress and the regulation of glutathione synthesis. Biol Chem **384:**527-37.

53. **Dienel, G. A., and L. Hertz.** 2005. Astrocytic contributions to bioenergetics of cerebral ischemia. Glia **50:**362-88.

54. **D'Onofrio, M., L. Cuomo, G. Battaglia, R. T. Ngomba, M. Storto, A. E. Kingston, F. Orzi, A. De Blasi, P. Di Iorio, F. Nicoletti, and V. Bruno.** 2001. Neuroprotection mediated by glial group-II metabotropic glutamate receptors requires the activation of the MAP kinase and the phosphatidylinositol-3-kinase pathways. J Neurochem **78:**435-45.

55. **Dringen, R.** 2000. Metabolism and functions of glutathione in brain. Prog Neurobiol **62:**649-71.

56. **Dringen, R., J. M. Gutterer, and J. Hirrlinger.** 2000. Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. Eur J Biochem **267:**4912-6.

57. **Du, S., B. McLaughlin, S. Pal, and E. Aizenman.** 2002. In vitro neurotoxicity of methylisothiazolinone, a commonly used industrial and household biocide, proceeds via a zinc and extracellular signal-regulated kinase mitogen-activated protein kinase-dependent pathway. J Neurosci **22:**7408-16.

58. **Ennis, S. R., N. Kawai, X. D. Ren, G. E. Abdelkarim, and R. F. Keep.** 1998. Glutamine uptake at the blood-brain barrier is mediated by N-system transport. J Neurochem **71:**2565-73.

59. **Fahey, J. W., X. Haristoy, P. M. Dolan, T. W. Kensler, I. Scholtus, K. K. Stephenson, P. Talalay, and A. Lozniewski.** 2002. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A **99:**7610-5.

60. **Ferraguti, F., B. Baldani-Guerra, M. Corsi, S. Nakanishi, and C. Corti.** 1999. Activation of the extracellular signal-regulated kinase 2 by metabotropic glutamate receptors. Eur J Neurosci **11:**2073-2082.

61. **Fisher, M.** 2004. The ischemic penumbra: identification, evolution and treatment concepts. Cerebrovasc Dis **17 Suppl 1:**1-6.

62. **Flor, P. J., G. Battaglia, F. Nicoletti, F. Gasparini, and V. Bruno.** 2002. Neuroprotective activity of metabotropic glutamate receptor ligands. Adv Exp Med Biol **513:**197-223.

63. **Forman, H. J., M. M. Shi, T. Iwamoto, R. M. Liu, and T. W. Robison.** 1995. Measurement of gamma-glutamyl transpeptidase and gamma-glutamylcysteine synthetase activities in cells. Methods Enzymol **252:**66-71.

64. **Foyer, C. H., and S. Ferrario.** 1994. Modulation of carbon and nitrogen metabolism in transgenic plants with a view to improved biomass production. Biochem Soc Trans **22:**909-15.

65. **Frodin, M., and S. Gammeltoft.** 1999. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. Mol Cell Endocrinol **151:**65-77.

66. **Fujimori, S., Y. Abe, M. Nishi, A. Hamamoto, Y. Inoue, Y. Ohnishi, C. Nishime, H. Matsumoto, H. Yamazaki, H. Kijima, Y. Ueyama, H. Inoue, and M. Nakamura.** 2004. The subunits of glutamate cysteine ligase enhance cisplatin resistance in human non-small cell lung cancer xenografts in vivo. Int J Oncol **25:**413-8.

67. **Fukuda, M., I. Gotoh, Y. Gotoh, and E. Nishida.** 1996. Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. J Biol Chem **271:**20024-8.

68. **Furukawa, M., and Y. Xiong.** 2005. BTB protein Keap1 targets antioxidant transcription factor Nrf2 for ubiquitination by the Cullin 3-Roc1 ligase. Mol Cell Biol **25:**162-71.

69. **Gabellini, N., R. M. Manev, P. Candeo, M. Favaron, and H. Manev.** 1993. Carboxyl domain of glutamate receptor directs its coupling to metabolic pathways. Neuroreport **4:**531-4.

70. **Gallagher, J. Z., F; Shinnick-Galagher, P.** 1994. Long-lasting modulation of synaptic transmission by metabotropic glutamate receptors. Metabotropic Glutamate Receptors**:**173-179.

71. **Gerber, U.** 2003. Metabotropic glutamate receptors in vertebrate retina. Doc Ophthalmol **106:**83-7.

72. **Gerlach, M., D. Ben-Shachar, P. Riederer, and M. B. Youdim.** 1994. Altered brain metabolism of iron as a cause of neurodegenerative diseases? J Neurochem **63:**793-807.

73. **Grant, E. R., M. A. Errico, S. L. Emanuel, D. Benjamin, M. K. McMillian, S. A. Wadsworth, R. A. Zivin, and Z. Zhong.** 2001. Protection against glutamate toxicity through inhibition of the p44/42 mitogen-activated protein kinase pathway in neuronally differentiated P19 cells. Biochem Pharmacol **62:**283-96.

74. **Greenamyre, J. T., and T. G. Hastings.** 2004. Biomedicine. Parkinson's--divergent causes, convergent mechanisms. Science **304:**1120-2.

75. **Grewal, S. S., R. D. York, and P. J. Stork.** 1999. Extracellular-signal-regulated kinase signalling in neurons. Curr Opin Neurobiol **9:**544-53.

76. **Griot, C., T. Burge, M. Vandevelde, and E. Peterhans.** 1989. Antibody-induced generation of reactive oxygen radicals by brain macrophages in canine distemper encephalitis: a mechanism for bystander demyelination. Acta Neuropathol (Berl) **78:**396-403.

77. **Grueter, B. A., H. B. Gosnell, C. M. Olsen, N. L. Schramm-Sapyta, T. Nekrasova, G. E. Landreth, and D. G. Winder.** 2006. Extracellular-signal regulated kinase 1-dependent metabotropic glutamate receptor 5-induced long-term depression in the bed

nucleus of the stria terminalis is disrupted by cocaine administration. J Neurosci **26:**3210-9.

78. **Grunewald, T., and M. F. Beal.** 1999. NOS knockouts and neuroprotection. Nat Med **5:**1354-5.

79. **Haberg, A., H. Qu, O. Saether, G. Unsgard, O. Haraldseth, and U. Sonnewald.** 2001. Differences in neurotransmitter synthesis and intermediary metabolism between glutamatergic and GABAergic neurons during 4 hours of middle cerebral artery occlusion in the rat: the role of astrocytes in neuronal survival. J Cereb Blood Flow Metab **21:**1451-63.

80. **Hall, A. G.** 1999. Review: The role of glutathione in the regulation of apoptosis. Eur J Clin Invest **29:**238-45.

81. **Halliwell, B.** 1992. Reactive oxygen species and the central nervous system. J Neurochem **59:**1609-23.

82. **Han, B. H., and D. M. Holtzman.** 2000. BDNF protects the neonatal brain from hypoxic-ischemic injury in vivo via the ERK pathway. J Neurosci **20:**5775-81.

83. **Hansen, J. M., E. Lee, and C. Harris.** 2004. Spatial activities and induction of glutamate-cysteine ligase (GCL) in the postimplantation rat embryo and visceral yolk sac. Toxicol Sci **81:**371-8.

84. **Heales, S. J., J. P. Bolanos, V. C. Stewart, P. S. Brookes, J. M. Land, and J. B. Clark.** 1999. Nitric oxide, mitochondria and neurological disease. Biochim Biophys Acta **1410:**215-28.

85. **Heales, S. J., S. E. Davies, T. E. Bates, and J. B. Clark.** 1995. Depletion of brain glutathione is accompanied by impaired mitochondrial function and decreased N-acetyl aspartate concentration. Neurochem Res **20:**31-8.

86. **Heiss, W. D., and R. Graf.** 1994. The ischemic penumbra. Curr Opin Neurol **7:**11-9.

87. **Heiss, W. D., A. Thiel, M. Grond, and R. Graf.** 1999. Which targets are relevant for therapy of acute ischemic stroke? Stroke **30:**1486-9.

88. **Hermans, E., and R. A. Challiss.** 2001. Structural, signalling and regulatory properties of the group I metabotropic glutamate receptors: prototypic family C G-protein-coupled receptors. Biochem J **359:**465-84.

89. **Hetman, M., and A. Gozdz.** 2004. Role of extracellular signal regulated kinases 1 and 2 in neuronal survival. Eur J Biochem **271:**2050-5.

90. **Hetman, M., K. Kanning, J. E. Cavanaugh, and Z. Xia.** 1999. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. J Biol Chem **274:**22569-80.

91. **Honig, L. S., and R. N. Rosenberg.** 2000. Apoptosis and neurologic disease. Am J Med **108:**317-30.

92. **Hori, K., M. Katayama, N. Sato, K. Ishii, S. Waga, and J. Yodoi.** 1994. Neuroprotection by glial cells through adult T cell leukemia-derived factor/human thioredoxin (ADF/TRX). Brain Res **652:**304-10.

93. **Houamed, K. M., J. L. Kuijper, T. L. Gilbert, B. A. Haldeman, P. J. O'Hara, E. R. Mulvihill, W. Almers, and F. S. Hagen.** 1991. Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. Science **252:**1318-21.

94. **Huang, C. S., L. S. Chang, M. E. Anderson, and A. Meister.** 1993. Catalytic and regulatory properties of the heavy subunit of rat kidney gamma-glutamylcysteine synthetase. J Biol Chem **268:**19675-80.

95. **Huang, H. C., T. Nguyen, and C. B. Pickett.** 2000. Regulation of the antioxidant response element by protein kinase C-mediated phosphorylation of NF-E2-related factor 2. Proc Natl Acad Sci U S A **97:**12475-80.

96. **Iacovelli, L., V. Bruno, L. Salvatore, D. Melchiorri, R. Gradini, A. Caricasole, E. Barletta, A. De Blasi, and F. Nicoletti.** 2002. Native group-III metabotropic glutamate receptors are coupled to the mitogen-activated protein kinase/phosphatidylinositol-3-kinase pathways. J Neurochem **82:**216-23.

97. **Iles, K. E., and R. M. Liu.** 2005. Mechanisms of glutamate cysteine ligase (GCL) induction by 4-hydroxynonenal. Free Radic Biol Med **38:**547-56.

98. **Ishii, T., K. Itoh, S. Takahashi, H. Sato, T. Yanagawa, Y. Katoh, S. Bannai, and M. Yamamoto.** 2000. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem **275:**16023-9.

99. **Itoh, K., T. Ishii, N. Wakabayashi, and M. Yamamoto.** 1999. Regulatory mechanisms of cellular response to oxidative stress. Free Radic Res **31:**319-24.

100. **Itoh, K., N. Wakabayashi, Y. Katoh, T. Ishii, K. Igarashi, J. D. Engel, and M. Yamamoto.** 1999. Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev **13:**76-86.

101. **Jaiswal, A. K.** 2004. Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med **36:**1199-207.

102. **Jha, N., O. Jurma, G. Lalli, Y. Liu, E. H. Pettus, J. T. Greenamyre, R. M. Liu, H. J. Forman, and J. K. Andersen.** 2000. Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity. Implications for Parkinson's disease. J Biol Chem **275:**26096-101.

103. **Joly, C., J. Gomeza, I. Brabet, K. Curry, J. Bockaert, and J. P. Pin.** 1995. Molecular, functional, and pharmacological characterization of the metabotropic glutamate receptor type 5 splice variants: comparison with mGluR1. J Neurosci **15:**3970-81.

104. **Kalehua, A. N., J. E. Nagel, L. M. Whelchel, J. J. Gides, R. S. Pyle, R. J. Smith, J. W. Kusiak, and D. D. Taub.** 2004. Monocyte chemoattractant protein-1 and macrophage inflammatory protein-2 are involved in both excitotoxin-induced neurodegeneration and regeneration. Exp Cell Res **297:**197-211.

105. **Kametsu, Y., S. Osuga, and A. M. Hakim.** 2003. Apoptosis occurs in the penumbra zone during short-duration focal ischemia in the rat. J Cereb Blood Flow Metab **23:**416-22.

106. **Kamsler, A., and M. Segal.** 2004. Hydrogen peroxide as a diffusible signal molecule in synaptic plasticity. Mol Neurobiol **29:**167-78.

107. **Katoh, Y., K. Itoh, E. Yoshida, M. Miyagishi, A. Fukamizu, and M. Yamamoto.** 2001. Two domains of Nrf2 cooperatively bind CBP, a CREB binding protein, and synergistically activate transcription. Genes Cells **6:**857-68.

108. **Kerr, J. F.** 1971. Shrinkage necrosis: a distinct mode of cellular death. J Pathol **105:**13-20.

109. **Kerr, J. F., A. H. Wyllie, and A. R. Currie.** 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer **26:**239-57.

110. **Ketterer, B.** 1982. The role of nonenzymatic reactions of glutathione in xenobiotic metabolism. Drug Metab Rev **13:**161-87.

111. **Kirino, T.** 1982. Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res **239:**57-69.

112. **Klein, J., K. G. Reymann, and G. Riedel.** 1997. Activation of phospholipases C and D by the novel metabotropic glutamate receptor agonist tADA. Neuropharmacology **36:**261-3.

113. **Knopfel, T., and P. Grandes.** 2002. Metabotropic glutamate receptors in the cerebellum with a focus on their function in Purkinje cells. Cerebellum **1:**19-26.

114. **Kobayashi, A., M. I. Kang, H. Okawa, M. Ohtsuji, Y. Zenke, T. Chiba, K. Igarashi, and M. Yamamoto.** 2004. Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2. Mol Cell Biol **24:**7130-9.

115. **Kobayashi, A., M. I. Kang, Y. Watai, K. I. Tong, T. Shibata, K. Uchida, and M. Yamamoto.** 2006. Oxidative and electrophilic stresses activate Nrf2 through inhibition of ubiquitination activity of Keap1. Mol Cell Biol **26:**221-9.

116. **Koistinaho, J., S. Miettinen, R. Keinanen, N. Vartiainen, R. Roivainen, and J. T. Laitinen.** 1996. Long-term induction of haem oxygenase-1 (HSP-32) in astrocytes and microglia following transient focal brain ischaemia in the rat. Eur J Neurosci **8:**2265-72.

117. **Kondo, T., K. Yoshida, Y. Urata, S. Goto, S. Gasa, and N. Taniguchi.** 1993. gamma-Glutamylcysteine synthetase and active transport of glutathione S-conjugate are responsive to heat shock in K562 erythroid cells. J Biol Chem **268:**20366-72.

118. **Kong, A. N., E. Owuor, R. Yu, V. Hebbar, C. Chen, R. Hu, and S. Mandlekar.** 2001. Induction of xenobiotic enzymes by the MAP kinase pathway and the antioxidant or electrophile response element (ARE/EpRE). Drug Metab Rev **33:**255-71.

119. **Krzywanski, D. M., D. A. Dickinson, K. E. Iles, A. F. Wigley, C. C. Franklin, R. M. Liu, T. J. Kavanagh, and H. J. Forman.** 2004. Variable regulation of glutamate cysteine ligase subunit proteins affects glutathione biosynthesis in response to oxidative stress. Arch Biochem Biophys **423:**116-25.

120. **Kuida, K., T. S. Zheng, S. Na, C. Kuan, D. Yang, H. Karasuyama, P. Rakic, and R. A. Flavell.** 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature **384:**368-72.

121. **Kulich, S. M., and C. T. Chu.** 2001. Sustained extracellular signal-regulated kinase activation by 6-hydroxydopamine: implications for Parkinson's disease. J Neurochem **77:**1058-66.

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

123. **Kyriakis, J. M., and J. Avruch.** 2001. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev **81:**807-69.

124. **Lamb, J. G., P. Marick, J. Sorensen, S. Haley, and M. D. Dearing.** 2004. Liver biotransforming enzymes in woodrats Neotoma stephensi (Muridae). Comp Biochem Physiol C Toxicol Pharmacol **138:**195-201.

125. **Lee, H. T., H. Xu, A. Ota-Setlik, and C. W. Emala.** 2003. Oxidant preconditioning protects human proximal tubular cells against lethal oxidant injury via p38 MAPK and heme oxygenase-1. Am J Nephrol **23:**324-33.

126. **Lee, J. I., J. Kang, and M. H. Stipanuk.** 2006. Differential regulation of glutamate-cysteine ligase subunit expression and increased holoenzyme formation in response to cysteine deprivation. Biochem J **393:**181-90.

127. **Lee, J. M., and J. A. Johnson.** 2004. An important role of Nrf2-ARE pathway in the cellular defense mechanism. J Biochem Mol Biol **37:**139-43.
128. **Lee, J. M., J. Li, D. A. Johnson, T. D. Stein, A. D. Kraft, M. J. Calkins, R. J. Jakel, and J. A. Johnson.** 2005. Nrf2, a multi-organ protector? Faseb J **19:**1061-6.
129. **Lee, T. H., H. Kato, S. T. Chen, K. Kogure, and Y. Itoyama.** 1998. Expression of nerve growth factor and trkA after transient focal cerebral ischemia in rats. Stroke **29:**1687-96; discussion 1697.
130. **Leis, J. A., L. K. Bekar, and W. Walz.** 2005. Potassium homeostasis in the ischemic brain. Glia **50:**407-16.
131. **Leist, M., and P. Nicotera.** 1998. Apoptosis, excitotoxicity, and neuropathology. Exp Cell Res **239:**183-201.
132. **Levinthal, D. J., and D. B. Defranco.** 2005. Reversible oxidation of ERK-directed protein phosphatases drives oxidative toxicity in neurons. J Biol Chem **280:**5875-83.
133. **Levinthal, D. J., and D. B. DeFranco.** 2004. Transient phosphatidylinositol 3-kinase inhibition protects immature primary cortical neurons from oxidative toxicity via suppression of extracellular signal-regulated kinase activation. J Biol Chem **279:**11206-13.
134. **Li, Y., P. Maher, and D. Schubert.** 1998. Phosphatidylcholine-specific phospholipase C regulates glutamate-induced nerve cell death. Proc Natl Acad Sci U S A **95:**7748-53.
135. **Li, Y., P. Maher, and D. Schubert.** 1997. A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron **19:**453-63.
136. **Liska, D. J.** 1998. The detoxification enzyme systems. Altern Med Rev **3:**187-98.
137. **Liu, R. M.** 2002. Down-regulation of gamma-glutamylcysteine synthetase regulatory subunit gene expression in rat brain tissue during aging. J Neurosci Res **68:**344-51.
138. **Liu, R. M., Z. Borok, and H. J. Forman.** 2001. 4-Hydroxy-2-nonenal increases gamma-glutamylcysteine synthetase gene expression in alveolar epithelial cells. Am J Respir Cell Mol Biol **24:**499-505.
139. **Liu, R. M., L. Gao, J. Choi, and H. J. Forman.** 1998. gamma-glutamylcysteine synthetase: mRNA stabilization and independent subunit transcription by 4-hydroxy-2-nonenal. Am J Physiol **275:**L861-9.
140. **Liu, R. M., H. Hu, T. W. Robison, and H. J. Forman.** 1996. Increased gamma-glutamylcysteine synthetase and gamma-glutamyl transpeptidase activities enhance resistance of rat lung epithelial L2 cells to quinone toxicity. Am J Respir Cell Mol Biol **14:**192-7.
141. **Love, S.** 1999. Oxidative stress in brain ischemia. Brain Pathol **9:**119-31.
142. **Lucius, R., and J. Sievers.** 1996. Postnatal retinal ganglion cells in vitro: protection against reactive oxygen species (ROS)-induced axonal degeneration by cocultured astrocytes. Brain Res **743:**56-62.
143. **Maher, P.** 2001. How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. J Neurosci **21:**2929-38.
144. **Maher, P., and J. B. Davis.** 1996. The role of monoamine metabolism in oxidative glutamate toxicity. J Neurosci **16:**6394-401.
145. **Makar, T. K., M. Nedergaard, A. Preuss, A. S. Gelbard, A. S. Perumal, and A. J. Cooper.** 1994. Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that

astrocytes play an important role in antioxidative processes in the brain. J Neurochem **62:**45-53.

146. **Mansour, S. J., W. T. Matten, A. S. Hermann, J. M. Candia, S. Rong, K. Fukasawa, G. F. Vande Woude, and N. G. Ahn.** 1994. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science **265:**966-70.

147. **Mao, L., and J. Q. Wang.** 2002. Glutamate cascade to cAMP response element-binding protein phosphorylation in cultured striatal neurons through calcium-coupled group I metabotropic glutamate receptors. Mol Pharmacol **62:**473-84.

148. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179-85.

149. **Martin, L. J.** 2001. Neuronal cell death in nervous system development, disease, and injury (Review). Int J Mol Med **7:**455-78.

150. **Martinou, J. C., M. Dubois-Dauphin, J. K. Staple, I. Rodriguez, H. Frankowski, M. Missotten, P. Albertini, D. Talabot, S. Catsicas, C. Pietra, and et al.** 1994. Overexpression of BCL-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron **13:**1017-30.

151. **Marz, P., K. Heese, B. Dimitriades-Schmutz, S. Rose-John, and U. Otten.** 1999. Role of interleukin-6 and soluble IL-6 receptor in region-specific induction of astrocytic differentiation and neurotrophin expression. Glia **26:**191-200.

152. **Masu, M., Y. Tanabe, K. Tsuchida, R. Shigemoto, and S. Nakanishi.** 1991. Sequence and expression of a metabotropic glutamate receptor. Nature **349:**760-5.

153. **Matthews, R. T., L. Yang, and M. F. Beal.** 1997. S-Methylthiocitrulline, a neuronal nitric oxide synthase inhibitor, protects against malonate and MPTP neurotoxicity. Exp Neurol **143:**282-6.

154. **Mazzucchelli, C., C. Vantaggiato, A. Ciamei, S. Fasano, P. Pakhotin, W. Krezel, H. Welzl, D. P. Wolfer, G. Pages, O. Valverde, A. Marowsky, A. Porrazzo, P. C. Orban, R. Maldonado, M. U. Ehrengruber, V. Cestari, H. P. Lipp, P. F. Chapman, J. Pouyssegur, and R. Brambilla.** 2002. Knockout of ERK1 MAP kinase enhances synaptic plasticity in the striatum and facilitates striatal-mediated learning and memory. Neuron **34:**807-20.

155. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem **244:**6049-55.

156. **McCulloch, J., and D. Dewar.** 2001. A radical approach to stroke therapy. Proc Natl Acad Sci U S A **98:**10989-91.

157. **Meister, A.** 1974. Glutathione synthesis. The Enzymes**:** 671–697.

158. **Meister, A., S. S. Tate, and O. W. Griffith.** 1981. Gamma-glutamyl transpeptidase. Methods Enzymol **77:**237-53.

159. **Mena, M. A., M. J. Casarejos, A. Carazo, C. L. Paino, and J. Garcia de Yebenes.** 1996. Glia conditioned medium protects fetal rat midbrain neurones in culture from L-DOPA toxicity. Neuroreport **7:**441-5.

160. **Mikawa, S., H. Kinouchi, H. Kamii, G. T. Gobbel, S. F. Chen, E. Carlson, C. J. Epstein, and P. H. Chan.** 1996. Attenuation of acute and chronic damage following traumatic brain injury in copper, zinc-superoxide dismutase transgenic mice. J Neurosurg **85:**885-91.

161. **Moellering, D., J. Mc Andrew, R. P. Patel, H. J. Forman, R. T. Mulcahy, H. Jo, and V. M. Darley-Usmar.** 1999. The induction of GSH synthesis by nanomolar

concentrations of NO in endothelial cells: a role for gamma-glutamylcysteine synthetase and gamma-glutamyl transpeptidase. FEBS Lett **448:**292-6.

162. **Moi, P., K. Chan, I. Asunis, A. Cao, and Y. W. Kan.** 1994. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci U S A **91:**9926-30.

163. **Moinova, H. R., and R. T. Mulcahy.** 1999. Up-regulation of the human gamma-glutamylcysteine synthetase regulatory subunit gene involves binding of Nrf-2 to an electrophile responsive element. Biochem Biophys Res Commun **261:**661-8.

164. **Morales, A., M. Miranda, A. Sanchez-Reyes, A. Colell, A. Biete, and J. C. Fernandez-Checa.** 1998. Transcriptional regulation of the heavy subunit chain of gamma-glutamylcysteine synthetase by ionizing radiation. FEBS Lett **427:**15-20.

165. **Moreira, P. I., K. Honda, Q. Liu, M. S. Santos, C. R. Oliveira, G. Aliev, A. Nunomura, X. Zhu, M. A. Smith, and G. Perry.** 2005. Oxidative stress: the old enemy in Alzheimer's disease pathophysiology. Curr Alzheimer Res **2:**403-8.

166. **Mori, T., X. Wang, J. C. Jung, T. Sumii, A. B. Singhal, M. E. Fini, C. E. Dixon, A. Alessandrini, and E. H. Lo.** 2002. Mitogen-activated protein kinase inhibition in traumatic brain injury: in vitro and in vivo effects. J Cereb Blood Flow Metab **22:**444-52.

167. **Murphy, T. F., and M. R. Loeb.** 1989. Isolation of the outer membrane of Branhamella catarrhalis. Microb Pathog **6:**159-74.

168. **Murray, B., A. Alessandrini, A. J. Cole, A. G. Yee, and E. J. Furshpan.** 1998. Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. Proc Natl Acad Sci U S A **95:**11975-80.

169. **Namura, S., K. Iihara, S. Takami, I. Nagata, H. Kikuchi, K. Matsushita, M. A. Moskowitz, J. V. Bonventre, and A. Alessandrini.** 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:**11569-74.

170. **Nguyen, T., P. J. Sherratt, H. C. Huang, C. S. Yang, and C. B. Pickett.** 2003. Increased protein stability as a mechanism that enhances Nrf2-mediated transcriptional activation of the antioxidant response element. Degradation of Nrf2 by the 26 S proteasome. J Biol Chem **278:**4536-41.

171. **Nguyen, T., C. S. Yang, and C. B. Pickett.** 2004. The pathways and molecular mechanisms regulating Nrf2 activation in response to chemical stress. Free Radic Biol Med **37:**433-41.

172. **Nijhawan, D., N. Honarpour, and X. Wang.** 2000. Apoptosis in neural development and disease. Annu Rev Neurosci **23:**73-87.

173. **Noor, R., S. Mittal, and J. Iqbal.** 2002. Superoxide dismutase--applications and relevance to human diseases. Med Sci Monit **8:**RA210-5.

174. **Northington, F. J., D. M. Ferriero, and L. J. Martin.** 2001. Neurodegeneration in the thalamus following neonatal hypoxia-ischemia is programmed cell death. Dev Neurosci **23:**186-91.

175. **Noshita, N., T. Sugawara, T. Hayashi, A. Lewen, G. Omar, and P. H. Chan.** 2002. Copper/zinc superoxide dismutase attenuates neuronal cell death by preventing extracellular signal-regulated kinase activation after transient focal cerebral ischemia in mice. J Neurosci **22:**7923-30.

176. **Nozaki, K., M. Nishimura, and N. Hashimoto.** 2001. Mitogen-activated protein kinases and cerebral ischemia. Mol Neurobiol **23:**1-19.
177. **Ochi, T.** 1995. Hydrogen peroxide increases the activity of gamma-glutamylcysteine synthetase in cultured Chinese hamster V79 cells. Arch Toxicol **70:**96-103.
178. **Pages, G., S. Guerin, D. Grall, F. Bonino, A. Smith, F. Anjuere, P. Auberger, and J. Pouyssegur.** 1999. Defective thymocyte maturation in p44 MAP kinase (Erk 1) knockout mice. Science **286:**1374-7.
179. **Papaiahgari, S., S. R. Kleeberger, H. Y. Cho, D. V. Kalvakolanu, and S. P. Reddy.** 2004. NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells. J Biol Chem **279:**42302-12.
180. **Parmentier, M. L., C. Joly, S. Restituito, J. Bockaert, Y. Grau, and J. P. Pin.** 1998. The G protein-coupling profile of metabotropic glutamate receptors, as determined with exogenous G proteins, is independent of their ligand recognition domain. Mol Pharmacol **53:**778-86.
181. **Patterson, S. L., C. Pittenger, A. Morozov, K. C. Martin, H. Scanlin, C. Drake, and E. R. Kandel.** 2001. Some forms of cAMP-mediated long-lasting potentiation are associated with release of BDNF and nuclear translocation of phospho-MAP kinase. Neuron **32:**123-40.
182. **Pearson, G., F. Robinson, T. Beers Gibson, B. E. Xu, M. Karandikar, K. Berman, and M. H. Cobb.** 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev **22:**153-83.
183. **Pettmann, B., and C. E. Henderson.** 1998. Neuronal cell death. Neuron **20:**633-47.
184. **Pong, K.** 2003. Oxidative stress in neurodegenerative diseases: therapeutic implications for superoxide dismutase mimetics. Expert Opin Biol Ther **3:**127-39.
185. **Poot, M., H. Teubert, P. S. Rabinovitch, and T. J. Kavanagh.** 1995. De novo synthesis of glutathione is required for both entry into and progression through the cell cycle. J Cell Physiol **163:**555-60.
186. **Pouyssegur, J., V. Volmat, and P. Lenormand.** 2002. Fidelity and spatio-temporal control in MAP kinase (ERKs) signalling. Biochem Pharmacol **64:**755-63.
187. **Przedborski, S., V. Jackson-Lewis, R. Yokoyama, T. Shibata, V. L. Dawson, and T. M. Dawson.** 1996. Role of neuronal nitric oxide in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced dopaminergic neurotoxicity. Proc Natl Acad Sci U S A **93:**4565-71.
188. **Przedborski, S., V. Kostic, V. Jackson-Lewis, A. B. Naini, S. Simonetti, S. Fahn, E. Carlson, C. J. Epstein, and J. L. Cadet.** 1992. Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. J Neurosci **12:**1658-67.
189. **Raghupathi, R., D. I. Graham, and T. K. McIntosh.** 2000. Apoptosis after traumatic brain injury. J Neurotrauma **17:**927-38.
190. **Rahman, I., A. Bel, B. Mulier, M. F. Lawson, D. J. Harrison, W. Macnee, and C. A. Smith.** 1996. Transcriptional regulation of gamma-glutamylcysteine synthetase-heavy subunit by oxidants in human alveolar epithelial cells. Biochem Biophys Res Commun **229:**832-7.
191. **Rangasamy, T., C. Y. Cho, R. K. Thimmulappa, L. Zhen, S. S. Srisuma, T. W. Kensler, M. Yamamoto, I. Petrache, R. M. Tuder, and S. Biswal.** 2004. Genetic

ablation of Nrf2 enhances susceptibility to cigarette smoke-induced emphysema in mice. J Clin Invest **114:**1248-59.

192. **Raps, S. P., J. C. Lai, L. Hertz, and A. J. Cooper.** 1989. Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. Brain Res **493:**398-401.

193. **Ratan, R. R., T. H. Murphy, and J. M. Baraban.** 1994. Oxidative stress induces apoptosis in embryonic cortical neurons. J Neurochem **62:**376-9.

194. **Reed, D. J.** 1990. Glutathione: toxicological implications. Annu Rev Pharmacol Toxicol **30:**603-31.

195. **Reid, M., and F. Jahoor.** 2001. Glutathione in disease. Curr Opin Clin Nutr Metab Care **4:**65-71.

196. **Rice, M. E., and I. Russo-Menna.** 1998. Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. Neuroscience **82:**1213-23.

197. **Richman, P. G., and A. Meister.** 1975. Regulation of gamma-glutamyl-cysteine synthetase by nonallosteric feedback inhibition by glutathione. J Biol Chem **250:**1422-6.

198. **Robinson, M. J., S. A. Stippec, E. Goldsmith, M. A. White, and M. H. Cobb.** 1998. A constitutively active and nuclear form of the MAP kinase ERK2 is sufficient for neurite outgrowth and cell transformation. Curr Biol **8:**1141-50.

199. **Rosen, D. R., T. Siddique, D. Patterson, D. A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J. P. O'Regan, H. X. Deng, and et al.** 1993. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature **362:**59-62.

200. **Rossler, O. G., K. M. Giehl, and G. Thiel.** 2004. Neuroprotection of immortalized hippocampal neurones by brain-derived neurotrophic factor and Raf-1 protein kinase: role of extracellular signal-regulated protein kinase and phosphatidylinositol 3-kinase. J Neurochem **88:**1240-52.

201. **Rouse, S. T., M. J. Marino, S. R. Bradley, H. Awad, M. Wittmann, and P. J. Conn.** 2000. Distribution and roles of metabotropic glutamate receptors in the basal ganglia motor circuit: implications for treatment of Parkinson's disease and related disorders. Pharmacol Ther **88:**427-35.

202. **Runden, E., P. O. Seglen, F. M. Haug, O. P. Ottersen, T. Wieloch, M. Shamloo, and J. H. Laake.** 1998. Regional selective neuronal degeneration after protein phosphatase inhibition in hippocampal slice cultures: evidence for a MAP kinase-dependent mechanism. J Neurosci **18:**7296-305.

203. **Sagara, Y., and D. Schubert.** 1998. The activation of metabotropic glutamate receptors protects nerve cells from oxidative stress. J Neurosci **18:**6662-71.

204. **Saito, A., C. M. Maier, P. Narasimhan, T. Nishi, Y. S. Song, F. Yu, J. Liu, Y. S. Lee, C. Nito, H. Kamada, R. L. Dodd, L. B. Hsieh, B. Hassid, E. E. Kim, M. Gonzalez, and P. H. Chan.** 2005. Oxidative stress and neuronal death/survival signaling in cerebral ischemia. Mol Neurobiol **31:**105-16.

205. **Sastry, P. S., and K. S. Rao.** 2000. Apoptosis and the nervous system. J Neurochem **74:**1-20.

206. **Sato, H., M. Tamba, T. Ishii, and S. Bannai.** 1999. Cloning and expression of a plasma membrane cystine/glutamate exchange transporter composed of two distinct proteins. J Biol Chem **274:**11455-8.

207. **Satoh, T., D. Nakatsuka, Y. Watanabe, I. Nagata, H. Kikuchi, and S. Namura.** 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:**163-6.

208. **Scholz, W., K. Schutze, W. Kunz, and M. Schwarz.** 1990. Phenobarbital enhances the formation of reactive oxygen in neoplastic rat liver nodules. Cancer Res **50:**7015-22.

209. **Seger, R., D. Seger, A. A. Reszka, E. S. Munar, H. Eldar-Finkelman, G. Dobrowolska, A. M. Jensen, J. S. Campbell, E. H. Fischer, and E. G. Krebs.** 1994. Overexpression of mitogen-activated protein kinase kinase (MAPKK) and its mutants in NIH 3T3 cells. Evidence that MAPKK involvement in cellular proliferation is regulated by phosphorylation of serine residues in its kinase subdomains VII and VIII. J Biol Chem **269:**25699-709.

210. **Sekhar, K. R., X. X. Yan, and M. L. Freeman.** 2002. Nrf2 degradation by the ubiquitin proteasome pathway is inhibited by KIAA0132, the human homolog to INrf2. Oncogene **21:**6829-34.

211. **Selcher, J. C., T. Nekrasova, R. Paylor, G. E. Landreth, and J. D. Sweatt.** 2001. Mice lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. Learn Mem **8:**11-9.

212. **Seo, S. R., S. A. Chong, S. I. Lee, J. Y. Sung, Y. S. Ahn, K. C. Chung, and J. T. Seo.** 2001. Zn2+-induced ERK activation mediated by reactive oxygen species causes cell death in differentiated PC12 cells. J Neurochem **78:**600-10.

213. **Serbest, G., J. Horwitz, M. Jost, and K. Barbee.** 2006. Mechanisms of cell death and neuroprotection by poloxamer 188 after mechanical trauma. Faseb J **20:**308-10.

214. **Shen, G., V. Hebbar, S. Nair, C. Xu, W. Li, W. Lin, Y. S. Keum, J. Han, M. A. Gallo, and A. N. Kong.** 2004. Regulation of Nrf2 transactivation domain activity. The differential effects of mitogen-activated protein kinase cascades and synergistic stimulatory effect of Raf and CREB-binding protein. J Biol Chem **279:**23052-60.

215. **Shi, M. M., T. Iwamoto, and H. J. Forman.** 1994. gamma-Glutamylcysteine synthetase and GSH increase in quinone-induced oxidative stress in BPAEC. Am J Physiol **267:**L414-21.

216. **Shih, A. Y., D. A. Johnson, G. Wong, A. D. Kraft, L. Jiang, H. Erb, J. A. Johnson, and T. H. Murphy.** 2003. Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. J Neurosci **23:**3394-406.

217. **Simonian, N. A., and J. T. Coyle.** 1996. Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol **36:**83-106.

218. **Simpson, E. P., A. A. Yen, and S. H. Appel.** 2003. Oxidative Stress: a common denominator in the pathogenesis of amyotrophic lateral sclerosis. Curr Opin Rheumatol **15:**730-6.

219. **Smythies, J. R.** 1997. Oxidative reactions and schizophrenia: a review-discussion. Schizophr Res **24:**357-64.

220. **Soltaninassab, S. R., K. R. Sekhar, M. J. Meredith, and M. L. Freeman.** 2000. Multi-faceted regulation of gamma-glutamylcysteine synthetase. J Cell Physiol **182:**163-70.

221. **Spencer, J. P., C. Rice-Evans, and R. J. Williams.** 2003. Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. J Biol Chem **278:**34783-93.

222. **Spillson, A. B., and J. W. Russell.** 2003. Metabotropic glutamate receptor regulation of neuronal cell death. Exp Neurol **184 Suppl 1:**S97-105.

223. **Spitz, D. R., E. I. Azzam, J. J. Li, and D. Gius.** 2004. Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: a unifying concept in stress response biology. Cancer Metastasis Rev **23:**311-22.
224. **Sporn, M. B., and K. T. Liby.** 2005. Cancer chemoprevention: scientific promise, clinical uncertainty. Nat Clin Pract Oncol **2:**518-25.
225. **Stanciu, M., and D. B. DeFranco.** 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:**4010-7.
226. **Stanciu, M., Y. Wang, R. Kentor, N. Burke, S. Watkins, G. Kress, I. Reynolds, E. Klann, M. R. Angiolieri, J. W. Johnson, and D. B. DeFranco.** 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:**12200-6.
227. **Stewart, D., E. Killeen, R. Naquin, S. Alam, and J. Alam.** 2003. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. J Biol Chem **278:**2396-402.
228. **Suh, J. H., S. V. Shenvi, B. M. Dixon, H. Liu, A. K. Jaiswal, R. M. Liu, and T. M. Hagen.** 2004. Decline in transcriptional activity of Nrf2 causes age-related loss of glutathione synthesis, which is reversible with lipoic acid. Proc Natl Acad Sci U S A **101:**3381-6.
229. **Sweatt, J. D.** 2004. Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol **14:**311-7.
230. **Tan, S., Y. Sagara, Y. Liu, P. Maher, and D. Schubert.** 1998. The regulation of reactive oxygen species production during programmed cell death. J Cell Biol **141:**1423-32.
231. **Tan, S., D. Schubert, and P. Maher.** 2001. Oxytosis: A novel form of programmed cell death. Curr Top Med Chem **1:**497-506.
232. **Tan, S., M. Wood, and P. Maher.** 1998. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem **71:**95-105.
233. **Taniguchi, N., and Y. Ikeda.** 1998. gamma-Glutamyl transpeptidase: catalytic mechanism and gene expression. Adv Enzymol Relat Areas Mol Biol **72:**239-78.
234. **Thompson, C. B.** 1995. Apoptosis in the pathogenesis and treatment of disease. Science **267:**1456-62.
235. **Thomsen, C.** 1996. Metabotropic glutamate receptor subtype 1A activates adenylate cyclase when expressed in baby hamster kidney cells. Prog Neuropsychopharmacol Biol Psychiatry **20:**709-26.
236. **Tokumine, J., O. Kakinohana, D. Cizkova, D. W. Smith, and M. Marsala.** 2003. Changes in spinal GDNF, BDNF, and NT-3 expression after transient spinal cord ischemia in the rat. J Neurosci Res **74:**552-61.
237. **Trendelenburg, G., and U. Dirnagl.** 2005. Neuroprotective role of astrocytes in cerebral ischemia: focus on ischemic preconditioning. Glia **50:**307-20.
238. **Trendelenburg, G., K. Prass, J. Priller, K. Kapinya, A. Polley, C. Muselmann, K. Ruscher, U. Kannbley, A. O. Schmitt, S. Castell, F. Wiegand, A. Meisel, A. Rosenthal, and U. Dirnagl.** 2002. Serial analysis of gene expression identifies metallothionein-II as major neuroprotective gene in mouse focal cerebral ischemia. J Neurosci **22:**5879-88.

239. **Tsuji, Y., Y. Shimada, T. Takeshita, N. Kajimura, S. Nomura, N. Sekiyama, J. Otomo, J. Usukura, S. Nakanishi, and H. Jingami.** 2000. Cryptic dimer interface and domain organization of the extracellular region of metabotropic glutamate receptor subtype 1. J Biol Chem **275:**28144-51.

240. **Urata, Y., H. Yamamoto, S. Goto, H. Tsushima, S. Akazawa, S. Yamashita, S. Nagataki, and T. Kondo.** 1996. Long exposure to high glucose concentration impairs the responsive expression of gamma-glutamylcysteine synthetase by interleukin-1beta and tumor necrosis factor-alpha in mouse endothelial cells. J Biol Chem **271:**15146-52.

241. **Vaishnav, D., P. Jambal, J. E. Reusch, and S. Pugazhenthi.** 2003. SP600125, an inhibitor of c-jun N-terminal kinase, activates CREB by a p38 MAPK-mediated pathway. Biochem Biophys Res Commun **307:**855-60.

242. **van Muiswinkel, F. L., and H. B. Kuiperij.** 2005. The Nrf2-ARE Signalling pathway: promising drug target to combat oxidative stress in neurodegenerative disorders. Curr Drug Targets CNS Neurol Disord **4:**267-81.

243. **Venugopal, R., and A. K. Jaiswal.** 1996. Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc Natl Acad Sci U S A **93:**14960-5.

244. **Vincent, A. M., Y. Mohammad, I. Ahmad, R. Greenberg, and K. Maiese.** 1997. Metabotropic glutamate receptors prevent nitric oxide-induced programmed cell death. J Neurosci Res **50:**549-64.

245. **Wang, X., C. Zhu, L. Qiu, H. Hagberg, M. Sandberg, and K. Blomgren.** 2003. Activation of ERK1/2 after neonatal rat cerebral hypoxia-ischaemia. J Neurochem **86:**351-62.

246. **Weiss, S. J., and A. F. LoBuglio.** 1982. Phagocyte-generated oxygen metabolites and cellular injury. Lab Invest **47:**5-18.

247. **Wild, A. C., H. R. Moinova, and R. T. Mulcahy.** 1999. Regulation of gamma-glutamylcysteine synthetase subunit gene expression by the transcription factor Nrf2. J Biol Chem **274:**33627-36.

248. **Wild, A. C., and R. T. Mulcahy.** 1999. Pyrrolidine dithiocarbamate up-regulates the expression of the genes encoding the catalytic and regulatory subunits of gamma-glutamylcysteine synthetase and increases intracellular glutathione levels. Biochem J **338 (Pt 3):**659-65.

249. **Wild, A. C., and R. T. Mulcahy.** 2000. Regulation of gamma-glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses. Free Radic Res **32:**281-301.

250. **Williams, R. T.** 1971. The metabolism of certain drugs and food chemicals in man. Ann N Y Acad Sci **179:**141-54.

251. **Woltjer, R. L., W. Nghiem, I. Maezawa, D. Milatovic, T. Vaisar, K. S. Montine, and T. J. Montine.** 2005. Role of glutathione in intracellular amyloid-alpha precursor protein/carboxy-terminal fragment aggregation and associated cytotoxicity. J Neurochem **93:**1047-56.

252. **Woods, J. S., and M. E. Ellis.** 1995. Up-regulation of glutathione synthesis in rat kidney by methyl mercury. Relationship to mercury-induced oxidative stress. Biochem Pharmacol **50:**1719-24.

253. **Wu, G., Y. Z. Fang, S. Yang, J. R. Lupton, and N. D. Turner.** 2004. Glutathione metabolism and its implications for health. J Nutr **134:**489-92.

254. **Wullner, U., J. Seyfried, P. Groscurth, S. Beinroth, S. Winter, M. Gleichmann, M. Heneka, P. Loschmann, J. B. Schulz, M. Weller, and T. Klockgether.** 1999. Glutathione depletion and neuronal cell death: the role of reactive oxygen intermediates and mitochondrial function. Brain Res **826:**53-62.

255. **Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg.** 1995. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science **270:**1326-31.

256. **Xia, Z., H. Dudek, C. K. Miranti, and M. E. Greenberg.** 1996. Calcium influx via the NMDA receptor induces immediate early gene transcription by a MAP kinase/ERK-dependent mechanism. J Neurosci **16:**5425-36.

257. **Yamagata, K., M. Tagami, K. Ikeda, S. Tsumagari, Y. Yamori, and Y. Nara.** 2002. Differential regulation of glial cell line-derived neurotrophic factor (GDNF) mRNA expression during hypoxia and reoxygenation in astrocytes isolated from stroke-prone spontaneously hypertensive rats. Glia **37:**1-7.

258. **Yang, D. D., C. Y. Kuan, A. J. Whitmarsh, M. Rincon, T. S. Zheng, R. J. Davis, P. Rakic, and R. A. Flavell.** 1997. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. Nature **389:**865-70.

259. **Yang, H., Y. Zeng, T. D. Lee, Y. Yang, X. Ou, L. Chen, M. Haque, R. Rippe, and S. C. Lu.** 2002. Role of AP-1 in the coordinate induction of rat glutamate-cysteine ligase and glutathione synthetase by tert-butylhydroquinone. J Biol Chem **277:**35232-9.

260. **Yao, K. S., A. K. Godwin, S. W. Johnson, R. F. Ozols, P. J. O'Dwyer, and T. C. Hamilton.** 1995. Evidence for altered regulation of gamma-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. Cancer Res **55:**4367-74.

261. **Yu, R., C. Chen, Y. Y. Mo, V. Hebbar, E. D. Owuor, T. H. Tan, and A. N. Kong.** 2000. Activation of mitogen-activated protein kinase pathways induces antioxidant response element-mediated gene expression via a Nrf2-dependent mechanism. J Biol Chem **275:**39907-13.

262. **Yu, R., W. Lei, S. Mandlekar, M. J. Weber, C. J. Der, J. Wu, and A. T. Kong.** 1999. Role of a mitogen-activated protein kinase pathway in the induction of phase II detoxifying enzymes by chemicals. J Biol Chem **274:**27545-52.

263. **Zanjani, H. S., M. W. Vogel, N. Delhaye-Bouchaud, J. C. Martinou, and J. Mariani.** 1996. Increased cerebellar Purkinje cell numbers in mice overexpressing a human bcl-2 transgene. J Comp Neurol **374:**332-41.

264. **Zeiss, C. J.** 2003. The apoptosis-necrosis continuum: insights from genetically altered mice. Vet Pathol **40:**481-95.

265. **Zhang, D. D., S. C. Lo, J. V. Cross, D. J. Templeton, and M. Hannink.** 2004. Keap1 is a redox-regulated substrate adaptor protein for a Cul3-dependent ubiquitin ligase complex. Mol Cell Biol **24:**10941-53.

266. **Zhu, M., and W. E. Fahl.** 2001. Functional characterization of transcription regulators that interact with the electrophile response element. Biochem Biophys Res Commun **289:**212-9.

267. **Zipper, L. M., and R. T. Mulcahy.** 2003. Erk activation is required for Nrf2 nuclear localization during pyrrolidine dithiocarbamate induction of glutamate cysteine ligase modulatory gene expression in HepG2 cells. Toxicol Sci **73:**124-34.

268. **Zipper, L. M., and R. T. Mulcahy.** 2000. Inhibition of ERK and p38 MAP kinases inhibits binding of Nrf2 and induction of GCS genes. Biochem Biophys Res Commun **278:**484-92.