NUTRITIONAL INFLUENCE ON OXIDATIVE STRESS IN GLOBAL ISCHEMIA

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ABSTRACT

Primary brain injury in stroke is followed by oxidative stress and further neural damage. Glutathione (GSH) is critical in antioxidant defense. Since cysteine is limiting in GSH synthesis, Phase 1 of this study investigated the effect of a dietary sulphur amino acid deficiency (-SAA) on neural damage in global hemispheric hypoxia-ischemia (GHHI). Rats were fed a -SAA or control diet for 6 days, and subjected to GHHI after 3 days. Histologically evaluated neural damage at 7 days post hypoxia-ischemia was greater in -SAA rats. Brain GSH concentration was decreased in -SAA rats 3 days after ischemia. A cysteine precursor, L-2-oxothiazolidine-4-carboxylic acid (OTC) administered to -SAA rats did not ameliorate neural damage. GSH is decreased by protein-energy malnutrition (PEM) in some tissues. Phase 2 investigated the effect of PEM on brain oxidative stress, neural damage and behaviour after global ischemia in adult male gerbils. In a 2x2 factorial design, gerbils were fed an adequate protein (12%; C) or low protein (2%; PEM) diet for 4 weeks, then subjected to transient ischemia (I) or sham surgery (S). After 12 hours of reperfusion, brain from half the gerbils was collected for biochemical analyses. Remaining gerbils were fed pre-surgery diets for 10 more days. To assess functional consequences of ischemia, gerbils were placed in an open field on Days 3, 7 and 10 after surgery. On Day 10, viable hippocampal CA1 neurons were counted. C-I gerbils did not habituate as readily in the open field on day 3 as C-S, but normalized by day 7. PEM-I gerbils failed to habituate by day 10, traveled greater distance than other gerbils and 7 of 12 displayed thigmotaxis, a 'wall-hugging' preference for the outer perimeter of the open field. CA1 neuron loss in I was 61.5% of S, but unaffected by PEM. Four of 12 PEM-I gerbils had marked increases in hippocampal glia. Hippocampus protein thiols were reduced by PEM and by ischemia, consistent with oxidative stress. GSH concentration, glutathione reductase activity and thiobarbituric acid reactive substances were not significantly affected by PEM or ischemia. Findings from these two studies suggest well-nourished but not nutritionally-deficient rodents tolerate a mild brain insult. This is clinically relevant because many elderly stroke victims suffer from PEM at the time of ischemia, which may compromise recovery.

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THE ROAD TO WISDOM

The road to wisdom? - Well, it's plain
and simple to express:
Err
and err
and err again
but less
and less
and less.

Piet Hein

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

4-HNE	4-hydroxynonenal
Acetyl CoA	acetyl coenzyme A
ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AGE	advanced glycation endproduct
AIN	American Institute of Nutrition
AMP	adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid
AP-1	Activator Protein-1
$AscH_2$	ascorbate
ATP	adenosine triphosphate
BCCAO	bilateral common carotid artery occlusion
CA1	cornu-ammonus
CNS	central nervous system
COX	cyclooxygenase
Cu^{+}	cuprous ion
DNA	deoxyribonucleic acid
EAA	excitatory amino acid
EAAT	excitatory amino acid transport
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
fEPSP	excitatory postsynaptic potential
FOOD trial	Feed Or Ordinary Diet trial
GCS	γ-glutamylcysteine synthase
GHHI	global hemispheric hypoxia-ischemia
GL	glutamate-cysteine ligase
GPx	glutathione peroxidase
GRed	glutathione reductase
$GS^{\bullet}$	glutathiyl radical

GSH	glutathione
GSSG	oxidized glutathione
H&E	hematoxylin and eosin
H^+	hydrogen ion
HIV	human immunodeficiency virus
HOOH	hydrogen peroxide
HPLC	high performance liquid chromatography
ICAM	intracellular adhesion molecule-1
IL-1B	interleukin-1B
IL-6	iInterleukin-6
I*κ*B	inhibitory component *kappa* B
$L^{\bullet}$	carbon-centred lipid radical
LH	unsaturated fatty acid
$LOO^{\bullet}$	lipid hydroperoxide
MAP-2	microtubule-associated protein 2
MCP-1	monocytes chemoattractant protein-1
MUN	Memorial University of Newfoundland
Na^+K^+-ATPase	sodium-potassium ATPase pump
NAC	N-acetylcysteine
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NF-*κ*B	nuclear factor *kappa* B
NHANES I	National Health and Nutrition Examination Study I
NMDA	N-methyl-D-aspartate
$NO^{\bullet}$	nitric oxide radical
NOS	nitric oxide synthase
$O_2^{\bullet-}$	superoxide anion
OH^-	hydroxyl ion
$OH^{\bullet}$	hydroxyl radical
$ONOO^-$	peroxynitrite

OTC	L-2-oxothiazolidine-4-carboxylic acid
PEM	protein-energy under- or malnutrition
P_i	inorganic phosphate
RANTES	regulated upon normal T-cell expressed and secreted protein
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAA	sulphur amino acid
SHRSP	spontaneously hypertensive stroke prone rat
SOD	superoxide dismutase
$T_{1/2}$	half-life
TBARS	thiobarbituric acid reactive substances
TBCAO	transient bilateral common carotid artery occlusion
TNF-α	tumour necrosis factor alpha
TOH	vitamin E, α-tocopherol
YM737	N-[N-r-L-glutamyl-L-cysteinyl]glycine 1-isopropyl ester sulphate monohydrate

CHAPTER 1

INTRODUCTION

1.1 RATIONALE

Health Canada and the Heart and Stroke Foundation of Canada define cerebrovascular disease (ICD-9, 430-438) as the sudden development of a focal neurologic deficit due to disease of one or more blood vessels of the brain. Stroke, either focal or global (ICD-9, 430-432, 434, 436) is simply a reduction in blood flow to the brain. Stroke can be thromboembolytic or ischemic, with blockage of a blood vessel supplying the central nervous system (CNS), or hemorrhagic, with bleeding into the parenchyma or subarachnoid space. Global ischemia results from conditions such as cardiac arrest, head trauma and shock, often secondary to an episode of significant systemic hypotension. At 7% of all deaths, stroke remains the third most common cause of death in Canada (Heart and Stroke Foundation of Canada,1999; Yager and Thornhill,1997). Direct and indirect costs of stroke in Canada total $2.8 billion annually, with mortality costs responsible for $1.2 billion or 44% of total costs (Heart and Stroke Foundation of Canada,1999). Although mortality rates for all cardiovascular disease have been declining since the 1960s, and rates of ischemic heart disease and acute myocardial infarction continue to decline modestly, mortality rates for stroke have not changed significantly in the last ten years in Canada. In Saskatchewan, 20% of stroke victims die and 30% suffer permanent disability (Juurlink,1999). There remains a need to identify both treatment strategies to improve outcome and compromised nutritional states that could worsen outcome after stroke. As in cardiovascular disease, diet and nutritional status may play a role in prevention of stroke and amelioration of deficits for survivors. The focus of this thesis is therefore on the effect of nutritional status on outcome in stroke.

Although stroke occurs in all age groups, the elderly are at high risk for stroke. Many elderly have compromised nutritional status due to a variety of factors such as anorexia, poor dental status, drug therapy, decreased activity levels, and chronic diseases (Abbasi and Rudman,1994; Lipschitz,1991; Marcus and Berry,1998). Several studies have reported evidence of compromised protein-energy status at the time of admission to hospital for stroke and deterioration of this status during the hospital stay (Axelsson *et al.*,1988; Choi-Kwon *et al.*,1998; Davalos *et al.*,1996; Gariballa *et al.*,1998b; Gariballa *et al.*,1998a). In a Canadian study, 49% of stroke patients suffered from protein-energy malnutrition (PEM) at the time of admission to rehabilitation units (Finestone *et al.*,1995; Finestone *et al.*,1996). Authors suggested there was inadequate nutritional intervention immediately post-injury which could compromise antioxidant defense mechanisms. A large, multicentre randomized trial evaluating feeding policies after stroke, the Feed Or Ordinary Diet Trial (FOOD), has determined nutritional status at baseline and early after stroke to be an important predictor of long term outcome (FOOD Trial Collaboration,2003). Patients undernourished immediately after stroke suffered reduced survival, functional ability and living circumstances six months later. The length of time to start of feeding after stroke varies, and stroke patients fed within seventy-two hours of injury have a shorter hospital stay (Nyswonger and Helmchen,1992). Low serum albumin, often an indicator of poor protein status, has been associated with increased length of stay, complications, and death in medical, surgical and stroke patients, and is considered an indicator of poor outcome (Aptaker *et al.*,1994; Axelsson *et al.*,1988; Choi-Kwon *et al.*,1998; Davalos *et al.*,1996; Finestone *et al.*,1996; Gariballa *et al.*,1998b; Gariballa *et al.*,1998a; Gariballa and Sinclair,1998).

Primary brain injury in stroke is followed by an excitotoxic cascade, production of reactive oxygen and nitrogen species, oxidative stress and further neural damage. The endogenous tripeptide glutathione (GSH) is critical in antioxidant defense, and depleted in conditions of oxidative stress. The sulphur amino acid cysteine, supplied primarily by dietary protein, is limiting in glutathione synthesis. Liver and erythrocyte glutathione are responsive to dietary protein or short-term food deprivation, while brain glutathione is relatively preserved (Bauman *et al.*,1988a; Benuck *et al.*,1995; Taylor *et al.*,1992). However, an acute dietary sulphur amino acid deficiency decreases brain glutathione

concentration in some brain regions (Paterson *et al.*,2001). Reduced availability of critical substrate for glutathione synthesis in the face of high demand for glutathione during oxidative stress may further deplete brain glutathione. Sub-optimal protein-energy status or sulphur amino acid status due to inadequate intake may thus compromise antioxidant defense in stroke and increase neural damage. Delivery of various cysteine precursors has been shown to enhance glutathione synthesis in some tissues, particularly in conditions of glutathione depletion (Jain *et al.*,1995; Kamencic *et al.*,2001; Taylor *et al.*,1992; Yao *et al.*,1997).

This research investigates first the effect of an acute dietary sulphur amino acid deficiency on brain and liver glutathione concentration and neural damage in a rat model of global hemispheric hypoxia-ischemia, followed by administration of a cysteine precursor in an attempt to ameliorate neural damage. Since it is extremely unlikely any human would be deficient only in sulphur amino acids, the second part of this research investigates the effect of a short-term, severe dietary protein-energy deficiency on functional outcome, neural damage, and some biochemical markers of oxidative stress in a gerbil model of transient bilateral carotid artery occlusion. Behaviour and function are investigated in the gerbil ischemia model, due to evidence suggesting histological assessment of neural damage is not necessarily reflective of cognitive and motor function (Bothe *et al.*,1986; Hori and Carpenter,1994; Ishimaru *et al.*,1995).

1.2 HYPOTHESIS

The research project described in this thesis tested the following hypotheses:

1. Sulphur amino acid-deficiency or protein-energy deficiency will decrease brain glutathione concentration in critical regions secondary to reduced synthesis of glutathione and uptake of key glutathione precursors, compromising antioxidant defense and increasing neural damage in stroke.
2. Administration of L-2-oxothiazolidine-4-carboxylic acid (OTC), a cysteine precursor, will enhance glutathione synthesis in brain and ameliorate neural damage due to stroke.

1.3 OBJECTIVES

The objectives of this research were divided into two experiments as follows:

Experiment 1: Sulphur amino acid deficiency, global hemispheric hypoxia-ischemia and OTC in the rat

1. To investigate the effect of sulphur amino acid deficiency on brain glutathione concentration after global hemispheric hypoxia-ischemia (GHHI) in the rat.
2. To investigate whether OTC protects against neural damage in the sulphur amino acid-deficient rat subjected to GHHI.

Experiment 2: Protein-energy malnutrition and temporary bilateral carotid artery occlusion in the Mongolian gerbil

1. To characterize a gerbil model of moderate protein-energy malnutrition (PEM).
2. To investigate whether PEM exacerbates neural damage in global ischemia, as measured by histological assessment and behavioural (open field) outcome.
3. To determine whether moderate PEM depletes brain glutathione and increases oxidative stress in a gerbil model of transient bilateral carotid artery occlusion (TBCAO).

1.4 SUMMARY

Oxidative stress causes secondary damage after initial brain injury in stroke. Availability of substrates for antioxidant defense may be compromised by dietary deficiencies. Epidemiological evidence suggests poor nutritional status at the time of stroke is a predictor of worse outcome for survivors. This thesis, entitled *Nutritional Influence on Oxidative Stress in Global Ischemia*, was investigated first with an acute, severe dietary sulphur amino acid deficiency in a rat model of hemispheric hypoxia-

ischemia, and secondly with moderate protein-energy malnutrition in a gerbil model of transient global ischemia with reperfusion.

CHAPTER 2

REVIEW OF LITERATURE

2.1 PATHOPHYSIOLOGICAL MECHANISMS IN STROKE

The brain comprises 2% of body weight, but receives 20% of cardiac output and accounts for 20% of total body oxygen consumed (Shivakumar *et al.*,1995). Under aerobic conditions, glucose is the major source of energy for metabolism in the brain, which has few endogenous reserves of either glucose or glycogen. Ketone bodies can act only as a limited energy source in times of chronic metabolic imbalance. Glucose crosses the blood brain barrier in an insulin-independent manner (Peters *et al.*,2002), is taken into neurons via a membrane transporter and phosphorylated, and enters glycolysis then the tricarboxylic acid pathway. Ultimately, reducing equivalents pass to oxygen in the mitochondria with release of adenosine triphosphate (ATP), the energy 'currency' of the cell, to the cytoplasm (Gunter *et al.*,1994). The mitochondria produce ~95% of cell ATP requirements. The brain cannot oxidize free fatty acids, and is therefore susceptible to injury resulting from disturbance of blood flow and interruption of oxygen and glucose supply, such as in stroke. Anaerobic metabolism is insufficient to meet the demands of nervous tissue. When lack of oxygen removes the final electron acceptor from the respiratory chain, the system fails.

Several secondary mechanisms activated following primary brain injury and stroke contribute to extensive damage. These mechanisms resulting from reduction in blood flow involve depletion of glucose, ATP and ADP, membrane depolarization and glutamate release from the cell initiating an excitotoxic cascade, calcium overload and finally production of strong oxidants leading to oxidative stress and activation of inflammatory pathways (Juurlink and Paterson,1998; Schmidt-Kastner and Freund,1991). Oxidative stress is a threshold phenomenon occurring when antioxidant

defenses are overwhelmed and free radical production exceeds elimination (Wilson,1997). Ischemia and subsequent reperfusion generate reactive oxygen (ROS) and nitrogen (RNS) species, toxic to the brain. During reperfusion, oxidative stress increases as oxygen is restored to the brain (Grace,1994; Juurlink and Paterson,1998; Shivakumar *et al.*,1995; Yano *et al.*,1998).

2.1.1 Excitatory amino acid transmitters in the central nervous system

In order to understand the mechanisms leading to oxidative stress, some background on neurotransmitters will be discussed. The predominant excitatory neurotransmitters (EAA) in the mammalian CNS are L-glutamate (glutamate) and L-aspartate, two structurally similar acidic amino acids. Glutamate is the most abundant free amino acid, able to stimulate almost every type of CNS neuron. Identified glutamatergic pathways include hippocampal, cerebellar, corticocortical and corticofugal (Greene and Greenamyre,1996). Glutamate is concentrated in synaptic vesicles at nerve terminals. Release of glutamate is calcium-dependent, with active, high-affinity, sodium-dependent re-uptake into perisynaptic astrocytes by excitatory amino acid transporter proteins (mainly EAAT-1, EAAT-2) (Milton *et al.*,1997; Rothstein *et al.*,1994). There is some re-uptake by neurons, but the majority is via the perisynaptic astrocytes, wherein glutamate is transaminated to glutamine via glutamine synthase, and diffused back into nerve terminals, where it is deaminated to glutamate via a mitochondrial phosphate-activated glutaminase (Greene and Greenamyre,1996).

Glutamate receptors are of two types, ionotropic and metabotropic. Ionotropic receptors are linked to cation channels and fall into three categories, named for the agonists that specifically stimulate them: α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA). Both AMPA and NMDA receptors are usually involved in excitatory post-synaptic potentials. Metabotropic receptors are coupled to second messenger systems.

2.1.1.1 Ionotropic receptors

AMPA and kainate receptors are mediators of fast, voltage-independent synaptic responses, and promote activation of voltage-dependent NMDA receptors (Nakanishi,1992). The AMPA receptors are usually heteromeric, made up of five

subunits in combinations of four types, GluR1-GluR4 (Nakanishi,1992; Nakanishi and Masu,1994). Receptors containing subunits GluR1, GluR3 or GluR4 are permeable to calcium, but presence of GluR2, which is in majority, will confer calcium impermeability (Hume *et al.*,1991). Each subunit consists of 4 transmembrane domains (TM-I - TM-IV). Substitution of arginine (as in GluR2) for a glutamine (as in GluR1,3,4) in TM-II confers calcium impermeability to the channel pore (Nakanishi,1992). The kainate receptors are similar in structure to AMPA receptors: subunits GluR5-GluR7 confer low-affinity kainate binding in brain membrane, while subunits KA-1 and KA-2 confer high affinity binding (Nakanishi and Masu,1994). AMPA/kainate receptors are found in both neurons and glia, including microglia (Noda *et al.*,2000; Steinhauser and Gallo,1996).

NMDA receptors are unique in that they are both voltage- and ligand-gated. At resting membrane potential, the ion channel is blocked by extra-cellular magnesium; post-synaptic depolarization is required for magnesium release. Both glutamate and glycine ('co-agonist') binding is required for activation (Nakanishi and Masu,1994). NMDA receptors are therefore activated by coincident post-synaptic depolarization and agonist binding. Unlike AMPA receptors, NMDA receptors have a high calcium conductance, and also can transduce sodium. Calcium is the primary mediator of both physiological and toxic properties of the NMDAs. Several NMDA subunits have been identified: NMDA R1, NMDA R2A-R2D, NMDA-L(c-1) (Ciabarra *et al.*,1995; Nakanishi *et al.*,1998).

2.1.1.2 Metabotropic Receptors

Metabotropic receptors are coupled to guanine nucleotide-binding proteins (G-proteins) and, depending on cell type and receptor subtype, can be linked to phosphoinositol turnover, arachidonic acid metabolism or cyclic AMP (Greene and Greenamyre,1996; Nakanishi and Masu,1994). Eight identified subunits have been named mGluR1-mGluR8. In contrast to ionotropic receptors, the metabotropic receptors have not been well-studied, since there are no specific, potent antagonists for

this class. The function of glutamate receptors is intimately involved in the pathophysiology of stroke.

2.1.2 Ischemic Brain Damage

Ischemic brain damage is thought to be due to passive processes involving reduced oxygen availability leading to decreased energy production and subsequent diminished survival, as well as active processes leading to the production of free radicals, activation of inflammatory responses, and finally apoptotic mechanisms (Love,1999). At the onset of ischemia, anaerobic metabolism leads to a fall in tissue pH, depletion of ATP and first slowing, then failure of the critical sodium-potassium ATPase pump (Na^+K^+-ATPase) (Sweeney *et al.*,1995). As a result of this failure, ion homeostasis is disturbed as ions move down their electrochemical gradients across plasma membranes, such that sodium moves into the cell and potassium moves out (Love,1999; Shuaib and Kanthan,1997). Although this is initially a gradual process, by two minutes after onset of ischemia, depolarization of the membrane is pronounced and ion movement is more rapid (Love,1999). The Na^+K^+-ATPase pump further depletes ATP as the cell attempts to correct the ion imbalance. Since even under normal conditions, 40% of neuronal ATP is used by this pump to maintain resting membrane potential and ion gradients, ATP can be rapidly depleted at a time when demand is high (Greene and Greenamyre,1996). As the membrane depolarizes, calcium also moves into the cell through voltage-gated channels, causing the release of excitatory neurotransmitters, especially glutamate. Extracellular glutamate activates post-synaptic glutamate receptors, allowing further sodium entry, depolarization and ATP consumption. Depolarization of the membrane releases magnesium from the voltage- and ion-gated NMDA receptors, which are then activated by glutamate and yet more sodium and calcium enter the cell. There are many consequences to calcium influx, such as activation of ATP-consuming calcium pumps, futile mitochondrial calcium cycling, activation of second messenger pathways resulting in changes in gene expression and calcium-dependent phospholipases, proteases, kinases, phosphatases and endonucleases, and finally production of ROS, leading to activation of phospholipase C, the arachidonic

acid cascade, production of inflammatory molecules and further ROS production (Alexi *et al.*,2000; Juurlink and Paterson,1998; Siesjo *et al.*,1995; Sweeney *et al.*,1995). There is a biphasic rise in intracellular calcium (Love,1999). The primary rise is coupled to accumulation of extracellular glutamate and activation of NMDA receptors, while the secondary rise occurs two to three hours after reperfusion and indicates irreversible cell damage. While not believed to be tied to glutamate release, the secondary calcium rise may be due to a post-ischemic potentiation of calcium influx through NMDA and AMPA receptors and/or recurrent spreading depression (Obrenovitch and Richards,1995; Walz,1997). Surrounding the primary infarct core of damage is an area of reduced blood flow, the penumbra (Hakim,1987). Waves of depolarization from the ischemic core spread across the penumbric region (spreading depression), leading to depletion of ATP in an area already compromised by poor circulation (Walz,1997). The penumbra may be rescuable, however, if this process can be minimized or halted (Juurlink and Sweeney,1997; Walz,1997).

2.1.2.1 The role of sodium

Sodium is an important mediator of secondary active transport in brain. In ischemia, glutamate re-uptake is impaired by the decrease in sodium gradient, leading to the calcium-independent release of glutamate via reversal of the sodium-dependent glutamate transporter (Blaustein *et al.*,1991). The sodium gradient is important for maintenance of intra-cellular calcium via the low-affinity, high-capacity calcium/sodium exchange, which is dependent on a large sodium gradient, and critical in reducing increased intracellular calcium levels, such as occur in ischemia (Mattson *et al.*,1989). Metabolic inhibition thus impairs the exchange, increasing neuronal intracellular calcium, with all its consequences (Mattson *et al.*,1989). The sodium /H^+ exchange is critical in maintenance of neuronal pH, and is also thus impaired by the decreased sodium gradient in ischemia, resulting in acidic intracellular conditions (Greene and Greenamyre,1996).

2.1.2.2 Mitochondrial calcium transport

Mitochondrial calcium transport is integral to the evolution of oxidative stress. Extracellular calcium concentration is 1-2mM, while cytoplasmic calcium concentration is often less than 100nM (Gunter and Pfeiffer,1990). These concentrations are maintained by the outward calcium ATPase pump and the calcium/sodium exchange. When cytoplasmic calcium increases above 200-300nM, mitochondria begin to accumulate calcium in the matrix. Influx of calcium into the matrix is via a passive uniporter in the membrane, dependent on membrane potential, and modulated by calcium, magnesium, and ADP. Efflux of calcium from mitochondria is energy-dependent via both sodium-dependent and sodium-independent mechanisms. Accumulation of calcium in mitochondrial matrix, as well as many other stimulants such as oxidative stress and decreased glutathione concentrations, activates a 'transition pore' in the membrane. Solutes can then move across the membrane by facilitated diffusion, driven by their concentration gradients, destroying membrane potentials and allowing the mitochondria to swell, resulting eventually in reduced ADP phosphorylation, and increased production of ROS (Gunter *et al.*,1994; Gunter and Pfeiffer,1990; Juurlink and Paterson,1998).

2.1.2.3 Glutamate

The release of glutamate to the extracellular space during ischemia is contributory to oxidative stress through more than one mechanism. Three classes of membrane proteins in the CNS mediate the physiological consequences of increased extracellular glutamate, namely ionotropic receptors, metabotropic receptors, and the cystine/glutamate X_c^- antiporter (Schubert and Piasecki,2001).

2.1.2.3.1 Excitotoxicity

The action of glutamate released at synapses is normally terminated via neuronal and predominantly glial uptake by glutamate transporter proteins. Glutamate and sodium are co-transported into the cell while potassium moves out, resulting in one net proton-equivalent into the cell (Love,1999). It remains unclear whether this proton-equivalent is

due to hydrogen ion (H^+) in or hydroxyl ion (OH^-) out. During ischemia, when the membrane is depolarized, this glutamate/sodium transporter cannot work efficiently, and glutamate thus accumulates extracellularly. Excessive stimulation of all types of ionotropic and even metabotropic glutamate receptors occurs, followed by neuronal damage or death (Greene and Greenamyre,1996). Calcium influx and loss of calcium homeostasis play an additional role in this process (Greene,1999; Greene and Greenamyre,1996; Siesjo *et al.*,1995), termed classic excitotoxic glutamate toxicity (Juurlink and Sweeney,1997; Tan *et al.*,1998).

2.1.2.3.2 Oxidative glutamate toxicity

Glutamate can also be toxic in the CNS via a transporter-mediated mechanism known as oxidative glutamate toxicity, which has characteristics of both necrotic and apoptotic cell death (Tan *et al.*,1998). Necrotic cell death is a passive process, cell 'murder' (Alexi *et al.*,2000), characterized by formation of vacuoles, cell swelling, random DNA degradation, and early loss of plasma membrane integrity (Ratan *et al.*,1994a; Tan *et al.*,1998). On the other hand, apoptotic cell death, or cell 'suicide' (Alexi *et al.*,2000), is associated with depolarization of the mitochondrial membrane, a rise in intracellular calcium, generation of ROS, chromatin condensation, cytoplasmic and nuclear shrinkage, 'laddering' of DNA into reproducible oligonucleosomal fragments, late loss of membrane integrity, and active RNA and protein synthesis (Pereira and Oliveira,2000; Tan *et al.*,1998). Oxidative glutamate toxicity appears to involve both a rapid necrotic phase and a delayed apoptotic phase that includes nuclear condensation and chromatin cleavage into oligonucleosomal fragments (Ratan *et al.*,1994a; Schubert and Piasecki,2001; Tan *et al.*,1998).

Almost all mammalian cells contain a sodium-independent, anionic amino acid transporter that is highly specific for cystine and glutamate (Sato *et al.*,1999). This X_c^- antiporter, enriched in brain membranes and concentrated in neurons, exchanges extracellular cystine for intracellular glutamate in a 1:1 fashion, and has a higher affinity for cystine than glutamate (Murphy *et al.*,1989; Sato *et al.*,1999). Mammalian extracellular brain cysteine concentration is less than 1μM, intracellular glutamate

concentration is greater than 3mM, and extracellular glutamate can reach 200-300μM after initial excitotoxic cell lysis (Murphy *et al.*,1990; Schubert and Piasecki,2001). Since the X_c^- antiporter is critical for intracellular supply of cystine, which is rapidly reduced to cysteine in the cell and used for synthesis of acetyl-CoA, protein and glutathione, the higher affinity for cystine is essential (Murphy *et al.*,1990). Transporter activity is induced by electrophilic agents, depletion of cystine, and oxygen, all of which can deplete glutathione (Sato *et al.*,1999). Cystine uptake is inhibited by glutamate. Thus, in conditions such as ischemia when there is excessive extracellular glutamate, transport of cystine to the cell via this mechanism is inhibited, there is a loss of cystine homeostasis, and intracellular glutathione concentrations fall (Pereira and Oliveira,2000; Tan *et al.*,1998). The subsequent rise in ROS leads to lipid peroxidation, decreased mitochondrial membrane potential, perturbation of calcium homeostasis, and ultimate mitochondrial dysfunction, decreased ATP production and cell death (Pereira and Oliveira,2000; Tan *et al.*,1998). This oxidative glutamate toxicity has been described in primary neuronal cultures (Davis and Maher,1994; Murphy *et al.*,1989; Murphy *et al.*,1990), neuronal cell lines (Davis and Maher,1994; Murphy *et al.*,1989), and tissue slices (Schubert *et al.*,1992). Oxidative glutamate toxicity can be blocked *in vitro* by antioxidants such as vitamin E (Murphy *et al.*,1990; Schubert *et al.*,1992), inhibition of arachidonic acid metabolism and 12-lipoxygenase (Li *et al.*,1997; Murphy *et al.*,1989) and by inhibition of macromolecular synthesis (Ratan *et al.*,1994a). It has been suggested that inhibitors of macromolecular synthesis may protect against oxidative glutamate toxicity via shunting of cysteine to glutathione synthesis (Ratan *et al.*,1994b). However, since apoptotic cell death requires some protein synthesis as well, perhaps inhibition of this synthesis may contribute to the observed protection.

2.1.3 Oxidative Stress

2.1.3.1 ROS/RNS

A free radical is any species having one or more unpaired electrons (Halliwell and Gutteridge,1990). Free radicals and other chemically reactive species include the ROS superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide, hydroxyl radical ($OH^{\bullet}$), and singlet

oxygen, and the RNS nitric oxide radical ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) (Juurlink and Paterson,1998).

The superoxide anion is produced in the cell by various mechanisms, including normal mitochondrial respiration, in which 3% of oxygen consumed is only partially reduced to superoxide instead of water (Juurlink,1997). The superoxide anion has a number of reactions in the cell: reaction with thiols, de-esterifying membrane lipids and releasing arachidonic acid, which initiates a metabolic cascade, leading to increased formation of superoxide anion; depletion of cellular NADH stores via a chain reaction with NADH bound to lactate dehydrogenase; reaction with nitric acid, producing peroxynitrite, a strong oxidant; dismutation to hydrogen peroxide and singlet oxygen with release of iron (ferrous ion) from ferritin stores ($Fe^{3+} \rightarrow Fe^{2+}$) (Fridovich,1986; Hall and Braughler,1993; Juurlink and Paterson,1998). Hypoxanthine, a metabolite of ATP via AMP, adenosine and inosine, is normally converted to xanthine and then uric acid by xanthine dehydrogenase, using NAD^{+} as an electron acceptor. During and following ischemia, intracellular calcium rise activates proteases that convert xanthine dehydrogenase to xanthine oxidase. Xanthine oxidase uses molecular oxygen as its electron acceptor, thus increasing the potential for superoxide anion production and/or hydrogen peroxide (Juurlink,1997; McCord,1985). Significant is that in ischemia, ATP is depleted, resulting in elevation of AMP and thus a ready supply of hypoxanthine substrate for xanthine oxidase (Juurlink,1997; McCord,1985), *i.e.*- in ischemia, not only does the harmful enzyme appear, but also its substrate. As well, reperfusion brings a ready supply of oxygen, the second substrate required by xanthine oxidase for free radical production (Sussman and Bulkley,1990). Superoxide dismutase can scavenge cellular superoxide by dismutation to hydrogen peroxide and molecular oxygen (Juurlink and Paterson,1998).

Hydrogen peroxide is also formed by other mechanisms, such as by oxidoreductases in the cellular peroxisome organelles (Juurlink and Paterson,1998). Hydrogen peroxide is not a free radical as it does not have an unpaired electron. Although relatively unreactive, hydrogen peroxide diffuses easily across biological membranes (Halliwell and Gutteridge,1990), and in the presence of ferrous ion (Fe^{2+}) or

cuprous ion (copper, Cu^{+}), hydrogen peroxide converts to the hydroxyl radical (Fenton, reaction 2.1). Oxidized iron (Fe^{3+}) can then be reduced by superoxide anion (reaction 2.1). Reactions 2.1 and 2.2 together are known as the Haber-Weiss reaction (Juurlink,1999). The hydroxyl radical is the most potent oxidant in biological systems. It will extract an electron from thiol-containing enzymes such as glutathione reductase and glutathione peroxidase, DNA and polyunsaturated lipids, or hydroxylate another molecule. Hydroxyl radical interferes with mitochondrial function, inactivating electron-carrying proteins and mitochondrial ATPase and peroxidizing membrane lipids (Halliwell,1992; Juurlink and Paterson,1998).

$$Fe^{2+} + HOOH \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (2.1)$$

$$Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2 \quad (2.2)$$

Singlet oxygen can be formed from the interaction of superoxide anion with superoxide anion, peroxynitrite with hydrogen peroxide, or superoxide anion with hydrogen peroxide. Singlet oxygen may inactivate calcium ATPase, denature proteins, inactivate superoxide dismutase and catalase, and oxidize polyunsaturated lipids to yield lipid hydroperoxides and endoperoxides. These peroxides, with transition ions, can initiate propagation of lipid peroxidation chains or produce more singlet oxygen (Juurlink and Paterson,1998).

Nitric oxide, a normally innocuous signaling molecule used by most cells, is synthesized from arginine by nitric oxide synthase (NOS). Two constitutive isoforms, NOS-I in neuronal and epithelial cells, and NOS-III in endothelial cells, are calcium and calmodulin dependent, while a third isoform, NOS-II or iNOS is inducible through cytokine activation of second messenger pathways and does not require elevation of cytosolic calcium (Hara *et al.*,1996; Juurlink,1999). Inducible NOS produces toxic levels of nitric oxide, and is expressed in CNS in pathological states such as cerebral ischemia (Iadecola *et al.*,1997). Nitric oxide can interact with superoxide anion to produce peroxynitrite, which generates nitrogen dioxide (an oxidant similar to hydroxyl radical in reactivity), oxidizes thiol-containing proteins, DNA bases and polyunsaturated lipids, and inhibits mitochondrial electron transport, leading to decreased ATP production and increased production of ROS (Halliwell,1992; Juurlink and

Paterson,1998). In brain injury, polymorphonuclear leukocytes can produce nitric oxide and superoxide. Neurons and oligodendrocytes are susceptible to nitric oxide and peroxynitrite effects on mitochondrial respiration (Juurlink and Paterson,1998).

2.1.3.2 Lipid peroxidation

Since the brain is rich in polyunsaturated fatty acids, ROS can easily propagate lipid peroxidation (Shivakumar *et al.*,1995). Superoxide anion can de-esterify membrane phospholipids to release fatty acids. Hydroxyl radical extracts a hydrogen atom from the methylene carbon of unsaturated fatty acids (LH), yielding a carbon-centred lipid radical ($L^{\bullet}$, reaction 2.3). The lipid radical reacts with molecular oxygen to form a peroxy radical (reaction 2.4), which is converted to a lipid hydroperoxide ($LOO^{\bullet}$) by abstraction of a hydrogen atom from the methylene carbon of an adjacent unsaturated fatty acid (reaction 2.5) (Juurlink,1997). The propagation (reactions 2.4 and 2.5) continues until either two lipid radicals interact, or an antioxidant (such as vitamin E) stops the chain reaction (Kinuta *et al.*,1989).

$$LH + OH^{\bullet} \rightarrow L^{\bullet} + HOH \quad (2.3)$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \quad (2.4)$$

$$LOO^{\bullet} + LH \rightarrow L^{\bullet} + LOOH \quad (2.5)$$

Lipid peroxidation can also be initiated by non-radical ROS such as singlet oxygen and hypochlorite. As well, in the presence of iron or iron complexes, lipid hydroperoxides can form alkoxy (reaction 2.6) or peroxy (reaction 2.7) radicals to propagate new chains of peroxidation (reactions 2.8 and 2.9), particularly in acidic conditions such as arise during and following ischemia (Hall and Braughler,1993; Juurlink,1997).

$$LOOH + Fe^{2+} \rightarrow Fe^{3+} + LO^{\bullet} + OH^{-} \quad (2.6)$$

$$LOOH + Fe^{3+} \rightarrow Fe^{2+} + LOO^{\bullet} + H^{+} \quad (2.7)$$

$$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet} \quad (2.8)$$

$$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet} \quad (2.9)$$

Lipid peroxidation can alter membrane fluidity and permeability, and function of membrane-bound ion pumps, further compromising ion homeostasis, resulting finally in loss of membrane integrity and cell rupture (Juurlink,1997). Lipid peroxides can also break down into harmful pro-inflammatory isoprostanoids and strong oxidants such as 4-hydroxynonenal (4-HNE) (Christman *et al.*,2000).

2.1.3.3 The Arachidonic Acid Cascade and Inflammation

Both normal physiological mechanisms and pathological conditions such as mechanical damage, lipid peroxidation, and increased cytosolic calcium can release the polyunsaturated 20-carbon arachidonic acid (5,8,1,14-*cis*-eicosotetraenoic acid) from esterified membrane phospholipids via activation of calcium-dependent phospholipase A_2 (Dayton and Major,1996; Juurlink and Paterson,1998; Shimizu and Wolfe,1990). Phosphatidylcholine and phosphatidylinositol, where arachidonate is in the sn-2 position, are the most important sources of arachidonic acid. Phospholipase C is also activated by high intracellular calcium, giving rise to diacylglycerol which can release arachidonic acid via the action of lipases (Juurlink and Paterson,1998). The resultant arachidonic acid cascade involves oxidation of arachidonic acid by several enzyme families to produce prostaglandins, thromboxanes, leukotrienes, lipoxins, epoxy-eicosatrienoic acids, and hydroperoxy acids, potent biological signaling molecules acting as short-range messengers (Shimizu and Wolfe,1990). Arachidonic acid can also be transformed into hydroperoxy acids by nonenzymatic autooxidation. Collectively termed eicosanoids, these arachidonate derivatives are responsible for a wide variety of modulatory physiological responses.

Two isoforms of the enzyme cyclooxygenase, constitutive (COX-1) and inducible (COX-2) are responsible for the conversion of arachidonic acid to prostaglandins and thromboxanes. COX-1 activity regulates prostaglandins and thromboxanes production under normal physiological conditions. COX-2 is rapidly inducible by mitogens, cytokines, lipopolysaccharide, and hypoxia/ischemia, and promotes pro-inflammatory prostaglandins and thromboxanes and free radicals, leading to permeability changes in the blood brain barrier, neutrophil activation, platelet and

leukocyte adhesion to endothelium (Juurlink and Paterson,1998; Sairanen *et al.*,1998). COX-2 is the main isoform in the CNS, and is present in both neuronal and glial cells (Sairanen *et al.*,1998).

Leukotrienes and lipoxins are produced by the lipoxygenase pathways, are potent mediators of inflammation, are released in response to cerebral trauma/ischemia, and stimulate leukocyte chemotaxis, neutrophil superoxide generation and release, vasospasm, and increased microvascular permeability (Feuerstein and Hallenbeck,1987; Juurlink and Paterson,1998). Five-lipoxygenase requires calcium and ATP for activity, thus is activated following ischemia and intracellular calcium rise, and will further deplete ATP. Free radicals may also act directly on arachidonic acid, with resultant isoleukotrienes, biologically active free radicals (Harrison and Murphy,1995).

Platelet-activating factor can be formed after acetylation of the sn-2 position of phosphatidylcholine following phospholipase A_2-mediated release of arachidonic acid from that position (Juurlink and Paterson,1998). Platelet-activating factor has several actions, including increasing permeability of microvessels, promotion of neutrophil transmigration and adhesion, acting as a second messenger to release more arachidonic acid and superoxide anion, and enhancing glutamate release (Juurlink and Paterson,1998).

Neutrophils become activated and invade CNS parenchyma following CNS injury and phopholipase-mediated lipid inflammatory molecule formation. Arachidonic acid stimulates neutrophil NADPH-oxidase, the 'respiratory burst oxidase' normally active in host defense against microbial invasion (Chanock *et al.*,1994). The consequent production of superoxide anion, however, can cause considerable tissue damage as well (Juurlink and Paterson,1998).

2.1.3.4 Nuclear factor *kappa* B

Nuclear factor *kappa* B (NF-κB) is a protein transcription factor, required for the transcription of pro-inflammatory molecules such as intracellular adhesion molecule-1 (ICAM-1), the enzymes inducible nitric oxide synthase (iNOS) and COX-2, cytokines

interleukin-1 B (IL-1B), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNF-α), and chemokines regulated upon activation normal T-cell expressed and secreted protein (RANTES) and monocytes chemoattractant protein-1 (MCP-1) (Christman *et al.*,2000; Schneider *et al.*,1999). NF-*κ*B is thus a mediator of the inflammatory cascade. In resting, unstimulated cells, NF-*κ*B is sequestered in the cytoplasm as a complex with an inhibitory component, I*κ*B. Upon stimulation, the I*κ*B is phosphorylated, polyubiquitinated and degraded by the 26S proteasome, unmasking a nuclear location signal on NF-*κ*B, which then locates to the nucleus to activate gene transcription (Schneider *et al.*,1999). NF-*κ*B is stimulated by both receptor-dependent signals such as lipopolysaccharide, TNF-α, and IL-1B, and by non-receptor activation such as ultraviolet radiation, physical stress/trauma, hydrogen peroxide and ischemia/reperfusion, whose mechanisms are not well understood.

2.1.3.5 Advanced Glycation Endproducts

Advanced glycation endproducts (AGEs) are formed by the nonenzymatic interaction of highly reactive dicarbonyls, or 2-oxo-aldehydes, with the nitrogen of protein-bound amino acids and nucleic acids (Juurlink,1999). The dicarbonyls glyoxal and methylglyoxal are derived from glucose via transition metal ion catalyzed oxidation, and glycolysis, respectively. Alternatively, glucose can undergo Amadori rearrangement to yield another dicarbonyl, 3-deoxyglucosone. Advanced glycation endproduct formation is accelerated in hyperglycemia and oxidative stress (Juurlink,1999). Advanced glycation endproducts inactivate proteins such as glutathione reductase, and act with receptors that increase ROS and NF-*κ*B activation, as well as with scavenger receptors whose activation causes release of arachidonic acid and ROS (Juurlink,2001; Shinpo *et al.*,2000).

2.1.4 Summary

Many complicated and as yet incompletely understood mechanisms contribute to tissue damage during and following stroke, and pathways are interrelated and

interwoven. Juurlink (1999) talks of four phenomena: glutamate excitotoxicity, intracellular calcium influx, oxidative stress and ATP depletion as responsible for activation of a vicious spiral ending in cell death, with additional influence of oxidative stress in promotion of inflammatory processes, also damaging. Alexi *et al.* (2000) describe the 'lethal triplet' of metabolic compromise, excitoxicity and oxidative stress in both acute and chronic pathological states. It is clear that cell death in stroke occurs by rapid necrotic and delayed apoptotic mechanisms, the latter providing some hope of intervention before irrevocable damage occurs.

2.2 ANTIOXIDANT DEFENSE

Antioxidant defense is essential to survival, since cells are exposed to free radicals from the environment and from normal physiological processes. Both enzymatic and non-enzymatic cellular systems exist, designed to scavenge or inactivate free radicals.

2.2.1 Enzymatic defense

The superoxide dismutase (SOD) family removes superoxide anion and converts it to hydrogen peroxide, according to reaction 2.10. Mammalian cells contain two types of SODs, a tetrameric mitochondrial SOD containing manganese (MnSOD), and a dimeric cytosolic SOD containing copper and zinc (CuZnSOD) (Halliwell,1994; Nordberg and Arner,2001). Leakage of electrons from the respiratory chain in mitochondria produces much of the superoxide anion encountered in the cell, and MnSOD can be induced by thioredoxin and by oxidative stress. In contrast, CuZnSOD is not induced by oxidative stress (Chan,1994; Nordberg and Arner,2001).

$$2\ O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad (2.10)$$

The catalase enzymes are mainly heme-containing, located in peroxisomes. They remove hydrogen peroxide (reaction 2.11) and can detoxify phenols and alcohols (reaction 2.12), but cannot affect lipid peroxides (Halliwell,1994; Nordberg and

Arner,2001). Catalase also lowers the risk of hydroxyl radical formed from hydrogen peroxide via the Fenton reaction, thus acting as an antioxidant. Binding of catalase to NADPH will protect the enzyme from inactivation as well as increase its efficiency (Nordberg and Arner,2001).

$$2\ H_2O_2 \rightarrow O_2 + H_2O \tag{2.11}$$

$$H_2O_2 + R'H_2 \rightarrow R' + 2H_2O \tag{2.12}$$

The peroxiredoxins (thioredoxin peroxidases), only recently discovered, can directly reduce peroxides such as hydrogen peroxide and alkyl hydroperoxides and can inhibit apoptosis (Chae *et al.*,1999). Oxidized peroxiredoxin is regenerated by thioredoxin. The glutaredoxins, whose function overlaps that of thioredoxins, can reduce glutathione mixed protein disulfides formed in oxidative stress, and are themselves reduced by glutathione (Nordberg and Arner,2001).

The selenocysteine-containing glutathione peroxidases (GPx) catalyze the reduction of both hydrogen and lipid peroxides using glutathione as a substrate (reaction 2.13). This is in contrast to catalase, which can act only on hydrogen peroxides. The cytosolic glutathione peroxidase 1 and the membrane-bound glutathione peroxidase 4 (phospholipid hydroperoxide glutathione peroxidase) isoforms are present in most tissues. Glutathione peroxidase 4 has broad specificity for membrane-bound hydroperoxides, fatty acid peroxides and others, including hydrogen peroxide (Ursini *et al.*,1985). Glutathione peroxidase 2 is the gastrointestinal isoform, and glutathione peroxidase 3, or plasma glutathione peroxidase, is mainly expressed in the kidney (Nordberg and Arner,2001), although has also been found in the eye, lung, brain, heart, breast, placenta, and liver (of humans, but not rodents) (Arthur,2000; Chu *et al.*,1992). Glutathione peroxidase 3 can be catalytically regenerated by the thioredoxin system.

$$ROOH + 2GSH \xrightarrow{GPx} ROH + GSSG + H_2O \tag{2.13}$$

The thioredoxin system consists of two oxidoreductase enzymes: thioredoxin, a general protein disulfide reductant, and thioredoxin reductase, which catalyzes reduction of the active site disulfide in thioredoxin using NADPH (Nordberg and Arner,2001).

Thioredoxin is ubiquitous in mammalian cells, targeting ribonucleotide reductase, protein-disulfide isomerase, and transcription factors including p53, NF-κB, and Activator Protein-1 (AP-1) for reduction. In humans, the isoform thioredoxin 1 is in cytosol, thioredoxin 2 in mitochondria, and Sp thioredoxin in spermatozoa, all containing a cysteine-glycine-proline-cysteine active site (Nordberg and Arner,2001). Thioredoxin is an electron donor for the peroxiredoxins, thus important in reduction of peroxides. When reduced, thioredoxin prevents apoptosis via inhibitory binding to apoptosis signal-regulating kinase 1. Expression of thioredoxin is induced by oxidative stress, some forms of which cause translocation of thioredoxin to the nucleus where it enhances DNA binding of NF-κB by reduction of its cysteine residue, exacerbating the production of pro-inflammatory molecules (Matthews *et al.*,1992).

All thioredoxin reductase isoenzymes contain selenocysteine, which is essential for their activity. Thioredoxin reductase is NADPH-dependent and directly reduces hydrogen peroxide and lipid peroxides, especially when peroxide levels are elevated. This function is thought to be handled normally by glutathione peroxidase 4 (Nordberg and Arner,2001). In addition to thioredoxin, some thioredoxin reductase substrates are protein-disulfide isomerases, L-cystine, dehydroascorbic acid, α-lipoic acid, ubiquinone, and glutathione peroxidase (Nordberg and Arner,2001). Inhibition of thioredoxin reductase impairs function of thioredoxin system-dependent or -regulated reactions, resulting ultimately in significant intracellular oxidative stress.

2.2.2 Non-enzymatic defense

2.2.2.1 Glutathione

2.2.2.1.1 Roles of glutathione

The tripeptide glutathione (L-γ-glutamyl-L-cysteinylglycine), the most prevalent nonprotein intracellular thiol, is involved in many functions in the body, including regulation of cellular redox balance, leukotriene and prostaglandin metabolism, deoxyribonucleotide synthesis, immune function, cell proliferation, transport and storage of cysteine, detoxification of xenobiotics, and antioxidation of reactive oxygen

species and free radicals (Akerboom and Sies,1981; Bray and Taylor,1993; Bray and Taylor,1994; Halliwell and Gutteridge,1990; Juurlink,1999; Philbert *et al.*,1991). Intracellular concentrations of glutathione range from ~1-2μmol/g in brain to ~8-10μmol/g in liver (Bauman *et al.*,1988a ; Bray and Taylor,1994; Paterson *et al.*,2001). The most abundant thiol in the CNS, glutathione, along with glutathione peroxidase, is the major protection against peroxide and reactive oxygen species in the cell, and is thus critical in antioxidant defense (Barker *et al.*,1996; Bray and Taylor,1993; Bray and Taylor,1994; Jain *et al.*,1991; Juurlink,1997; Wullner *et al.*,1999).

As mentioned, glutathione peroxidase converts any peroxide (including lipid peroxides) to water and molecular oxygen (or hydroxy group) through oxidation of glutathione, which acts as the electron donor (reaction 2.13). Oxidized glutathione (GSSG) can then be reduced to glutathione, dependent on glutathione reductase (GRed) and NADPH (reaction 2.14). The activity of glutathione reductase and availability of NADPH thus can regulate the activity of glutathione peroxidase. The ability of glutathione peroxidase to scavenge peroxides is dependent on the concentration of glutathione, increasing markedly with small increases in glutathione (Juurlink,1999; Thorburne and Juurlink,1996).

$$GSSG + NADPH + H^+ \xrightarrow{GRed} 2GSH + NADP^+ \quad (2.14)$$

$$2GSH + 2R^\bullet \rightarrow 2RH + 2GS^\bullet \quad (2.15)$$

$$GS^\bullet + GS^\bullet \rightarrow GSSG \quad (2.16)$$

Glutathione can also scavenge free radicals directly, forming glutathiyl radicals ($GS^\bullet$), which interact to form GSSG (reactions 2.15, 2.16) (Dringen *et al.*,2000). Vitamin E (α-tocopherol, TOH) can inactivate lipid radicals, forming the tocopherol radical which is in turn reduced by ascorbate ($AscH_2$) (reactions 2.17, 2.18). Two ascorbate radicals dismutate to ascorbate and dehydroascorbate (oxidized), which is reduced back to ascorbate by glutathione (reactions 2.19, 2.20) (Juurlink,1997). Glutathione and ascorbate together prevent oxidative damage to mitochondria. Meister and co-workers found glutathione can spare ascorbate, and ascorbate can spare

glutathione (Martensson *et al.*,1993; Martensson *et al.*,1991a; Martensson and Meister,1989; Martensson *et al.*,1991b; Meister,1991; Meister,1994). Ascorbate increased mitochondrial glutathione in glutathione-deficient animals, while administration of glutathione esters delayed the onset of scurvy in ascorbate-deficient animals. Ascorbate may provide an alternative cytosolic antioxidant defense in neuronal somata (Philbert *et al.*,1991).

$$LOO^{\bullet} + TOH \rightarrow LOOH + TO^{\bullet} \quad (2.17)$$

$$TO^{\bullet} + AscH_2 \rightarrow TOH + AscH^{\bullet} \quad (2.18)$$

$$2AscH^{\bullet} \rightarrow AscH_2 + Asc \quad (2.19)$$

$$2GSH + Asc \rightarrow GSSG + AscH_2 \quad (2.20)$$

The hydroperoxides produced in reaction 2.17, in the presence of free iron, can be converted to alkoxyl and peroxyl radicals, potentially initiating more lipid peroxidation (reactions 2.6, 2.7) (Juurlink and Paterson,1998). Glutathione and the glutathione peroxidase family scavenge these radicals. Glutathione also forms glutathione S-conjugates, nonenzymatically or via glutathione S-transferase enzymes, important in detoxification of endogenous and exogenous compounds, as well as in normal cellular metabolism, such as leukotriene C formation (Anderson and Luo,1998). Lipid peroxides can break down into strong oxidant aldehydes such as 4-hydroxynonenal, which can be inactivated through the action of glutathione S-transferase to form glutathiyl adducts (Christman *et al.*,2000). Glutathione S-transferases also form these adducts with lipid epoxides and hydroperoxides (Christman *et al.*,2000).

The transcription factor NF-κB mediates the inflammatory response in brain ischemia, and, through its redox capacity, glutathione can inhibit the signal transduction pathway that results in NF-κB activation (Christman *et al.*,2000). Glutathione plays an important role in prevention of advanced glycation endproduct formation, via reaction of glutathione with methylglyoxal and glyoxalase I, for example, to produce a hemithioacetyl, which is then converted to D-lactic acid and glutathione with glyoxalase II (Thornalley,1988).

Glutathione deficiency leads to mitochondrial damage in tissues, including lung, gastrointestinal tract, eye, skeletal muscle, liver, kidneys, and brain (Meister,1995a). Decreased tissue glutathione levels have been associated with several diseases, including hereditary glutathione deficiencies, hepatitis C, AIDS, myocardial infarction, stroke, ischemic reperfusion injury, Parkinson's disease, adult respiratory distress syndrome, chronic digestive diseases and burns (Anderson and Luo,1998; Bray and Taylor,1994). Deficient glutathione status contributes to a weakened antioxidant defense system (Jain *et al.*,1991; Juurlink *et al.*,1998; Mizui *et al.*,1992; Thorburne and Juurlink,1996) and decreased immune response in malnourished individuals, especially when associated with these diseases (Bray and Taylor,1994).

In summary, glutathione plays many roles in antioxidant defense, such as direct scavenging of free radicals, scavenging of hydrogen and lipid peroxides as well as their peroxidation products, interaction with vitamin E and ascorbate in lipid peroxidation defense, prevention of advanced glycation endproduct formation, and finally redox regulation of NF-κB activation.

2.2.2.1.2 Metabolism and homeostasis of glutathione

Glutathione is synthesized in two ATP-requiring steps in almost all animal cells (Meister,1995a). Step 1 (reaction 2.21) requires the enzyme glutamate-cysteine ligase (GL, γ-glutamylcysteine synthetase, GCS), is the rate-limiting step in glutathione synthesis, and is regulated by glutathione via negative feedback inhibition (Juurlink *et al.*,1998). The feedback inhibition of γ-glutamylcysteine synthetase can be alleviated by glutamate, suggesting that high intracellular glutamate may be able to increase intracellular glutathione (Juurlink,1999). Step 2 (reaction 2.22) is catalyzed by glutathione synthetase.

$$\text{L-glutamate} + \text{L-cysteine} + \text{ATP} \rightarrow \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + P_i \quad (2.21)$$

$$\text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{glycine} + \text{ATP} \rightarrow \text{glutathione} + \text{ADP} + P_i \quad (2.22)$$

Glutathione is synthesized in the cytoplasm (Anderson and Luo,1998; Meister,1995a; Wullner *et al.*,1999). Since mitochondria do not contain the enzymes required for glutathione synthesis, about 10-20% of total cellular glutathione is transported into mitochondria from the cytoplasm by at least two transport systems (Cooper,1997). Activity of γ-glutamylcysteine synthetase and availability of cysteine are the rate-limiting factors for glutathione synthesis (Bauman *et al.*,1988a; Lu,1999). Glutamate and glycine are readily synthesized via several metabolic pathways, and are not believed to limit the rate of glutathione synthesis (Bannai and Tateishi,1986). The most important source of cysteine is dietary, but it can also be supplied by cleavage of cystine or by trans-sulphuration of methionine via the cystathionine pathway in the liver (Bauman *et al.*,1988a; Bray and Taylor,1994). Although glutathione is exported from many cells under normal conditions, and exported glutathione enters plasma (Meister and Anderson,1983), the liver is the main source of plasma glutathione (Cooper,1997). Sources of glutathione in the intestine include the diet, hepatic glutathione exported into bile, desquamated epithelial cells, and export from epithelial cells of the stomach and intestine (Bray and Taylor,1994; Cooper,1997). Generally, glutathione does not freely enter cells, but uptake depends on γ-glutamyl transpeptidase, an enzyme present on the external surface of the cell membrane in many tissues (Anderson and Luo,1998; Bray and Taylor,1994; Jain *et al.*,1991). However, there is some evidence for direct absorption and local use of intact glutathione in the small intestine (Bray and Taylor,1994; Hagen and Jones,1987), and transport of intact glutathione from the blood into the brain by carriers identified in capillaries and endothelial cells (Kannan *et al.*,2000; Kannan *et al.*,1990). Cellular glutathione concentration is determined by several factors including consumption by formation of conjugates via glutathione S-transferase, oxidation to GSSG, *de novo* synthesis and by reduction of oxidized glutathione by glutathione reductase (Juurlink,1999). Changes in γ-glutamylcysteine synthetase activity and gene expression will also alter glutathione concentration, and are mediated by factors such as oxidative stress, Phase II enzyme inducers, and antioxidants (Juurlink,2001; Lu,1999).

At the centre of glutathione homeostasis is the γ-glutamyl cycle. The enzyme γ-glutamyl transpeptidase is bound to the outer surface of most cell membranes, is

enriched in secretory and absorptive cells, and has also been found in brain (Jain *et al.*,1991; Meister and Anderson,1983). Breakdown of glutathione, oxidized glutathione or S-substituted glutathione can occur at the cell membrane in the presence of γ-glutamyl transpeptidase, which transfers the γ-glutamyl moiety to acceptors such as cystine, glutamate, methionine, some dipeptides, water, or even glutathione (Meister and Anderson,1983). Plasma or extracellular glutathione can react with amino acids or water to form γ-glutamyl amino acids or glutamate, respectively, and cysteinylglycine. The γ-glutamyl amino acids are transported into the cell and split into amino acid and 5-oxoproline by γ-glutamylcyclotransferase. Five-oxoproline is then ring-opened by 5-oxoprolinase, with ATP, to glutamate. If the γ-glutamyl moiety is transferred to cystine by γ-glutamyl transpeptidase, then γ-glutamylcystine transported into the cell can enter Step 2 of glutathione synthesis directly, thus by-passing the glutathione feedback inhibition of γ-glutamyl cysteine synthetase (Anderson and Luo,1998). Cysteinylglycine can be hydrolyzed by dipeptidases at the cell membrane to free amino acids for transport, or can be transported as the dipeptide and cleaved by intracellular dipeptidases to cysteine and glycine to be used in protein or glutathione synthesis (Anderson and Luo,1998). Inhibition of γ-glutamyl transpeptidase will increase plasma glutathione, while inhibition of γ-glutamyl cysteine synthetase will decrease plasma glutathione, confirming that glutathione is exported from the cell (Meister and Anderson,1983). Nonenzymatic or enzymatic (via glutathione S-transferase) reaction of glutathione with electrophilic compounds forms S-substituted glutathione derivatives. Gamma-glutamyl transpeptidase can remove the γ-glutamyl moiety of these compounds, resulting in a γ-glutamyl amino acid and an S-substituted cysteinylglycine moiety. The dipeptide can then be cleaved by dipeptidase to yield corresponding S-substituted cysteines which may be either N-acetylated or undergo a further transpeptidation to yield a γ-glutamyl derivative (Meister and Anderson,1983). By these mechanisms, referred to as the 'salvage pathway', there is transport of various amino acids across cell membranes, as well as conservation and redistribution of glutathione constituents (Anderson and Luo,1998; Meister and Anderson,1983). If dietary cysteine is decreased, glutathione may be used to supply cysteine for critical proteins, limiting glutathione available for antioxidant defense (Hunter and Grimble,1997).

2.2.2.1.3 Brain glutathione and glutathione peroxidase

Brain glutathione and glutathione peroxidase activity help regulate the extent of tissue damage in stroke. Brain glutathione arises from synthesis from amino acids and reduction of oxidized glutathione, and concentration decreases with age (Benuck *et al.*,1995). Jain *et al.* (1991) suggest brain glutathione may exist in several distinct pools, such as mitochondria, neurons and glial cells. Philbert and co-workers (1991) examined cellular and regional distribution of glutathione in the nervous system of the rat. They found glutathione in the CNS appears localized in the non-neuronal components of neuropil and in the white matter tracts, with only small amounts in the neuronal somata, confirming results demonstrated by others (Slivka *et al.*,1987). Brain cells have been shown to release glutathione into the extracellular space during conditions such as ischemia (Janaky *et al.*,1999). Neurons contain lower concentrations of glutathione than astrocytes, and in culture, neurons cannot utilize exogenous cystine, relying on cysteine either in the culture medium or released by astrocytes/glia for glutathione synthesis (Dringen and Hamprecht,1999; Dringen *et al.*,1997; Iwata-Ichikawa *et al.*,1999; Juurlink,1996; Raps *et al.*,1989). Wang and Cynader (2000) suggest cystine is transported from blood to the CNS extracellular fluid, where it reacts in a thiol/disulphide exchange with glutathione released from astrocytes. The resultant formation of cysteine and a cysteine-glutathione disulphide allows neurons access to cysteine. There is some evidence that neurons can utilize cysteine, or cysteinylglycine and γ-glutamylcysteine as cysteine precursors at lower concentrations than those required by astrocytes (Dringen *et al.*,1999). As well, buthionine sulfoximine, an inhibitor of γ-glutamyl cysteine synthetase, inhibits utilization of both cysteinylglycine and γ-glutamylcysteine, suggesting these dipeptides are hydrolyzed before glutathione synthesis in neurons (Dringen *et al.*,1999). In contrast, in cultured astroglial cells, the first step of glutathione synthesis can be bypassed (Dringen *et al.*,1997). There is some breakdown and re-synthesis of glutathione via γ-glutamyl transpeptidase on the luminal side of brain capillaries (Jain *et al.*,1991).

Husain and Juurlink (1995) cultured rat astrocytes and oligodendrocyte precursors under conditions of anoxia and hypoxia. Both cell types survived anoxia, but

only astrocytes survived twenty-four hours of hypoxia. More lipid peroxidation was seen under hypoxia than anoxia, suggesting free radical induced damage. Oligodendroglial precursors in culture contain more iron, less glutathione, and suffer more oxidative stress than astrocytes in the same culture medium (Thorburne and Juurlink,1996). Chelating iron or increasing glutathione content protects oligodendroglial precursors from oxidative damage, suggesting glutathione plays an important role in antioxidant defense in these cells. Wullner *et al.* (1999) studied glutathione in cerebellar granule neurons *in vitro*, and found inhibition of cytosolic synthesis of glutathione by buthionine sulfoximine had little effect on mitochondrial glutathione or mitochondrial transmembrane potential. Direct conjugation of glutathione with ethacrynic acid resulted in a rapid depletion of both cytosolic and mitochondrial glutathione, and subsequent generation of ROS with impairment of the electrochemical mitochondrial gradient. When mitochondrial respiration was inhibited with rotenone, ROS production was suppressed, confirming mitochondria as the source of ROS in neurons. Mizui *et al.* (1992) depleted rat brain glutathione with buthionine sulfoximine and found increased damage due to ischemic insult.

Ushijima *et al.* (1986), using a peroxidase-anti-peroxidase immunohistochemical method, studied the distribution of glutathione peroxidase in rat brain. They found glutathione peroxidase was localized in the nuclei of some nerve cells in the cerebral cortex, hippocampus, and cerebellar cortex, and absent in the Purkinje cells of the cerebellum. Distribution of cells lacking glutathione peroxidase coincided with those cells more vulnerable to hypoxia. They concluded differences in the distribution of glutathione peroxidase in brain might contribute to the selective vulnerability of neurons in post-hypoxic damage. Juurlink *et al.* (1998) examined astrocytes and oligodendrocyte precursors in culture, and found oligodendrocyte precursors to have 50% of the glutathione reductase activity and 15% of the glutathione peroxidase activity of astrocytes.

2.2.2.1.4 Response of tissue glutathione to dietary protein, energy and sulphur amino acids

Hepatic glutathione concentration is responsive to dietary protein (Bauman *et al.*,1988a). There is a diurnal rhythm to liver glutathione in rats, with the level rising during the night as feeding proceeds, and falling during the day. When animals are fasted or given pure sucrose or fat diets, glutathione levels are low and the diurnal rhythm disappears (Beck *et al.*,1958). Liver glutathione responds to the sulphur amino acid (SAA) content of protein, and increasing protein levels above normal will not increase glutathione above those levels found when adequate protein is fed (Bauman *et al.*,1988a). When rats, however, were first fed low-protein diets and hepatic glutathione was depleted, supplementation with a cysteine precursor increased hepatic glutathione beyond physiological maximum (Bauman *et al.*,1988b; Taylor *et al.*,1992). Benuck *et al.* (1995) deprived both young and aged rats of food for forty-eight hours, and found loss of hepatic protein and glutathione, but no loss of brain glutathione and little change in brain protein or amino acid levels. They suggested there is relative stability of protein, amino acids and glutathione in brain in short-term food deprivation, and brain protein may be spared even in extreme conditions. In food deprivation, there appears to be recycling of glutathione constituents within the brain and/or transportation of precursors across the blood brain barrier (Dringen,2000). Seemingly in contrast, but likely due to the acute nature of the dietary treatment, our laboratory has recently shown that glutathione concentration is decreased in certain brain regions by an acute dietary SAA deficiency (Paterson *et al.*,2001). Although plasma glutathione and cysteine levels are determined by efflux of hepatic glutathione, which is decreased in protein deficiency (Adachi *et al.*,1992), levels and function of liver glutathione-synthesizing enzymes appear to be maintained in food deprivation (Tateishi *et al.*,1974). Enzymes involved in glutathione synthesis are present in brain (Dringen,2000), but their function in food deprivation has not been investigated.

2.3 SULPHUR AMINO ACID METABOLISM

2.3.1 Methionine

Methionine and cysteine have important roles in organism survival in their capacity as amino acids for protein synthesis, precursors to essential metabolites, and for their catalytic roles in the active sites of enzymes, primarily due to their sulphur content

(Griffith,1987). Homocysteine is readily converted to methionine in mammals, and is the intermediate in the transsulphuration pathway to cysteine, but is absent from mammalian diets, rendering methionine the only indispensable sulphur amino acid (Finkelstein,2000). One essential function of methionine is the synthesis of the primary methyl group donor in biological systems, S-adenosylmethionine, via the action of methionine adenosyltransferase. Cellular methionine is partitioned between protein synthesis and S-adenosylmethionine synthesis. Although S-adenosylmethionine was not thought to be exported from its cell of origin, recent evidence suggests the liver may export some S-adenosylmethionine to the CNS and other tissues (Finkelstein,2000). Approximately 95% of S-adenosylmethionine forms S-adenosylhomocysteine by transmethylation with an acceptor such as glycine, guanidinoacetate, phosphatidylethanolamine, the lysine residues in some proteins, pyrimidine and purine bases of tRNA, and some xenobiotics (Griffith,1987). The next step is the formation of homocysteine via adenosylhomocysteinase. If homocysteine is in excess, it is irreversibly converted to cystathionine with the addition of serine via cystathionine-β-synthase, and cystathionine is cleaved to α-ketobutyrate and cysteine with γ-cystathionase (Finkelstein,2000; Griffith,1987). Thus, cysteine is unable to serve as a precursor to homocysteine or methionine in mammals. Alternatively, excess homocysteine can be exported to the liver and other tissues. When in short supply, the homocysteine moiety is conserved in the methionine cycle, wherein homocysteine is converted to methionine by accepting a methyl group from betaine, which becomes dimethylglycine; or from 5-methyltetrahydrofolate, which becomes tetrahydrofolate. There is some competition for homocysteine between cystathionine-β-synthase and the two enzymes involved in homocysteine conservation, betaine homocysteine methyltransferase and methylfolate methyltransferase (Finkelstein,2000). Activity of cystathionine-β-synthase is increased in an oxidative environment, but methylfolate methyltransferase is vulnerable to oxidation. It is therefore plausible that conditions of oxidative stress tip the balance in favour of trans-sulphuration, cysteine production, and glutathione synthesis (Finkelstein,2000). As well, the enzymes of the methionine conserving cycle tend to be inhibited by their products, while the trans-sulphuration pathway enzymes tend to be activated by their metabolites.

Methionine is transported by the sodium-independent system L, subject to *trans*-stimulation, meaning an amino acid on one side of a membrane can stimulate transport of another amino acid on the other side (Bannai and Tateishi,1986; Lu,1999; Lu,2000). System L appears to consist of both a high affinity, low capacity phase and a low affinity, high capacity phase. In hepatocytes, methionine is transported by both phases (Lu,2000).

2.3.2 Cysteine

Cysteine is a dispensable amino acid in mammals, but only if methionine intake meets the sulphur amino acid requirement. It follows, then, that the requirement for methionine in the diet is reduced if cysteine intake is adequate. Cysteine is unique in that it occurs predominantly in the oxidized form extracellularly as the disulphide cystine, but as the reduced sulphhydryl cysteine intracellularly.

Cysteine is transported across membranes as a neutral amino acid via the ASC system, which is pH sensitive and decreased in acidic conditions (Kranich *et al.*,1996; Lu,1999). This may be significant in acidosis of ischemia, especially if cysteine has been depleted in diet, for example. The ASC system also transports other neutral amino acids of short length, such as alanine and serine, in a competitive fashion. This system is subject to *trans*-stimulation: intracellular cysteine depends on the intracellular and extracellular concentrations of other ASC amino acids. As well, increased extracellular cysteine will increase intracellular concentrations of other ASC amino acids in addition to cysteine because of competitive inhibition, termed *cis*-inhibition, thereby stimulating efflux of cysteine via *trans*-stimulation (Lu,1999).

Cystine, with four ionizable groups, is present at neutral pH as the tetrapolar ion, while approximately 20% will exist as the tripolar ion at physiological pH. As covered under ‘oxidative glutamate toxicity’, cystine is generally transported via the sodium-independent X_c^- antiporter in a 1:1 exchange with glutamate. In culture, activity of the X_c^- antiporter can be induced by exposure to electrophilic agents or oxygen, and inhibited by extracellular glutamate or homocysteate (Bannai,1986). In brain, transport of cystine is complex and not yet completely elucidated. In cultures of fetal or neonatal rat brain, both astrocytes and neurons appear to have a high affinity, low capacity

transport system for cystine, identified as the X_c^- system (Allen *et al.*,2001; Murphy *et al.*,1990). In addition, astroglia have a sodium-dependent, low affinity, high capacity X_{AG}^- transport system that is inhibited by glutamate and D-aspartate (Allen *et al.*,2001; Murphy *et al.*,1990). Although the X_c^- antiporter is enriched in brain membranes and concentrated in neurons, (Murphy *et al.*,1989; Sato *et al.*,1999), Sagara's group (1993a;1993b) found neurons cannot take up cystine, but rely on astroglial cystine uptake and subsequent release of cysteine into the extracellular medium. Wade and Brady (1981) reported that they found no evidence for carrier-mediated uptake of plasma cystine in rat brain.

Cystine entering the cell or released during protein catabolism is reduced by transhydrogenation with glutathione to cysteine. Cysteine is used for protein or glutathione synthesis, or catabolized by two distinct pathways. Both of these pathways generate pyruvate and sulphate, but only the cysteine sulphinate-dependent pathway, the major pathway for cysteine catabolism in mammals, can produce taurine (Griffith,1987). In the first step, cysteine dioxygenase catalyzes the formation of cysteine sulphinate, which then can be transaminated to *β*-sulfinylpyruvate ending in the production of pyruvate and sulphate. Alternatively, cysteine sulphinate can undergo decarboxylation via cysteine sulphinate decarboxylase to hypotaurine and finally oxidation to taurine (Bella and Stipanuk,1996). Partitioning between transamination and decarboxylation varies between mammalian species (Griffith,1987). The cysteine sulphinate-independent pathway involves γ-cystathionase and formation of pyruvate, ammonia and hydrogen sulphide from cysteine, or pyruvate, ammonia and S-mercaptocysteine from cystine. The S-mercaptocysteine is then reduced to cysteine and hydrogen sulphide. Cysteine is required for synthesis of pantetheine and enzyme CoA, which can be catabolyzed to cysteamine and further to hypotaurine and taurine. If intracellular cysteine accumulates above its normal 30-200μM concentration, it can react with the co-enzyme pyridoxal phosphate to produce a thiazolidine derivative, thus depleting pyridoxal phosphate. The multiple pathways of cysteine degradation may serve to prevent its toxic accumulation (Griffith,1987).

2.3.3 Cysteine delivery systems

2.3.3.1 Cysteine

A number of methods of increasing intracellular glutathione in conditions of oxidative stress have been investigated. Cysteine, the limiting amino acid in glutathione synthesis, can be administered, but it is toxic to neonatal rodents and cultured cells (Anderson and Luo,1998; Olney *et al.*,1971; Olney *et al.*,1972). Neonatal rats given a single intravenous dose of cysteine (1.52 or 1.14g/kg body weight) or OTC (1.8 or 1.35g/kg body weight) had mortality rates of 80%, 50%, 10% and 0% respectively, demonstrating cysteine toxicity compared to OTC (White *et al.*,1993). Cysteine is rapidly oxidized to cystine which has limited solubility and poses physical administration problems (Anderson and Luo,1998).

2.3.3.2 Glutathione

Administration of glutathione has had limited success, since glutathione is generally not absorbed or transported intact, but is broken down to its constituents by γ-glutamyl transpeptidases at the cell membrane. Administration of glutathione to rats increased plasma and urinary glutathione, but not tissue glutathione (Anderson and Luo,1998). Ozaki *et al.* (1994) starved rats overnight and gave an intraperitoneal dose of glutathione one hour before a one-hour hepatic ischemia epsiode. Glutathione had no effect on liver glutathione levels or lipid peroxidation. Gotoh and co-workers (1994) gave rats an intraperitoneal injection of glutathione after two and one-half hours of middle cerebral artery occlusion ischemia. They examined brain sodium, water and glutathione levels, and found no changes with glutathione administration compared to controls.

2.3.3.3 N-acetylcysteine

N-acetylcysteine (NAC) is an effective intracellular cysteine delivery system. NAC is used clinically in acetaminophen overdose, as an antimucolytic agent, and more recently has been investigated in HIV infection. Oral NAC is absorbed, deacetylated and catabolyzed in the intestinal wall and liver, resulting in about 10% bioavailability (Bray

and Taylor,1994). NAC can also be given parenterally. Banks and Stipanuk (1994) cultured rat hepatocytes with [^{35}S]NAC and found NAC was taken up by cells and converted to cysteine. Dringen and Hampbrecht (1999) cultured embryonal rat brain neurons with NAC and found increased intracellular glutathione, concluding neurons in culture contain acylase which can cleave NAC to cysteine for intracellular synthesis. However, NAC does not appear to cross the blood brain barrier (McLellan *et al.*,1995). Pretreatment of rats with intraperitoneal NAC before bilateral common carotid artery occlusion (BCCAO) increased hippocampal neuronal survival at seven days post-insult, but post-ischemic administration of NAC only partially improved neuronal survival in moderate insult (45mmHg hypotension during BCCAO) and offered no protection in severe insult (30mmHg) (Knuckey *et al.*,1995). High oral doses of NAC can cause nausea, vomiting and diarrhea, and intravenous doses can produce anaphylactic reactions such as angioedema, bronchospasm, flushing, and hypotension (Bray and Taylor,1994).

2.3.3.4 L-2-oxothiazolidine-4-carboxylic acid

2.3.3.4.1 *In vitro* studies

A thiazolidine analog of 5-oxoproline, OTC has been extensively studied as a cysteine precursor. OTC is transported into most cells and ring-opened by 5-oxoprolinase to yield 5-carboxycysteine (rapidly decarboxylated to cysteine) and carbon dioxide, which is exhaled (Anderson and Luo,1998). All tissues except erythrocytes and the ocular lens contain 5-oxoprolinase (Bray and Taylor,1994), and incubation of 5-oxoprolinase with OTC and ATP yields rapid formation of ADP and cysteine (Williamson and Meister,1981). Guinea pig liver and kidney homogenates incubated with OTC increased intracellular cysteine (Nishina *et al.*,1987). Coloso *et al.* (1991) cultured each of [^{35}S]OTC, [^{35}S]methionine, and [^{35}S]cysteine with rat hepatocytes, renal tubule cells, and enterocytes. All three compounds were metabolized to glutathione, inorganic sulphur and taurine by renal cells and enterocytes, although the transport and metabolic rate for OTC was slower than for the amino acids. Hepatocytes from weanling rats fed a protein-poor diet were incubated with OTC, methionine or cysteine. Glutathione levels depressed by the low protein diet (82% lower than controls)

were increased to normal levels in culture (Goss *et al.*,1994). Banks and Stipanuk (1994) incubated [^{35}S]OTC, [^{35}S]NAC, or [^{35}S]cysteine with rat hepatocytes and measured intracellular sulphates, taurine and glutathione. OTC was transported into the cells, converted to cysteine, and glutathione was synthesized. [^{35}S]glutathione accounted for 78% of OTC metabolism. Embryonal rat brain neurons cultured with OTC did not show increased glutathione levels (Dringen and Hamprecht,1999).

2.3.3.4.2 Animal studies

Fasted mice given an intraperitoneal injection of OTC increased liver glutathione (Williamson and Meister,1981). Buthionine sulfoximine is a compound which irreversibly inhibits γ-glutamylcysteine synthetase (Pileblad and Magnusson,1992). Mice given intraperitoneal acetaminophen (to deplete glutathione) and then OTC had liver glutathione levels two and one-half times higher than controls, even when OTC was given two hours after acetaminophen. Subsequent administration of buthionine sulfoximine decreased liver glutathione, with no recovery after OTC addition, demonstrating OTC can be synthesized to glutathione (Williamson *et al.*,1982). Guinea pigs given intra-peritoneal OTC had increased liver and kidney glutathione and increased kidney cysteine levels compared to controls (Nishina *et al.*,1987). Both chicks and rats fed a cysteine-free diet with the addition of OTC had increased liver glutathione levels (Chung *et al.*,1990). Weanling PEM rats fed OTC and exposed to hyperoxia (85% oxygen) had increased glutathione levels in liver and lung, but not kidney and blood. These levels exceeded those in rats on a normal diet exposed to hyperoxia. Authors suggest oral OTC given to PEM rats is effective in protecting the lung against oxygen toxicity (Taylor *et al.*,1992). Jain *et al.* (1995) fed rats a sulphur amino acid-deficient diet supplemented with OTC for three weeks. Bronchoalveolar lavage fluid, liver, lung and lymphocyte glutathione levels increased over those of rats fed sulphur amino acid-deficient diet alone. Rats supplemented with OTC gained more weight than sulphur amino acid-deficient rats (Jain *et al.*,1995). Weanling rats fed a protein deficient diet for fourteen days, then supplemented with OTC and exposed to hyperoxia (85% oxygen) or normoxia for four days showed decreased lung/body weight ratio (oxidative damage in lung increases the lung/body weight ratio) and increased lung glutathione compared to

protein deficient or protein sufficient rats (Levy *et al.*,1998). Elevation of lung glutathione via OTC was more effective than protein repletion in protecting against hyperoxia-induced lung damage in PEM rats.

Rats and mice given intraperitoneal [^{35}S]OTC at various doses showed increased brain cysteine but little or no change in brain glutathione(Anderson and Meister,1989). OTC did appear to cross the blood brain barrier. Authors concluded the rate of transport of OTC to the brain is greater than the rate of conversion to cysteine, glutathione synthesis in the brain is slow, and cysteine concentration is not a factor in brain glutathione synthesis. In contrast, Mesina *et al.* (1989) gave rats subcutaneous injections of OTC and found brain glutathione to increase significantly over controls. Intraperitoneal administration of OTC to rats after a spinal cord crush injury decreased oxidative stress with a sparing of white matter at the site of injury, leading to partial return of function, while vehicle-treated animals remained paraplegic (Kamencic *et al.*,2001).

2.3.3.4.3 Human studies

OTC has been studied in humans. Porta (1991) and co-workers gave single oral doses of OTC to healthy volunteers and found increased plasma OTC and cysteine, no change in plasma glutathione, and increased lymphocyte glutathione and cysteine. OTC given twice weekly for six weeks to asymptomatic HIV positive volunteers increased whole blood glutathione (Kalayjian *et al.*,1994). In contrast, a single intravenous OTC infusion (4500mg) or multiple infusions (70 or 100mg/kg every eight hours for four doses) were given to fasting male volunteers. OTC increased total blood cysteine but not glutathione. OTC was 84% converted to cysteine in this study. Small frequent doses were considered more efficient than a single large dose (Gwilt *et al.*,1998). Cudkowicz *et al.* (1999) gave a single intravenous OTC dose (4500mg) or oral OTC (3000mg three times a day for 29 days or [mean] 4.9 months) to volunteers with amyotrophic lateral sclerosis. Cerebrospinal fluid glutathione levels are not known to be depressed in amyotrophic lateral sclerosis, and there was no change after OTC administration. Both oral and intravenous OTC were well tolerated and OTC was found to enter

cerebrospinal fluid efficiently. Authors also noted cerebrospinal fluid glutathione level decreased with age.

2.3.3.5 Esters and related compounds

There has been recent interest in a number of γ-glutamylcysteine esters and a glucose-cysteine adduct. Anderson and Meister (1987) have reported administration of γ-glutamylcystine leads to increased renal glutathione. Gamma-glutamylcystine is transported intracellularly and hydrolyzed to γ-glutamylcysteine and cysteine, both of which can be synthesized to glutathione. Isolated rat hepatocytes pretreated with diethyl maleate (to deplete glutathione) and incubated with γ-glutamyl cysteine ester or glutathione (at two doses) demonstrated increased intracellular glutathione only with γ-glutamyl cysteine ester (Nishida *et al.*,1996).

Kobayashi *et al.* (1992) pretreated rats with γ-glutamylcysteine ethyl ester or glutathione before exposure to two hours of ischemia with or without one hour of reperfusion. Gamma-glutamylcysteine ethyl ester maintained liver glutathione and mitigated post-ischemic injury while glutathione had no effect. Rats starved overnight and then given an intraperitoneal injection of γ-glutamylcysteine ethyl ester, γ-glutamylcysteine ethyl ester plus glycine, glycine or glutathione one hour before a one-hour period of hepatic ischemia with reoxygenation showed decreased thiobarbituric acid reactive substances (TBARS), decreased lipid peroxidation and increased liver glutathione with γ-glutamylcysteine ethyl ester and γ-glutamylcysteine ethyl ester plus glycine (Ozaki *et al.*,1994). Hoshida and group (1994) exposed dogs to ninety minutes of coronary occlusion followed immediately by intravenous γ-glutamylcysteine ethyl ester (at two doses) and five hours of reperfusion. Infarct size was reduced and myocardial glutathione levels increased in dogs given γ-glutamylcysteine ethyl ester compared to controls.

YM737 (N-[N-r-L-glutamyl-L-cysteinyl]glycine 1-isopropyl ester sulphate monohydrate) was given as an intra-peritoneal injection zero, one, and two hours post-ischemia in bilateral common carotid artery occluded rats, and inhibited lipid peroxidative responses in brain (Yamamoto *et al.*,1993). Gotoh *et al.* (1994) exposed rats to two and one-half hours of middle cerebral artery occlusion ischemia and gave

intraperitoneal YM737 or glutathione. They found the post-ischemic brain increases in sodium and water were suppressed by YM737. There was amelioration of post-ischemic decrease in brain glutathione with YM737, but no change with glutathione. Yao *et al.* (1997) investigated the effect of a glucose-cysteine adduct, 2-(D-gluco-pentahydroxypentyl)-thiazolidine-4-carboxylic acid, as an intraperitoneal injection in rats alone or after pre-treatment with diethyl maleate, on liver and kidney cysteine and glutathione. Liver and kidney cysteine increased and although there was no change in glutathione, depressed glutathione due to pre-treatment with diethyl maleate was restored.

Summary

In summary, various methods of intracellular cysteine delivery have been investigated. Cysteine is considered toxic and difficult to administer. Glutathione is broken down to its constituents before intracellular resynthesis. NAC is effective in increasing cysteine and glutathione in some tissues, but does not readily cross the blood brain barrier, and is associated with some adverse effects in humans. OTC is transported into many tissues, including brain, and its cysteine moiety can be used to synthesize glutathione. OTC moderately increases cellular glutathione in cultured cells, animal studies and human clinical trials. The γ-glutamylcysteine esters appear promising, and some glutathione mono and diesters have also been studied.

2.4 ANIMAL MODELS OF STROKE

Although many different animal models of stroke have been developed, no single model has been able to successfully mimic the diverse etiologies and characteristics of human stroke. There are, however, advantages to animal models, in that physiological parameters can be strictly controlled, allowing for reproducible and systematic study of the mechanisms involved in ischemic damage (Ginsberg and Busto,1989). Ideally, an animal model would closely parallel human anatomy, physiology and function, suggesting the primate as most closely approximating the human condition. Financial and animal welfare considerations render this and most large animal models all but impossible to study extensively. Rodents remain the most

accepted compromise for several reasons: cerebrovascular anatomy and physiology closely resemble that of higher species (Ginsberg and Busto,1989); they are small, easy to handle and relatively inexpensive to house, allowing for large numbers; their brain size is suitable for rapid fixation; intra-strain variability is relatively low (Ginsberg and Busto,1989; Seta *et al.*,1992). Disadvantages to the use of rodents include difficulty with physiological monitoring due to size constraints (blood gases, pressure, sampling, etc.); difficulty with neurological and functional assessments; respiratory, seizure and consciousness problems (Seta *et al.*,1992). The most commonly used models produce either focal or global ischemia.

2.4.1 Focal ischemia

Embolism models generally do not require craniotomy, but lesions are unpredictable and permanent. They include such methods as homologous blood clot fragment injection, microsphere injection, arachidonate-induced thrombosis and photochemically initiated thromboembolism (Ginsberg and Busto,1989). Middle cerebral artery occlusion is focal, reversible, and highly mimics human ischemic stroke (Seta *et al.*,1992). The lesion presents with an ischemic core and a penumbric region, allowing for intervention strategies. The contralateral hemisphere conveniently provides an internal 'control'. Surgical procedures, however, are invasive and traumatic, requiring skill and specialized equipment. Several techniques have been developed to produce middle cerebral artery occlusion, ranging from ligation of the artery to laser illumination after rose bengal injection to insertion of a microfilament of defined size (Seta *et al.*,1992). Ginsberg and Busto (1989) provide a comprehensive description of many methods. Cerebral hemorrhages and infarcts occur spontaneously in ~80% of males in the stroke-prone spontaneously hypertensive rat (SHRSP) strain (Okamoto *et al.*,1974). This rat strain has been extensively investigated and brain damage characterized in both spontaneous 'strokes' (Okamoto *et al.*,1974; Sadoshima *et al.*,1981; Yamasaki *et al.*,1991) and induced ischemia of various types (Brint *et al.*,1988; Carswell *et al.*,1999; Coyle,1984; Duverger and MacKenzie,1988; Gemba *et al.*,1992; Nordborg and Johansson,1996; Sadoshima *et al.*,1988; Shima *et al.*,1994; Slivka *et al.*,1995; Tagami *et al.*,1999; Watanabe *et al.*,1998).

2.4.2 Global ischemia

Induction of global cerebral ischemia in rodents involves mainly either two or four vessel occlusion. Since rats have an intact circle of Willis (posterior communicating arteries) in the brain, the four-vessel occlusion model was developed to produce forebrain ischemia. This involves occlusion, often by coagulation, of the vertebral arteries in a first procedure, followed about twenty-four hours later by isolation and transient occlusion of the common carotid arteries (Pulsinelli and Buchan,1988). This procedure induces a high-grade incomplete forebrain ischemia, but requires considerable expertise to avoid death from brainstem ischemia and post-ischemic seizures after the first stage (Ginsberg and Busto,1989).

The two-vessel common carotid artery occlusion produces reversible forebrain ischemia similar in histopathology to the four-vessel occlusion. In the rat and other animals with an intact circle of Willis, the procedure is combined with systemic hypotension to reduce forebrain blood flow. A similar model, of global hemispheric hypoxia-ischemia (GHHI), developed by Yager and group (Yager *et al.*,1996; Yager and Thornhill,1997), is a modification of the Levine preparation (Levine,1960). The right common carotid artery is exposed, ligated and severed, and the animal is subjected to a 35-minute period of hypoxia (12% oxygen). Body temperature is controlled throughout. The advantages of this model are several: its size makes physiological monitoring possible, the surgical procedure is relatively minor and not subject to a hypermetabolic response, the model has been well-characterized, and the hemispheric nature of damage allows internal comparison with the contralateral side of the brain. However, this stroke model subjects the entire brain to a period of hypoxia, and ischemia is achieved by ligation and severing of the carotid artery. Thus reperfusion is somewhat compromised in this model. Results are variable, partially due to lack of control of blood glucose and head/brain temperature, both of which can contribute to variability (Dijk *et al.*,1994; Ginsberg and Busto,1989; Mhairi Macrae,1992).

The Mongolian gerbil is unique in that it lacks a circle of Willis (Seta *et al.*,1992), thus avoiding the necessity of subjecting the animal to global hypoxia, hypotension or four-vessel occlusion to achieve mimicking of human stroke (Ginsberg

and Busto,1989). Bilateral transient occlusion of the common carotid arteries results in reproducible, consistent and well-characterized forebrain ischemic damage (Corbett and Crooks,1997; Corbett and Nurse,1998; Corbett *et al.*,1997; Nurse and Corbett,1994). Ischemia is of more abrupt onset and reversal than in the rat GHHI model. Surgical procedures are minor, as in the rat model, but due to the small size of the gerbil, physiological monitoring is usually limited to body and head/brain temperature. Functional and histological endpoints for this model have been published (Colbourne and Corbett,1994; Colbourne and Corbett,1995; Corbett and Nurse,1998; Ginsberg and Busto,1989). As well, oxidative stress has been demonstrated in susceptible brain regions by this model of ischemia (Baek *et al.*,2000; Candelario-Jalil *et al.*,2001; Park *et al.*,2000; Stanimirovic *et al.*,1988). Unilateral common carotid artery occlusion has also been used (Ginsberg and Busto,1989). The hippocampus is selectively vulnerable in models of transient ischemia (Akai and Yanagihara,1993; Nunn and Hodges,1994; Pulsinelli *et al.*,1982a; Schmidt-Kastner and Freund,1991). Temporary bilateral common carotid artery occlusion (TBCAO) in the gerbil produces delayed neuronal death mainly in the CA1 region of the hippocampus (Domanska-Janik *et al.*,1999; Kirino,1982; Kirino and Sano,1984; Kirino *et al.*,1986; Mitani *et al.*,1991), similar to vulnerability of human hippocampus to ischemia (Petito *et al.*,1987; Zola-Morgan *et al.*,1986).

2.4.3 Evaluation of deficit after ischemia

Evaluation of the effects and impact of ischemia in laboratory animals has traditionally been confined to histological examinations such as quantifying damage via cell counts, assessing infarct size *etc*., and biochemical, immunocytochemical and Southern/Northern/Western blot methods to elucidate pathways and regulatory mechanisms. All of these are valuable in characterizing the damage wreaked by ischemia in the various animal models, but animals subjected to ischemia show learning and locomotor function deficits (Colbourne and Corbett,1995; Wang and Corbett,1990). Morphological damage does not necessarily correlate with functional outcome (DeVries *et al.*,2001). Neurons may have a normal or near-normal histological appearance but compromised function (Colbourne and Corbett,1994; Colbourne and Corbett,1995;

Colbourne *et al.*,1999; Corbett and Nurse,1998; Dong *et al.*,2001). Recently, researchers have been characterizing the electrophysiological properties of neurons, particularly the vulnerable CA1 neurons of the hippocampus, in an effort to better understand the functional state of neurons before, during and after ischemia, both in short-term and long-term survival (Dong *et al.*,2001; Hori and Carpenter,1994; Nurse and Corbett,1994; Shinno *et al.*,1997; Urban *et al.*,1989; Xu and Pulsinelli,1994).

Ischemia in humans can result in profound impairments in memory, sensorimotor and cognitive function (DeVries *et al.*,2001; Petito *et al.*,1987; Zola-Morgan *et al.*,1986). In Saskatchewan, 30% of stroke victims suffer permanent disability (Juurlink,1999). In the United States, stroke is the most common cause of permanent sensorimotor and cognitive disability (DeVries *et al.*,2001). Ultimately, it is brain function after stroke that is the most clinically significant outcome, and behavioural assessment of learning, memory and locomotor skills in animal ischemia models is essential to understanding implications of deficits. Many neurobehavioural tests have been developed, some of which are as follows: (1) tactile stimulation test, to measure recovery from lesion-induced somatosensory deficits (DeVries *et al.*,2001); (2) rotarod task, to assess fine motor and postural control (Hogg *et al.*,1998); (3) the elevated plus maze, to measure anxiety and motivation (Hogg *et al.*,1998); (4) the open field, to measure ability to habituate to a novel environment and possibly spatial mapping (Wang and Corbett,1990). The open field apparatus is simply a white box approximately 75cm square, with high walls. The animal is placed in one corner of the apparatus and activity recorded for ten minutes. An intact animal will typically investigate the entire apparatus, then decrease activity over the ten-minute period, or *habituate*, and on repeated exposure habituate more quickly. An animal that has suffered ischemia will tend not to habituate, or habituate more slowly, even with repeated exposure (Dowden and Corbett,1999; Dowden *et al.*,1999). There is some evidence the degree of CA1 neural loss is correlated with the inability to habituate, making this test a good indicator of ischemic damage to the hippocampus (Babcock *et al.*,1993; Mileson and Schwartz,1991; Wang and Corbett,1990). It is easy to perform, requires minimal equipment, and is non-invasive.

2.5 NUTRITIONAL REQUIREMENTS OF THE MONGOLIAN GERBIL

The advantages of using the gerbil as an animal model of transient bilateral common carotid artery occlusion have been discussed, but little is known of the nutritional requirements of the gerbil, and the requirement for protein is not well established. The Mongolian gerbil (*Meriones unguiculatus*) is a desert rodent, used in research since 1893 (Cheal,1986). The gerbil is herbivorous in its wild habitat, consuming little water, eating leaves in summer and seeds in winter. Leaves and seeds have approximate protein contents of 2% and 11% wet weight respectively (Edwards *et al.*,1983). The relatively low protein content of the natural diet can be an advantage in the desert environment when water supply is minimal, since the gerbil will not have to dispose of excessive amounts of urea, a process which requires water for osmotic reasons (Edwards *et al.*,1983). Laboratory gerbils, however, grow well on laboratory rodent chow, which is about 16-24% protein (water content usually less than 10%) (Arrington *et al.*,1973; Edwards *et al.*,1983). When protein content of a diet is higher than that of laboratory chow, gerbil intake will decrease and growth declines (Arrington *et al.*,1973). As well, consumption of purified diet tends to be low for the first two to three days, then increases to that of laboratory chow with satisfactory catch-up growth (Arrington *et al.*,1973).

The metabolic pathways for methionine, cyst(e)ine, choline are interdependent. While the S-adenosylmethionine pathway can fulfill the requirement for cyst(e)ine and choline in most laboratory animals, gerbils appear to have a requirement for choline (Otken and Yolanda,1983). When choline is absent from the diet, liver lipid increases (Otken,1984). When the dietary choline is adequate, but methionine is deficient, methionine deficiency develops very slowly in the gerbil, in contrast to the rat, suggesting gerbil sulphur amino acid metabolism may differ from that of the rat (Otken and Yolanda,1983). In the absence of cystine and choline, no amount of methionine supports good growth in the gerbil (Otken and Yolanda,1983), although methionine metabolism should provide some cysteine. Cereal grains have more cystine than methionine, in roots and nuts the proportion is about equal, and in meat methionine is greater than cystine (Otken,1984). Otken suggests the gerbil may have adapted to a diet

high in cystine. The gerbil also appears to have a requirement for taurine in the diet (Otken *et al.*,1985).

2.6 HUMAN PROTEIN REQUIREMENT: DIETARY REFERENCE INTAKES

2.6.1 Background

Protein is a critical structural and functional component of all cells, comprising enzymes, hormones, transport molecules, intracellular matrices, collagen, keratin, portions of membranes, hair, and fingernails (Institute of Medicine of the National Academies,2002b). Mammalian proteins are macromolecules made up of long chains of α-amino acids and the α-imino acid proline. The amino acids are also precursors to nucleic acids, hormones, coenzymes and other physiologically important molecules such as glutathione, for example. The physical structure of proteins is complex: the primary structure is determined by the sequence of amino acids in the chain; the secondary structure, coiling and pleating, is due to hydrogen bonding between side chain residues; disulphide bonding and hydrophobic interactions between non-polar side chains are responsible for the tertiary structure that results in folding of helices on each other; and finally quaternary structure involves interaction of more than one protein molecule (subunit) with another, forming complex units with specific structures and functions (Institute of Medicine of the National Academies,2002b).

Dietary protein is essential as a supply of amino acids. The following amino acids are considered indispensable, meaning they either cannot be synthesized in the body, or cannot be synthesized in sufficient quantities to meet metabolic needs, and must be supplied in the diet: histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine (Laidlaw and Kopple,1987). Some amino acids are conditionally indispensable, meaning a dietary source is required when endogenous production does not meet metabolic needs, such as in some physiological conditions or disease states; these are arginine, cysteine, glutamine, glycine, proline and tyrosine (Laidlaw and Kopple,1987). Finally, alanine, aspartic acid, asparagine, glutamic acid, serine and selenocysteine (Leinfelder *et al.*,1999) are dispensable amino acids, and can be synthesized in the body from other amino acids or complex nitrogenous metabolites (Laidlaw and Kopple,1987).

2.6.2 Protein digestion and absorption: a brief overview

Dietary protein is denatured by stomach acid and cleaved into peptide fragments by pepsin, which is activated by the decrease in stomach pH upon feeding. The pancreas releases proteolytic enzymes such as trypsin, chymoptrypsin, elastase and carboxypeptidases into the small intestine, where peptides are hydrolyzed to smaller peptides and free amino acids. By various mechanisms, these small peptides and free amino acids enter intestinal mucosal cells and are subjected to further hydrolysis, whereupon free amino acids are either metabolized directly within the gut or released into the portal blood and transported to the liver. Some of these amino acids may be used within the liver, or secreted into the systemic circulation for use in peripheral tissues (Institute of Medicine of the National Academies,2002b).

2.6.3 Body protein and obligatory losses

Approximately half of body protein reserve is as skeletal muscle, about 15% each as skin and blood, and the remainder as liver, kidney, brain, lung, heart and body (Lentner, 1981). There exists in the body a small 'labile protein reserve', believed to be mainly in the liver and visceral tissues, which can be gained or lost as a short-term store to temper day-to-day variations in protein intake (Swick and Benevenga,1977). Body protein is continuously turned over, and determination of maintenance needs must take into account recycling of amino acids as well as obligatory losses of nitrogen.

Protein secretion into the intestinal lumen, primarily from sloughed mucosal cells and proteolytic enzymes, occurs even in the absence of dietary protein intake. Fecal nitrogen losses (as bacteria in the feces) account for approximately 25% of obligatory nitrogen loss, and intestinal loss of amino acids is significant to maintenance protein needs (Fuller and Reeds,1998; Taverner *et al.*,1981). Other losses of intact amino acids occur in urine, sweat, hair and skin, all of which may be significant, especially when estimating protein requirements in disease states (Matthews,1999).

2.6.4 Protein synthesis, degradation and turnover

The sequence of nucleotides on messenger RNA synthesized in the nucleus from DNA via transcription determines the sequence of individual amino acids in a protein. Transfer RNA in the cytoplasm binds each amino acid to the messenger RNA in a process known as translation, which is regulated by hormones and amino acids such as leucine. In the steady state, although there is no net growth or loss of protein, protein synthesis is in balance with protein degradation. In conditions of inadequate protein intake, or imbalance of amino acid intake, the balance shifts such that the rate of some protein synthesis decreases, while protein degradation proceeds to effect an endogenous source of those amino acids most in need.

Intracellular protein degradation occurs via two systems, lysosomal and proteasomal. The lysosomal system consists of membrane-enclosed, proteolytic enzyme-containing intracellular vesicles which engulf portions of cytoplasm and degrade proteins, usually unselectively (Cuervo and Dice,1998). Regulation of the lysosomes requires an acidic pH and insulin or glucocorticoids (Inubushi *et al.*,1996). The proteasomal system is ATP-dependent (Goldberg and Rock,1992). A molecule of ubiquitin 'targets' a specific protein for degradation by binding to its lysine residues. The proteasome is a protein complex which recognizes the targeted protein and degrades it. This process is selective and usually involves abnormal, damaged or regulatory proteins in the cell.

Approximately 250g of protein are turned over daily in the adult male body, although daily intake is in the range of 55-100g (Waterlow,1984). Tissues differ in their protein turnover rate, such that liver and intestine account for almost 50% of the total (McNurlan and Garlick,1980; Waterlow,1984). Skeletal muscle, on the other hand, accounts for about 43% of body protein, but only 25% of protein turnover (Reeds and Garlick,1984; Waterlow,1984). Turnover is generally greater in infants and lower in the elderly.

2.6.5 Dietary Reference Intakes for protein

The Dietary Reference Intakes (DRIs) have defined adequacy of requirement as the lowest daily intake value for a nutrient that will meet the need for apparently healthy individuals (Institute of Medicine of the National Academies,2002b). The DRIs consist

of the Recommended Dietary Allowance , Adequate Intake, Tolerable Upper Intake Level, and Estimated Average Requirement, defined as follows (Institute of Medicine of the National Academies,2002a):

> ***Recommended Dietary Allowance (RDA)*: the average daily dietary nutrient intake level sufficient to meet the nutrient requirement of nearly all (97-98 percent) healthy individuals in a particular life stage and gender group.**
> ***Adequate Intake (AI)*: the recommended average daily intake level based on observed or experimentally determined approximations or estimates of nutrient intake by a group (or groups) of apparently healthy people that are assumed to be adequate- used when an RDA cannot be determined.**
> ***Tolerable Upper Intake Level (UL)*: the highest average daily nutrient intake level that is likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the potential risk of adverse effects may occur.**
> ***Estimated Average Requirement (EAR)*: the average daily nutrient intake level estimated to meet the requirement of half the healthy individuals in a particular life stage and gender group.[a]**
>
> **[a] In the case of energy, an Estimated Energy Requirement (EER) is provided; it is the average dietary energy intake that is predicted to maintain energy balance in a healthy adult of a defined age, gender, weight, height and level of physical activity, consistent with good health. In children and pregnant and lactating women, the EER is taken to include the needs associated with the deposition of tissues or the secretion of milk at rates consistent with good health.**

While protein is essential for proper function, diet must also be adequate in non-protein components so that amino acids are not used for energy. Further, ingested amino acids must be in the correct balance so that utilization is maximized. To determine the DRIs for protein, the committees examined both factorial and nitrogen balance methods to estimate requirements, and in adults, conclusions were heavily drawn from a large meta-analysis by Rand *et al.* (2003). Conflicting reports of energy requirements of the elderly being greater (Roberts,1996) or less (Zanni *et al.*,1979) than younger adults were resolved by Rand's analysis, which concluded there was no significant effect of age on

the protein requirement of older adults, recognizing that lean body mass as percent of body weight, and protein content of the body decrease with age. A summary of recommended protein intakes for all age groups is presented in Table 2.1 (Institute of Medicine of the National Academies,2002b).

2.7 PROTEIN-ENERGY MALNUTRITION (PEM)

2.7.1 Characteristics of PEM

Undernutrition is defined as a disorder of nutritional status resulting from reduced nutrient intake, impaired metabolism, increased metabolic demands, or increased nutrient losses (Corish and Kennedy,2000). Undernutrition can cause alterations in

Table 2.1. Dietary Reference Intakes for Protein*

Age	Gender	AI	EAR	RDA
			g/kg/day	
0-6 months	M/F	1.52		
7-12 months	M/F		1.1	1.5
1-3 years	M/F		0.88	1.1
4-8 years	M/F		0.76	0.95
9-13 years	M/F		0.76	0.95
14-18 years	M		0.73	0.85
14-18 years	F		0.71	0.85
19-30 years	M/F		0.66	0.8
31-50 years	M/F		0.66	0.8
51-70 years	M/F		0.66	0.8
>70 years	M/F		0.66	0.8
Pregnancy	F		0.88	1.1
Lactation	F		1.05	1.1

*Institute of Medicine of the National Academies,2002b

structure and function of the gastrointestinal system leading to atrophy and impairment of epithelial integrity. Deterioration occurs in three stages: (1) availability of nutrients becomes inadequate due to poor diet, increased requirement, decreased utilization, or excessive loss, (2) nutrient stores become depleted and there is impairment of physiological and/or biochemical processes, (3) severe nutrient deficiency leads to cellular or tissue deterioration (Corish and Kennedy,2000). The consequences of undernutrition can include increased risk of respiratory and cardiac problems, immune dysfunction, infection, deep vein thromboses, pressure ulcers, peri-operative mortality, and multi-organ failure (Omran and Morley,2000). The prevalence of intake of too few macronutrients is estimated at 1-15% of the ambulatory population, 25-60% of institutionalized patients, and 35-65% of hospitalized patients (Omran and Morley,2000).

PEM is characterized by muscle wasting and loss of subcutaneous tissue. In severe PEM, or kwashiorkor, particularly pediatric, serum albumin is decreased, skin and hair suffer dyspigmentation, sodium and water are retained resulting in edema, and the liver becomes fatty (Corish and Kennedy,2000; Rana *et al.*,1996; Swails *et al.*,1996; Waterlow,1975). In kwashiorkor, energy intake may be somewhat sufficient, but protein content is minimal. It has been suggested, but not proven, that the fatty liver results from a diet providing a relative excess of carbohydrate compared to protein (Waterlow,1975). Fatty liver cis believed to result from one of two main mechanisms: (1) factors leading to an increase in triglyceride synthesis beyond the capacity for their removal, or (2) factors blocking utilization of liver triglycerides which are normally excreted into plasma (Flores *et al.*,1970). It is thought the fatty liver in kwashiorkor is due to decreased ability for synthesis of the apolipoprotein required for low density lipoprotein transport of triglycerides out of the liver (Flores *et al.*,1970; Truswell *et al.*,1969; Waterlow,1975). Serum lipids, particularly triglycerides and *β*-lipoprotein cholesterol, are often decreased in kwashiorkor (Flores *et al.*,1970; Truswell *et al.*,1969). With recovery, serum lipids increase and liver fat decreases. Erythrocyte glutathione can be decreased in kwashiorkor (Golden and Ramdath,1987). Rana *et al.* (1996) fed rats a low protein (5%) or adequate protein (20%) diet for four weeks. They included a 20% protein group pair-fed to the intake of the 5% protein group, since appetite was

decreased by 65% of control in the 5% protein group. They found fatty liver and low blood glutathione in the 5% protein and 20% protein pair-fed groups.

In marasmus, there is general insufficient energy intake with muscle wasting and loss of subcutaneous fat, but edema, hypoalbuminemia, or fatty liver are not seen (Corish and Kennedy,2000; Golden and Ramdath,1987). Erythrocyte glutathione is normal in marasmus (Golden and Ramdath,1987). A combination of both conditions, marasmic kwashiorkor, characterized by wasting of muscle and fat with hypoalbuminemia, is most often seen in hospitalized patients (Corish and Kennedy,2000).

Protein synthesis is necessarily decreased in PEM, for preservation of essential protein-dependant functions (Torun and Chew, 1999). In early PEM, there is some loss of visceral protein, but loss is primarily from skeletal muscle as the condition progresses, until nonessential tissue proteins are depleted, at which point further depletion of visceral protein may signal imminent death. When dietary protein is reduced, recycling of the free amino acid pool (from diet and body tissues) increases, while amino acid catabolism decreases compared to normal conditions. Albumin synthesis initially decreases, and within days its half-life increases. There is a shift of albumin from the extravascular pool into the blood. With severe protein depletion, however, albumin and other serum proteins decrease, reducing intravascular oncotic pressure, resulting in extravasation of water and edema, as is typical of advanced kwashiorkor.

Hormonal changes and altered cellular responses to hormones occur in PEM in order to maintain energy homeostasis via increased glycolysis and lipolysis, mobilization of amino acids, preferential breakdown of skeletal muscle for preservation of visceral protein, decreased glycogen, fat and protein storage, and reduced energy metabolism (Torun and Chew, 1999). More specifically, there is reduced insulin secretion, tissue insulin sensitivity, somatomedin activity, and active thyroid hormone levels and higher glucagon secretion, epinephrine release, glucocorticoid production, and growth hormone levels (favouring amino acid recycling).

Reduction of lean body mass in PEM and lowered physical activity level contribute to decreased oxygen consumption and need for hematopoiesis, further

conserving amino acids for more essential protein synthesis. Other body functions, however, are not as well-adapted. There can be reduced cardiac output and peripheral blood flow, immune system defects with depression of T lymphocytes and complement components, alterations in monokine response (mediators of response to injury), electrolyte imbalances, impaired gastrointestinal function, and compromised brain and central nervous system function.

2.7.2 Assessment of PEM

Nutritional status may be assessed by anthropometric measurements such as weight for height (current status) and height/length for age (past history); body mass index (Quetelet's index) for adolescents and adults; triceps skin fold; mid-arm circumference; and upper-arm muscle circumference (Axelsson *et al*.,1988; Newmark *et al*.,1981; Torun and Chew,1999). Urinary creatinine excretion can be used to estimate body muscle mass. Serum protein concentrations may provide a useful estimation of protein status, but caution must be exercised in interpreting results. Serum albumin, as discussed above, may or may not be depressed in PEM, and with a half-life of eighteen to twenty days, may not provide information on more recent status. As well, albumin synthesis rate, distribution, and turnover rate can be altered by PEM (Benjamin,1989). Serum proteins with shorter half-lives, such as transferrin ($T_{1/2}$ 8-9 days), prealbumin (transthyretin, $T_{1/2}$ 2-3 days), retinol-binding protein ($T_{1/2}$ 12 hours), and somatomedin C (insulin-like growth factor-1, $T_{1/2}$ 2-6 hours) may be more sensitive indicators of recent status (Benjamin,1989). Tests of immunocompetence using injection of antigen to determine delayed cutaneous hypersensitivity can also be indicators of PEM (Newmark *et al.*,1981). Of course, diet histories, if obtainable, can also yield useful information. The Mini Nutritional Assessment has been developed as a quick, convenient tool for rapid assessment of the elderly in hospitalized settings (Guigoz *et al.*,1996; Vellas *et al.*,1999). No single parameter is sufficient to evaluate nutritional status, and depending on clinical situation, appropriate combinations of available tools need to be employed.

2.8 STROKE AND PROTEIN-ENERGY STATUS

There has been a decrease in cerebrovascular disease mortality in Japan since 1970, and increased per capita consumption of protein, especially animal protein (beef, eggs, dairy products) (Kodama,1993; Omura *et al.*,1987). The Honolulu Heart Program showed an inverse association between protein intake and stroke incidence, and a sixteen year follow-up showed consumption of animal protein was inversely associated with the incidence of thromboembolic stroke, independent of other risk factors (Kagan *et al.*,1985; Lee *et al.*,1988). There is a paradox of high risk of stroke in populations with a low risk of cardiovascular disease (Reed,1990). This is attributed to a diet low in animal sources. Animal studies with SHRSP rats have mimicked human epidemiology (Sarwar *et al.*,1999; Yamori *et al.*,1984). SHRSP rats fed a Japanese diet (15% protein) had higher incidence of stroke than those fed an American diet (22% protein), despite no differences in blood pressure. The American diet contained more methionine and lysine than the Japanese, suggesting more animal protein in this formulation.

Of more relevance to this thesis are those studies that investigated the effect of PEM on stroke outcome. Although two studies did not find a relationship between protein intake and stroke mortality (Khaw and Barrett-Connor,1987; Lapidus *et al.*,1986), Klag and Whelton (1993) suggest the discrepancy with other studies could be due to the overall higher level and narrower range of protein intake in these two groups. The elderly are a group at high risk for stroke (Gariballa and Sinclair,1998), and many elderly have compromised nutritional status due to a variety of factors such as anorexia, poor dental status, drug therapy, decreased activity levels, and chronic diseases (Abbasi and Rudman,1994; Lipschitz,1991; Marcus and Berry,1998). Several studies have reported evidence of compromised protein/energy status at the time of admission to hospital for stroke and deterioration of this status during the hospital stay (Axelsson *et al.*,1988; Choi-Kwon *et al.*,1998; Davalos *et al.*,1996; FOOD Trial Collaboration,2003; Gariballa *et al.*,1998b; Gariballa *et al.*,1998a). Data from the National Health and Nutrition Examination Study I (NHANES I) suggest low serum albumin is a risk factor for stroke (Gillum *et al.*,1994). Although serum albumin is not necessarily the best indicator of protein status, low serum albumin has been associated with increased length of stay, complications, and death in medical, surgical and stroke patients, and is considered an indicator of poor outcome (Aptaker *et al.*,1994; Axelsson *et al.*,1988;

Choi-Kwon *et al.*,1998; Davalos *et al.*,1996; Finestone *et al.*,1996; Gariballa *et al.*,1998b; Gariballa *et al.*,1998a; Gariballa and Sinclair,1998). Finestone and co-workers studied Canadian stroke patients at the time of admission to rehabilitation units, and reported PEM in 49% of admissions, with dysphagia in 47%. Authors suggested there was inadequate nutritional intervention immediately post-injury which could compromise antioxidant defense mechanisms (Finestone *et al.*,1995; Finestone *et al.*,1996). Nyswonger *et al.* (1992) determined the length of time to start of feeding after stroke varies, and stroke patients fed within seventy-two hours of injury have a shorter hospital stay.

Compromised nutritional status resulting in low protein status, particularly in the elderly, may be an important risk factor for stroke and for poorer outcome after stroke. Feeding after a stroke is often delayed, further exacerbating pre-existing poor nutritional status. There may be a 'window of opportunity' for optimizing survival and recovery from stroke with adequate nutritional support, especially with respect to increasing glutathione status.

CHAPTER 3

OVERVIEW OF EXPERIMENTAL PROGRESSION OF THESIS

This thesis was investigated in two phases. Since glutathione is critical in antioxidant defense, and the sulphur amino acid cysteine, supplied primarily by dietary protein, is limiting at the cellular level for glutathione synthesis, we wanted first to explore one mechanism by which a dietary protein deficiency might limit antioxidant defense under the increased demands of oxidative stress in cerebral ischemia. In Experiment 1 of Phase 1, described in Chapter 5, a rat model of GHHI was used to examine the effect of an acute dietary sulphur amino acid deficiency on glutathione status and neural damage. In this model, the right common carotid artery was ligated, followed by a period of hypoxia, resulting in unilateral damage, providing an internal control with the contralateral (unaffected) hemisphere. The rat GHHI stroke model was well-established at the University of Saskatchewan (Yager *et al.*,1996). Our focus was on removing the dietary supply of the limiting glutathione precursor, cysteine. A rat dietary sulphur amino acid deficiency model had been previously established in our laboratory (Paterson *et al.*,2001). Neural damage was assessed in neocortex, hippocampus, striatum and thalamus, brain regions vulnerable to damage in this stroke model, and previously shown to be sensitive to dietary sulphur amino acid deficiency. We initially used a previously published semi-quantitative system of neural damage assessment, but as a more sensitive system of detecting damage was desirable, our laboratory also designed a more quantitative scoring system. This system, called the *hippocampal grid score*, became the preferred assessment tool. Rat brain sections, stained with hematoxylin and eosin (H&E), were evaluated with both systems, and results obtained with the two methods were well-correlated. Sections adjacent to those used for H&E were stained with antibody against microtubule-associated protein 2 (MAP-2), the absence of which is an early indicator of neural damage (Kitagawa *et*

al.,1989; Matesic and Lin,1994). Although not quantified, areas of MAP-2 deficit correlated visually with areas of H&E-evaluated damage. This experiment demonstrated that neural damage in GHHI was exacerbated by an acute dietary sulphur amino acid deficiency.

To determine if the increased neural damage in sulphur amino acid deficient rats exposed to GHHI was due to glutathione depletion, glutathione concentration was determined in neocortex, hippocampus, striatum and thalamus at six and seventy-two hours after GHHI (for assay procedure, see Appendix A). Glutathione concentration was unchanged at six hours. At seventy-two hours after GHHI, glutathione concentration in the ipsilateral hemisphere was decreased in neocortex and striatum, with a trend towards significance in hippocampus. Glutathione concentration, however, was not different between neocortex taken from the two hemispheres at seventy-two hours, suggesting diet, but not GHHI, decreased glutathione in susceptible regions. These results were in agreement with those obtained previously in our laboratory in sulphur amino acid deficient rats not exposed to GHHI (Paterson *et al.*,2001).

Given the hypothesis that a sulphur amino acid deficiency combined with an hypoxic-ischemic insult would deplete glutathione in specific brain regions, the effect of a cysteine precursor, OTC, was also tested, using the experimental conditions of Experiment 1, repeated in a two by two factorial design. Rats were fed an adequate or sulphur amino acid deficient diet, and given injections of OTC or saline immediately after GHHI, and every twelve hours for six additional doses. At seven days post-GHHI, brains were collected, sectioned and stained with H&E as in the first experiment. Assessment of neural damage was with the two scoring systems used previously. OTC did not have any effect on neural damage. The results of this experiment in rats fed a sulphur amino acid deficient diet were published in (Bobyn *et al.*,2002) and are discussed in Chapter 5. Appendix B describes results obtained in rats fed a sulphur amino acid sufficient diet. It was intended that this experiment be repeated with collection of tissue for glutathione concentration, but this was not pursued further due to the negative findings described in the paragraph above and the identification of a histological artifact, discussed below.

During H&E slide assessment, it became clear that damage appeared in regions not theoretically sensitive to GHHI, and in some cases there was more damage in the hemisphere contralateral to the occlusion. We were later able to attribute this phenomenon to the existence of 'dark' neuron, an artifact of post-mortem handling of tissue before complete fixation (Cammermeyer,1962). Chapter 4 of this thesis describes this artifact. All slides in Experiments 1 and 2 were re-evaluated, and although differences between groups remained relatively unchanged, overall damage in this GHHI model was concluded to be very mild and virtually non-existent in rats fed the control diet. The pattern of brain damage, however, was now consistent with what had been expected. At this point, it was also concluded that the GHHI model was not suitable for further investigations, since: 1) severing of the carotid artery precluded true reperfusion and thus perhaps generation of oxidative stress, 2) the level of hypoxia was mild and did not generate much neural damage, and 3) the damage was extremely variable.

The experiments of Phase 1 investigated the effect of an acute dietary sulphur amino acid deficiency on outcome in global ischemia, recognizing the unlikelihood of humans suffering this selective deficiency. Part of the rationale for this thesis was based on evidence that approximately 16% of the elderly admitted to hospital for stroke suffer from PEM, and that this condition deteriorates during the course of hospital stay (Gariballa *et al.*,1998b; Gariballa *et al.*,1998a; Gariballa and Sinclair,1998). Elderly stroke victims who are malnourished have a longer hospital stay and worse outcome than well-nourished patients, such that almost half of those admitted to long-term care facilities after stroke have PEM (Finestone *et al.*,1995; Finestone *et al.*,1996). For these reasons, combined with mechanistic reasons previously discussed, Phase 2, described in Chapter 6, investigated the effect of PEM on outcome after global ischemia. Since oxidative stress and glutathione depletion is primarily expected during and after reperfusion, a global model of transient ischemia in the Mongolian gerbil was chosen to test this hypothesis. Because the gerbil lacks a circle of Willis, a transient bilateral occlusion of the common carotid arteries is sufficient to effect a reproducible, consistent model of global ischemia producing mainly CA1 hippocampal damage, well-characterized in the literature (Kirino and Sano,1984). Functional (behavioural) and

histological outcomes in this ischemia model are well-documented (Babcock *et al.*,1993; Colbourne and Corbett,1995; Wang and Corbett,1990). Typically, brain temperature falls during ischemia, and can be neuroprotective (Babcock *et al.*,1993; Colbourne and Corbett,1995; Colbourne *et al.*,1993b; Wang and Corbett,1990). This can be overcome, since regulation of brain temperature is non-invasive, achievable, and reduces variability in this stroke model. The author spent two weeks at the Memorial University of Newfoundland learning the gerbil transient bilateral carotid artery occlusion model, histological evaluation (hippocampal neuron counting), and behavioural outcome (the open field and T-maze tests) from Dr. Dale Corbett. His laboratory uses a five-minute occlusion with controlled brain temperature, achieving consistent loss of CA1 hippocampal neurons in the range of 90-95%. Since we theorized PEM might exacerbate neural damage, it was first necessary to vary ischemic conditions to achieve less neuronal loss. A pilot study tested various occlusion time and brain temperature combinations (four minutes at 37°C; three and one-half, four and five minutes at 36.5°C). Variability was high with lower occlusion times, and maintaining constant brain temperature above 36.5°C was difficult, especially with a shorter occlusion time. Ultimately a five-minute occlusion at 36.5°C produced the most consistent results, presented in Appendix C.

The nutritional requirements of the Mongolian gerbil, including protein, are not well investigated. It is known this animal does well on standard rodent laboratory chow, which is about 16-24% protein. An early pilot study, testing whether the response of the gerbil to a purified sulphur amino acid deficient diet was similar to that of the rat, determined that 1) the animals did as well on the 'control' (sulphur amino acids included) crystalline amino acid modified AIN-93G rat diet (for diet composition, see Appendix D) as on rodent laboratory chow, and 2) liver and brain neocortex glutathione concentrations were decreased by the sulphur amino acid deficient diet. For description of the pilot study and results, see Appendix D. Gerbils in this pilot study were housed individually in suspended stainless steel cages for six days. Without bedding and companionship, the gerbils appeared ungroomed and 'jumpy'. These animals normally groom each other, construct nests out of their bedding, and sleep together buried within.

Phase 2 studies would therefore house gerbils in groups of three in shoebox cages with bedding material.

A second pilot feeding study was designed, using a modified AIN-93M pelleted rodent diet without the antioxidant tertiary butyl hydroquinone (TBHQ), containing adequate or low protein[*], fed for four weeks as described in Appendix E. Gerbils on the low protein diet ate less, lost weight, and had increased liver lipid compared to the adequate protein group, confirming a model of PEM. These diets were thus used in Phase 2 experiments.

In Phase 2 experiments, described in Chapter 6, two sets of gerbils were treated identically pre-ischemia/sham surgery. Gerbils were housed in groups of three in shoebox cages and randomized to adequate protein (control diet, C) or low protein (PEM[§]) pelleted diet, both without TBHQ. The diet was modified from the AIN-93M rodent diet (Reeves *et al.*,1993) and fed for four weeks. On day 28, gerbils were subjected to a five-minute bilateral carotid artery occlusion (I) or sham surgery (S). Following surgery, group one was continued on randomized diet for ten days, and tested in the open field on Days 3, 7, and 10. The open field test was chosen as an indicator of hippocampal function, well-documented in the literature as mentioned previously. Brain was harvested for histology after the Day 10 open field exposure. The brains from gerbils in group 2 were collected twelve hours after ischemia/sham surgery for biochemical analyses of indicators of oxidative stress. Glutathione concentration (see Appendix A), soluble protein content, glutathione reductase activity, and protein thiols concentration were determined in brain hippocampus and neocortex, while thiobarbituric acid reactive substances were determined in neocortex only, due to availability of tissue. Liver glutathione concentration and lipid were determined as measures of PEM. Phase 2 experiments showed that 1) a gerbil model of moderate PEM was achieved; 2) ischemic gerbils fed the control diet recovered normal behaviour in the open field by Day 7 post-ischemia, while PEM ischemic gerbils did not recover even by Day 10; 3) although PEM did not affect the survival of hippocampal CA1

[*] Adequate protein and low protein diets were formulated to contain 12% and 2% protein as casein, respectively.
[§] Since gerbils fed the 2% protein diet voluntarily reduced intake, leading to a reduction in both protein and energy, the group fed the low protein diet was given the abbreviation of PEM.

neurons after ischemia, four of twelve PEM-ischemic gerbil brains had marked increases in hippocampal glia; 4) hippocampus protein thiol concentration was decreased by PEM and by ischemia. These results suggest PEM gerbils recover more slowly from ischemia and may experience more oxidative stress than ischemic animals fed a diet adequate in protein.

A concurrent study, described in Appendix F, was conducted to characterize the temporal change in neocortex and hippocampus glutathione concentration in response to brain global ischemia in gerbils fed a nutritionally adequate diet. Glutathione concentration was determined in neocortex and hippocampus immediately after sham surgery, and after zero and thirty minutes, one, two, six, twelve, and twenty-four hours of reperfusion following ischemia. Although the literature reports decreases in brain glutathione concentration at early reperfusion periods (Baek *et al.*,2000; Candelario-Jalil *et al.*,2001; Park *et al.*,2000; Shivakumar *et al.*,1992), we were unable to reproduce these data.

CHAPTER 4

DARK NEURON

Confronted, however, with the vast literature which has accumulated in the last 10 years on the dark and light liver cells, the statement that they are artifacts settles a question on which much work has been wasted. Ernest Scharrer, "On Dark and Light Cells in the Brain and in the Liver", p.57 (Scharrer,1938), quoted by Jan Cammermeyer (Cammermeyer,1962).

A study in our laboratory of rats subjected to global hemispheric hypoxia-ischemia (GHHI) yielded some unexpected results in brain tissue stained with hematoxylin and eosin (H&E). Dark, shrunken, and shriveled neurons were noticed during evaluations of tissue for neuronal damage. Initially these cells were thought to be damaged neurons and assessed as such, even though there was neither cytoplasmic eosinophilia nor chromatin clumping characteristic of injured neurons (Petito *et al.*,1987). There was no pattern to the occurrence of these 'damaged' neurons. They were seen randomly, in the hemisphere contralateral to carotid artery ligation (the control), in areas not vulnerable to damage in this stroke model, such as the dentate gyrus, or surrounding obvious tissue tears at the edge of the neocortex. We were unable to explain this pattern of 'damage'. Further investigation yielded a wealth of literature on the 'artifact complex' and 'dark' neuron. The literature descriptions matched what we had seen on our slides. We had removed formalin:acetic acid:methanol-perfused brains from skulls immediately after perfusion with fixative, resulting in many postmortem artifactual 'dark' neurons. Re-evaluation of brain damage, armed with this new information, yielded quite different patterns and extent of neuronal damage that now fit the expected results for the model. Before recognition of 'dark' neuron, two

groups of rats subjected to GHHI had apparent damage scores of 1.9 and 3.2 out of maximum 8 using one scoring system, and 9.7% and 11.1% using a second scoring system, both described in Bobyn *et al.*, 2002 (Table 4.1, Groups 2, 4). After re-assessment, these scores were 0.1 and 0.4 out of 8, and 0.1% and 0%, showing essentially no brain damage from this stroke model once the artifact was identified. An additional two groups of rats (Table 4.1, Groups 1, 3) fed a sulphur amino acid-deficient diet for three days before and after GHHI had similar reductions in damage after artifact identification, although overall damage was greater than in Groups 2 and 4 in Table 4.1.

In a subsequent experiment with gerbils, intact heads were refrigerated in phosphate buffered 10% formalin overnight after trans-cardiac whole body perfusion with saline followed by fixative. Brains were carefully removed from the skulls 18-24 hours later. Only one brain of forty-five had some 'dark' neurons, this attributed (in retrospect) to forcing the severed head into a container whose opening was too small. In another study in our laboratory, dark neurons were noted in one brain from a gerbil improperly perfused with fixative (Figures 4.1, 4.2).

Jan Cammermeyer has written many papers on the 'dark' neuron, and in his review (Cammermeyer,1962) discusses the history of observation of this phenomenon, identified as early as 1894 by Nissl, and again in 1903 (Turner), 1922 (Spielmeyer), 1932 (Scherer), 1938 (Greenfield) and 1946 (Weil). These researchers attributed the 'dark' neuron to both pathology and postmortem artifactual change, but did not distinguish between the two. Others thought these neurons were a normal cell type, since they were found in normal brains as well as those exhibiting pathologies, and ignored them. By 1916, rapid perfusion of fixative was known to largely avoid 'dark' neuron, but even slight errors in technique could not wholly prevent its occurrence (Scharrer,1938). Cammermeyer demonstrated that 'dark' neurons could be avoided if several hours elapsed between perfusion fixation and removal of brain from the skull (Cammermeyer,1960). Following is a description of the 'dark' neuron as reviewed by Cammermeyer (Cammermeyer,1961; Cammermeyer,1962). Whatever the mode of death, fixation technique or staining method, 'dark' neurons have the same appearance in humans and animals. These neurons are more abundant near a cut or tear, and in compressed, bent or pulled areas. Pyknosis of nuclei in neuroglia and cerebral blood

Table 4.1. The effect of 'dark' neuron identification on neural damage scores in rats exposed to global hemispheric hypoxia-ischemia‡ (GHHI), evaluated with two assessment systems†

	Original Global Score*	Revised Global Score*	Original Hippocampal Grid Score** %	Revised Hippocampal Grid Score** %
Group 1#	4.1 ± 0.6	2.5 ± 0.7	44.0 ± 7.9	34.9 ± 9.3
Group 2§	1.9 ± 0.3	0.1 ± 0.1	9.7 ± 3.3	0.1 ± 0.1
Group 3#	4.5 ± 0.4	1.9 ± 0.6	32.3 ± 9.1	25.6 ± 8.7
Group 4§	3.2 ± 0.3	0.4 ± 0.2	11.1 ± 2.3	0

Mean ± SEM

‡ Table shows original scores (before 'dark' neuron identification) and revised scores (after 'dark' neuron identification).

†For detailed explanation of scoring systems, see Bobyn *et al.*(2002).

Groups 1 & 3: rats fed sulphur amino acid deficient diet for 3 days before and after GHHI.

§ Groups 2 & 4: rats fed purified crystalline amino acid defined AIN-93G diet (Paterson *et al.*,2001)

*Global score: 0 = no damage; 1 = <50% damage; 2 = >50% damage; maximum score of 2 in each region (striatum, thalamus, neocortex, hippocampus) for total maximum score of 8.

** Hippocampal Grid Score: a grid was superimposed on an image of the hippocampus; any grid square containing one or more damaged cells of 15-20 cells per grid was counted as one damaged square and the damage score was expressed as percentage of baseline squares.

vessels are often seen as well, contributing to the broader 'artifact complex', of which the 'dark' neuron is only a part. The cytoplasm of the 'dark' cell is shrunken, the compacted basophil material therein causing the dark appearance. The cell may be separated from the parenchyma by a vacuole of varying size and shape. Often the shrunken nucleus is indistinguishable from the dark cytoplasm, yet is sometimes within a rim of clear cytoplasm. The nucleolus appears larger than normal because of the shrunken cytoplasm. The apical processes may have a corkscrew-like configuration. While there is often random occurrence of single 'dark' neurons (Cammermeyer,1978), haphazard mingling of 'dark' and unaffected neurons is common, as is a more ordered arrangement "lined up like schools of fish" (Cammermeyer,1961). Similarly, before

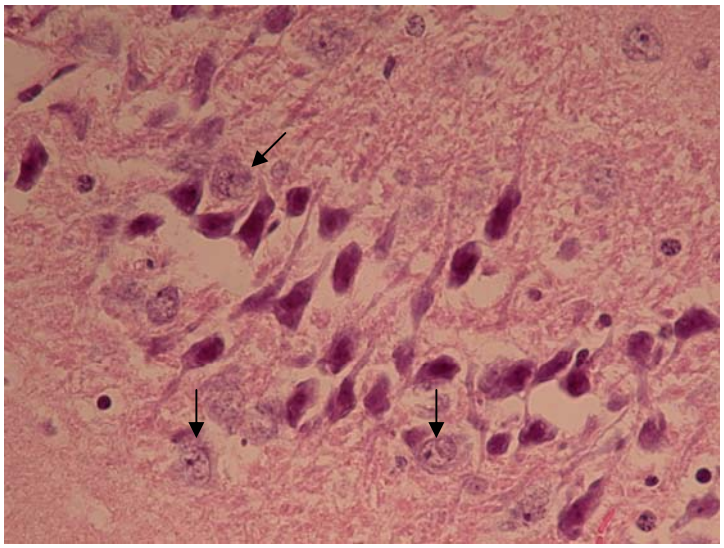

Figure 4.1. Gerbil hippocampus CA4 neurons. Abundant ‘dark’ neurons with twisted apical processes and surrounding vacuoles are shown. Arrows point to normal neurons.

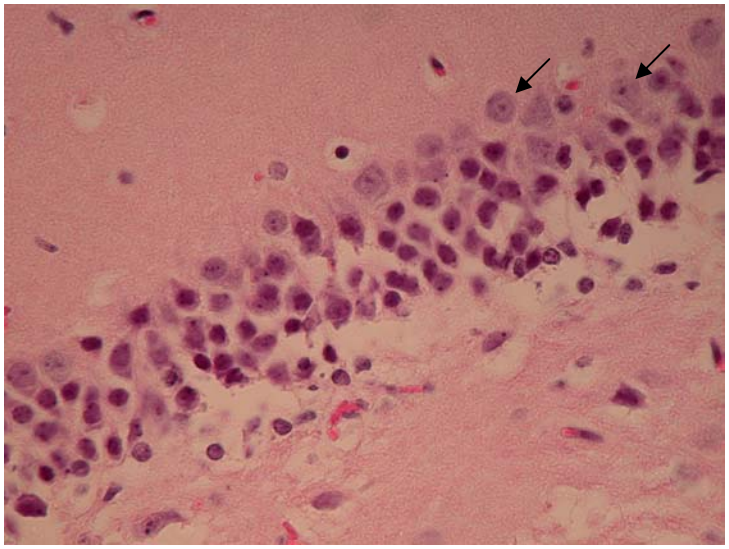

Figure 4.2. Gerbil hippocampus dentate gyrus. Abundant shrunken ‘dark’ neurons with surrounding vacuoles are shown. Arrows point to normal neurons.

awareness of the ‘dark’ neuron in our laboratory, this ordered arrangement was described by the author of this thesis as ‘soldiers standing at attention’, and was prominent in the dentate gyrus. The exact mechanism by which the artifact complex occurs has been a subject of much debate over the years, and is beyond the scope of this thesis. The reader is referred to reviews (Brierley *et al.*,1973; Brown and Brierley,1968; Cammermeyer,1960; Cammermeyer,1961; Cammermeyer,1962; Cammermeyer,1978; Cammermeyer,1979; Kepes *et al.*,1995).

Others have discussed this artifact as well. Brown and Brierley (1968) talk of an “histological artefact of hyperchromatic neurons”, the result of trauma at the time of brain removal and immersion in fixative. They say this artifact can easily be confused with early ischemic damage. Described are several brains exhibiting dark cells, shrunken, heavily stained, with corkscrew-like apical dendrites, occurring in brains removed immediately or soon after perfusion-fixation, and seen more frequently in ipsilateral hemispheres, likely due to inadequate perfusion of fixative. Levy *et al.* (1975) describe the location of artifactual ‘dark’ or ‘hydropic’ neurons occurring in brains with only fair perfusion-fixation, and provide an image as example. They also reiterate, described in their previous paper (Brierley *et al.*,1973), that 1) distribution of ‘dark cells’ may bear no relation to pattern of regions vulnerable to hypoxia, and 2) that the incidence of the ‘dark cells’ is not proportional to severity of hypoxia, as is true ischemic cell damage. Petito *et al.* (1987) describe a study of postmortem human brains after cardiac arrest, identifying criteria by which neuronal necrosis is distinguished from ‘artifactually dark neurons’. Halsey *et al.* (1991) placed rat skulls in fixative overnight, recognizing that fixation artifact must be differentiated from severe neuron damage in evaluation, but did not elaborate further.

In our laboratory, ‘dark’ neuron has now been largely avoided in experimental animals by trans-cardiac saline perfusion followed by buffered formalin and immersion of intact skull in refrigerated buffered formalin for eighteen to twenty-four hours before gentle removal of brain. Although the artifact complex is well-documented, its existence may not be widely appreciated or acknowledged. This was significant to our laboratory in that once the artifact was recognized and removed from tissue assessment, a model of

global ischemia thought to be inducing mild damage was found to result in no damage unless the animals were also subjected to a nutritional insult.

Care to allow time for adequate tissue fixation before handling will be rewarded with specimens free of confounding ‘dark’ neurons. If conditions make this artifact unavoidable, familiarity with its description will assist in distinguishing it from true neuronal necrosis.

CHAPTER 5

THE EFFECTS OF DIETARY SULFUR AMINO ACID DEFICIENCY ON RAT BRAIN GLUTATHIONE CONCENTRATION AND NEURAL DAMAGE IN GLOBAL HEMISPHERIC HYPOXIA-ISCHEMIA*

5.1 ABSTRACT

Primary brain injury in stroke is followed by an excitotoxic cascade, oxidative stress and further neural damage. Glutathione is critical and depleted in oxidative stress. Since cysteine is limiting in glutathione synthesis, this study investigated the effect of dietary sulphur amino acid deficiency on neural damage in a rat model of global hemispheric hypoxia-ischemia. Animals were fed a sulphur amino acid deficient ('deficient') or control diet for 3 days, subjected to right common carotid artery ligation and hypoxia, and diet continued for 3 more days. Histologically evaluated neural damage at 7 days post hypoxia-ischemia was greater in 'deficient' rats, shown by mean (± SEM) global and hippocampal grid scores of 2.5 ± 0.7 and 34.9% ± 9.3 respectively vs. controls' scores of 0.1 ± 0.1 and 0.1% ± 0.1 respectively. Mean brain (± SEM) reduced glutathione was not different between groups at 6 hours post hypoxia-ischemia, but was decreased in 'deficient' animals 3 days later in neocortex (1.46 μmole/g wet weight ± 0.05 *vs.* 1.67 ± 0.04 in controls) and thalamus (1.60 μmole/g wet weight ± 0.05 *vs.* 1.78 ± 0.03 in controls). Administration of a cysteine precursor to 'deficient' animals did not ameliorate neural damage. These findings suggest that well-nourished but not 'deficient' animals tolerate a mild brain insult. The decline in brain glutathione in the 'deficient' animals may be one of several contributing mechanisms.

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* A modification of this chapter was published: Bobyn, P. J., J. L. Franklin, C. M. Wall, J. A. Thornhill, B. H. Juurlink and P. G. Paterson (2002). The effects of dietary sulfur amino acid deficiency on rat brain glutathione concentration and neural damage in global hemispheric hypoxia-ischemia. Nutr. Neurosci. 5(6): 407-416., and is reproduced in this thesis by permission of Taylor and Francis Group, http://www.tandf.co.uk.

5.2 INTRODUCTION

Stroke (ICD-9, 430-438), is a reduction in blood flow to an area of the brain, and the third most common cause of death in the developed world. Primary brain injury due to stroke is followed by activation of several mechanisms contributing to extensive further damage (Juurlink and Paterson,1998). These mechanisms include depletion of glucose, ATP and ADP, membrane depolarization, and glutamate release, initiating an excitotoxic cascade, calcium overload and finally production of reactive oxygen (ROS) and nitrogen species, leading to oxidative stress (Juurlink and Paterson,1998). The acidic conditions promote formation of the potent hydroxyl radical which can initiate peroxidation of membrane polyunsaturated lipids, a self-propagating process that ultimately alters membrane fluidity and function (Juurlink and Paterson,1998). Calcium influx activates an inflammatory cascade, resulting in production of more ROS and also isoleukotrienes, biologically active free radicals (Harrison and Murphy,1995). ATP depletion, excitotoxicity, increased intracellular calcium and oxidative stress eventually lead to cell death (Juurlink,1999).

Glutathione (γ-glutamylcysteinylglycine) is the most prevalent cellular thiol, critical in antioxidant defense (Juurlink,1999). Vitamin E, whose regeneration is dependent on ascorbate and reduced glutathione, can convert lipid peroxyl radicals into less reactive lipid hydroperoxides. These hydroperoxides, however, in the presence of free iron released under acidic conditions, can be converted to alkoxyl and peroxyl radicals, potentially initiating more lipid peroxidation (Juurlink and Paterson,1998). Glutathione and the glutathione peroxidase family scavenge these radicals, and the GSH S-transferases detoxify the aldehyde breakdown products (Juurlink,1999). The transcription factor nuclear factor *kappa* B (NFκB) mediates the inflammatory response in brain ischemia, and glutathione can inhibit the signal transduction pathway that results in NFκB activation (reviewed in Christman *et al.*,2000; Juurlink,1999). That maintaining optimal glutathione levels is of principal importance in minimizing cell damage in stroke is supported by a report that glutathione depletion with buthionine sulfoximine exacerbates injury in a rat focal ischemia model (Mizui *et al.*,1992). N-acetylcysteine, a cysteine precursor, or glutathione ester administration are also both neuroprotective in brain ischemia (Gotoh *et al.*,1994; Knuckey *et al.*,1995).

Tissue glutathione concentration is determined by its utilization, reduction of oxidized glutathione (GSSG) by glutathione reductase, and *de novo* synthesis. Glutathione will be depleted in conditions of severe oxidative stress such as stroke. The sulphur amino acid (SAA) cysteine is limiting for glutathione synthesis. It has long been known that liver and lung GSH is responsive to dietary protein and SAA content of protein (Bauman *et al.*,1988a), and our laboratory has recently shown that glutathione concentration is decreased in certain brain regions by an acute dietary SAA deficiency (Paterson *et al.*,2001). Intracellular cysteine can also be increased by the administration of a cysteine precursor such as L-2-oxothiazolidine-4-carboxylic acid (OTC) (Juurlink,1999). OTC is transported into many tissues, incorporated into glutathione (Meister *et al.*,1986), crosses the blood brain barrier and increases brain cysteine (Anderson and Meister,1989) and glutathione concentrations (Mesina *et al.*,1989). Oral OTC given to protein-energy malnourished rats increases liver, lung and spleen glutathione, and protects against hyperoxia-induced lung damage (Bray and Taylor,1994; Taylor *et al.*,1992). Intraperitoneal administration of OTC to rats after a spinal cord crush injury decreases oxidative stress with a sparing of white matter at the site of injury and partial return of function, while vehicle-treated animals remain paraplegic (Kamencic *et al.*,2001). These encouraging results suggest OTC may have a beneficial effect in stroke, particularly if nutritional substrate for glutathione synthesis is compromised.

Our study investigated whether SAA deficiency would decrease rat brain glutathione and exacerbate neural damage in a rat model of global hemispheric hypoxia-ischemia (GHHI). This model of stroke has been shown to cause neuronal cell loss in the hippocampus, striatum, thalamus and neocortex (Thornhill and Asselin,1999). We also investigated if OTC administration to SAA deficient animals would ameliorate neural damage due to GHHI.

5.3 MATERIALS AND METHODS

5.3.1 Experiment 1

5.3.1.1 Animals, diets and surgical procedures

Male Long-Evans rats aged 9-10 weeks were acclimated for 10 days, then housed individually in suspended stainless steel cages and randomized to an amino acid-based diet as previously described (Paterson *et al.*,2001), but without the antioxidant tertiary butylhydroquinone. Sulphur amino acids methionine and cysteine were omitted from the formulation for the sulpur amino acid deficient (-SAA) diet. The control diet (+SAA) was formulated to contain L-methionine (6g/kg diet) and L-cystine (4g/kg diet), levels recommended for a crystalline amino acid-based diet (Baker and Boebel,1981). Diets were made isocaloric and isonitrogenous by varying sucrose and glycine. Animals had free access to food and water, and daily body weights and feed intake were recorded. Diets were obtained from Dyets, Inc. (Bethlehem, PA, USA). The animals were maintained at 22°C with a 12-hour light/dark cycle. All animal care and procedures adhered to the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Committee on Animal Care and Supply. Control or -SAA diets were fed for 3 days before and after surgery (total 6 days), after which all animals were fed the control diet. Following 3 days on experimental diet, animals (aged 9-11 weeks) were subjected to GHHI according to a modification of the Levine preparation (Levine,1960) described by Yager *et al.* (1996). Under halothane anesthesia, the right common carotid artery was isolated, ligated in 2 positions and severed. The wound was closed and after 5 minutes of stabilization, the animal was exposed to 35 minutes of 12% oxygen in nitrogen (hypoxic period) under light halothane anesthesia. Animals were recovered and returned to their cages. Throughout surgery, core temperature was monitored with a rectal probe and maintained at 36.6 ± 0.5°C with a thermal blanket. There were no spontaneous deaths in any experiments (n=70). Any animals experiencing seizures post-GHHI (n=2; 3%), cessation of breathing during surgery (n=5; 7%), or opening of incision sites (n=2; 3%) were eliminated for an overall failure rate of 9 of 70 (13%).

5.3.1.2 Global scoring system for neural damage

Seven days after GHHI, rats were anesthesized with halothane and perfused transcardially with normal saline to remove blood contamination, followed by FAM fixative (formaldehyde 1 part, glacial acetic acid 1 part, methanol 8 parts) (*n*=15 per group). Brains were removed intact and stored in FAM. Brains were then embedded in paraffin, sectioned at coordinates of approximately -0.4mm (anterior section) and -3.1mm (posterior section) from bregma, and stained with hematoxylin and eosin (H&E). Neural damage to neocortex, striatum, hippocampus and thalamus was assessed in H&E stained sections in the hemisphere ipsilateral to the ligated artery according to the semi-quantitative method of Thornhill and Asselin (Thornhill and Asselin,1999): 0 = no damage; 1 = <50% damage; 2 = >50% damage; maximum score of 2 in each region for total maximum score of 8. Damage in the ipsilateral hemisphere was assessed against the contralateral hemisphere, to account for a possible effect of global hypoxia. To increase the sensitivity for detecting more subtle forms of neuronal dysfunction, adjacent sections were stained with a mouse monoclonal anti-microtubule-associated protein 2 (MAP-2) IgG, using the avidin-biotin method of immunocytochemistry with 3,3'-diaminobenzidine tetrachloride as the chromagen (Miller,1996). All slides were assigned a blinded number to avoid assessment bias.

5.3.1.3 Hippocampal grid scoring system

Since the global hemispheric hypoxia-ischemia model produces mainly forebrain damage, and the hippocampus is particularly vulnerable (Schmidt-Kastner and Freund,1991), our laboratory also developed a more quantitative scoring system restricted to the hippocampal region. The hippocampal area of the H&E stained posterior section of rat brain (approximately -3.1mm from bregma) was visualized at 100 x magnification on a Zeiss microscope with attached SonyPowerHAD colour video camera, and images were captured on computer with Northern Eclipse 2.0 software. A grid was superimposed on each image, and a hardcopy composite image of the entire hippocampal area constructed (about 5-9 images per collage) using Microsoft PowerPoint (for representative image with superimposed grid, see Appendix G). The densest regions of the subiculum/CA1, CA2/CA3, CA4 and dentate gyrus were defined

on the collage and baseline numbers of grid squares in each area counted. Each grid square held approximately 15-20 cells. To score damage, slides were viewed under a microscope at 400 x magnification and damaged cells located and marked by hand on the corresponding composite image. Any grid square containing one or more damaged cells was counted as one damaged square and the damage score was expressed as percentage of baseline squares in each area of the hippocampus. This score will be called the 'hippocampal grid score' in this paper to differentiate from the score assigned to the hippocampal region in the original global scoring system.

5.3.1.4 Brain and liver glutathione concentration

At 6 hours (*n*=8 per group) or 3 days (*n*=8, -SAA; *n*=7, +SAA) after GHHI, rats were anesthesized with halothane and perfused transcardially with cold phosphate-buffered saline (PBS). Brains were quickly removed and cut coronally into 2mm sections (brain slicer from Zivic-Miller, Portersville, PA, USA), and neocortex, striatum, hippocampus and thalamus from the ipsilateral hemisphere dissected, quickly frozen in liquid nitrogen and stored at -70°C until analysis. Slicing and dissection were on ice. To examine whether any change in glutathione in brain was due to diet alone or exacerbated by the GHHI, glutathione level in neocortex was determined in the contralateral hemisphere at 3 days post-GHHI. Small portions of liver were also harvested, frozen and stored as for brain. All tissue collection was between 1100 hours and 1300 hours to minimize diurnal variation in glutathione content. Tissues were weighed, homogenized in 5% 5-sulfosalicylic acid containing 0.1mM EDTA (on ice) and centrifuged at 4°C, at approximately 12,000 x g for 15 minutes to separate the protein precipitate. The supernatant was stored at -70°C until analysis. Final dilutions for brain and liver were 1:50 and 1:200, respectively. Glutathione was analyzed in brain regions and liver by reverse-phase high performance liquid chromatography (HPLC) with ultraviolet detection and precolumn derivatization with 5,5'-dithio-bis(2-nitrobenzoic acid) as previously described (Paterson *et al.*,2001). A standard curve was run with samples daily, all in duplicate.

5.3.2 Experiment 2

Under the same housing and surgical conditions as Experiment 1, a separate group of SAA deficient animals was randomized to receive injections of OTC (n=14) or placebo (PBS vehicle, n=15). Fifteen minutes after the end of hypoxia, animals were administered a subcutaneous injection of vehicle or 1 mol/L OTC (Sigma) in PBS, adjusted to pH 7-7.4 with NaOH at a dose of 12mmol/kg body weight, followed by 4mmol/kg body weight every 12 hours for 6 doses. Injection regimen concluded at the same time as the test diet. Neural damage was assessed histologically at 7 days after GHHI by the methods described under Experiment 1.

5.3.3 Statistical analysis

Global score of H&E stained slides was analyzed by Mann-Whitney *U*. The hippocampal grid score and GSH levels were analyzed by unpaired, 2-tailed Student's *t*-test. Differences between groups were considered significant at $p<0.05$. SPSS 10 for Windows (SPSS Inc., Chicago, IL) was used for all statistics.

5.4 RESULTS

5.4.1 Experiment 1

Body weight at entry into the study was the same for both groups of rats. Animals on the -SAA test diet lost a mean (± SEM) of 64g (± 1.7) over the 6 day period while the +SAA animals gained a mean of 20g (± 2.6). Feed intake of the -SAA rats was about 40% that of the +SAA rats (Table 5.1). At three days post-GHHI, -SAA rats were given the +SAA diet for 4 days until tissue harvest for histology. During this time, their intake normalized (mean 4-day intake [± SEM] 130.5g ± 2.8 *vs*. original +SAA group 120.9g ± 2.5) and they gained weight (51.4g ± 2.4 *vs*. original +SAA group 21.1g ± 3.1). Animals in both experimental groups ate little on the day of surgery, but returned to normal intake for the group within 24 hours (data not shown).

Using the global scoring system, rats in the -SAA group had significantly more damage in neocortex ($p<0.05$), striatum ($p<0.01$) and hippocampus ($p<0.01$) and a higher total score ($p<0.01$) when compared to those in the +SAA group (Table 5.2). Total damage scores in -SAA and +SAA animals ranged from 0 to 6 and 0 to 1 respectively. Figure 5.1 illustrates total scores of (a) 0 and (b) 8. Adjacent sections

Table 5.1. Effect of sulfur amino acid deficiency on weight gain and food intake[*]

	Sulfur amino acid deficient (-SAA)	Sulfur amino acid sufficient (control, +SAA)
Initial body weight (g)	356.1 ± 3.0	357.3 ± 2.2
Total weight gain/6 days (g)	-64.2 ± 1.7[†]	20.1 ± 2.6
Total food intake/6 days (g)	70.2 ± 2.5[†]	177.0 ± 3.4

[*]Values are expressed as mean ± SEM; *n* includes animals for histology (15/group) and for tissue GSH analysis (*n*=8 -SAA; *n*=7 +SAA) totaling *n*=23 -SAA and *n*=22 +SAA.
[†]Indicates significant difference between groups by unpaired *t*-test ($p<0.001$).

Table 5.2. Global score of neural damage in GHHI with dietary SAA deficiency[*]

	Sulfur amino acid deficient (-SAA)	Sulfur amino acid sufficient (control,+SAA)
Neocortex	0.5 ± 0.2[†]	0
Striatum	0.7 ± 0.2[†]	0.1 ± 0.1[#]
Hippocampus	1.1 ± 0.3[†]	0.1 ± 0.1[#]
Thalamus	0.2 ± 0.1	0
Total Score	2.5 ± 0.7[†]	0.1 ± 0.1

[*]Values are expressed as mean ± SEM; *n*= 15/dietary treatment group; Global score: 0 = no damage; 1 = <50% damage; 2 = >50% damage; maximum score of 2 in each region for total maximum score of 8.
[†]Indicates significant difference between groups by Mann-Whitney *U* ($p<0.02$).
[#]Only 1 animal of 15 showed damage to the striatum, and 1 animal showed damage to the hippocampus.

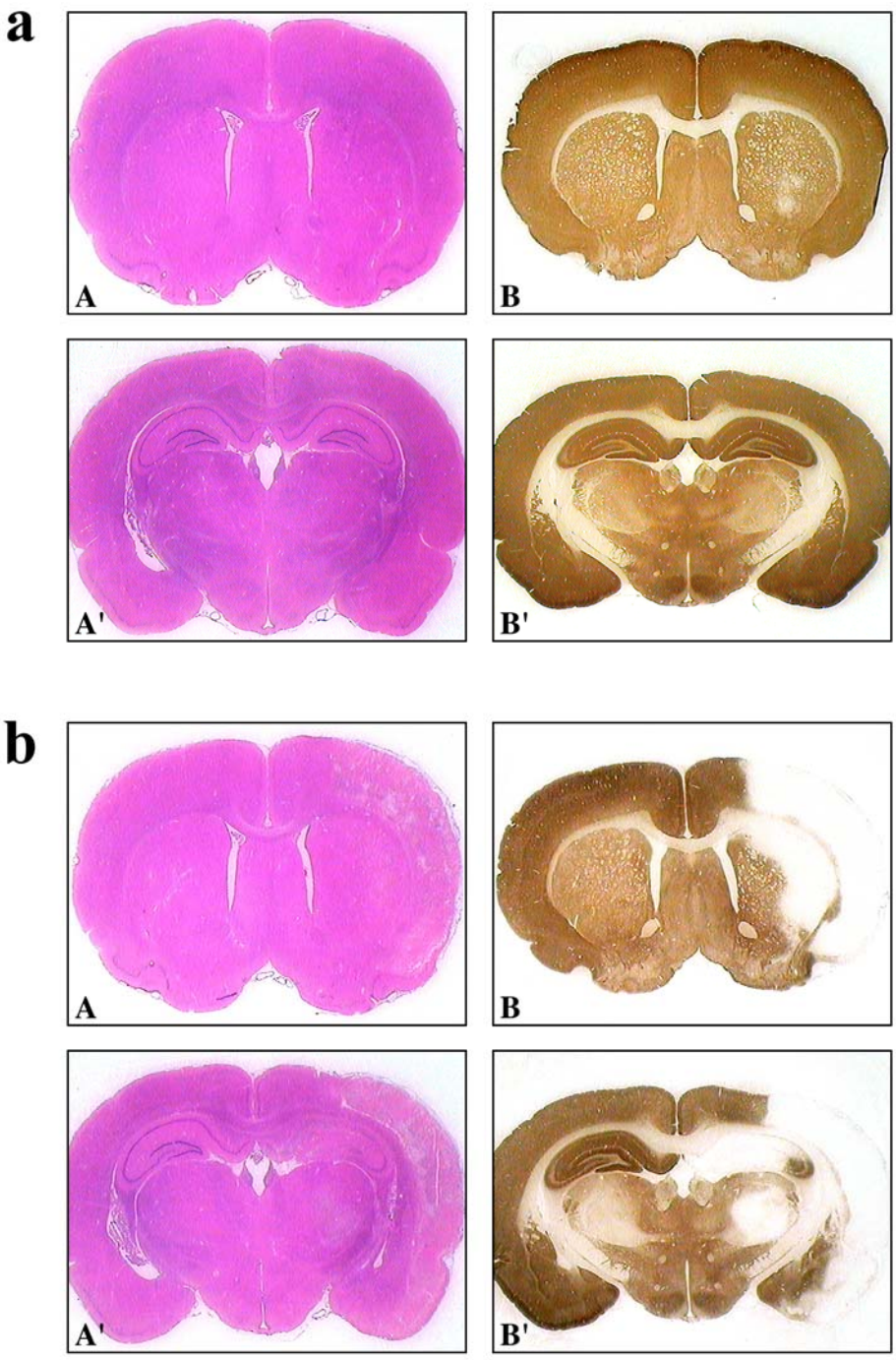


Figure 5.1. Hematoxylin and eosin stained anterior (~ -0.4mm from bregma, A) and posterior (~ -3.1mm from bregma, A') sections of rat brain 7 days post global hemispheric hypoxia-ischemia showing (a) a global neural damage score of 0, and (b) a global neural damage score of 8; with corresponding adjacent MAP-2 stain (anterior, B, and posterior, B'). Global scoring: 0 = no damage; 1 = <50% damage; 2 = >50% damage; maximum score of 2 in each region for total maximum score of 8.

stained for MAP-2 were compared visually to the matched H&E stained slides. By visual inspection alone, areas and amounts of staining of MAP-2 appeared to correlate well with areas of damage on H&E stains (Figure 5.1). Using the hippocampal grid score, damage to the hippocampal area was significantly greater ($p<0.01$) in -SAA animals than in +SAA animals in the subiculum/CA1, CA2/CA3, CA4, hippocampus proper and total hippocampus areas (Table 5.3). Damage to the dentate gyrus was highly variable in -SAA animals (range 0-100%) and completely absent in +SAA animals.

At 6 hours after GHHI, representing approximately 3 days of dietary treatment, there was no significant difference in glutathione concentration between dietary treatment groups in any area of the brain analyzed (Figure 5.2a). Three days after GHHI (6 days of dietary treatment), there was a significant decrease in mean glutathione concentration of neocortex and thalamus ($p < 0.01$) in -SAA animals compared to +SAA, while the difference in glutathione in hippocampus approached significance at $p = 0.06$ (Figure 5.2b). There was no apparent difference in glutathione concentrations in striatum between groups.

At 3 days post-GHHI, mean glutathione (±SEM) concentration in ipsilateral and contralateral neocortex was 1.46 ± 0.05 and 1.39 ± 0.05 μmol/g wet weight respectively in -SAA animals, and 1.67 ± 0.04 and 1.67 ± 0.02 μmol/g wet weight in +SAA animals. Thus, there was no difference in neocortex glutathione between left and right hemispheres at 3 days post-GHHI.

Mean (± SEM) liver glutathione concentration was dramatically lowered in the -SAA group at 3 days post-GHHI (-SAA 2.7μmol/g wet weight ± 0.4 *vs.* +SAA 6.6μmol/g wet weight ± 0.5).

5.4.2 Experiment 2

Animals on the -SAA diet with vehicle or OTC injection lost a mean (± SEM) of 57.1g ± 1.9 and 53.2g ± 3.0 respectively over the 6 day test diet period, and this was not statistically different between groups. Food intake was not different from that of -SAA animals in experiment 1 (data not shown). There was no effect of OTC on neural

Table 5.3. Hippocampal grid score of neural damage after GHHI and dietary SAA deficiency[*]

	SAA deficient (-SAA)	Control (+SAA)
	%	
Subiculum/CA1	51.9 ± 13.0 [†]	0.2 ± 0.2[#]
CA2/CA3	24.1 ± 7.7[†]	0
CA4	45.7 ± 12.7[†]	0
Dentate Gyrus	15.2 ± 8.8	0
Hippocampus Proper[‡]	44.3 ± 11.3[†]	0.1 ± 0.1[#]
Hippocampus Total[¶]	34.9 ± 9.3[†]	0.1 ± 0.1[#]

[*]Values are expressed as mean percent damaged grid squares per region ± SEM [(number of damaged grid squares / number of baseline grid squares) x 100]; *n*= 15/dietary treatment group.
[‡]Hippocampus Proper = Subiculum/CA1 + CA2/CA3 + CA4.
[¶]Hippocampus Total = Hippocampus Proper + Dentate Gyrus.
[†]Indicates significant difference between groups by unpaired *t*-test ($p<0.01$).
[#] In +SAA, only 1 animal of 15 showed damage, in the subiculum/CA1 region.

damage in any brain region assessed by the global scoring system (Figure 5.3a), or by the hippocampal grid score (Figure 5.3b).

5.5 DISCUSSION

Rats fed the SAA deficient diet lost weight and consumed less food. The phenomenon of appetite loss is characteristic of amino acid imbalance (Harper *et al.*,1970), and would be expected to cause generalized reduced protein synthesis and enzyme function. Consistent with other studies of reduced sulphur amino acid or protein intake (Bauman *et al.*,1988a; Hum *et al.*,1992), liver glutathione was dramatically lowered after 6 days on the sulphur amino acid deficient diet, confirming that a sulphur amino acid deficiency was achieved. Brain glutathione was unaltered by a sulphur amino acid deficient diet fed for 3 days, when measured 6 hours after the hypoxic-ischemic insult. By 3 days post-ischemia (6 days of dietary treatment), brain glutathione was significantly reduced in neocortex and thalamus of sulphur amino acid deficient rats, and a similar trend ($p = 0.06$) was observed in the hippocampus. Striatal

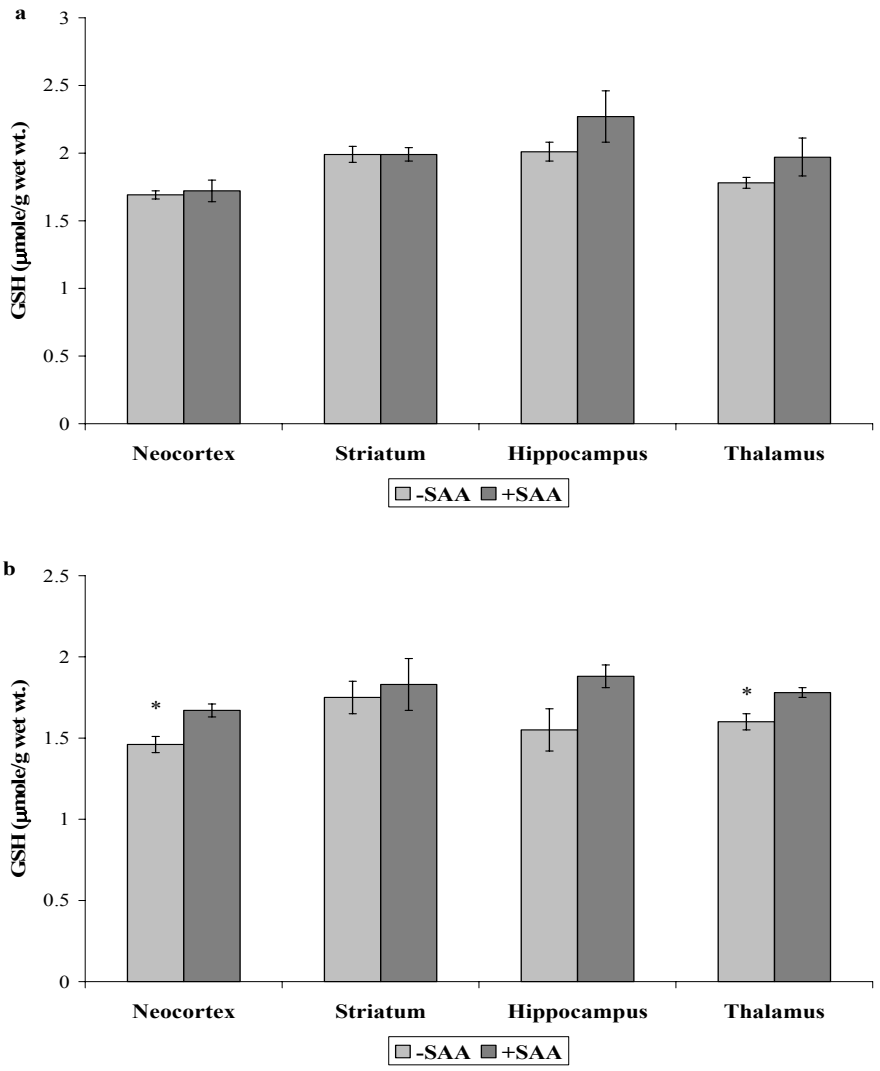


Figure 5.2. Effect of dietary SAA deficiency on brain glutathione concentration. Values are expressed as mean ± SEM. At (a) 6 hours post-GHHI, there are no significant differences in any brain region between groups, *n*= 8/ group; (b) 3 days post-GHHI, *n*=8 -SAA, *n*=7 +SAA. *Significantly different from control (+SAA) by unpaired *t*-test ($p<0.01$). Hippocampal GSH approached significance ($p=0.06$).

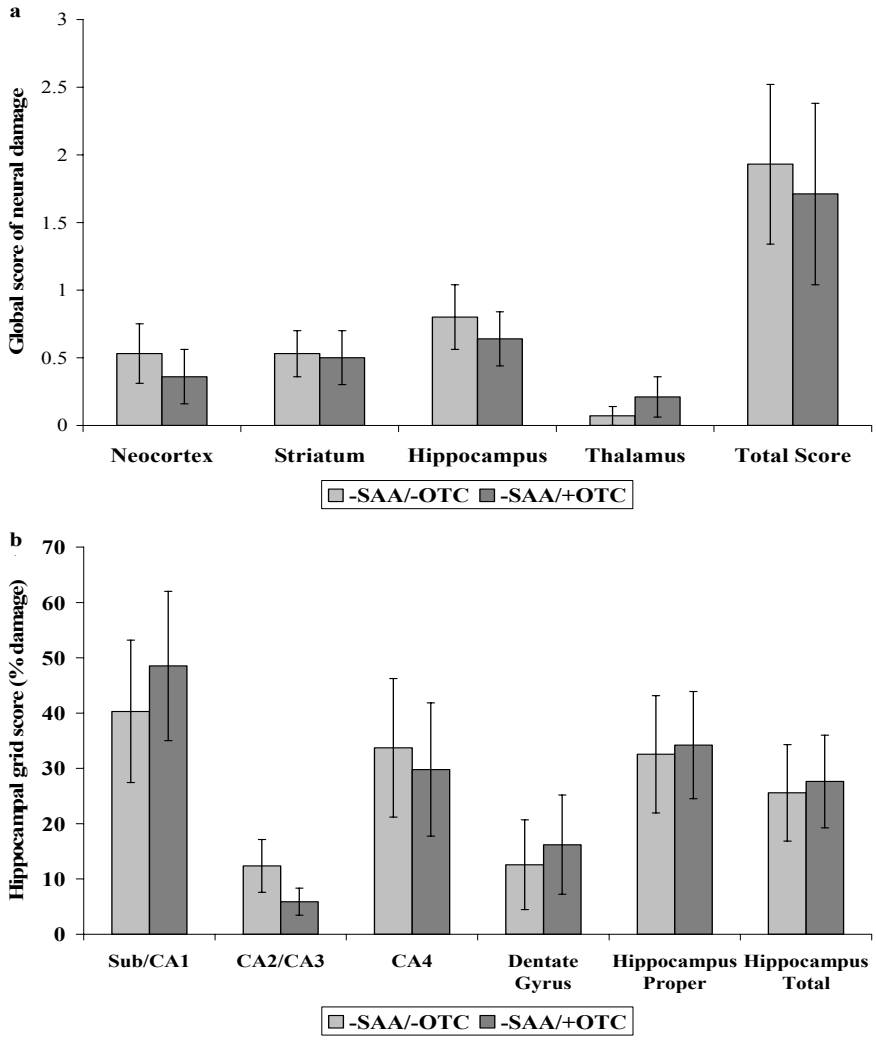


Figure 5.3. Effect of subcutaneous administration of L-2-oxothiazolidine-4-carboxylic acid (OTC) on neural damage in GHHI with dietary SAA deficiency. Values are expressed as mean ± SEM; *n*= 15 -SAA/-OTC; *n*=14 -SAA/+OTC. There was no effect of OTC in any brain region evaluated in -SAA animals by (a) global score: 0 = no damage; 1 = <50% damage; 2 = >50% damage; maximum score of 2 in each region for total maximum score of 8; using Mann-Whitney *U*; or (b) hippocampal grid score: percent damaged grid squares per region [(number of damaged grid squares/number of baseline grid squares) x 100]; Sub/CA1 = Subiculum/CA1, Hpc Proper = Hippocampus Proper (Sub/CA1 + CA2/CA3 + CA4), Hpc Total = Hippocampus Total (Hpc Proper + Dentate gyrus); using unpaired *t*-test.

glutathione did not respond to the dietary treatment. These regional differences are similar to what we have previously found (Paterson *et al.*,2001), suggesting the dietary sulphur amino acid deficiency decreased availability of cysteine for glutathione synthesis. Glutathione is synthesized in two steps in the cytosol of most cells, and concentration is highly regulated (Taylor *et al.*,1996). The formation of γ-glutamylcysteine in the first step is catalyzed by glutamate-cysteine ligase (GL, γ-glutamylcysteine synthetase, γ-GCS) and regulated by glutathione via negative feedback inhibition (Anderson,1997). Availability of cysteine from diet and/or trans-sulphuration of methionine in the liver and activity of γ-GCS are the rate-limiting factors in glutathione synthesis (Lu,1999). Glutamate and glycine are readily synthesized via several metabolic pathways, and are not believed to limit the rate of glutathione synthesis (Bannai and Tateishi,1986). When dietary cysteine is decreased, glutathione may be used to supply cysteine for critical proteins, whose synthesis is favoured over that of glutathione in response to cellular stress, limiting glutathione available for antioxidant defense (Hunter and Grimble,1997). Brain cells have been shown to release glutathione into the extracellular space during conditions such as ischemia (Janaky *et al.*,1999). Extracellular cysteine rapidly auto-oxidizes to cystine, which is transported into the cell via System Xc^- in exchange for glutamate (Bannai and Tateishi,1986). If the normally steep glutamate concentration gradient is unbalanced, such as in the glutamate excitoxicity occurring following stroke, there may be temporary compromise of cystine transport. This could reduce intracellular glutathione synthesis at a time when demand for glutathione is critical (Griffith,1999; Schubert and Piasecki,2001). Changes in γ-GCS activity and gene expression will also alter glutathione concentration, and are mediated by factors such as oxidative stress, Phase II enzyme inducers, and antioxidants (Juurlink,2001; Lu,1999).

We suggest the decrease in glutathione concentration in certain brain regions of sulphur amino acid deficient rats is one of several mechanisms contributing to their greater neural damage following hypoxia-ischemia. Neural damage assessed by two scoring systems was significantly higher in the sulphur amino acid deficient rats than in controls. Cellular damage visible on MAP-2-stained slides mirrored that seen with hematoxylin and eosin staining. MAP-2, abundant in the dendrites of neurons, provides

information on dendritic function (Schwartz,1991). Absence of MAP-2 has been used as an early marker of ischemic damage (Kitagawa *et al.*,1989; Matesic and Lin,1994). Variability in brain damage was high among animals. Although core body temperature was controlled, variation in brain temperature may have contributed to variability in our model. Damage in this stroke model was mild, as shown by the mean score of 2.5 and 0.1 out of 8 in sulphur amino acid deficient and control animals respectively. As well, there was no significant difference in GSH levels between ipsilateral and contralateral neocortex. This suggests the observed depletion of GSH in some brain regions of sulphur amino acid deficient rats was due primarily to decreased synthesis resulting from absence of cysteine in the diet; increased GSH utilization resulting from the hypoxia-ischemia appears to have made little contribution. GSH as an antioxidant becomes critical in reperfusion after stroke, as large quantities of reactive oxygen species are produced during this period, contributing to oxidative stress (Juurlink and Paterson,1998). Oxidative stress may have been milder than anticipated in the stroke model studied, as the brain may not be truly ischemic (Ginsberg and Busto,1989), and reperfusion may be compromised since the carotid artery was severed. We suggest this model did not adequately test the effect of nutrition on GSH homeostasis in ischemic brain. In contrast, some other rodent models of stroke deplete brain GSH markedly (Rehncrona *et al.*,1980; Shivakumar *et al.*,1995).

A pair fed control group was not included in this experiment. Since food intake was substantially decreased, we cannot be certain that SAA deficiency was the sole determinant of the increased neural damage and modest reduction in brain glutathione concentration that developed sometime between 6 hours and 3 days post-insult. Generalized lack of other essential nutrients may have exacerbated the findings. However, brain glutathione, in comparison with liver glutathione, is relatively protected in food deprivation (Benuck *et al.*,1995), when there appears to be recycling of glutathione constituents within the brain and/or transportation of precursors across the blood brain barrier (Dringen,2000). Although plasma glutathione and cysteine levels are determined by efflux of hepatic glutathione, which is decreased in protein deficiency (Adachi *et al.*,1992), levels and function of liver glutathione-synthesizing enzymes appear to be maintained in food deprivation (Tateishi *et al.*,1974). Enzymes involved in

glutathione synthesis are present in brain (Dringen,2000), but their function in food deprivation has not been investigated.

Histological assessment of neural damage showed no effect of administration of a cysteine precursor to SAA deficient animals subjected to global hemispheric hypoxia-ischemia. Although we did not measure brain glutathione concentration following OTC administration, the dose given has been shown to partially replete spinal cord glutathione concentrations following a spinal cord crush injury (Kamencic *et al.*,2001). Activity of 5-oxoprolinase appears to be maintained during protein energy malnutrition (Tateishi *et al.*,1974; Taylor *et al.*,1992), and it was expected brain intracellular cysteine and glutathione levels would be increased. OTC releases cysteine slowly in the cell, avoiding the induction of cysteine dioxygenase, although feedback inhibition of glutathione synthesis can occur (Bray and Taylor,1994). However, since OTC cannot increase tissue glutathione concentration above a physiological maximum (Bauman *et al.*,1988b) and brain glutathione was not yet depleted at the time of hypoxia-ischemia by either sulfur amino acid deficiency or the ischemic insult, our study failed to adequately test the efficacy of OTC.

Several studies have reported compromised protein-energy status at the time of admission to hospital for stroke (Axelsson *et al.*,1988; Choi-Kwon *et al.*,1998; Davalos *et al.*,1996; Gariballa *et al.*,1998b). Feeding after a stroke is often delayed, further exacerbating pre-existing poor nutritional status. In our study, the almost complete absence of neural damage after GHHI in animals fed the control diet suggests a mild insult to the brain may be tolerated in an adequate nutritional state. Sulphur amino acid deficiency, in conjunction with poor food intake, however, rendered the animals in this study less able to cope with mild ischemia. Recognizing that acute sulphur amino acid deficiency is uncommon, this study attempted to examine a mechanism whereby protein-energy deficiency, which will be next examined, might play a role in neural damage due to stroke. Glutathione can be increased with nutritional intervention in animal studies of protein or sulphur amino acid deficiency, and animals 'preconditioned' with decreased protein intake show an overshoot phenomenon of glutathione synthesis upon introduction of a cysteine precursor (Bauman *et al.*,1988b).

There may exist a ‘window of opportunity’ for nutritional support after stroke, especially with respect to optimizing glutathione status in malnourished patients.

CHAPTER 6

PROTEIN-ENERGY MALNUTRITION ALTERS OXIDATIVE STRESS AND FUNCTIONAL OUTCOME, BUT NOT ISCHEMIC CELL DEATH IN A GERBIL MODEL OF STROKE

6.1 ABSTRACT

Primary brain injury in stroke is followed by an excitotoxic cascade, oxidative stress and further neural damage. Since glutathione (GSH), a key component of antioxidant defense, is sensitive to protein-energy malnutrition (PEM), we investigated whether PEM would deplete brain GSH concentration, increase oxidative damage, and exacerbate neural damage after global ischemia. In a 2 x 2 factorial design, adult male gerbils were fed an adequate protein (12%;C) or low protein (2%;PEM) diet for 4 weeks, then subjected to 5 minutes of bilateral carotid artery occlusion (I, ischemia) or sham surgery (S). After 12 hours of reperfusion, hippocampus and neocortex, brain regions selectively damaged by this stroke model, and liver were collected from half the gerbils in each group for biochemical analyses. The remaining gerbils were continued on pre-surgery diets for 10 more days. To assess functional consequences of global ischemia, gerbils were placed in an open field on Days 3, 7 and 10 after I or S. On Day 10, viable hippocampal CA1 neurons were counted in 3 stained brain sections. By surgery day, PEM gerbils consumed 15% less food, lost 13% of initial body weight, and had 62% higher liver lipid and 49% lower liver GSH concentration than C gerbils, confirming PEM. C-I gerbils did not habituate as readily in the open field on Day 3 as sham-operated gerbils, but normalized by Day 7. PEM-I gerbils did not habituate well by Day 10, traveled greater distance than all other gerbils, and 7 of 12 displayed thigmotaxis in the open field. Mean CA1 neuron loss in I was 61.5% of S, but not different between PEM and C. Four of 12 PEM-I gerbils had marked increases in hippocampal glia. Hippocampus protein thiols were significantly reduced in PEM

compared to C (p<0.05), and in I compared to S. There was a trend for ischemia to reduce hippocampus GSH concentration (p=0.08), but diet or surgery did not alter neocortex GSH concentration. Neocortex thiobarbituric acid reactive substances were unaffected by any treatment. There was a trend for ischemia to reduce hippocampus GSH reductase activity in PEM but not C gerbils (p=0.07). Although ability to maintain brain GSH concentration was not altered by PEM, these results suggest PEM gerbils experienced more oxidative stress and recovered normal activity more slowly following global ischemia than well-nourished animals. This is clinically relevant because many elderly stroke victims suffer from PEM at the time of ischemia, which may compromise recovery.

6.2 INTRODUCTION

Stroke (ICD-9, 430-432, 434, 436), usually associated with the elderly, occurs in all age groups and at 7% of all deaths remains the third most common cause of death in Canada. Although mortality rates for all cardiovascular disease have been declining since the 1960s, and rates of ischemic heart disease and acute myocardial infarction continue to decline modestly, mortality rates for stroke have not changed significantly in the last ten years in Canada. At present, no specific therapy for stroke is available, with the exception of thrombolytic treatment (del Zoppo *et al.*,2000). There is a need to identify potential factors that either have detrimental effects on or improve outcome after stroke.

Primary brain injury in stroke is followed by an excitotoxic cascade, calcium overload, production of reactive oxygen and nitrogen species, oxidative stress, and eventual cell death extending into the penumbra surrounding the infarct region (Juurlink and Paterson,1998). Post-ischemic inflammatory processes are also important contributors to secondary brain damage in stroke (for reviews, see del Zoppo *et al.*,2000; Feuerstein *et al.*,1997; Hallenbeck,1996; Kogure *et al.*,1996; Tomita and Fukuuchi,1996).

Glutathione (γ-glutamylcysteinylglycine) is the most prevalent cellular thiol, critical in antioxidant defense (Lu,1999) and depleted in conditions of oxidative stress such as stroke (Candelario-Jalil *et al.*,2001; Rehncrona *et al.*,1980; Shivakumar *et*

al.,1995). Synthesis of glutathione is limited by availability of the sulphur amino acid cysteine, supplied primarily by dietary protein (Anderson,1997). Liver and lung glutathione concentration is sensitive to dietary protein and to sulphur amino acid content of dietary protein (Bauman *et al.*,1988a), and is reduced by severe protein-energy malnutrition (PEM) (Taylor *et al.*,1992). Although brain glutathione concentration is not responsive to decreased dietary protein intake alone (Zhang *et al.*,2002), dietary protein deficiency may limit brain glutathione synthesis during oxidative stress, when the demand for glutathione is high. This laboratory has previously shown that glutathione concentration in some brain regions is decreased by an acute, severe dietary sulphur amino acid deficiency (Paterson *et al.*,2001), and that rats fed a sulphur amino acid-deficient diet suffer more neuronal loss after a mild global ischemia than well-nourished rats (Bobyn *et al.*,2002). Further, animals fed a diet containing protein-deficient (6% protein or less) diet will voluntarily reduce intake (Eisenstein and Harper,1991; Hum *et al.*,1992; Rana *et al.*,1996), resulting in both protein and energy malnutrition.

Compromise of antioxidant defense in brain by PEM at the time of a stroke is a clinically relevant problem. The elderly are a group at high risk for stroke (Gariballa *et al.*,1998a), and many elderly have compromised nutritional status due to a variety of factors (Abbasi and Rudman,1994; Lipschitz,1991; Marcus and Berry,1998). Several studies have reported evidence of PEM in the elderly at the time of admission to hospital for stroke and deterioration of this status during the hospital stay (Axelsson *et al.*,1988; Choi-Kwon *et al.*,1998; Davalos *et al.*,1996; Gariballa *et al.*,1998b; Gariballa *et al.*,1998a). Pre-existing PEM, particularly in the elderly, may be an important risk factor for poorer outcome after stroke, when feeding is often delayed.

This study investigated the effect of PEM on functional, histological and biochemical outcome in a gerbil model of global ischemia. We hypothesized PEM would increase neural damage due to increased oxidative stress secondary to limited supply of essential precursors for glutathione antioxidant defense, and negatively affect functional outcome. The ischemia model used in our study produces neural damage mainly in the CA1 region of the hippocampus (Kirino and Sano,1984; Pulsinelli *et al.*,1982a), and we assessed neuronal survival in this brain region by counting viable

neurons ten days after ischemia. The ability to habituate to an open field was utilized as a measure of hippocampal function (Babcock *et al.*,1993; Wang and Corbett,1990). Glutathione concentration, protein thiols, glutathione reductase activity, and thiobarbituric acid reactive substances were measured as indices of oxidative stress in susceptible brain regions after twelve hours of reperfusion following ischemia.

6.3 METHODS

6.3.1 Animals and diets

Adult male Mongolian gerbils (*Meriones unguiculatus*, Charles River Canada, Saint-Constant, QC, Canada), age eleven to twelve weeks, were acclimated for seven days, and then randomized to adequate protein[¶] (control diet, C) or low protein[¶] (PEM[*]) pelleted diet (D*ye*ts, Inc., Bethlehem, PA, USA). The basal diet was modified from the AIN-93M rodent diet (Reeves *et al.*,1993), and did not contain the antioxidant tertiary butyl hydroquinone (Table 6.1**)**. Diets were made isocaloric by varying cornstarch and dextrinized cornstarch. Animals were housed at 22°C with a twelve-hour light/dark cycle in groups of three in shoebox cages with CareFRESH® (Absorption Corp, Bellingham, WA, USA) bedding and free access to food and water. Biweekly body weights and daily food intakes were recorded. Food pellets were white, facilitating retrieval of food wastage from the brown bedding. All animal care and procedures adhered to the Canadian Council on Animal Care guidelines and were approved by the University of Saskatchewan Committee on Animal Care and Supply. Diets were fed for twenty-eight days.

6.3.2 Surgical Procedures

On day 28, gerbils were subjected to a five minute bilateral carotid artery occlusion (ischemia, I) or sham surgery (S) according to the procedure of Dowden and Corbett (1999). Under 1.5%-2.0% isoflurane anesthetic with 30% O_2, the common carotid arteries were isolated through a ventral midline incision. A continuous thread of surgical silk, passed under both arteries, was looped to allow gentle lifting of the arteries. Core

[¶] Adequate and low protein diets were formulated to contain 12% and 2% protein as casein, respectively.
[*] Since gerbils fed 2% protein will voluntarily reduce intake, leading to a reduction in both protein and energy, the group fed the low protein diet was given the abbreviation of PEM.

Table 6.1. Modified AIN-93M Rodent Diet

Component	Adequate protein[§] (Control, C) g/kg	Low protein[§§] (PEM) g/kg
Vitamin free casein	140	22.4
L-cystine	1.8	0.29
Sucrose	100	100
Cornstarch	465.7	543.049
Dextrinized Cornstarch	155	181.001
Soybean oil	40	40
Cellulose	50	50
Mineral mix*	35	35[¶]
Calcium phosphate dibasic	0	12.4
Calcium carbonate	0	3.36
Vitamin mix[†]	10	10
Choline bitartrate	2.5	2.5

[§] Control diet was formulated to contain 12% protein, 10% fat, 78% carbohydrate.
[§§] PEM diet was formulated to contain 2% protein, 10% fat, 88% carbohydrate.
* AIN-93M mineral mix (Reeves *et al.*,1993).
[¶] AIN-93M modified mineral mix: calcium and phosphorus deleted, potassium citrate·H_2O increased from 28 to 226.55g/kg, sucrose increased from 209.806 to 618.256 g/kg mineral mix.
[†] AIN-93M vitamin mix (Reeves *et al.*,1993).

body temperature was monitored with a rectal probe and maintained at 37 ± 0.5°C with a homeothermic blanket (Harvard Apparatus Canada, Saint-Laurent, QC, Canada). Brain temperature was approximated with a tympanic membrane probe (Barnant Type T Digi-Sense Thermometer) and maintained at 36.5 ± 0.2°C throughout occlusion with a Mul-T-Pad® water-heated blanket (Global Medical Products, Inc., Burlington, ON, Canada) wrapped around the head. When brain temperature was stable at 36.5°C, 8 x 1.5mm micro-aneurysm clips providing 60g pressure (World Precision Instruments, Inc., Sarasota, FL, USA) were applied to the arteries for five minutes. Blockage of blood

flow was visually verified. After clip removal, carotid artery reflow was visually confirmed and the incision closed. Animals were warmed with a red 40 watt bulb in an overhead lamp for thirty to sixty minutes until they became active, and returned to their shoebox cages. For sham surgery animals, procedures were identical: arteries were isolated, looped with silk and released, but not occluded, and the incision was closed. Groups were as follows: control diet with sham surgery (C-S, n=21), control diet with ischemia (C-I, n=24), PEM with sham surgery (PEM-S, n=21), and PEM with ischemia (PEM-I, n=24).

6.3.3 Assessment of Protein-Energy Malnutrition

On day 28, twelve hours after surgery, half the gerbils in each group were anesthesized with isoflurane, perfused trans-cardially with heparinized saline, and livers and brains collected on ice (C-S, n=10; C-I, n=13; PEM-S, n=12; PEM-I, n=12). Whole organ weights were recorded. Since liver glutathione concentration is responsive to PEM (Taylor *et al.*,1992), samples of liver (C-S, n=7; C-I, n=8; PEM-S, n=8; PEM-I, n=8) were analyzed for glutathione using reverse-phase high performance liquid chromatography with ultraviolet detection and pre-column derivatization with 5,5´-dithio-bis(2-nitrobenzoic acid) as previously described (Paterson *et al.*,2001) (See appendix A). Brain hippocampus and neocortex regions were dissected on ice and frozen at -70°C until biochemical analyses (see below, Biochemical Assessment of Oxidative Stress). Liver lipid can be elevated in PEM (Rana *et al.*,1996; Waterlow,1975), and was analyzed using a modification of the methods of Folch *et al.*(1956), and Miyazawa *et al.* (1994) (C-S, n=10; C-I, n=13; PEM-S, n=12; PEM-I, n=12). Liver was homogenized with 0.1M NaCl, and lipid extracted with chloroform:methanol 2:1. After centrifugation at 1000 x g for ten minutes, the upper layer was removed with a glass pipet and discarded. Anhydrous Na_2SO_4 was added to remaining tissue/chloroform layer to dehydrate, and tube contents filtered through #1 Whatman paper into a pre-weighed glass tube. Solvent was evaporated under N_2 gas for fifteen to thirty minutes at 37°C, until dry. The tube was re-weighed to determine lipid content.

6.3.4 Behavioural testing

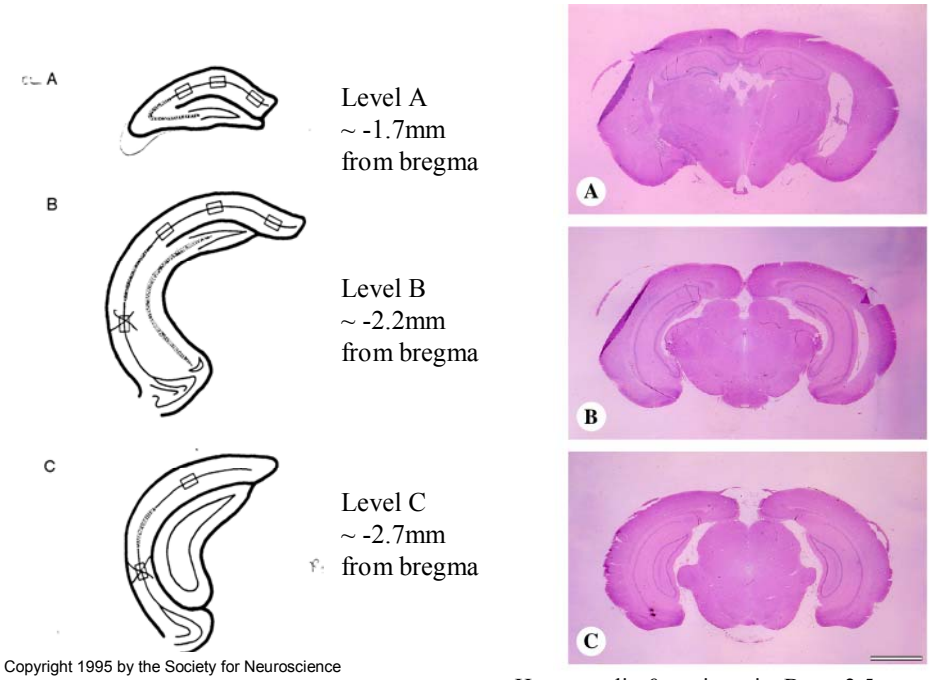
The remaining gerbils in each group were continued on pre-surgery diet for ten additional days. On Days 3, 7, and 10 after surgery, animals were weighed and placed in an open field (75 x 75 x 75cm) for ten minutes of exploration, similar to the protocol of Colbourne and Corbett (1995). The open field was surrounded by sound-absorbing free-standing partitions in a secluded room with constant lighting conditions during testing. Activity was recorded continuously for ten minutes with a suspended video camera, and analyzed later with an EthoVision Basic Program (Version 2.3.19, Noldus Information Technology): (1) by using the entire field, and (2) by dividing the field into an inner zone and a perimeter zone measuring approximately one and one-half times gerbil body width at mid-hip.

6.3.5 Histology

Following the Day 10 open field exploration, gerbils were anesthesized with isoflurane, and perfused trans-cardially with heparinized saline (four minutes at 12mL/min) followed by 10% phosphate-buffered formalin (eight minutes at 12mL/min). To minimize the occurrence of dark neuron artifact (Cammermeyer,1962), intact heads were refrigerated in the formalin for eighteen to twenty-four hours, then brains gently removed and stored in formalin until paraffin embedding. Brains were sectioned in 6μm thicknesses and stained with hematoxylin and eosin (H&E). Viable-looking neurons, non-eosinophilic with defined cell membrane and nucleus, were counted bilaterally in a 200μm square grid (10 x 10) at median, middle and lateral sectors in the CA1 region of the hippocampus at levels ‘A’ (anterior, ~-1.7mm from bregma) and ‘B’ (middle, ~-2.2mm from bregma) and in the middle sector at level ‘C’ (posterior, ~-2.7mm from bregma) at 400 x magnification (Colbourne and Corbett,1995) (Figure 6.1). The slides were assigned a blinded number to avoid assessment bias.

6.3.6 Biochemical Assessment of Oxidative Stress

Twelve hours after surgery, brain hippocampus and neocortex (collected as described under Assessment of Protein-Energy Malnutrition) were analyzed for reduced



Hematoxylin & eosin stain. Bar = 2.5 mm

Figure 6.1. Hippocampal CA1 neuron regions for histological assessment.

GSH using the same method as for liver. Although the hippocampus is selectively vulnerable in this model of ischemia, variable damage can also be seen in the neocortex in similar models (Pulsinelli *et al.*,1982a; Stanimirovic *et al.*,1988). The gerbil hippocampus is very small, providing limited amounts of tissue for biochemical analyses, and thus neocortex collection allowed for additional analyses. Since glutathione reductase activity can be reduced by oxidative stress (Barker *et al.*,1996; Powell and Puglia,1987; Stanimirovic *et al.*,1988), glutathione reductase was analyzed in hippocampus and neocortex using a modification of the methods of Carlberg and Mannervik (1975;1985) and Racker (1955). Tissue was homogenized 1:10 in 20mM sodium phosphate buffered saline containing 1mM $Na_2EDTA{\cdot}2H_2O$ (pH 7.6) on ice and centrifuged at 4°C at 16,000 x g for thirty minutes. Supernatant was further diluted to 1:40 with incubation buffer (0.1M sodium phosphate buffer, pH 7.6 containing 0.5mM $Na_2EDTA{\cdot}2H_2O$). The reaction mixture of 140µL incubation buffer, 30µL 1mM

NADPH in 0.1% $NaHCO_3$, and 100µL sample was incubated at room temperature in a 96-well plate for 10 minutes, followed by addition of 30µL 10mM oxidized glutathione (GSSG) in incubation buffer and immediate reading at thirty second intervals for four minutes at 340nm in a Spectramax 190® plate reader, analyzed with SOFTmax PRO® software (both Molecular Devices Corp., Sunnyvale, CA, USA). A standard calibration curve was prepared using incubation buffer and NADPH, and a molar absorption coefficient for NADPH of $\varepsilon = 6.22 \times 10^3 M^{-1} cm^{-1}$. All analyses were run in triplicate.

Protein thiols are susceptible to oxidation and thus decreased under conditions of oxidative stress (Castilho *et al.*,1996; Netto *et al.*,2002). Protein thiols were analyzed in hippocampus and neocortex with a modification of the methods of Netto *et al.* (2002) and Visser *et al.* (2002). Tissue was homogenized on ice, 1:25 in 10mM HEPES, pH 7.4, containing 137mM NaCL, 4.6mM KCL, 1.1mM KH_2PO_4, 0.6mM $MgSO_4$, and 1.1mM $Na_2EDTA{\cdot}2H_2O$, with the following protease inhibitors added immediately before homogenization (final dilutions): 0.5µg/mL leupeptin, 0.7µg/mL pepstatin A, 40µg/mL phenylmethylsulfonylfluoride (PMSF), and 0.5µg/mL aprotinin. Leupeptin and aprotinin were initially dissolved in HEPES buffer pH 7.4, pepstatin A in methanol and PMSF in isopropanol. Homogenate was centrifuged at 4°C at 16,000 x g for fifteen minutes. To precipitate proteins, equal volumes (100µL) of supernatant and 4% sulphosalicylic acid were allowed to stand at room temperature for fifteen minutes, centrifuged, and supernatant discarded. The protein pellet was re-suspended in 50µL 10% sodium dodecyl sulphate (sodium lauryl sulphate) with vortexing, then incubated in the dark for twenty minutes after addition of 950µL 0.5M TRIS buffer, pH 8.3 containing 0.5mM $Na_2EDTA{\cdot}2H_2O$ and 100µM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Ellman's reagent). Absorbance was read at 412nm in a 96-well plate, in triplicate, with the plate reader and software described above. A standard calibration curve was prepared using glutathione and the same TRIS buffer containing EDTA and DTNB.

As a measure of lipid peroxidation, thiobarbituric acid reactive substances (TBARS) were analyzed in neocortex only, due to availability of tissue samples, using a modification of the method of Ohkawa *et al.* (1979). Tissue was homogenized on ice with 1.15% KCl (1:9), then 50µL added to a glass tube containing 100µL 8.1% SDS,

750µL 20% acetic acid at pH 3.5, 20µL 2% butylated hydroxytoluene in methanol and 330µL deionized, distilled water, followed by addition of 750µL 0.8% thiobarbituric acid. A glass marble was placed on the top of the tube during incubation for one hour at 95°C. After cooling, 500µL deionized, distilled water was added, followed by 2500µL n-butanol:pyridine 15:1. The glass tube was stoppered, shaken vigourously and centrifuged at 1000 x g for 10 minutes. The tube was allowed to stand for thirty to sixty minutes until the two layers were completely clear. Absorbance of the upper, organic layer was read in triplicate in a 96-well plate with reader and software as described, at 532nm. A standard calibration curve was prepared using 1,1,3,3-tetramethoxypropane (malondialdehyde bis[dimethylacetal]) reacted with 1% H_2SO_4 to yield malondialdehyde, and 1.15% KCl for dilutions.

Neocortex and hippocampus soluble protein content was determined with each assay using the bicinchoninic acid (BCA) method of Smith *et al.* (1985), and the appropriate homogenization buffer. Tissue was homogenized ~1:10 in buffer, centrifuged, and supernatant diluted to 1:250. To 20µL sample in a 96-well plate was added 200µL working BCA solution (1 part 4% cupric sulphate pentahydrate:50 parts stock BCA solution [Sigma B-9643]), in triplicate. The plate was incubated for thirty minutes at 37°C, cooled to room temperature, and absorbance read at 562nm with the above microplate reader and software as described. Standard calibration curves were prepared for each assay using dilutions of 1mg/mL bovine serum albumin in the homogenization buffers specific to each assay.

6.3.7 Statistical analyses

All statistical analyses were conducted using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). Body weights, feed intake, and organ weights used for the assessment of the PEM model were analyzed by unpaired Student's *t*-test. Open field data were analyzed using General Linear Model Repeated Measures or two-way ANOVA accordingly, followed by *posthoc* LSD means comparison where appropriate. Biochemical data, including liver lipid and glutathione concentration, were analyzed by two-way ANOVA, followed by *posthoc* LSD means comparison where appropriate.

Differences were considered statistically significant at $p < 0.05$. All data are presented as means ± SEM.

6.4 RESULTS

6.4.1 Exclusions

There were ninety-six gerbils at the beginning of the study. Two gerbils were excluded due to inadequate brain temperature control during occlusion, three died within twenty-four hours of surgery (two PEM-I and one C-S), and one gerbil was excluded due to abnormal toenail formations, enlarged spleen and liver growths. The overall failure rate was 6.25%.

6.4.2 PEM Model

Except for liver lipid (*vide infra*), there were no differences between sham-operated and ischemic animals within each dietary treatment group (data not shown) for any of the parameters used to assess PEM. Initial mean body weight (± SEM) was not different between experimental groups, but by day 28, PEM mean weight was significantly lower than that of gerbils fed control diet ($p<0.001$) (Table 6.2). PEM gerbils lost 13% of initial body weight, and mean feed intake of PEM was 85% that of gerbils fed control diet over the 28-day period. Liver gross weight or weight per g body weight, and gross brain weight of PEM were significantly lower than those of gerbils in the control diet group, while brain weight per g body weight was significantly higher in PEM (all $p<0.001$). Liver lipid content was higher in PEM gerbils compared to those fed control diet ($p<0.001$), and was increased by ischemia in both dietary treatment groups ($p=0.01$). Liver from PEM gerbils contained a lower concentration of glutathione than that of gerbils fed control diet ($p<0.001$), but this parameter was unaffected by ischemia.

Body weight on Day 10 after surgery was lower in PEM gerbils than those fed the control diet ($p<0.001$), and lower in ischemic gerbils than sham-operated ($p=0.03$), but there was no interaction of diet and surgery (Table 6.3). During the ten post-surgical days, PEM gerbils ate less than those fed control diet ($p<0.001$), but there was no difference between PEM-ischemic and PEM sham-operated animals. On the other hand, ischemic gerbils fed control diet ate significantly less than sham-operated ($p<0.001$).

Table 6.2. Effect of experimental diet on body weight, feed intake, organ weights, liver lipid and liver glutathione concentration

Parameter	Adequate Protein Diet (Control diet, C)		Low Protein Diet (PEM)	
Initial body weight (g)[†]	67.5±0.7		69.1±0.5	
Day 28 body weight (g) [†]	72.1±0.7		59.5±0.4*	
28-day feed intake (g) [†]	111.2±1.3		94.7±0.7*	
Liver weight (g)[¶]	2.4±0.1		1.7±0.1*	
Liver weight (mg/g body wt.) [¶]	34.5±0.7		28.7±0.4*	
Brain weight (g) [¶]	1.03±0.01		0.99±0.01*	
Brain weight (mg/g body wt.) [¶]	14.8±0.2		16.7±0.1*	
	C-S	C-I	PEM-S	PEM-I
Liver lipid (mg/g wet wt.)[‡]	35.4±1.4[a]	46.5±2.9[b]	58.0±3.2[c]	77.7±3.8[d]
Liver GSH (μmole GSH/g wet wt.)[§]	5.6±0.4	5.5±0.2	3.1±0.4[Δ]	2.6±0.2[Δ]

Mean ± SEM.
[†] n=45 per group; [¶] C, n=22; PEM, n=24; tissue weights obtained after whole body perfusion with heparinized saline; [‡] C-S, n=10; C-I, n=12; PEM-S, n=13; PEM-I, n=12; [§] C-S, n=7; C-I, n=8; PEM-S, n=8; PEM-I, n=8.
* Indicates significant difference from control group by unpaired *t*-test, $p<0.05$.
Letters indicate significant difference between groups by two way ANOVA followed by *posthoc* LSD test of means, $p<0.05$.
[Δ] Indicates significant difference between dietary treatment groups by two way ANOVA, $p<0.05$.

Table 6.3. Effect of ischemia on post-surgical feed intake and body weight

	C-S	PEM-S	C-I	PEM-I
Body weight Day 10 post-surgery	74.3 ± 1.3	$59.9 \pm 1.2^{\Phi}$	$72.8 \pm 1.1^{\#}$	$55.4 \pm 0.9^{\#\Phi}$
Feed intake:10 post-surgical day total	36.4 ± 0.9^{a}	26.6 ± 0.8^{c}	32.6 ± 1.0^{b}	27.3 ± 0.9^{c}

Mean ± SEM; C-S, n=11; PEM-S, n=9; C-I n=11; PEM-I, n=12.
[#] Indicates significant difference between surgical treatment groups by two way ANOVA.
[Φ]Indicates significant difference between dietary treatment groups by two way ANOVA.
Letters indicate significant differences between groups by two way ANOVA followed by *posthoc* LSD test of means, $p<0.05$.

6.4.3 Behavioural Testing

Decreasing activity level during an exposure to the open field, measured as total distance traveled, is an indicator of ability to adapt or *habituate* to a novel environment. Sham-operated gerbils in both dietary treatment groups habituated well in the open field beginning on Day 3 post-surgery (Figure 6.2). Ischemic gerbils did not habituate on Day 3, but by Day 7, ischemic gerbils fed control diet habituated as well as their sham-operated controls. PEM ischemic gerbils failed to habituate even by Day 10. Although not statistically different from gerbils fed the control diet, PEM sham-operated gerbils tended to have a lower overall activity level. Total distance traveled significantly decreased on repeated exposure to the open field ($p<0.001$) in all groups (within-subjects effects). Collapsed across days, total distance traveled was greater in ischemic than in sham-operated gerbils ($p<0.001$), and in PEM ischemic gerbils compared to ischemic gerbils fed the control diet ($p=0.005$, between-subjects effects) (Figure 6.3). The three-day average of total distance traveled was significantly greater for PEM ischemic gerbils compared to all other groups ($p=0.002$).

Seven of twelve PEM ischemic gerbils, and one of eleven ischemic gerbils fed control diet displayed marked thigmotaxis, a wall-hugging behaviour, readily apparent

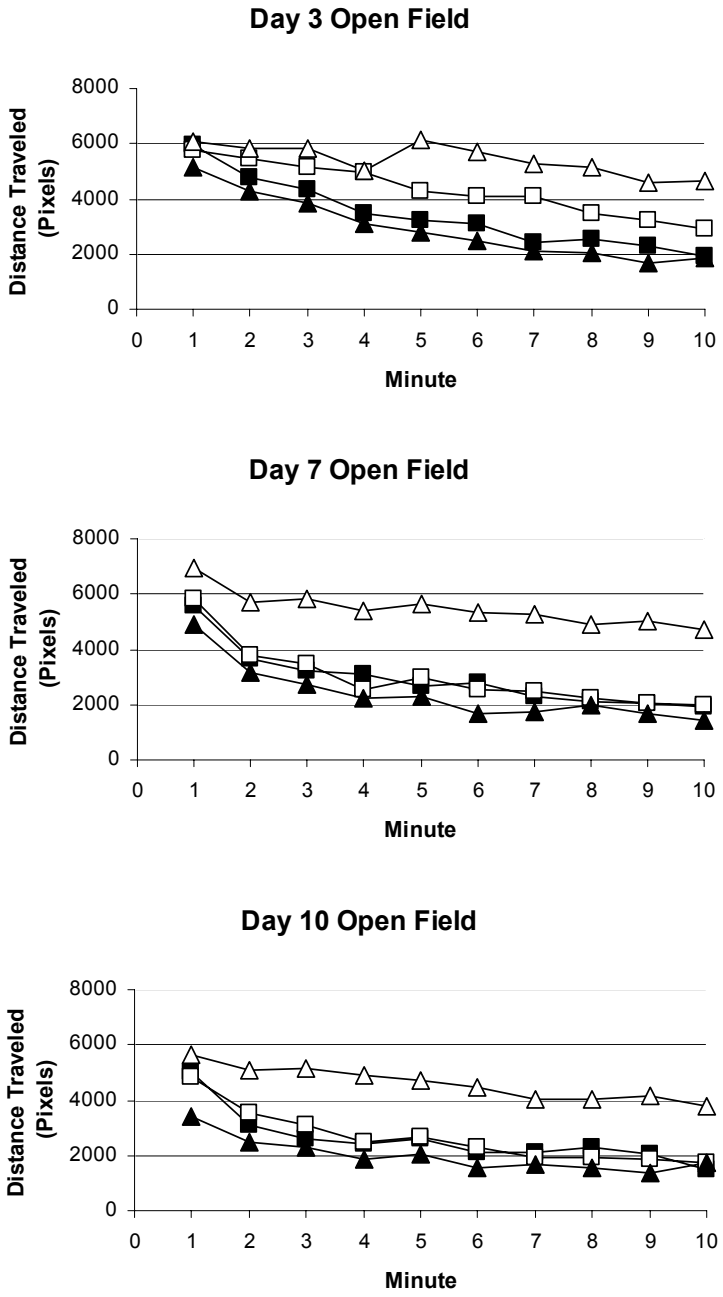


Figure 6.2. Distance traveled in the open field. Closed squares: control diet, sham surgery, n=11; open squares: control diet, ischemia, n=11; closed triangles: PEM, sham surgery n=9; open triangles: PEM, ischemia, n=12.

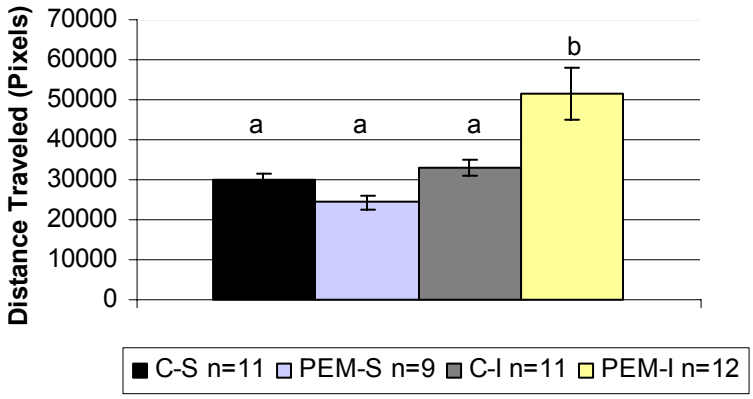


Figure 6.3. Three-day average of total distance traveled in the open field. C-S: control diet with sham surgery; PEM-S: PEM with sham surgery; C-I: control diet with ischemia; PEM-I: PEM with ischemia

on videotape (Figure 6.4). The percent of distance traveled spent in the outer zone remained constant in sham-operated gerbils from day to day, but decreased on repeated exposure to the open field in ischemic gerbils (Figure 6.5). Analysis of between-subjects effects showed the percent of distance traveled spent in the outer zone was greater in PEM than in gerbils fed the control diet (p=0.03), and greater in ischemic than in sham-operated gerbils (p=0.005)(Figure 6.6). *Post hoc* LSD means comparison of percent of distance traveled in the outer zone also showed significantly greater values for PEM ischemic gerbils compared to all other groups.

To examine the pattern of habituation during each open field exposure from day to day, the approximated slopes of the ten-minute activity patterns within each day were calculated using the difference between distance traveled in minute one and minute ten, normalized by dividing this value by the sum of distance traveled in minute one and minute ten (Figure 6.7). Repeated measures analysis of within-subjects effects showed the effect of ischemia on pattern of habituation from day to day was altered by diet (p=0.01). A repeated measures analysis of pattern of habituation by individual group showed a change in pattern of activity over repeated exposures for the.PEM

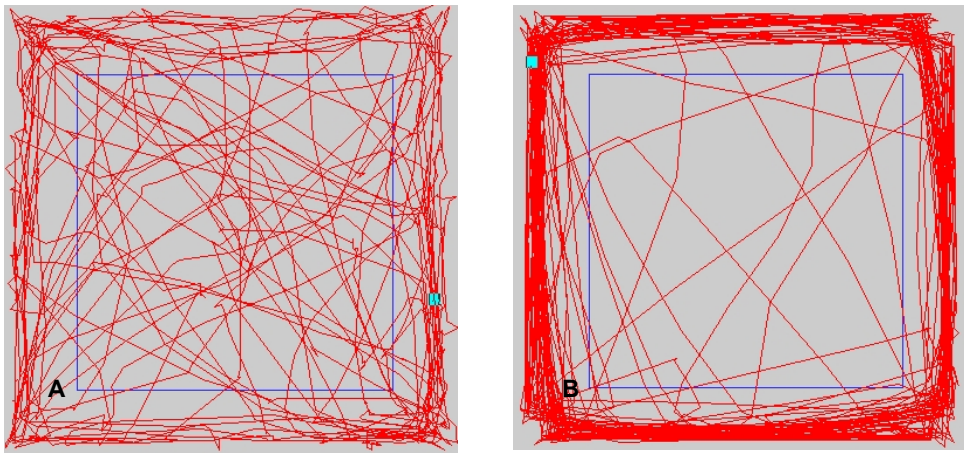


Figure 6.4. Tracks from videotape of ten-minute exposure to the open field. (A): Normal activity pattern of sham-operated gerbil fed control diet; (B): Thigmotaxis observed in protein-energy malnourished ischemic gerbil.

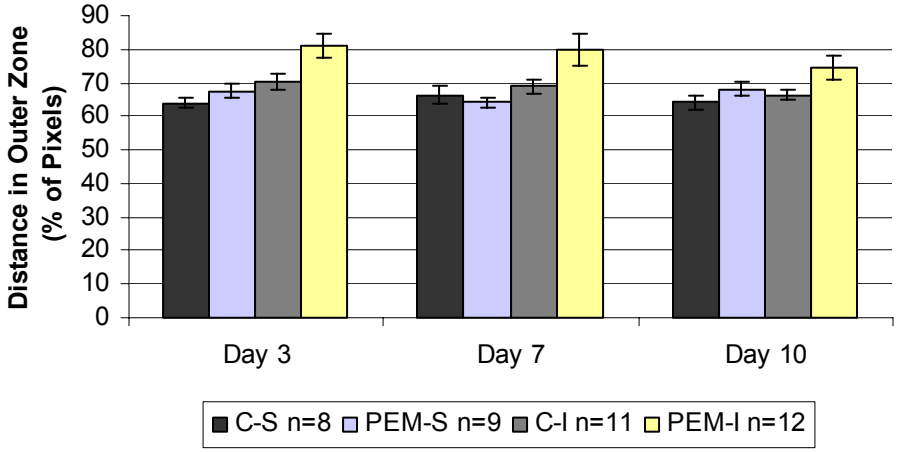


Figure 6.5. Open field: percent of total distance traveled spent in the outer zone. C-S: control diet with sham surgery; PEM-S: PEM with sham surgery; C-I: control diet with ischemia; PEM-I: PEM with ischemia.

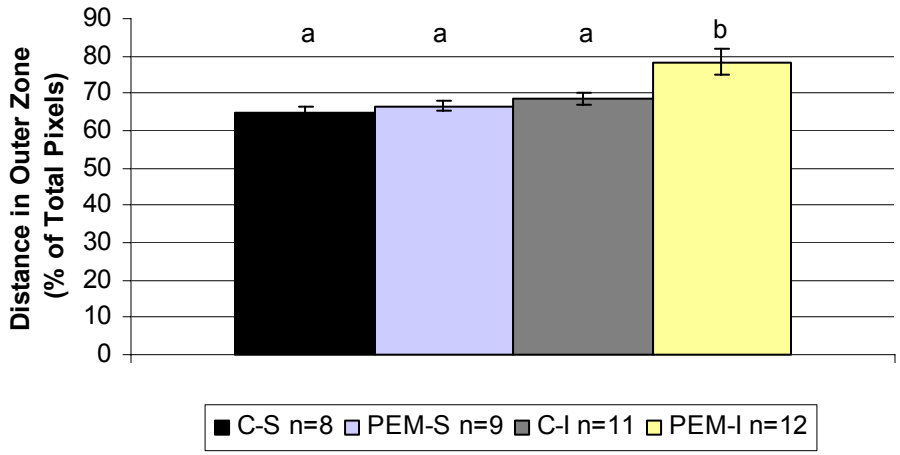


Figure 6.6. Open field: three-day average of percent of total distance traveled spent in the outer zone. C-S: control diet with sham surgery; PEM-S: PEM with sham surgery; C-I: control diet with ischemia; PEM-I: PEM with ischemia. Letters indicate significant differences among groups by two way ANOVA followed by *posthoc* LSD test of means, $p<0.05$.

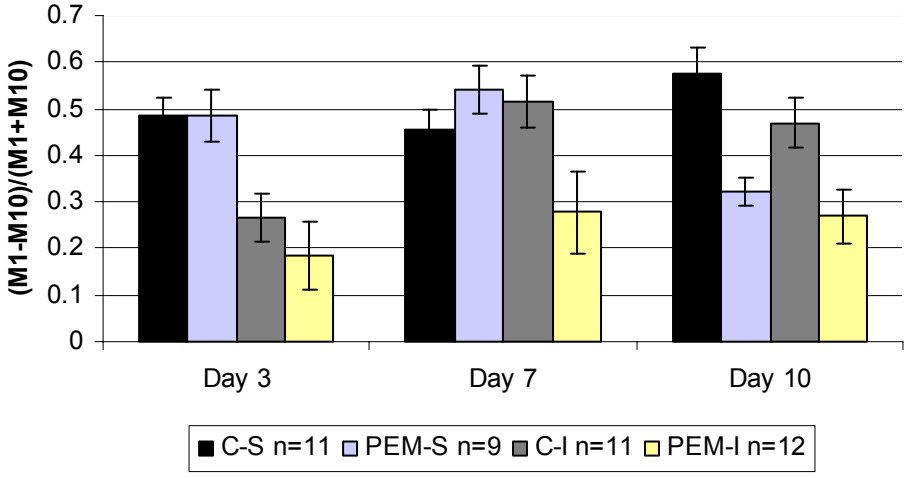


Figure 6.7. Open field: pattern of habituation. C-S: control diet with sham surgery; PEM-S: PEM with sham surgery; C-I: control diet with ischemia; PEM-I: PEM with ischemia.

sham-operated group only. This group had a flattening of the approximated 'slope' by Day 10, due to lower activity in the first few minutes of placement in the open field. This was in sharp contrast to the PEM ischemic group, which did not change the pattern of activity over all three days. Looking at each day individually using two-way ANOVA, surgical treatment was significant (p=0.001) for Day 3, while the interaction between diet and surgery was significant for Day 7 (p=0.03). By Day 10, only dietary treatment had an effect on the pattern of habituation ($p<0.001$). Between groups, collapsing all three days, the 'slope' of habituation was independently flattened by PEM (p=0.005) and ischemia (p=0.02) (Figure 6.8).

6.4.4 Histology

The number of viable hippocampal CA1 neurons at each level, and totalled was unaffected by dietary treatment, in sham or ischemic gerbils. Ischemia resulted in a mean of ~63% and ~ 60% total loss of CA1 neurons in the representative sectors in gerbils fed control diet and PEM gerbils, respectively (Figure 6.9). There was no

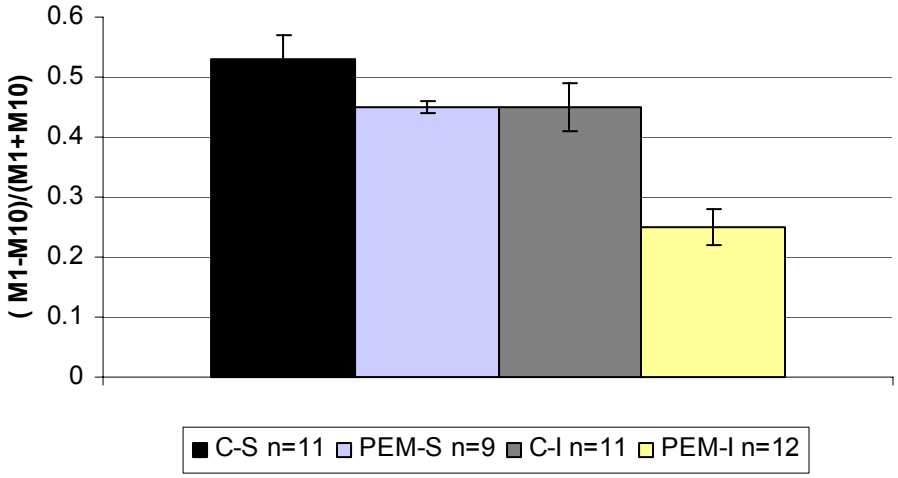


Figure 6.8. Open field: pattern of habituation averaged over three days of exposure. C-S: control diet with sham surgery; PEM-S: PEM with sham surgery; C-I: control diet with ischemia; PEM-I: PEM with ischemia.

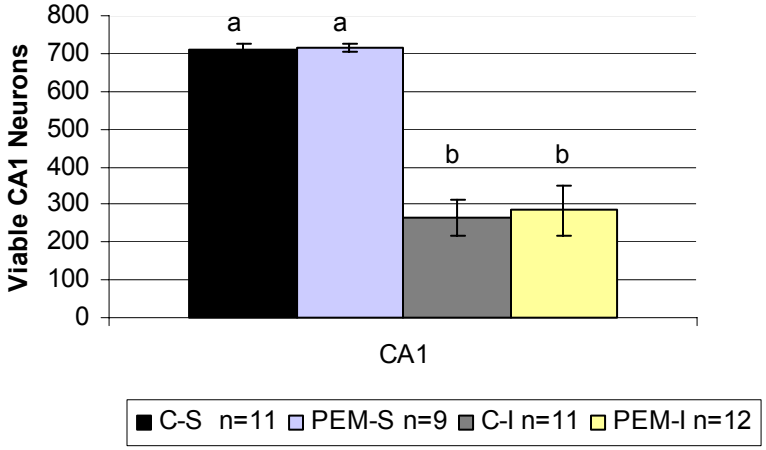


Figure 6.9. Viable hippocampal CA1 neurons ten days post-surgery. C-S: control diet with sham surgery; PEM-S: PEM with sham surgery; C-I: control diet with ischemia; PEM-I: PEM with ischemia. Letters indicate significant differences among groups by two way ANOVA followed by *posthoc* LSD test of means, $p<0.05$.

significant interaction between diet and ischemia in effects on histological outcome. Four of twelve PEM ischemic brains had a marked increase in numbers of hippocampal glia, a phenomenon not seen in any other group (Figure 6.10). Among a subset of ischemic gerbils displaying thigmotaxis in the open field (n=1, C-I and n=7, PEM-I), which included all four gerbils with increased glial numbers, viable CA1 neuron numbers at any level were not different between gerbils with and without increased glial numbers (data not shown). All ischemic gerbils displaying visually observed thigmotaxis, however, had significantly fewer surviving CA1 neurons than those ischemic gerbils not displaying thigmotaxis, at levels B (p=0.01) , C (p=0.01)and total of A,B and C (p=0.02), while level A approached significance at p=0.07 (Table 6.4).

6.4.5 Biochemical assessment of tissue protein and oxidative stress

Protein content of hippocampus was higher in ischemic than in sham-operated gerbils in both dietary treatment groups (p=0.04), while neocortex protein was not affected by diet or ischemia (Table 6.5). Hippocampus protein thiols were significantly

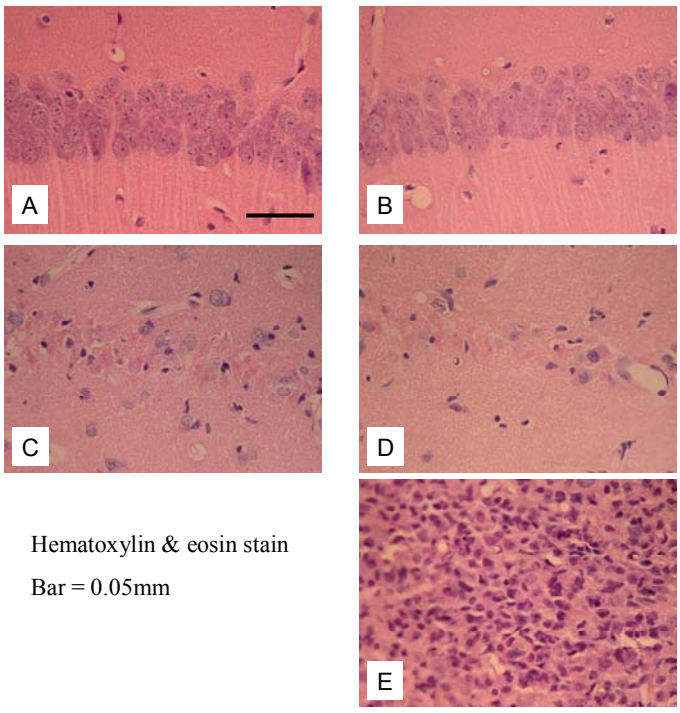


Figure 6.10. Representative images of hippocampal CA1 neurons at ~ - 1.7mm from bregma: intact neurons from sham-operated gerbil fed (A) control diet, (B) PEM diet; neuronal damage in ischemic gerbil fed (C) control diet, (D) PEM diet; and (E), increased number of glial cells in ischemic gerbil fed PEM diet.

Table 6.4. Numbers of viable hippocampal CA1 neurons in ischemic gerbils, categorized by observation of thigmotaxis in the open field

Ischemic gerbils	Level A	Level B	Level C	Total all levels
Thigmotaxis[‡]	60 ± 4	66 ± 5[*]	26 ± 3[*]	152 ± 9[*]
No thigmotaxis[§]	124 ± 24	158 ± 24	59 ± 9	341 ± 55

Mean ± SEM.
[‡] Control diet, n=1; PEM, n=7.
[§] Control diet, n=10; PEM, n=5.
[*] Indicates significant difference between groups by unpaired by *t*-test, $p < 0.05$.

Table 6.5. Effect of dietary treatment and ischemia on tissue soluble protein and biochemical assessment of oxidative stress in hippocampus and neocortex

Assessment	Region[¶]	Experimental Groups[§]			
		C-S	PEM-S	C-I	PEM-I
Protein[*] (mg/g wet wt)	HPC	42.4 ± 2.1	43.6 ± 2.1	46.0 ± 2.6[#]	49.3 ± 1.5[#]
	NC	46.5 ± 2.9	49.7 ± 3.0	45.2 ± 1.0	47.4 ± 2.3
GSH[*] (mMol GSH/mg protein)	HPC	26.4 ± 1.9	24.9 ± 1.4	22.8 ± 1.6	22.2 ± 2.0
	NC	27.7 ± 4.4	26.4 ± 2.7	28.6 ± 1.5	25.2 ± 2.1
GSH Reductase[†] (nMol NADPH/min/ mg protein)	HPC	24.9 ± 2.1	26.3 ± 1.1	25.9 ± 1.8	21.6 ± 0.7
	NC	19.7 ± 1.4	17.0 ± 1.1	19.0 ± 0.8	20.9 ± 1.3
Protein Thiols[*] (nMol GSH equiv/mg protein)	HPC	93.5 ± 6.8	83.5 ± 1.1[Φ]	87.2 ± 3.4[#]	75.8 ± 3.2[Φ#]
	NC	78.2 ± 1.0	75.1 ± 2.5	82.6 ± 1.9[#]	84.0 ± 3.4[#]
TBARS[‡] (nMol MDA/g wet wt.)	NC	371.9 ± 71.9	415.9 ± 70.8	282.7 ± 57.2	339.3 ± 60.0

Mean ± SEM.
[§] C-S = control diet, sham surgery; PEM-S = PEM, sham surgery; C-I = control diet, ischemia; PEM-I = PEM, ischemia.
[¶] HPC = hippocampus; NC = neocortex.
[#] Indicates significant difference between surgical treatment groups by two way ANOVA.
[Φ] Indicates significant difference between dietary treatment groups by two way ANOVA.
[*] C-S, n=7; PEM-S, n=8; C-I, n=8; PEM-I, n=8.
[†] C-S, n=7; PEM-S, n=8; C-I, n=8; PEM-I, n=7.
[‡] C-S, n=5; PEM-S, n=7; C-I, n=7; PEM-I, n=4.

reduced in PEM gerbils compared to those fed control diet (p=0.004), and in ischemic gerbils compared to sham-operated (p=0.04), but there was no interaction between effects of diet and ischemia . Neocortex protein thiols were increased by ischemia (p=0.01) but unaffected by diet; there was no interaction between diet and ischemia. There was a trend for ischemia to reduce hippocampus glutathione concentration (p=0.08), however diet had no effect. Reduction of hippocampus glutathione reductase activity by ischemia approached significance in PEM (p=0.07), but not in gerbils fed control diet. In contrast, neocortex glutathione reductase activity tended towards an increase in PEM (p= 0.06) compared to gerbils fed control diet. There were no independent or interactive effects of diet or surgery on neocortex TBARS.

6.5 DISCUSSION

Little is known of the nutritional or protein requirements of the Mongolian gerbil, but this animal appears healthy when fed laboratory rodent chow containing 16-24% protein (Arrington *et al.*,1973; Edwards *et al.*,1983). The decreased feed intake, loss of body weight, decreased liver glutathione and increased liver lipid seen in this study all suggest a gerbil model of moderate PEM was achieved by feeding 2% protein for four weeks. Various other rodent models of dietary protein deficiency have shown voluntary decreases in food intake and decreased liver glutathione concentration, consistent with our findings (Bauman *et al.*,1988a; Bauman *et al.*,1988b; Eisenstein and Harper,1991; Hum *et al.*,1992; Taylor *et al.*,1992). Our model can be distinguished from that of milder protein deficiency (7.5% protein) where food intake did not decrease (Zhang *et al.*,2002; Bauman *et al.*,1988a), or more severe PEM, where rats developed edema, diarrhea, steatorrhea, mucosal lesions, sparse hair and staggering gait after eating a protein-free diet fed for about two weeks (Leme-Brasil *et al.*,1980; Flores *et al.*,1970). PEM is characterized by muscle wasting and lossof subcutaneous tissue. Rana *et al.* (1996) fed rats a low protein (5%) or adequate protein (20%) diet for four weeks. Appetite and body weight decreased, and the liver was ‘fatty’ in the 5% protein group. It has been suggested, but not proven, that the fatty liver results from a diet providing a relative excess of energy from carbohydrate compared to protein or from decreased ability for synthesis of the apolipoprotein required for transport of triglycerides out of

the liver (Flores *et al.*,1970; Truswell *et al.*,1969; Waterlow,1975). The increase in liver lipid in ischemic gerbils may be a response to surgical stress. Surgery stimulates adrenocorticotropic hormone (ACTH) release from the pituitary, which elevates circulating corticosteroids such as cortisone, corticosterone and cortisol for up to twenty-four hours after insult, and activates the sympathetic system to release epinephrine and norepinephrine (Guyton, 1982; Souba and Wilmore, 1999). Epinephrine, norepinephrine, ACTH and cortisteroids activate triglyceride lipase, causing rapid breakdown of stored triglycerides and mobilization of fatty acids, such that the blood free fatty acid concentration rises. Epinephrine and norepinephrine also suppress insulin and raise glucagon, stimulating hepatic gluconeogenesis and glycogenolysis. Higher liver lipid after ischemia may be only transient, reflecting corticosteroid influence on lipid metabolism.

Hippocampal CA1 neurons are particularly sensitive to brief periods of global ischemia, surviving initially, but succumbing to programmed (delayed) neuronal death over a period of two to four days after the insult (Kirino,1982; Pulsinelli *et al.*,1982a) This delayed death provides a window of opportunity for intervention and neuronal preservation. In humans, a brief episode of global ischemia results in severe hippocampal CA1 injury with resultant anterograde amnesia (Zola-Morgan *et al.*,1986). In gerbils, certain behaviour tests, such as the open field, are effective in assessing hippocampal function after an ischemic insult (Babcock *et al.*,1993; Colbourne and Corbett,1995; Corbett and Nurse,1998; Wang and Corbett,1990). Decreasing activity level during exposure to the open field is an indicator of ability to adapt or *habituate* to a novel environment, testing working memory (Mileson and Schwartz,1991; Nurse and Corbett,1994). An increase in open field activity, or failure to habituate, can be a predictor of histological outcome and selective injury to hippocampal CA1 neurons (Dowden *et al.*,1999), although after ischemia, morphologically normal-appearing cells may be functionally incompetent (Bothe *et al.*,1986; Hori and Carpenter,1994; Ishimaru *et al.*,1995). Ischemic gerbils in both dietary treatment groups did not habituate well on novel exposure to the open field, three days after surgery. By the second exposure, one week after surgery, well-nourished ischemic gerbils habituated as well as all sham-operated gerbils, suggesting recovery or adaptation. Hippocampal damage in our

ischemia model was less severe and more variable than expected. Others have reported more severe, consistent damage with the same global ischemia model, where, in contrast to our results, ischemic gerbils fed a normal diet could be differentiated from sham-operated animals on all three post-surgical exposures to the open field (Colbourne and Corbett,1995; Dowden and Corbett,1999).

Protein-energy malnourished ischemic gerbils in our study, however, still did not habituate well by Day 10 post-surgery. Indeed, the activity of these gerbils could be differentiated from PEM sham-operated gerbils on all three days of open field exposure. This was in contrast to gerbils fed the control diet, despite almost identical CA1 neuron loss from ischemia in both dietary treatment groups. Seven of the twelve gerbils in the PEM ischemic group spent over 87% of the total distance traveled in the outer perimeter of the open field. This was in sharp contrast to findings in the ischemic gerbils fed control diet, where only one of eleven showed this increased affinity for the outer perimeter. All other gerbils showed a range of 65-70%, and no sham-operated gerbils exhibited this behaviour. Thigmotaxis is considered indicative of 'behaviour trapping' or anxiety (Schallert *et al.*,1980; Simon *et al.*,1994).

All gerbils except those in the PEM sham-operated group had a similar pattern of habituation over each ten-minute exposure from one test day to the next. The lack of habituation on first exposure to the open field in ischemic gerbils fed the control diet lost significance when averaged over all three exposures. The PEM sham-operated group, however, became less active over repeated exposures, and by Day 10 post-ischemia, although the approximated 'slope' of activity over ten minutes suggested no habituation, the activity level was slightly lower than that of all gerbils fed the control diet, and dramatically lower than that of PEM ischemic gerbils. This suggests the continuation of the PEM diet for an additional ten days after sham surgery has a depressing effect on level of activity, independent of ischemia. Similar reductions in level of physical activity and/or ability to perform manual labour in conditions of PEM have been reported in monkeys (Chopra *et al.*,1987) and humans (reviewed in Shetty,1999). In contrast, PEM ischemic gerbils maintained a higher than normal level of activity throughout the post-ischemic period, suggesting abnormal hippocampal function.

Ischemia resulted in a significant loss of hippocampal CA1 neurons, but PEM had no additional effect on neuronal death. Unexpectedly, four of twelve PEM ischemic gerbils had marked increases in numbers of hippocampal glia, suggesting inflammation, although these cells have not yet been stained to identify glial cell types. After ischemia, glia are activated to form reactive microglia and reactive astrocytes (reviewed by Liu *et al.*,2001). In transient global ischemia, there is microglial activation very early in reperfusion, with a return to normal conditions by seven days post-injury in the penumbral region, though microglia may persist in the hippocampal CA1 region for up to one month (reviewed by Kato and Walz,2000). Reactive astrocyte proliferation appears later, reaching maximal accumulation in hippocampus at three to seven days post-ischemia (Domanska-Janik *et al.*,2001; Kato *et al.*,1994; Liu *et al.*,1998). This response to ischemia has not been studied in PEM. While lowered immune response is one component of PEM, chronic inflammation can occur (reviewed in Mora,1999). Inflammatory response in PEM can be abnormal and/or prolonged (Leme-Brasil *et al.*,1980; Wan *et al.*,1989). A prolonged inflammatory response in PEM may explain in part the glial proliferation seen throughout the hippocampus and neocortex in the four PEM ischemic gerbil brains. All four gerbils with increased hippocampal glial numbers exhibited thigmotaxis in the open field. Ischemic gerbils not displaying thigmotaxis in both dietary treatment groups had more surviving CA1 neurons in caudal sections (levels B and C) than ischemic gerbils exhibiting thigmotaxis, linking more extensive damage with abnormal behaviour in the open field.

While we did not observe any effect of PEM, in the absence of ischemia, on surviving CA1 neurons, others have shown that prolonged PEM in postnatal or adult life in rats for fifty days and six months, respectively, resulted in decreased numbers of neurons and altered dendritic trees and synapses (Lima *et al.*,1999; Lukoyanov and Andrade,2000). Brock and Prasad (1992) found dendritic spine densities in some brain regions of rats were sensitive to dietary protein, while others were spared. To our knowledge, there have been no studies on the effect of short term PEM on hippocampal neuron numbers, and we did not expect the diet alone to affect this parameter in the adult brain.

Behaviour in the open field in this experiment was not reflected by extent of neuronal loss. Histology cannot predict communication within and between cells, making correlation between morphology and functional outcome difficult (DeVries *et al.*,2001). Using the same ischemia model as in this study, Corbett and Crooks preconditioned gerbils with two short occlusions seventy-two hours before five minutes of ischemia. Ten days later, preconditioned ischemic gerbils had lost only 19% of hippocampal CA1 neurons, yet showed significant impairments in the open field (Corbett and Crooks,1997). Similar results have been shown by others (Bothe *et al.*,1986; Dooley and Corbett,1998; Kudo *et al.*,1990). In another study from Corbett's laboratory, the amplitude of CA1 dendritic field excitatory postsynaptic potentials (fEPSP) recorded ten days after ischemia in preconditioned gerbils was significantly lower than that of sham animals, although CA1 neuron loss was only about 12%. The lower fEPSP amplitudes correlated well, however, with increased activity in the open field (Dowden and Corbett,1999). These findings emphasize the importance of functional testing as an outcome measure in global ischemia models in addition to examining morphology.

The brain, responsible for 20% of body oxygen consumption, is rich in polyunsaturated fatty acids and susceptible to free radical damage and lipid peroxidation (Comporti,1993; Siesjo *et al.*,1989). Production of reactive oxygen species during reperfusion after transient ischemia can ultimately lead to (1) oxidation of thiol groups on proteins and on susceptible enzymes such as glutathione reductase, essential for regenerating GSSG, and (2) initiation of lipid peroxidation with resultant decomposition of membrane fatty acids (Comporti,1993). Measurement of protein thiols, glutathione reductase activity, glutathione concentration and lipid peroxidation products may be used as indicators of oxidative stress, a condition in which free radical production exceeds antioxidant capacity (Shivakumar *et al.*,1995). We investigated whether the abnormal brain function of PEM gerbils measured post-ischemically could be partially attributable to glutathione depletion and increased oxidative stress. To our knowledge, the combined influence of PEM and ischemia on brain glutathione concentration and markers of oxidative stress has not been studied.

Hippocampus protein thiols were independently reduced by PEM and by ischemia, but the magnitude of the response to ischemia was not affected by diet. Protein thiol groups are susceptible to oxidation (Balijepalli *et al.*,2000; Sen and Packer,2000) and have been shown to decrease at one hour of reperfusion after thirty minutes of ischemia and return to normal levels at twenty-four hours (Shivakumar *et al.*,1995). That PEM alone decreases protein thiols may be partially supported by the hypothesis of Golden and Ramdath (1987), who suggest some of the pathogenesis in PEM is due to favourable conditions for production of free radicals and lipid peroxidation, in the milieu of low glutathione status due to high antioxidant demand and poor nutritional intake. Surprisingly, neocortex protein thiols did not appear to have been oxidized and were increased after ischemia in both dietary treatment groups. The neocortex is variably and less vulnerable to damage compared to the hippocampus in this model of ischemia. Soluble protein in this region was unchanged by ischemia (*vide infra*), thus it is unlikely there was overall increased protein synthesis or extravasation of plasma proteins into the parenchyma to account for the protein thiol content. It is possible there was selective synthesis but not inactivation, of sulphhydryl-containing proteins important in antioxidant defense, such as glutathione reductase, for example, in response to adjacent hippocampal injury or stress. The trend for increased glutathione reductase activity in PEM gerbil neocortex would support this suggestion.

There was a trend for ischemia to reduce hippocampus glutathione reductase in PEM, but not in adequately-nourished gerbils. Glutathione reductase is a flavoprotein utilizing NADPH to catalyze the reduction of GSSG to glutathione (Carlberg and Mannervik,1985). The thiol groups on glutathione reductase can be oxidized, inactivating the enzyme in conditions of oxidative stress (Barker *et al.*,1996). There is some evidence, however, that ischemia may need to be longer than five minutes to see a change in the hippocampus (Shivakumar *et al.*,1995; Stanimirovic *et al.*,1988; Stanimirovic *et al.*,1994). An interesting theory is proposed by Powell and Puglia (1987), who found that a 75% reduction in glutathione reductase activity did not alter brain glutathione concentration in a study of oxygen-induced CNS toxicity, and suggest under normal conditions, the brain may have an excess capacity for reduction of oxidized glutathione.

Compared to liver glutathione, brain glutathione is relatively protected in forty-eight-hour food deprivation (Benuck *et al.*,1995), and is not altered in mice or rats fed a low protein diet (Adachi *et al.*,1992; Li *et al.*,2002; Stipanuk *et al.*,2002; Zhang *et al.*,2002). Various rodent models of stroke have been shown to deplete brain glutathione at early reperfusion timepoints (Baek *et al.*,2000; Candelario-Jalil *et al.*,2001; Park *et al.*,2000; Rehncrona *et al.*,1980; Shivakumar *et al.*,1992; Shivakumar *et al.*,1995), followed by a return to normal levels four to twenty-four hours later (Candelario-Jalil *et al.*,2001; Shivakumar *et al.*,1992; Shivakumar *et al.*,1995). We expected that the high demand for glutathione in oxidative stress might deplete brain glutathione when challenged with limited availability of precursors for glutathione synthesis in PEM. Although there was a trend for ischemia to reduce hippocampus glutathione, differences among groups were not significant. The extent of lipid peroxidation in neocortex measured by TBARS was not affected by diet or ischemia, and variability was high, consistent perhaps with variability of this region to damage in this stroke model. Since the hippocampus is the region most affected by this model of global ischemia, it would have been valuable to assess hippocampus TBARS.

Although not a marker of oxidative stress, tissue soluble protein was used as a means of standardizing cellular content of other measured indices. Neocortex protein content was not affected by any treatment, but ischemia significantly increased hippocampus protein content in both dietary treatment groups. This may be due to increased production of pro-inflammatory enzymes/proteins such as adhesion molecules and growth factors, and even immune complement components in response to ischemia (reviewed in Petty and Wettstein,2001). Further, post-ischemic edema, with extravasation of plasma and accompanying proteins into the parenchyma across a compromised blood-brain barrier has been well-documented, and may have contributed to the increased hippocampal protein content (Lo *et al.*,2001; Okada *et al.*,1994; Petty and Lo,2002; Petty and Wettstein,2001; Sato *et al.*,2003). The change in hippocampus protein content may have influenced results of the markers of oxidative stress.

Our assessment of oxidative stress is limited to one time-point post-surgery. It is possible this single timepoint missed detection of critical transient changes in the parameters measured, since other rodent models of ischemia have shown hippocampal

glutathione concentration (Baek *et al.*,2000; Candelario-Jalil *et al.*,2001; Park *et al.*,2000), glutathione reductase activity (Shivakumar *et al.*,1992), and protein thiols (Shivakumar *et al.*,1995) to be decreased during the first six hours of reperfusion. Tissue concentration is only a snapshot, and cannot give information about transport, synthesis, and efflux, further limiting assessment of the influence of ischemia on oxidative stress. Enzymes important for glutathione synthesis, γ-glutamyl cysteine synthetase and glutathione synthetase, are present in brain (Dringen,2000), but to our knowledge, their function in ischemia has not been investigated *in vivo*. Brain γ-glutamyl cysteine synthetase activity was found not to respond to a low protein diet fed for two weeks (Stipanuk *et al.*,2002).

The gerbil brain is fragile and the hippocampus is very small, presenting difficulties with dissection and assays, possibly contributing to increased variability within groups. As well, since the CA1 region of the hippocampus is selectively damaged with this ischemia model, we might not see widespread biochemical changes in the hippocampus. The entire hippocampus was harvested from each brain hemisphere and used for one analysis, hence our assays might not have been sensitive enough to detect small changes primarily limited to the CA1 region. Moreover, our ischemia model produced a relatively mild and variable degree of hippocampal damage as evidenced from neuron counts, thus may not have generated sufficient oxidative stress to adequately test the hypothesis. Variability could also be attributed to individual variation amongst gerbils, since there may be a partial or complete circle of Willis in up to half of gerbils (Delbarre *et al.*,1991; Field and Sibold,1999; Ginsberg and Busto,1989). Since not all parameters used to assess oxidative stress are likely to be temporally matched, further studies should investigate both earlier and later timepoints.

Although ability to maintain brain glutathione concentration was not significantly altered by PEM, the overall results of this experiment indicate PEM gerbils recovered normal activity more slowly following global ischemia than well-nourished animals. These results are even more dramatic given that significant changes in mean functional outcome were observed in PEM gerbils despite the high variability in their response to the ischemic insult. While PEM gerbils subjected to global ischemia may experience more oxidative stress, the evidence for this was not strong. Although the

evidence for increased oxidative stress may be strengthened by decreasing variability in the ischemia model and examining other timepoints post-ischemia, other mechanisms by which PEM exacerbates neural injury following stroke must be explored.

Compromised nutritional status resulting in PEM, particularly in the elderly, is an important risk factor for stroke and for poorer outcome after stroke. Finestone and co-workers (1995;1996) studied Canadian stroke patients at the time of admission to rehabilitation units, and reported PEM in 49% of admissions, with dysphagia in 47%. Authors suggested there was inadequate nutritional intervention immediately post-injury, further exacerbating pre-existing poor nutritional status and compromising antioxidant defense mechanisms at a time when demand for antioxidants and their precursors is high. The Feed Or Ordinary Diet trial (FOOD), a robust multicentre randomized trial evaluating various feeding policies after stroke, has shown that nutritional status early after stroke is independently associated with long-term outcome (FOOD Trial Collaboration,2003). Undernutrition immediately after stroke reduced survival, functional ability and living circumstances six months later. Nyswonger *et al.* (1992) determined the length of time to start of feeding after stroke varies, and stroke patients fed within seventy-two hours of injury have a shorter hospital stay. There may be a 'window of opportunity' for optimizing survival and recovery from stroke with adequate nutritional support, especially with respect to increasing glutathione and antioxidant defense status. Dietary supplementation with cysteine prodrugs were shown to increase tissue glutathione, restore redox status, and normalize NFκB activation and pro-inflammatory cytokine production in mice with protein malnutrition (Li *et al.*,2002a; Li *et al.*,2002b). This promising alternative to protein repletion may be especially beneficial when feeding difficulties occur after stroke. The additional influence of inflammation, chronic due to PEM, and acute due to ischemia, must not be overlooked.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Reports in the literature suggest a proportion of the elderly admitted to hospital for stroke suffer from protein-energy malnutrition at the time of admission, and that this condition not only deteriorates after the stroke, but lengthens hospital stay and worsens outcome (Axelsson *et al.*,1988; Gariballa *et al.*,1998a). Much of the secondary brain damage in ischemia-reperfusion injuries such as stroke can be attributed to generation of reactive oxygen and nitrogen species during reperfusion, leading to oxidative stress in the penumbra surrounding the primary lesion and delayed neuronal death. The experiments described in this thesis examined whether part of the mechanism of worsened outcome in stroke with pre-existing protein-energy malnutrition could be due to impaired antioxidant defense secondary to reduced availability of precursors for glutathione synthesis. Glutathione, critical in antioxidant defense, is the most abundant thiol in the CNS, and its synthesis depends in large part on supply of amino acids from dietary protein.

The first experiments with rats were designed to examine the role of dietary sulphur amino acids, methionine and cysteine, in maintaining brain glutathione concentration following global hemispheric hypoxia-ischemia, when demand for antioxidant defense and glutathione synthesis is high. Since cysteine is limiting in glutathione synthesis, and the indispensable amino acid methionine can supply cysteine, removal of these amino acids from the diet for only five days is sufficient to induce weight loss, reduced intake, and reduced glutathione concentration in liver and some brain regions (Paterson *et al.*,2001). The effect of dietary sulphur amino acid deficiency on brain glutathione concentration and neural damage after stroke had not been previously assessed. It was hypothesized the dietary deficiency would compromise antioxidant defense after hypoxia-ischemia, secondary to glutathione depletion, thereby

exacerbating neural damage. It was further hypothesized that administration of a cysteine precursor, OTC, would restore brain glutathione depleted by dietary treatment and hypoxia-ischemia and ameliorate neural damage. Administration of OTC to rats after spinal cord trauma restored tissue glutathione depleted by the insult, decreasing damage and improving lower limb locomotion (Kamencic *et al.*,2001). A number of animal studies have shown that OTC can restore glutathione in various tissues after dietary sulphur amino acid or protein deficiencies (Chung *et al.*,1990; Jain *et al.*,1995; Levy *et al.*,1998; Taylor *et al.*,1992).

Although dietary sulphur amino acid deficiency did decrease glutathione concentration in rat brain neocortex and thalamus after six days, the hypoxia-ischemia administered on day three did not appear to exacerbate this decline, since there was no difference in neocortex glutathione concentration in the contralateral hemisphere in both deficient and control dietary treatment groups three days later. Neural damage, however, was increased in rats fed the sulphur amino acid deficient diet for six days compared to those fed control diet, suggesting an effect of diet as opposed to ischemia. That this finding was due to compromised antioxidant defense and increased oxidative stress in the animals fed the deficient diet was not supported by brain glutathione concentration. Measurement of glutathione concentration alone, however, may not have been sufficient to predict glutathione status or antioxidant status. The sulphur amino acid deficient diet was adequate in other components, but the rats fed this diet voluntarily reduced intake, and may therefore have been lacking all essential nutrients to some degree. Possible contribution of this added factor to the increased neural damage in the deficient group cannot be discounted. There was neither a sham-operated group nor a control group pair-fed adequate diet at the intake level of the deficient diet rats. Thus the effect of diet independent of ischemia, or reduced intake of all nutrients with and without ischemia on neuronal survival could not be evaluated. It was not possible to attribute observed results to lack of sulphur amino acids alone. Moreover, since the model of ischemia produced virtually no damage in adequately nourished rats, generation of reactive species and oxidative stress may have been limited. Reactive species are produced primarily during reperfusion (Grace,1994; Juurlink and Paterson,1998; Shivakumar *et al.*,1995; Yano *et al.*,1998). Although restoration of oxygen supply after thirty-five minutes of hypoxia

should generate some oxidative stress, severing of the common carotid artery compromised reperfusion in this stroke model. The brain also may not have been truly ischemic (Ginsberg and Busto,1989). Another difficulty with this model was that the hypoxia was by necessity applied to the entire brain, while only one carotid artery was severed, and the contralateral hemisphere was used as an internal control. Hypoxia alone of the degree and duration used in this experiment is not sufficient to produce neural damage (Levine,1960), but the lack of control groups (sham-operated, hypoxia alone, artery ligation alone) may have clouded assessment. Body temperature was controlled during ischemia, but brain temperature was not. Brain temperature is known not to be reflected by body temperature (Busto *et al.*,1987; Ginsberg and Busto,1989; Moyer *et al.*,1992; Warner *et al.*,1993), and typically falls during ischemia, which can be neuroprotective (Babcock *et al.*,1993; Colbourne and Corbett,1995; Colbourne *et al.*,1993b; Wang and Corbett,1990). Lack of brain temperature regulation during hypoxia-ischemia may have lessened neural damage and increased variability in the model. Other factors unregulated in this experiment may also have influenced variability and extent of damage: 1) hyperglycemia during hypoxia-ischemia as a stress response (Dijk *et al.*,1994; Ginsberg and Busto,1989), 2) blood gases and blood pressure can influence cerebral blood flow, introduce variability and confound results (Ginsberg and Busto,1989; Mhairi Macrae,1992).

There was no demonstrable effect of OTC administration to rats fed either sulphur amino acid deficient or sufficient diet. Given that there was no observed damage from ischemia in rats fed the control diet, OTC could not have had any effect in this group. The lack of brain glutathione depletion at the time of ischemia and mildness of the insult likely did not generate much, if any, oxidative stress, explaining the lack of effect of OTC in the sulphur amino acid deficient group. Glutathione concentration was not evaluated in the OTC experiment, but it would have been helpful to determine if OTC corrected the decreased brain glutathione seen three days after ischemia in the sulphur amino acid deficient rats. Although mouse brain glutathione and glutathione redox status were unaffected by three weeks of a low protein diet followed by addition of various dietary cysteine prodrugs, other tissues were responsive to the diet and to precursor repletion, suggesting OTC may have an effect if glutathione is first depleted

(Li et al.,2002a; Li et al.,2002b). The rat global hemispheric hypoxia-ischemia model failed to adequately test the hypothesis of dietary sulphur amino acid deficiency increasing oxidative stress. Repeating this experiment using either a focal model of ischemia or a global model producing more severe damage, and evaluating additional parameters such as protein and lipid oxidation and activity of enzymes sensitive to oxidative stress could yield data valuable to the original hypothesis.

The first experiments progressed to completion before awareness of the existence of the 'dark' neuron, an artifact of post-mortem handling of brain tissue before adequate fixation. Initially, 'dark' neurons were evaluated as neural damage. Subsequent reassessment of damage after identification of the 'dark' neuron revealed virtually no brain damage in the adequately nourished rats, and only mild damage in sulphur amino acid deficient animals, confirming that this ischemia model may not have adequately tested the hypothesis.

The rat sulphur amino acid deficient diet produced a severe, acute deficiency. It is unlikely a similar situation could occur in humans. More clinically relevant is moderate protein-energy malnutrition, relatively common in the elderly with stroke. It was hypothesized that protein-energy malnutrition would limit supply of essential precursors for glutathione production, compromising antioxidant defense and exacerbating neural damage in transient global ischemia-reperfusion. A gerbil model of temporary bilateral carotid artery occlusion is known to produce selective hippocampal damage similar to that observed in humans and primates after global ischemia (Zola-Morgan *et al.*,1986; Zola-Morgan *et al.*,1992). Most gerbils lack branches connecting the basilar artery branches and the posterior cerebral arteries, resulting in an incomplete circle of Willis (Ginsberg and Busto,1989). Forebrain ischemia is easily achieved with a two-vessel occlusion and minimal surgical intervention, and brain temperature is easily controlled with the use of a water-heated blanket in close contact with the head (Nurse and Corbett,1994). Tympanic temperature approximates brain temperature (Brambrink *et al.*,1999; Mariak *et al.*,1994; Schuhmann *et al.*,1999) and allows minimal trauma to the animal. Neural damage and functional outcome in this gerbil transient ischemia model have been well-characterized (Colbourne and Corbett,1995; Corbett and Crooks,1997; Kirino and Sano,1984). The gerbil model of transient bilateral carotid

artery occlusion was thus chosen for the next phase of this research, even though the small size of the gerbil does preclude intra-ischemic monitoring and control of blood glucose, gases and pressure, introducing the possibility of more variability than desired.

Gerbil nutritional and protein requirements are not well known (Arrington *et al.*,1973), and only one paper describing the response of the gerbil to a low protein diet was found in the literature (Leitch *et al.*,1993). Since the response was unpredictable, young adult gerbils were chosen as being more robust than older animals to establish protein-energy malnutrition, recognizing this as a limitation in clinical relevance of findings with respect to the basis for the hypothesis. Gerbils were fed adequate protein (control) or low protein diets. The voluntary reduction in food intake in the low protein group constituted the energy reduction needed to produce a model of protein-energy malnutrition. A limitation of reduced intake is possible inadequacy of other nutrients besides protein, and the unknown contribution of same to measured outcomes. This was considered unlikely since reduction of intake was not severe and micronutrients are provided at greater than requirement levels in the diet formulation. This is in contrast to the rat experiments where intake of the sulphur amino acid deficient diet was greatly reduced. Based on food intake, weight loss, liver lipid and liver glutathione concentration, a gerbil model of moderate protein-energy malnutrition was achieved. The gerbils remained well-groomed and active, with no signs of fur abnormalities or skin lesions characteristic of more severe malnutrition (Golden and Ramdath,1987).

Functional, histological and biochemical outcomes were evaluated in a two by two factorial design, with two dietary treatments (control and low protein), and two surgical treatments (sham and ischemia). The effect of protein-energy malnutrition on brain damage after transient global ischemia, assessed by the stated parameters, has not been investigated. The open field was used as a test of hippocampal function, testing working memory (Babcock *et al.*,1993; Mileson and Schwartz,1991; Nurse and Corbett,1994; Wang and Corbett,1990). Gerbils were exposed to the open field for ten minutes on Days 3, 7, and 10 after surgery. Typically a gerbil will explore the field vigourously for the first half of the exposure, and decrease activity, or *habituate* during the latter half. A gerbil suffering hippocampal damage will not habituate (Dowden *et al.*,1999). Ischemic gerbils fed the control diet did not habituate on novel exposure to

the open field, but recovered similar activity to sham-operated animals one week after ischemia. Protein-energy malnourished ischemic gerbils, however, failed to habituate even by Day 10, traveling significantly more distance than other groups. As well, half of these animals exhibited a wall-hugging behaviour known as thigmotaxis, indicative of anxiety or behaviour-trapping (Schallert *et al.*,1980; Simon *et al.*,1994). Only one well-nourished ischemic gerbil exhibited this activity. Sham-operated protein-energy malnourished gerbils were lethargic in the open field by Day 10, consistent with reports of reduced energy expenditure in similarly malnourished humans (Shetty,1999). Protein-energy malnutrition had no effect on numbers of surviving CA1 neurons ten days after ischemia. This finding strengthens the importance of conducting functional as well as histological assessments, as discussed by others (Bothe *et al.*,1986; Colbourne and Corbett,1995; Dowden and Corbett,1999; Hori and Carpenter,1994). Surprisingly, dramatically increased numbers of glial cells were evident in brain sections of one third of the ischemic protein-energy malnourished gerbils, suggesting inflammation. Pro-inflammatory cytokine production and NFκB activation has been shown in mice with protein malnutrition (Li *et al.*,2002a). Chronic inflammation is often seen in humans with protein-energy malnutrition (Mora,1999), and pro-inflammatory responses to ischemia have been identified (Kato and Walz,2000; Petty and Wettstein,2001). Identification of the glial cell types is beyond the scope of this thesis, but is ongoing in our laboratory at the time of writing. This phenomenon was not seen in any other groups.

Biochemical evidence for increased oxidative stress in ischemic malnourished gerbils compared to those well-nourished was not overwhelming, but nevertheless noteworthy. Hippocampus protein content was increased in all ischemic gerbils, possibly explained by an inflammatory response, compromise of the blood brain barrier and extravasation of plasma proteins (Petty and Wettstein,2001). Protein thiols, sensitive to oxidative stress, were reduced by ischemia and further by protein-energy malnutrition. Although not significant, there was a trend for ischemia to decrease hippocampus glutathione concentration and glutathione reductase activity in malnourished gerbils. Taken together, these findings indicate protein-energy malnourished gerbils suffered more oxidative stress than adequately-nourished gerbils.

The hypothesis of the high demand for glutathione in oxidative stress depleting brain glutathione concentration in protein-energy malnutrition was not strongly supported by the data, however.

The temporal change in brain glutathione concentration in rodent models of ischemia has been reported in the literature as decreasing during early reperfusion timepoints (Park *et al.*,2000), followed by a return to normal levels within twenty-four hours (Shivakumar *et al.*,1992; Shivakumar *et al.*,1995), and a further decrease at later timepoints (Candelario-Jalil *et al.*,2001). Protein thiols have been shown to decrease at one hour of reperfusion after thirty minutes of ischemia and return to normal levels at twenty-four hours (Shivakumar *et al.*,1995). The glutathione reductase enzyme is susceptible to oxidative damage because of its thiol groups, and varied temporal changes have been reported (Shivakumar *et al.*,1995; Stanimirovic *et al.*,1988). Hippocampus glutathione reductase activity is decreased at forty-eight to ninety-six hours (Candelario-Jalil *et al.*,2001), and thiobarbituric acid reactive substances are increased at four to fourteen days (Haba *et al.*,1991) after ischemia, following initial early decrease and subsequent return to normal levels. The timing suggests these results may be linked to delayed neuronal death. The above-mentioned ischemia models did not control brain temperature and were of varying durations of ischemia and reperfusion, and none was identical to the model used in this research. Comparison of findings therefore cannot be made directly. Only one timepoint was examined in the study for this thesis. Critical transient changes in measured parameters could easily have been missed. The gerbil hippocampus is small, and tissue dissection difficult due to the fragility of gerbil brain. The entire hippocampus tissue from each hemisphere was used for one assay, and if changes in biochemical indices were concentrated mainly in the susceptible CA1 region, the relatively small differences may not have been detectable. As well, it could not be expected that all parameters were temporally matched in response to ischemia. In order to construct a more complete picture of the evolution of oxidative stress in the ischemia model used in this research, it would be helpful to examine biochemistry at multiple reperfusion timepoints, in addition to assessment of oxidized glutathione and oxidation ratio (to assess glutathione redox status) (Baek *et al.*,2000), glutathione synthesis, and glutathione reductase up/down-regulation. Other markers such as 4-hydroxynonenal-

modified proteins (Urabe *et al.*,2000), 8-hydroxy-2'-deoxyguanosine (Baek *et al.*,2000), protein carbonyls (Evans *et al.*,1999), 4-hydroxyalkenals (Kawai *et al.*,1998), lipid hydroperoxides (Nourooz-Zadeh,1999) could also be considered in order to provide a broader spectrum of oxidative stress.

The damage in this gerbil ischemia model was milder and more variable than expected, yet there were still significant effects of protein-energy malnutrition on behaviour in the open field and moderate effects on oxidative stress. It was expected that the dietary deficiency would exacerbate neuronal damage, and thus length of ischemia was chosen to produce less than 90% neuronal loss as observed with the same model in another laboratory (Corbett and Crooks,1997). Greater variability can be expected with milder damage. Up to half of gerbils may have a partial or complete circle of Willis, and it was impossible to detect vascular anomalies in this research. Selecting only those gerbils without the circle of Willis, through examination of retinal blood flow for example, would ensure greater homogeneity in response to ischemia (Delbarre *et al.*,1991). The experiments could be repeated with a more severe ischemia, increasing likelihood of detecting changes in oxidative stress and perhaps reducing variability.

In humans, feeding after a stroke is often delayed, exacerbating pre-existing protein-energy malnutrition. Secondary brain damage in stroke occurs through delayed neuronal death, providing a potential window of opportunity for intervention and amelioration of this damage. Further studies designed to feed protein-energy malnourished animals an adequate protein diet after ischemia could assess the effect of early dietary intervention. The increases in glial cells in some malnourished ischemic gerbil brains suggested an inflammatory process. Future studies should incorporate assessment of these cell types and testing for markers of inflammation. The open field test is a good general indicator of hippocampal malfunction. More specific tests designed to detect anxiety, irritability, fine motor function, and memory would add greatly to understanding functional behaviour. Young adult gerbils were used in this study, but the initial hypothesis was based on epidemiological evidence in the elderly. Repeating the experiments with aged gerbils would be more clinically relevant. Ischemic gerbils fed the control diet recovered normal behaviour in the open field one week after the insult. It is possible that malnourished gerbils may also have recovered or

adapted, given dietary repletion and/or enough time. Extending the post-ischemic period to thirty to sixty days or longer would also be more clinically relevant (see FOOD trial below), and the long-term effects of protein-energy malnutrition and ischemia on neuronal survival could be evaluated.

In summary, the research described in this thesis was designed to test the hypothesis of dietary deficiencies influencing oxidative stress and antioxidant defense after global ischemia. The hypothesis was investigated in two rodent species with two models of ischemia. The first phase examined the effect of an acute sulphur amino acid deficiency on neural damage and brain glutathione concentration after hemispheric ischemia in the rat, while the second phase encompassed the influence of protein-energy malnutrition on functional, histological and biochemical outcomes after transient global ischemia in the gerbil. Both ischemia models produced relatively mild brain damage, and well-nourished animals generally tolerated this insult well. In both phases, however, malnourished animals suffered more brain damage and, although evidence was not robust, may have experienced more oxidative stress. Behaviour remained abnormal in malnourished ischemic gerbils ten days after the brain insult. The results of this research are clinically relevant to those suffering from protein-energy malnutrition at the time of stroke, and support the compromised outcome observed in this group of individuals. The Feed Or Ordinary Diet trial (FOOD), a robust multicentre randomized trial evaluating various feeding policies after stroke, has shown that nutritional status early after stroke is independently associated with long-term outcome (FOOD Trial Collaboration,2003). Undernutrition immediately after stroke reduced survival, functional ability and living circumstances six months later. Although the mechanism for sub-optimal recovery has not been clearly elucidated by the research described in this thesis, increased oxidative stress may be a contributing factor, emphasizing the need to intervene during the window of opportunity immediately after stroke. Future studies with swift dietary repletion may determine if oxidative stress can be reduced, delayed neuronal death diminished and outcome improved for malnourished stroke victims.

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APPENDIX A

Analysis of tissue glutathione concentration

Methods

Tissue homogenization

Frozen liver sample was weighed and homogenized on ice with 10 volumes of cold 5% sulphosalicylic acid containing 0.1mM $Na_2EDTA \cdot 2H_2O$, using a Brinkmann® Polytron Model PT10-35, at 5 intervals of 6 seconds each, pausing for 30 seconds between intervals to allow cooling of homogenate. The homogenate was centrifuged at 16,000 x g for 15 minutes in a refrigerated Eppendorf® centrifuge model 5415C, and the supernatant frozen in liquid nitrogen and stored at -70ºC until analysis. Brain tissue was treated in the same fashion as liver, using a Skil®Pistol Grip Drill Model 6225 instead of the Polytron.

Analysis of GSH

Phase 1- Rat Sulphur Amino Acid Study

GSH (as free reduced sulphhydryl groups) was assayed using a modification of the method of Komuro *et al.* (Komuro *et al.*,1985), described by Katrusiak *et al.* (Katrusiak *et al.*,2001) and Paterson *et al.* (Paterson *et al.*,2001), by reverse-phase high performance liquid chromatography (HPLC) using pre-column derivatization with 5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) and ultraviolet detection at 330nm. Sulphhydryl-DTNB derivatives were detected using isocratic elution at 37ºC on a Supelco LC-18T (150 x 4.6mm, 3µm; Supelco, Bellefonte, PA, USA) with a Supelguard LC-18T 2cm pre-column. The mobile phase was 12.5% v/v methanol / 87.5 % 100mmol/L KH_2PO_4 (pH 3.89), run for 9 minutes at a flow rate of 1.1mL/minute. Excess DTNB was eluted from the column over 7 minutes with 40% methanol / 60% 100mmol/L KH_2PO_4 (pH 3.89), and the system was flushed for 12 minutes with the 12.5% methanol mobile phase before injection of the next sample. The HPLC system was Shimadzu® (Tokyo, Japan) SCL-10A system controller, SPD-10A variable wavelength spectrophotometric detector, SIL-10A automatic sample injector, LC-10AT solvent delivery system, and X-Act-4-channel degasser. A Shimadzu EZChrom version

3.2 software program was used to collect data. The program measured peak areas and results were expressed as the ratio of peak area of sample or standard to peak area of internal standard. A standard (containing L-cysteine, cysteinyl-glycine, glutathione and DL-homocysteine) curve was prepared and run daily with each set of samples, all in duplicate.

The reaction mixture, prepared on ice, contained 500µL Tris-HCl buffer (pH 8.9), 130µL sample or standard, 20µL deionized distilled water, and 20µL internal standard (400µM D(-)penicillamine in 5% sulphosalicylic acid containing 0.1mM $Na_2EDTA{\cdot}2H_2O$). After addition of 350µL 10mM DTNB in 0.5M K_2HPO_4 (pH 7.2), the mixture was allowed to sit off ice for 5 minutes for derivatization. The reaction mixture was acidified by the dropwise addition of 50µL 7N orthophosphoric acid (H_3PO_4) while vortexing, and 50µL injected into the HPLC system for analysis. Representative chromatograms of standard (Figure A.1) and a rat liver sample (Figure A.2) follow.

Phase 2- Gerbil PEM study

Analysis of GSH concentration in gerbil tissues was identical to the procedures used in Phase one, with the following exceptions:

1- Mobile phases were prepared at pH 3.85.
2- The final reaction mixture contained 45 µL 7N H_3PO_4.
3- DL-homocysteine was omitted from the standard curve.
4- The HPLC system consisted of the following: Waters® (Waters Canada, Missisauga, ON) Automated Gradient Controller, 486 Tunable Absorbance Detector, 717plus Autosampler, 510 HPLC Pump, Temperature Control Module. Data were collected with Waters Millenium®32 Chromatography Software, Version 4.00(© 2001 Waters Corporation).

Representative chromatograms of standard (Figure A.3) and a gerbil brain (hippocampus) sample (Figure A.4) follow.

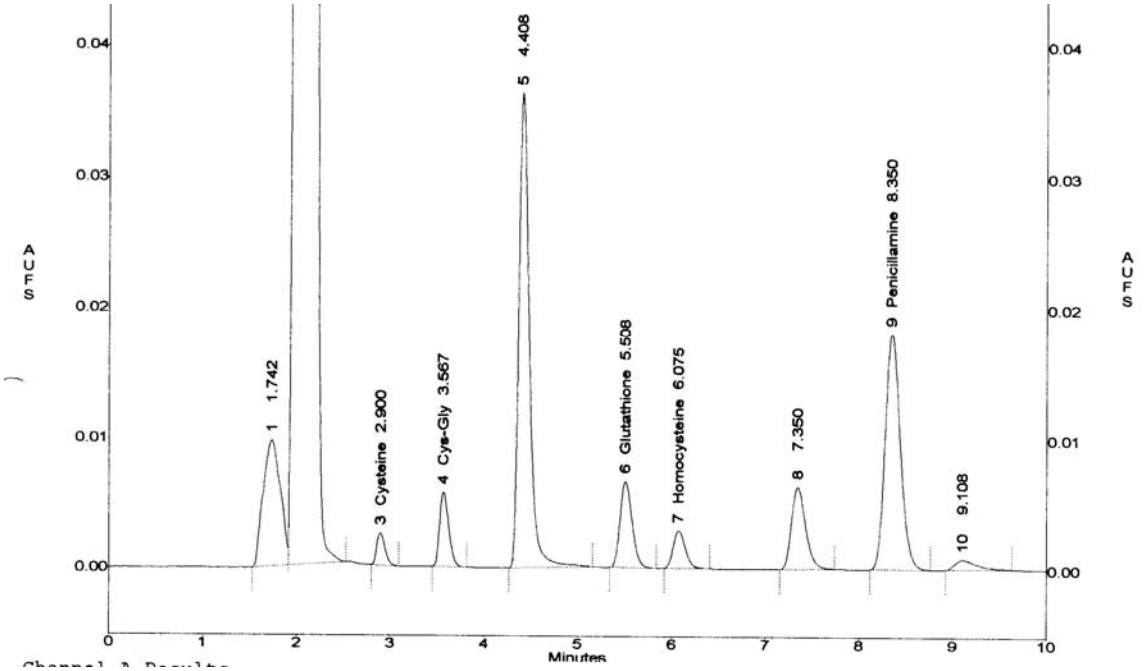

Figure A.1. Representative HPLC chromatogram of a thiol standard containing cysteine, cysteinyl-glycine (Cys-gly), glutathione and homocysteine

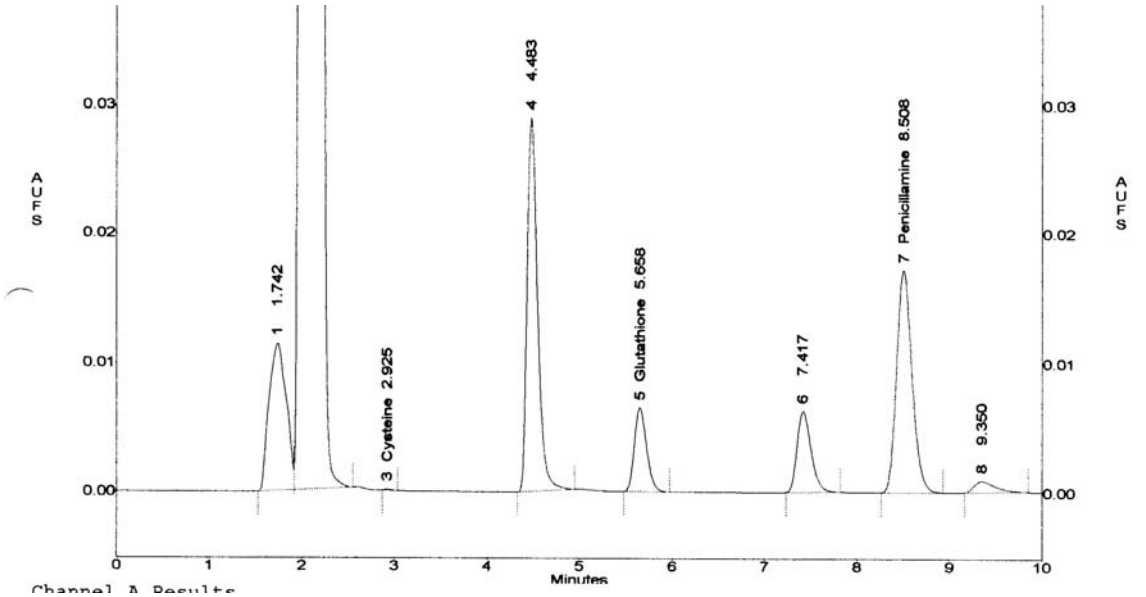

Figure A.2. Representative HPLC chromatogram of thiols in rat liver diluted 1:200

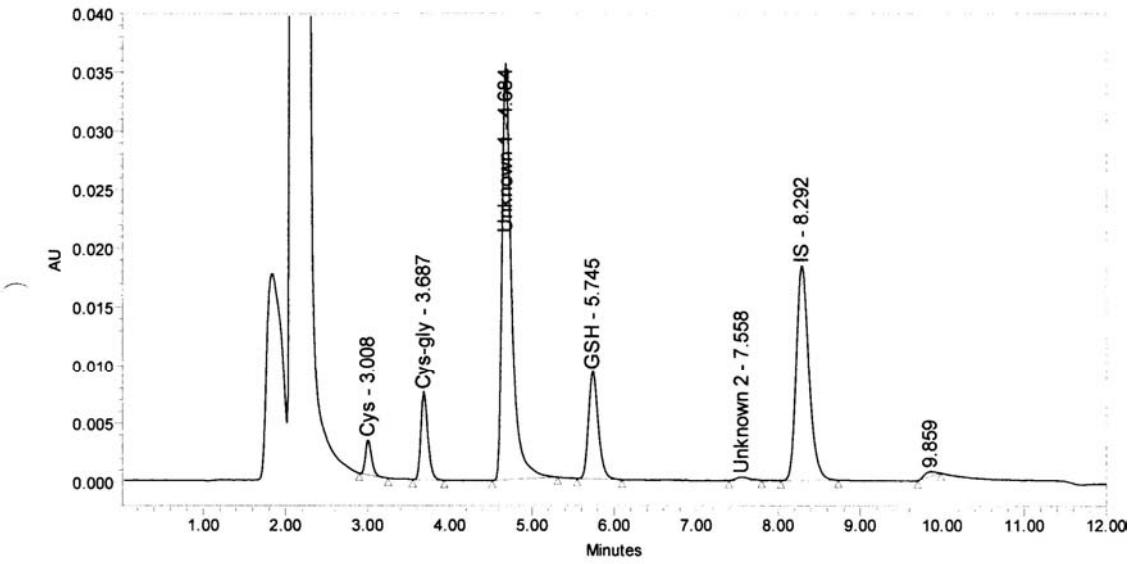

Figure A.3. Representative HPLC chromatogram of a thiol standard containing cysteine (Cys), cysteinyl-glycine (Cys-gly) and glutathione (GSH)

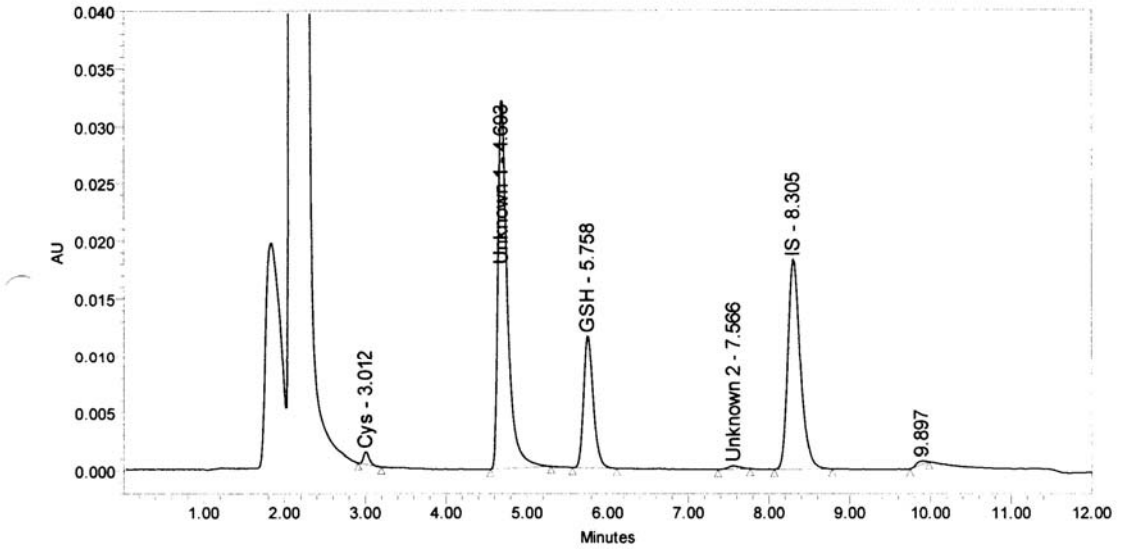

Figure A.4. Representative HPLC chromatogram of thiols in gerbil hippocampus diluted 1:50

APPENDIX B

Administration of L-2-oxothiazolidine-4-carboxylic acid (OTC) following global hemispheric hypoxia-ischemia (GHHI) in the Long-Evans rat

Purpose

The purpose of this experiment was to investigate whether the administration of a cysteine precursor, OTC, after GHHI would ameliorate neural damage secondary to increased availability of cysteine and enhanced synthesis of glutathione.

Methods

Under the same housing, surgical, and OTC treatment regimen described in Experiment 2 of Chapter 5 of this thesis, rats were fed a sulphur amino acid sufficient crystalline amino acid defined AIN-93G diet without antioxidant (for diet composition see Appendix D). After 3 days on diet, rats were subjected to GHHI, and randomized to receive injections of OTC (12mmol/kg body weight) or placebo (phosphate-buffered saline,equivalent volume) 15 minutes after completion of GHHI and every 12 hours thereafter for 6 additional doses at 4mmol/kg body weight. As described in Chapter 5, neural damage was assessed by 2 scoring systems and data were analyzed with SPSS for Windows 11.0, using Mann-Whitney *U* for global score, and unpaired student's *t*-test for hippocampal grid score.

Results

GHHI in adequately-nourished rats did not result in any appreciable neural damage, assessed by the semi-quantitative global score (Table B.1) or the more quantitative hippocampal grid score (Table B.2), and thus there was no significant effect of OTC.

Table B.1. Effect of global hemispheric hypoxia-ischemia and L-2-oxothiazolidine-4-carboxylic acid on global score* of neural damage in adequately nourished rats

	Neocortex	Striatum	Hippocampus	Thalamus	Total score
Placebo	0.1 ± 0.1	0.3 ± 0.2	0	0	0.4 ± 0.2
OTC	0	0	0.1 ± 0.1	0	0.1 ± 0.1

Mean ± SEM; Placebo, n=15; OTC, n=14.
*Global score: 0 = no damage; 1 = <50% damage; 2 = >50% damage; maximum score of 2 in each region (striatum, thalamus, neocortex, hippocampus) for total maximum score of 8.

Table B.2. Effect of global hemispheric hypoxia-ischemia and L-2-oxothiazolidine-4-carboxylic acid on hippocampal grid score* of neural damage in adequately nourished rats

	Subiculum/CA1	CA2/CA3	CA4	Dentate gyrus	Hippocampus Proper†	Hippocampus Total‡
Placebo (%)	0	0	0	0	0	0
OTC (%)	4.6 ± 4.6	0	0	0	2.5 ± 2.5	1.7 ± 1.7

Mean ± SEM; Placebo, n=15; OTC, n=14.
*Hippocampal Grid Score: a grid was superimposed on an image of the hippocampus; any grid square containing one or more damaged cells of 15-20 cells per grid was counted as one damaged square and the damage score was expressed as percentage of baseline squares.
†Hippocampus Proper = Subiculum/CA1 + CA2/CA3 + CA4.
‡Hippocampus Total = Hippocampus Proper + Dentate Gyrus.

Conclusion

Since the model of ischemia used in this experiment did not produce any neural damage in adequately-nourished rats in the areas evaluated, it was impossible to demonstrate any improvement with the administration of a cysteine precursor. The reasons for the failure of the model have been discussed in Chapter 5. This experiment was undertaken before the awareness of the 'dark' neuron artifact, discussed in Chapter 4, at a time when it was thought there was a mild degree of neural damage produced by the GHHI model, and that administration of a cysteine precursor might enhance glutathione synthesis and ameliorate neural insult. This experiment was conducted in

conjunction with a second group of rats fed a diet deficient in sulphur amino acids, and randomized to receive placebo or OTC injection post-GHHI. That portion of the experiment has been discussed in Chapter 5.

APPENDIX C

Transient bilateral carotid artery occlusion pilot study

The gerbil model of transient bilateral carotid artery occlusion (TBCAO) is well-established in the literature, as discussed in the body of this thesis. The author visited the laboratory of Dr. Dale Corbett at the Memorial University of Newfoundland (MUN) to learn the model. His laboratory uses a 5-minute occlusion with controlled brain temperature (36.5°C), achieving consistent loss of CA1 hippocampal neurons in the range of 90-95%. Since we theorized PEM would exacerbate neural damage, we required a stroke model with baseline neuronal loss in the range of 70-75%.

Purpose

To vary duration of ischemia and/or brain temperatures during ischemia to achieve 70-75% hippocampal CA1 neuronal loss while maintaining low variability with the model.

Methods

Male Mongolian gerbils aged 16-18 weeks were subjected to a 5-minute TBCAO, with brain temperature (estimated by tympanic temperature) maintained at 36.5ºC throughout ischemia, or sham surgery, as described in Chapter 6.
The following variations of the model were evaluated:

1) 4-minute ischemia with brain temperature of 37°C (at MUN)
2) 3.5-minute ischemia with brain temperature of 36.5°C (at U of S)
3) 4-minute ischemia with brain temperature of 36.5°C (at U of S)
4) 5-minute ischemia with brain temperature of 36.5°C (at U of S)

On day 10 after surgery, gerbils were anesthesized with isoflurane and perfused trans-cardially with heparinized saline followed by 10% phosphate buffered formalin. Heads were refrigerated in formalin for 18-24 hours before brain removal and embedding in paraffin. Brains were sectioned in 6 micron thicknesses and hippocampal CA1 neurons counted as described in Chapter 6.

Results (Table C.1)

1) 4-minute ischemia with brain temperature of 37°C

Neuronal loss was consistent at ~89%. Maintaining constant brain temperature at 37°C was difficult, especially with an ischemia time of 4 minutes. Target mean brain temperature (37°C ± 0.2°C) was achieved in only 5 of 10 gerbils.

2) 3.5-minute or 4-minute ischemia with brain temperature of 36.5°C

Variability in neuronal loss was high with these ischemia times (range 0-80%, 3.5-minute; 0-88%, 4-minute) and mean neuronal loss was low.

3) 5-minute ischemia with brain temperature of 36.5°C

This model produced an acceptable level of damage with lower variability than other durations of ischemia.

Table C.1. Hippocampal CA1 neuron loss: varied ischemia times and brain temperatures

Duration of ischemia	4 minutes	3.5 minutes	4 minutes	5 minutes
Brain temperature (°C)	37	36.5	36.5	36.5
% CA1 Neuron Loss*	89 ± 2.2	28.7 ± 8.8	43.2 ±14.4	68.2 ± 5.6
Laboratory	MUN	U of S	U of S	U of S
n	5	10	7	7

*Mean ± SEM

Conclusion

To allow room for the PEM model to exacerbate brain injury in the TBCAO model of stroke, we aimed to achieve neuron loss of 70-75%, with low variability. Although the 4-minute, 37°C model had consistent damage, the neuron loss was considered too high, and the failure rate of 50% in maintaining target brain temperature was unacceptable. The 3.5-minute and 4-minute, 36.5°C models produced little mean damage with high variability. In our laboratory, a 5-minute ischemia at 36.5°C produced the most consistent results with an acceptable level of neuronal loss. The 5-minute

ischemia with brain temperature maintained at 36.5°C was used for the main gerbil study.

APPENDIX D

Gerbil pilot feeding study: A comparison of laboratory chow with purified crystalline amino acid defined diets with and without sulphur amino acids

At the beginning of Phase 2 of this thesis, whether to use a sulphur amino acid deficiency in the gerbil was investigated. The response of the rat to a purified dietary sulphur amino acid deficiency had been established in our laboratory. The response of the gerbil to the same diet was unknown. The nutritional requirements of the gerbil are not well-investigated, but since this animal does well on laboratory rodent chow, we wanted to compare chow to the sulphur amino acid sufficient and deficient diets used in the rat.

Purpose

1) To test the response of the gerbil to an amino acid defined diet compared to laboratory rodent chow.
2) To test whether the response of the gerbil to a sulphur amino acid deficient diet was similar to that of the rat.

Methods

Male Mongolian gerbils aged 19-20 weeks were housed individually in suspended stainless steel cages and randomized to standard laboratory rodent chow, or AIN-93G diet modified to contain crystalline amino acids, with or without sulphur amino acids cystine and methionine (n=5 per group) (Table D.1). Diets were fed *ad libitum* for 6 days, with free access to water. Gerbils were maintained at 22°C with a 12-hour light/dark cycle. Daily food intake, food wastage and body weights were recorded. On day 6, gerbils were anesthetized with isoflurane and perfused trans-cardially with saline. Brain neocortex and liver were collected on ice, and analyzed for glutathione concentration as described in Chapter 5 and Appendix A. Data were analyzed using one way ANOVA followed by LSD means test where appropriate, significance $p<0.05$.

Table D.1. Composition of the purified crystalline amino acid based diets

Component	Sulphur amino acid deficient (g/kg)	Sulphur amino acid sufficient (g/kg)
L-amino acids*	161.3	167
Cornstarch	419.086	419.086
Dextrinized cornstarch	140	140
Sucrose	105.7	100
Cellulose	50	50
Soybean oil	70	70
Mineral mix†	35	35
Sodium bicarbonate	6.4	6.4
Vitamin mix¶	10	10
Choline bitartrate	2.5	2.5

* Sulphur amino acid deficient diet supplied the following amino acids (g/kg diet): L-arginine, 12; L-histidine, 6; L-lysine-HCl, 14; L-tyrosine, 4; L-tryptophan, 2; L-phenylalanine, 8; L-threonine, 8; L-leucine, 12; L-isoleucine, 8; L-valine, 8; glycine 24.3; L-proline, 5; L-glutamic acid, 30; L-alanine, 5; L-asparagine·H_2O, 5; L-serine, 5; L-glutamine, 5. The sulphur amino acid sufficient diet was supplemented with 4g/kg L-cystine and 6g/kg L-methionine, and L-glycine content was reduced to 20g/kg diet.
† Modified from that used in the AIN-93G diet to be sulphur-free.
¶ Vitamin mix was identical to that used in the AIN-93G diet (Reeves *et al.*,1993).

Results

Initial body weights were the same in each group (Table D.2). There was no significant difference in weight gain, food intake, liver or neocortex glutathione concentration between laboratory chow and sulphur amino acid sufficient diet ($p>0.05$). Gerbils on the sulphur amino acid deficient diet, however, lost weight, ate less food, and had reduced liver and neocortex glutathione concentration compared to those in the other 2 groups.

Table D.2. The effects of feeding adult gerbils purified crystalline amino acid-based diets with and without sulphur amino acids

	Laboratory Chow	+SAA Diet	-SAA Diet
Initial Weight (g)	73.1 ± 1.2[a]	75.6 ± 1.3[a]	75.2 ± 0.5[a]
Total weight gain (g)	1.2 ± 1.1[a]	- 1.7 ± 1.1[a]	-10.9 ± 1.2[b]
Total food intake (g)	44.1 ± 2.9[a]	36.8 ± 3.5[a]	23.0 ± 2.5[b]
Liver [GSH] (μmole/g wet weight)	6.1 ± 0.3[a]	5.9 ± 0.2[a]	3.3 ± 0.2[b]
Neocortex [GSH] (μmole/g wet weight)	1.9 ± 0.1[a]	1.7 ± 0.0[a]	1.6 ± 0.1[b]

Mean ± SEM; 6 days total treatment; n=5 per group
[a,b] Letters indicate significant difference between groups by one way ANOVA, followed by *posthoc* LSD means test, $p<0.05$

Conclusions

According to the parameters measured, gerbils fed the purified crystalline sulphur amino acid sufficient diet maintained comparable status to those fed rodent chow. The response of the gerbil to a dietary sulphur amino acid deficiency was similar to that of the rat (for rat data, see Chapter 5).

Gerbils are communal animals, grooming each other frequently. In the laboratory, gerbils dig in and shred their bedding. They sleep together, hidden inside nests of bedding material. By the end of 6 days in suspended individual cages, all gerbils were 'jumpy' and appeared ungroomed. For this reason, gerbils in Phase 2 of this study were housed in shoebox cages, in groups of three, with bedding.

APPENDIX E

Gerbil pilot feeding study: A comparison of laboratory chow with purified diets containing adequate or low protein

To test the hypothesis that PEM would exacerbate brain damage in stroke, we needed to establish a gerbil PEM model. In the sulphur amino acid pilot feeding study, gerbils were found to do well on rodent laboratory chow and an amino acid defined diet previously used in the rat. Since the protein requirements of the gerbil are not well-established, the diet composition in this pilot study was based on rat requirements.

Purpose

1) To test the response of the gerbil to a rodent casein-based diet compared to laboratory rodent chow.
2) To establish a gerbil model of PEM.

Methods

Male Mongolian gerbils aged 11-12 weeks were housed individually in suspended stainless steel cages and randomized to standard laboratory rodent chow, or a modified AIN-93M pelleted rodent diet without antioxidant, formulated to contain 12% protein (adequate) or 2% protein (low) as casein, fed for 4 weeks (n=4 per group). For diet composition, see Chapter 6. Diets were fed *ad libitum* for 28 days, with free access to water. Gerbils were maintained at 22°C with a 12-hour light/dark cycle. Daily food intake and food wastage and weekly body weights were recorded. On day 28, gerbils were anesthetized with isoflurane and perfused trans-cardially with saline. Liver, and brain neocortex and hippocampus were collected on ice, and analyzed for glutathione concentration as described in Chapter 5. Liver lipid was analyzed as described in Chapter 6. Tissue analyses were conducted on adequate and low protein groups only. Data were analyzed using one way ANOVA followed by *posthoc* LSD means tests where appropriate, significance $p<0.05$.

Results

Initial body weights were the same across groups (Table E.1). Feed intake was significantly different between all 3 groups, and highest in gerbils fed laboratory chow. Weight gain was the same in gerbils fed laboratory chow and those fed the adequate protein diet. Gerbils fed the low protein diet lost more weight than those fed chow, ate less than those on both other diets, and had increased liver lipid compared to the group fed adequate protein. Glutathione concentration in gerbils fed the low protein diet was decreased in liver, unchanged in hippocampus, and increased in neocortex compared to gerbils fed the adequate protein diet.

Table E.1. Influence of feeding a low protein diet to the gerbil

	Laboratory Chow	Adequate Protein	Low Protein
Initial weight (g)	63.1 ± 1.2^{a}	63.9 ± 0.6^{a}	62.1 ± 0.9^{a}
Weight gain (g/28days)	12.4 ± 3.5^{a}	$5.5 \pm 5.7^{a,b}$	-4.2 ± 1.7^{b}
Food intake (g/day)	8.2 ± 0.3^{a}	6.3 ± 0.2^{b}	5.5 ± 0.2^{c}
Liver lipid (mg/g wet wt)	N/A	37.3 ± 0.7^{a}	50.7 ± 1.7^{b}
Liver [GSH] (μmol/g wet wt)	N/A	6.8 ± 0.6^{a}	3.5 ± 0.4^{b}
Hippocampus [GSH] (μmol/g wet wt)	N/A	2.6 ± 0.3^{a}	2.5 ± 0.2^{a}
Neocortex [GSH] (μmol/g wet wt)	N/A	2.1 ± 0.1^{a}	2.5 ± 0.1^{b}

Mean ± SEM; n=4 per group
Letters indicate significant difference across groups by one-way ANOVA, followed by *posthoc* LSD means test, $p<0.05$

Conclusion

The response of measured parameters was similar in gerbils fed the adequate protein diet and those fed laboratory chow. Gerbils fed the low protein diet voluntarily reduced intake and as such were lacking in both protein and energy, a phenomenon observed in other rodent models of dietary protein content less than 6% (Eisenstein and Harper,1991; Hum *et al.*,1992; Rana *et al.*,1996). The results measured in gerbils fed the low protein diet suggest a moderate model of PEM was achieved, and this diet was used for the Phase 2 gerbil study. That neocortex glutathione concentration was elevated in the low protein group cannot be explained. As in the sulphur amino acid pilot feeding

study, these gerbils were housed individually in suspended steel cages, and were not well groomed. Communal housing in shoebox cages with bedding is preferable.

APPENDIX F

Characterization of temporal change in brain glutathione concentration at various reperfusion time-points after temporary bilateral carotid artery occlusion (TBCAO)

With several rodent global ischemia models, the literature reports decreases in brain glutathione concentration at early time-points of reperfusion, with a return to normal levels between 4-24 hours(Baek *et al.*,2000; Candelario-Jalil *et al.*,2001; Park *et al.*,2000; Shivakumar *et al.*,1992). We wanted to compare the temporal change in neocortex and hippocampus glutathione concentration in our model of TBCAO and controlled brain temperature to other models in the literature.

Purpose

To determine brain neocortex and hippocampus glutathione concentrations at various reperfusion time-points after a 5-minute TBCAO with brain temperature maintained at 36.5 ± 0.2ºC.

Methods

Male Mongolian gerbils aged 14-18 weeks were acclimated for 10 days on a purified sulphur amino acid sufficient AIN-93G diet modified to contain crystalline amino acids (for diet composition, see Appendix D). On day 10, gerbils were subjected to a 5-minute TBCAO with brain (tympanic) temperature maintained at 36.5± 0.2ºC throughout ischemia or sham surgery, as described in Chapter 6. Reperfusion time-points were as follows: 0, 0.5, 1, 2, 6, 12, and 24 hours after occlusion (n=6 per time-point; exception, n=7 at 1 hour). At each time-point gerbils were re-anesthetized with isoflurane and perfused trans-cardially with heparinized saline. Brain neocortex and hippocampus were collected on ice and analyzed for glutathione concentration as described in Chapter 5 and Appendix C. Data were analyzed by one way ANOVA followed by *posthoc* LSD means test where appropriate, significance $p<0.05$.

Results

There was no significant difference in neocortex glutathione concentration at any timepoint compared to that in sham-operated animals (Table F.1). Hippocampus glutathione concentration was elevated compared to that in sham-operated controls at 0.5, 1 and 2 hours of reperfusion.

Table F.1. Hippocampus and neocortex glutathione concentration at various reperfusion times after 5-minute TBCAO*

Reperfusion time	Hippocampus	Neocortex
	μmoleGSH/g wet wt.	
Sham	2.5 ± 0.2^{a}	2.4 ± 0.03
0 hour	2.5 ± 0.1^{a}	2.1 ± 0.1
0.5 hour	2.8 ± 0.1^{b}	2.2 ± 0.1
1 hour	2.9 ± 0.1^{b}	2.4 ± 0.04
2 hours	2.9 ± 0.1^{b}	2.4 ± 0.02
6 hours	2.6 ± 0.1^{a}	2.2 ± 0.1
12 hours	2.5 ± 0.1^{a}	2.3 ± 0.1
24 hours	2.7 ± 0.1^{a}	2.3 ± 0.2

* Brain temperature (estimated by tympanic temperature) maintained at 36.5± 0.2ºC. Mean ± SEM; n=6 per reperfusion time except n=7 at 1 hour.
[a,b] Letters indicate significant differences from sham surgery within brain region by one way ANOVA followed by *posthoc* LSD means test, $p<0.05$.

Conclusion

We were unable to reproduce temporal changes in brain glutathione concentration reported in the literature after TBCAO, although our model varied somewhat from those in the literature. The increase in hippocampal glutathione concentration at some time-points was unexpected. In tissues such as artery endothelium, lung epithelium and kidney, exposure to oxidative stress has been shown to upregulate mRNA expression and activity of γ-GCS, the rate-limiting enzyme in glutathione synthesis, with subsequent increases in glutathione concentration in these cells (Day *et al.*,2002; Diaz *et al.*,2001; Rahman and MacNee,2002; Ray *et al.*,2002; Woods and Ellis,1995; Woods *et al.*,1999). It is possible a similar response occurs in

brain, explaining the findings in this study. This is further supported by the lack of response in neocortex, a region less vulnerable to damage in this ischemia model.

APPENDIX G

Rat hippocampus: representative image used to evaluate hippocampal grid score of neural damage

The hippocampal area of the H&E stained posterior section of rat brain (approximately -3.1mm from bregma) was visualized at 100 x magnification on a Zeiss microscope with attached SonyPowerHAD colour video camera, and images were captured on computer with Northern Eclipse 2.0 software. A grid was superimposed on each image, and a hardcopy composite image of the entire hippocampal area constructed (about 5-9 images per collage) using Microsoft PowerPoint. The densest regions of the subiculum/CA1, CA2/CA3, CA4 and dentate gyrus were defined on the collage and baseline numbers of grid squares in each area counted. Each grid square held approximately 15-20 cells. To score damage, slides were viewed under a microscope at 400 x magnification and damaged cells located and marked by hand on the corresponding composite image. Any grid square containing one or more damaged cells was counted as one damaged square and the damage score was expressed as percentage of baseline squares in each area of the hippocampus.

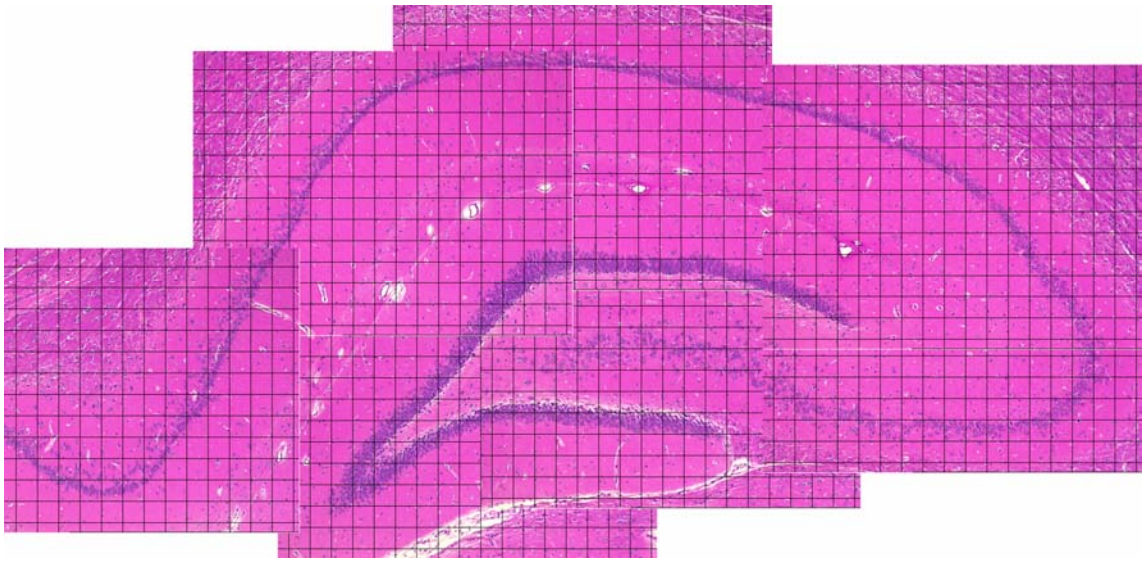

Figure G.1. Rat hippocampus: representative image used to evaluate hippocampal grid score of neural damage