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A role for IL-1ß in oxidative stress - mediated neuronal impairment.

By

Eamonn O'Donnell BSc., MSc.

Thesis submitted for the degree of Doctor of Philosophy at

the University of Dublin, Trinity College

Submitted September 1999

Department of Physiology,

Trinity College,

Dublin 2

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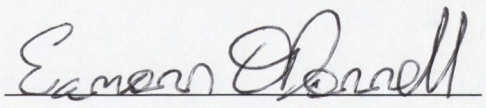
This thesis is dedicated to Camillus O'Donnell with much love.

IV Declaration

I declare this thesis is entirely my own work with the following exceptions; the 3 month antioxidant diet on young and aged rats referred to in chapter 3 was organised and managed by Dr. Ciara Murray; all LTP experiments were conducted by Dr. Marina Lynch. This work has not been previously submitted as an exercise for a degree to this or any other university. I give permission to the library to lend or copy this thesis.



Eamonn O'Donnell BSc., MSc.

V Abstract

The aims of this study were to; (1) investigate age-related changes in the antioxidant defence system in the rat cortex and hippocampus; (2) investigate the role of IL-1ß in influencing oxidative stress in brain tissue of aged and stressed rats; (3) to establish the efficacy of dietary supplementation with antioxidants in reversing the functional impairment seen in the hippocampus of stressed and IL-1ß-treated rats.

The data presented showed a consistent increase in the activity of superoxide dismutase (SOD) and a general trend toward a decline in other antioxidant defences in cortical and hippocampal tissue from aged rats, providing evidence of oxidative stress. Confirmation of this hypothesis is provided by the finding that lipid peroxidation, a marker for oxidative stress, was increased in these areas. Furthermore the concentration of the polyunsaturated fatty acid (PUFA), arachidonic acid, was decreased indicating that this fatty acid is one target molecule in the lipid peroxidation process. Previous evidence suggested that IL-1ß may play a role in mediating the oxidative stress and it was observed that concentrations of this cytokine were elevated in cortical and hippocampal tissue of the aged rat. The data presented showed that incubation of hippocampal tissue in the presence of IL-1ß increased reactive oxygen species (ROS) production and also increased SOD activity, suggesting a link between increased activity of this enzyme and increased ROS production. Dietary supplementation of vitamin C and vitamin E reversed these changes. The data showed that cortical tissue prepared from aged rats fed a antioxidant supplemented diet had; increased vitamin E concentration, decreased SOD activity, decreased IL-1ß concentration and decreased lipid peroxidation. These results suggest that dietary supplementation removed the oxidative stress on cortical tissue prepared from aged rats and further suggests a role for IL-1ß in mediating this stress, possibly by enhancing SOD activity.

To investigate the role of IL-1ß further this cytokine was injected intracerebroventricularly and its effects on neuronal function, oxidative defences and oxidative status were examined in the hippocampus. The data confirmed the hypothesis that IL-1ß imposes oxidative stress on tissue and that this action impairs neuronal function. The data revealed that IL-1ß induced an increase in SOD activity without compensatory increases in other components of antioxidant defence and an increase in lipid peroxidation. Long-term potentiation (LTP), used as a marker of neuronal function, was also impaired in these animals. However dietary supplementation, which increased vitamin C concentration in hippocampal tissue, reversed the impairment in LTP and the increase in lipid peroxidation implying that oxidative stress was responsible for the impairment in LTP and providing further evidence that IL-1ß is a mediator of oxidative stress in neuronal tissue.

Previous studies have shown an increase in IL-1ß in the dentate gyrus region of the hippocampus of socially isolated rats and an impairment of LTP in this region (Murray & Lynch, 1999). The possibility that this impairment in neuronal function might be mediated by oxidative stress was assessed. The data presented show the impairment in LTP was coupled with an increase in SOD activity and a decrease in glutathione (GSH) concentration in hippocampal tissue prepared from rats which had undergone a mild stress. Dietary supplementation decreased SOD activity, IL-1ß concentration and restored GSH concentration while the impairment in LTP was reversed. These data indicate that stress-mediated impairment of neuronal function may, in part, be due to oxidative stress.

Using hippocampal cells it was established that short-term incubation with IL-1ß enhanced caspase activation while co-incubation with H_2O_2 causes a further enhancement. Since increased caspase activity is one indicator of apoptotic cell death it may be speculated that sudden increases in oxidative stress accompanied by increases in IL-1ß

may contribute to neuronal death *in vivo*. The evidence presented is therefore consistent with the hypothesis that increased IL-1ß concentration in hippocampal and cortical tissue induces oxidative stress and that this, in turn, results in impairment in neuronal function, an example of which is a compromise in LTP in the hippocampus.

VI List of Abbreviations

AA	Arachidonic Acid
ACTH	Adrenocorticotrophic Hormone
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
BBB	Blood Brain Barrier
BHT	Butylated Hydroxytoluene
BSA	Bovine Serum Albumin
$CaCl_2$	Calcium Chloride
CaM Kinase II	Calcium/calmodulin Kinase II
CNS	Central Nervous System
CRF	Corticotropin Releasing Factor
$CuSO_4$	Copper Sulfate
DAG	Diacylglycerol
DCF	2'7'-Dichlorofluorescin
DETAPAC	Diethylenetriaminepentaacetic Acid
DMSO	Dimethylsulfoxide
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
ECL	Enhanced Chemiluminesence
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol bis (b-aminoethyl ether)N,N' tetraacetic Acid

EPSP	Excitatory Postsynaptic Potential
$FeSO_4.7H_2O$	Ferrous Sulfate
GABA	Gamma-Aminobutyric Acid
GC	Glucocorticoids
GPx	Glutathione Peroxidase
GRs	Glucocorticoid Receptor
GSH	Glutathione
GSSG	Glutathione Disulphide
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HFS	High Frequency Stimulation
HPA	Hypothalamic-Pituitary-Adrenal Axis
H_2SO_4	Sulfuric Acid
ICE	Caspase-1/Interleukin1ß-Converting Enzyme
IL-1	Interleukin-1
IL-1ra	Interleukin-1 Receptor Antagonist
KCl	Potassium Chloride
KOH	Potassium Hydroxide
KSCN	Potassion Thiocyanate
LTD	Long-Term Depression
LTP	Long-Term Potentiation
MDA	Malondialdehyde
$MgSO_4$	Magnesium Sulfate
MRs	Mineralocorticoid Receptors

NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NaN_3	Sodium Azide
NaOH	Sodium Hydroxide
NBM	Neurobasal Medium
NBT	Nitroblue Tetrazolium
NGF	Nerve Growth Factor
NMDA	N-Methyl-D-Aspartate
NPH-HCL	2-Nitrophenylhydrazine Hydrochloride
$NO^{\bullet}$	Nitric Oxide
NOS	Nitric Oxide Synthase
$O_2^{\bullet-}$	Superoxide Radical
$OH^{\bullet}$	Hydroxyl Radical
$ONOO^-$	Peroxynitrite
PB	Primed Burst Potentiation
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl Fluoride
PUFA	Polyunsaturated Fatty Acid
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
STBI	Soyabean Trypsin Inhibitor

TBA	Thiobarbituric Acid
TCA	Trichloroacetic Acid
TGF-ß	Transforming Growth Factor-ß
TMP	Tetramethoxypropane
TNF	Tumour Necrosis Factor

Chapter 1

General Introduction

1.1 Reactive Oxygen Species (ROS)

All mammalian cells require molecular oxygen, O_2, for oxidative phosphorylation. However by-products of this process, termed reactive oxygen species (ROS) can damage the cellular environment. It has been reported that between 2-5% of O_2 consumed by mitochondria leaks from the mitochondrial electron transport chain and is capable of interacting with cellular constituents (Fridovich, 1978). The cell has evolved a defence system against these "pro-oxidants" and it consists of a battery of enzymes and molecules that neutralise these ROS before they can cause damage to the cell. However if there is an increase in the production of ROS or a decrease in the efficacy of this antioxidant defence system then an "oxidative stress" is exerted on the cell that can adversely effect its ability to function efficiently. Prolonged and severe oxidative stress can eventually overwhelm the antioxidant defence system and destroy a cell.

1.1.1 Free Radicals

All atoms and molecules have electrons that occupy regions of space surrounding the nucleus termed orbitals. These orbitals can hold only 2 electrons. An orbital containing only one electron is termed "unpaired" and a free radical is an atom or molecule that is capable of independent existence with one or more unpaired electrons. These molecules are intrinsically unstable as there is a thermodynamic drive for them to fill their unpaired orbital(s).

O_2 is a free radical that possesses 2 unpaired electrons in different orbitals but "rotating" in the same direction. They are said to have parallel spin. This prevents O_2 from reacting with most molecules as it can only accept 2 electrons with opposite parallel spin to those in its unpaired orbitals.

90% of O_2 taken up by the human body is used by mitochondrial cytochrome oxidase present in mitochondria and this enzyme overcomes the spin restriction of O_2

by adding 4 electrons in conjunction with 4 protons onto each O_2 molecule in a step-wise fashion to yield energy and water.

$$O_2 + 4H^+ + 4e^- \longrightarrow 2H_2O$$

This process is carried out by a number of protein complexes embedded in the membranes of mitochondria.

1.1.2 The Mitochondrial Electron Transport Chain

The primary function of this system is to produce ATP by coupling its production with the free energy obtained from the transfer of electrons from NADH and $FADH_2$ to O_2. This system is comprised of over 70 polypeptide components which are grouped into 4 enzyme complexes present in the inner mitochondrial membrane. Complex I is termed NADH ubiquinone reductase and contains one molecule of flavin mononucleotide (FMN) surrounded by several iron-sulfur protein complexes. Complex I passes electrons from NADH to Co-enzyme Q. Complex II is known as succinate ubiquinone reductase and also passes electrons from succinate to co-enzyme Q. Complex III is termed Co-enzyme Q-Cytochrome c Reductase and passes electrons from reduced co-enzyme Q to cytochrome c. It contains 2 b-cytochromes and one iron-sulfur protein cluster. Cytochromes are highly conserved heme proteins present in nearly all organisms that are able to alter their oxidative state due to their Fe atom core. Each group of cytochromes contain a structurally different porphyrin ring close to the central Fe atom. It is still controversial but this complex is believed to be the initial site of free radical production. Cytochrome c is a peripheral membrane protein that is loosely bound to the outer surface of the inner mitochondrial membrane. It alternatively binds to cytochrome c1 of Complex III and to cytochome c oxidase and functions to shuttle electrons between these two structures.

Complex IV is known as Cytochrome c Oxidase and catalyses the one-electron oxidations of four consecutive reduced cytochrome c molecules and the concomitant four-electron reduction of one O_2 molecule

$$4 \text{ cytochrome } c^{2+} + 4H^+ + O_2 \longrightarrow 4 \text{ cytochrome } c^{3+} + 2H_2O$$

The energy produced from the interactions of Complexes I, III and IV allows the transport of H^+ across the inner mitochondrial membrane from the matrix, a region of low H^+ concentration into the intermembrane space, a region of high H^+ concentration. This creates a thermodynamic energy, termed the proton-motive force, that drives ATP synthesis. The free energy formed by the creation of the proton gradient is dissapated by inducing a conformational change in the active site of Complex V (ATP Syntase) that allows the synthesis of ATP (Voet & Voet, 1990).

1.1.3 Biologically Produced Free Radicals

Reactive oxygen species (ROS) are both free radicals and non-free radicals that are derived from oxygen and are capable of interacting with cellular constituents. This interaction is generally deleterious to the macromolecules of the cell. The acceptance of 1 electron by an O_2 molecule results in the formation of the superoxide radical, $O_2^{-\bullet}$. This molecule does not have the spin restriction of O_2 and is capable of reacting with a wider range of molecules. However it is believed to have limited reactivity. The main biological source of $O_2^{-\bullet}$ generation is the oxidaton by O_2 of semiquinone at Complex III (Demin *et al.*, 1998). However $O_2^{-\bullet}$ can also be generated from the leakage of electrons from endoplasmic recticulum electron transport chains (Fridovich, 1989). $O_2^{-\bullet}$ can interact and damage only a few enzymes directly and these include the NADH dehydrogenase complex of the mitochondrial electron transport chain (Zhang *et al.*, 1990) and creatine kinase (Cerutti *et al.*, 1994). Activated phagocytes are

capable of producing large amounts of $O_2^{-\bullet}$ and this is believed to be one mechanism employed by these cells for killing bacteria (Curnutte *et al.*, 1987). There may also be a role for $O_2^{-\bullet}$ in growth regulation and intracellular signalling with $O_2^{-\bullet}$ hypothesised to mediate vascular endothelial constriction antagonising the action of another free radical, nitric oxide ($NO^{\bullet}$) (Halliwell, 1992). However $O_2^{-\bullet}$ can lead to the formation of many other ROS that are more damaging to the cellular environment. These include hydrogen peroxide, H_2O_2 and the hydroxyl radical, $OH^{\bullet}$ (Halliwell & Gutteridge, 1986).

The transformation of $O_2^{-\bullet}$ to H_2O_2 by the superoxide dismutase (SOD) family of enzymes is a major source of ROS (Fridovich, 1989). H_2O_2 is not a free radical and has limited reactivity but it can cause severe oxidative damage to the cell by its interaction with transition metals which generates toxic hydroxyl ($OH^{\bullet}$) radicals. It can also diffuse through lipid membranes and initiate cellular damage removed from its site of production (Aruoma *et al.*, 1989). H_2O_2 is also produced by other enzymes in neuronal cells including L-amino acid oxidase and monoamine oxidase (Cohen *et al.*, 1996).

The $OH^{\bullet}$ radical is highly reactive and this is reflected in its very short half life (in the order of $1x10^{-9}$ sec) (Yu, 1996). It readily reacts with a variety of molecules especially polyunsaturated lipids and causes severe oxidative stress to cells. It is derived primarily from the interaction of H_2O_2 with transition metals, primarily Cu^{2+} and Fe^{2+}.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

Or by interaction of $O_2^{-\bullet}$ with H_2O_2

$$O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + H_2O + OH^{\bullet}$$

(Fong *et al.*, 1976).

Transition metals are tightly bound to specific protein transporters to minimise the risk of interaction with endogenously produced H_2O_2. Cu^{2+} is bound to ceruloplasmin while Fe^{2+} is bound to transferrin and homosiderin (Halliwell, 1992). In stroke there is a release of transition metals primarily from ruptured red blood cells and this is believed to initiate widespread oxidative damage to neuronal tissue (Chan, 1996).

Nitric oxide is an endogenously produced free radical that stimulates guanylate cyclase mediated cGMP synthesis (Hebeiss & Kilbinger, 1998). It is produced by the enzyme nitric oxide synthase (NOS) which catalyses the conversion of arginine to $NO^{\bullet}$ and citrulline (Knowles & Moncada, 1994). There are three isoforms of this enzyme with neurons possessing a constituitive, calcium dependent NOS isoform and astrocytes having a calcium independent inducible form that requires *de novo* synthesis of the protein (Lincoln *et al.*, 1997). There is also a particulate isoform present in vascular endothelial cells. $NO^{\bullet}$ is able to react with $O_2^{-\bullet}$ to form the cytotoxic peroxynitrite species $ONOO^-$ (Beckman *et al.*, 1990). At physiological pH, $ONOO^-$ degrades spontaneously to $OH^{\bullet}$ and nitrogen dioxide. $ONOO^-$ and its breakdown products are capable of interacting with cellular constituents including polunsaturated lipids, proteins and DNA (Beckman *et al.*, 1990).

1.2 Cellular damage caused by ROS

1.2.1 Protein Damage

All proteins are vulnerable to the actions of free radicals. The oxidation of amino acids leads to physical changes to the overall protein structure. It is well established that fragmentation can occur in large proteins such as collagen, albumin and α-globulins when exposed to free radicals. The amino acids proline, histidine and arginine are particularly susceptible to attack from $OH^{\bullet}$ as they are generally closely associated with transition metal atoms that are capable of being sites of $OH^{\bullet}$ generation

(Wolff, 1987). Fragmentation of protein can lead to protein aggregation and this is believed to be due to cross linkage reactions mediated by free radicals (Marx & Chevion, 1986). The oxidation of amino acids within a protein structure generally leads to loss of function and a susceptibility to degradation by proteolytic cleavage (Yu, 1996).

1.2.2 Membrane Damage

Biological membranes consist of a lipid bilayer embedded with proteins. There are 3 major kinds of membrane lipids: phospholipids, glycolipids and cholesterol. Glycolipids are sugar molecules containing lipids and are derived from sphingosine. Cholesterol generally makes up a small proportion of the total lipid in membranes. It functions to stabilise membranes by modulating the mobility of fatty acid chains. Therefore a high cholesterol content in lipid membranes decreases membrane fluidity. Phospholipids are derived from either glycerol or sphingosine and are called either phosphoglycerides or sphingomyelin. Phosphoglycerides consist of a glycerol backbone, 2 fatty acid side chains and a phosphorylated alcohol. The fatty acid side chains generally contain an even number of carbon atoms usually between 14-24 with 16 and 18 carbon fatty acids the most common. The fatty acid side chains are unbranched and can be either saturated or unsaturated. The fluidity of a lipid membrane is primarily governed by the fatty acid composition of phosphoglycerides and the cholesterol content with low cholesterol content, short fatty acid chain length and unsaturation enhancing fluidity (Voet & Voet, 1990).

Lipid molecules are vulnerable to attack from free radicals. The initiation phase of lipid peroxidation involves the abstraction of a hydrogen atom from a carbon-carbon double bond of a fatty acid. This allows the formation of a lipid radical capable of interacting with molecular oxygen (Aruoma *et al.*, 1989). This interaction forms a lipid hydroperoxide which can degrade to form peroxyl and alkoxyl radicals. These

moieties can attack amino acids within membrane-associated proteins or cause the abstraction of another hydrogen atom from neighbouring lipid molecules thus propagating the lipid peroxidation reaction (Halliwell & Gutteridge, 1984). Polyunsaturated fatty acids are especially vunerable to peroxidation due to the number of carbon-carbon double bonds within their structure. The major fatty acids susceptible to free radical attack are linoleic acid, arachidonic acid and docosahexanoic acid (Halliwell, 1992). When these fatty acids undergo peroxidation different hydroperoxide species are generated depending on the lipid substrate. These species can be biologically active. A by-product of arachidonic peroxidation is 4-hydroxynonenal which is both cytotoxic and mutagenic (Siu & Draper, 1982). These aldehyde by-products are also capable of causing protein cross-linkage which can disrupt protein function (Gutteridge & Draper, 1990).

Lipid peroxidation causes a disruption to lipid structures such as the plasma and mitochondrial membranes leading to an increase in permeablity and a decrease in fluidity in these structures. The alteration in permeability can lead to an increase in intracellular calcium and sodium and the consequent initiation of apoptosis or cell death by lysis (Orrenius *et al.*, 1989). Proteins associated with these structures can also be affected either directly, by attack by peroxyl radicals, or indirectly through the alteration in membrane fluidity (Halliwell, 1992).

1.2.3 DNA Damage

DNA is also vulnerable to the actions of free radicals. ROS can initiate chemical alterations in the deoxyribose sugar component and in the purine and pyrimidine bases (Cochrane, 1991). The $OH^{\bullet}$ radical has been shown to induce base alterations and strand breakage; however genomic DNA has very efficient repair processes that limit accumulative oxidative damage (Imlay *et al.*, 1988). It has been estimated that there were only between 8-83 residues/10^6 of oxidatively damaged bases

in liver and kidney cells of young rats (Fraga *et al.*, 1990). However a consequence of lipid peroxidation can be a rise in intracellular Ca^{2+} which can lead to the inappropriate activation of nuclear enzymes that may disrupt DNA structure and function (Orrenius *et al.*, 1989). Mitochondrial DNA is more vulnerable to free radical damage as it is near a major site of $O_2^{-\bullet}$ generation and it possesses very poor DNA repair mechanisms. It is speculated that mitochondrial genomic damage accumulates with time and that this alters the efficiency of mitochondria leading to an increase in the generation of free radicals with age (Cortopassi *et al.*, 1990).

1.3 Antioxidant Defence System

The brain is very metabolically active. It is estimated that it makes up 2% of the body mass but uses 20% of the available O_2 (Halliwell *et al.*, 1992). Neuronal cells have a multi-component defence against the constant barrage of ROS being produced by mitochondria. This system contains enzymatic and non-enzymatic elements that function to neutralise ROS before they can damage cellular constituents.

1.3.1 Superoxide Dismutase (SOD)

This enzyme catalyses the following reaction.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

In mammalian species there are two types of SOD differentiated by the metal atom present on their active sites. These 2 isoforms are CuZnSOD and MnSOD. The CuZnSod is a homodimeric enzyme with a molecular weight of 32kDaltons and contains one Cu^{2+} atom and one Zn^{2+} atom per subunit. The subunits are linked by disulphide bonds and the enzyme itself is very stable. It is found in the cytosol of all cells but a high molecular weight extracellular isoform has been described in human

plasma and has been termed EcSOD. There is a 55% homology between the CuZnSOD of yeast and cow demonstrating that the protein is highly conserved and very important for efficient cell functioning (Shaw *et al.*, 1997; Halliwell *et al.*, 1992). MnSOD is a tetrameric protein with subunits of 23kDaltons. It has 1 Mn^{2+} atom per subunit and this enzyme resides exclusively in the mitochondrial membrane. There is no homology between the CuZnSOD and MnSOD isoforms and this strongly suggests that they evolved separately (Fridovich, 1997).

The activity of SOD can be increased in response to elevated tissue oxygenation and toxic chemicals known to generate $O_2^{\bullet-}$, such as paraquat (Gregory & Fridovich, 1973). The protective role of these enzymes has been demonstrated in animal transgenic experiments. Increased activity of CuZnSOD in the brains of transgenic mice protects neurons from ischemia-reperfusion injury and also lessens the cerebral edema caused by traumatic brain injury (Chan *et al.*, 1996; Mikawa *et al.*, 1996). Also chronically increased CuZnSOD activity promoted cell survival and neuronal process development in postnatal midbrain neurons from transgenic mice overexpressing this enzyme (Przedborski *et al.*, 1996). Furthermore the downregulation of CuZnSOD activity leads to apoptotic neuronal cell death (Troy *et al.*, 1996). Mice deficient in MnSOD suffer from early neurodegeneration and overexpression of the enzyme prevents neuronal apoptosis in primary neuronal cultures and decreases ischemic brain injury (Keller *et al.*, 1998). However there is also substantial evidence that enhanced basal activity of the SOD isoforms is related to neurodegenerative disease. This may occur due to an increase in H_2O_2 production. The brains of CuZnSOD transgenic mice produce higher levels of H_2O_2 than wild type mice (Przedborski *et al.*, 1992). Also CuZnSOD can itself be a pro-oxidant as it is capable of catalysing $OH^{\bullet}$ production from H_2O_2 (Yim *et al.*, 1990). A familial form of amyotrophic lateral sclerosis, a motor neuron degenerative disease, is associated with a mutation in the gene for CuZnSOD which causes an increase in the activity of

the protein (Rosen *et al.*, 1993). An increase in the activity of MnSOD and enhanced expression of the gene for CuZnSOD has been reported in the substantia nigra of patients with Parkinson's disease (Saggu *et al.*, 1989; Ceballos *et al.*, 1990). Individuals with Down's Syndrome demonstrate pronounced neurodegeneration and this has been linked to an overexpression of CuZnSOD protein (De la Torre *et al.*, 1996). It appears that this enzyme has paradoxical actions on neuronal tissue. An increase in its activity in response to oxidative stress is beneficial and substantially reduces oxidative damage while an abnormal increase in its basal activity can actually initiate neuronal damage and cell death.

1.3.2 Glutathione Peroxidase (GPx)

This enzyme catalyses the decomposition of H_2O_2 or organic hydroperoxides by the following reactions

$$2GSH + H_2O_2 \longrightarrow GSSG + 2H_2O$$

or

$$2GSH + ROOH \longrightarrow GSSG + H_2O + ROH$$

(Yu, 1996)

There are two isoforms of this enzyme. One is selenium-dependent and present in the cytosol, the other selenium-independent and associated with the membrane. Both enzyme isoforms can catalyse the above reactions but they have different substrate specificities. The membrane associated GPx preferentially metabolises organic hydroperoxides (Wendel, 1981). This enzyme plays an important role in the protection of neurons from peroxide-mediated oxidative damage. For example, the nigrastriatal region in transgenic mice overexpressing cytosolic glutathione peroxidase was partially protected from ROS mediated damage upon the introcerebroventricular injection of the neurotoxin 6-hydroxydopamine (Bensadoun *et al.*, 1998) while there

was significantly reduced neuronal damage induced by cerebral ischemia/reperfusion. (Weisbrot-Lefkwitz *et al.*, 1998).

1.3.3 Catalase

This enzyme is responsible for the decomposition of H_2O_2 by the following reaction

$$2H_2O_2 \longrightarrow O_2 + 2H_2O$$

(Yu, 1994)

There is a high activity of this enzyme in the liver and kidney however catalase activity is low in brain tissue. It is believed that glutathione peroxidase is largely responsible for the decomposition of H_2O_2 in the brain (Cohen *et al.*, 1996).

1.3.4 Vitamin C (Ascorbic acid)

Vitamin C is widely distributed in the body but is present in the highest concentrations in the adrenal glands and central nervous system. Primates and guinea pigs are unable to synthesise this molecule but most other mammals produce ascorbic acid in the liver via the action of L-gulonlactone oxidase on L-glucolactone (Chatterjee *et al.*, 1975). Therefore all ascorbic acid in the brain is derived from the blood stream.

Ascorbic acid entry into the brain occurs at the choroid plexus by means of a saturable, carrier-mediated, energy dependent mechanism which is stereopecific (Spector, 1981). Recent evidence indicates that vitamin C is transported through the blood brain barrier by the glucose transporter GLUT 1 in the form of its oxidised derivative dehydroascorbic acid. It is then reduced in the CNS to ascorbic acid (Agus *et al.*, 1997) and further concentrated in nerve cells. Its intracellular concentration is estimated to be 1-3mM compared to 0.05-0.5mM in the CSF. In the rat it is present in

the highest concentrations in the amygdala, hippocampus and hypothalamus (Spector & Lorenzo, 1973).

Vitamin C functions as an antioxidant in the CNS. It decreases infarct volume in primates after ishemia-reperfusion experiments (Ranjan *et al.*, 1993). It has also been observed to decrease brain injury in excitatory amino acid toxicity experiments (Rebec *et al.*, 1994). It appears that there is active continuous uptake and secretion of vitamin C in the CNS and it has been suggested that glutamate uptake induces vitamin C release from nerve terminals by the actions of a glutamate/ascorbate exchanger. Its function is believed to neutralise the ROS generated upon glutamate receptor binding (Wilson, 1997). It has been shown that vitamin C is capable of modulating neuronal function as intrastriatal infusions of vitamin C attenuate the behavioural response of rats to amphetamine (White *et al.*, 1990) and using intracellular recording techniques there is a reversible depression of post-synaptic potentials at physiological concentrations of vitamin C in the rat cerebral cortex. It has been suggested that vitamin C may modulate the functioning of the NMDA receptor as experiments have demonstrated that vitamin C impairs binding of [^{3}H]-glutamate to the NMDA receptor (Majewski *et al.*, 1990).

Vitamin C is required for the maintenance of vascular and connective tissue integrity as well as normal hematopoesis and leukocyte function. In the brain it is involved in catecholamine biosynthesis and has a very important role as part of the antioxidant defence system. It is believed to be the most important antioxidant in the extracellular fluid but also very important intracellularly. It interacts directly with $O_2^{\bullet -}$, $OH^{\bullet}$ and various peroxyl radicals, neutralising them before they can damage macromolecules in the cellular environment (Sies & Stahl, 1995). However it can be a pro-oxidant if present in high concentrations and in the presence of transition metals (Aust *et al.*, 1985).

Vitamin C can also have a sparing effect on the concentration of vitamin E in different organs. One study reported that rats deficient in vitamin C were correspondingly deficient in vitamin E (Tanaka *et al.*, 1997). Dietary supplementation with high doses of vitamin C is believed to have beneficial effects as it was observed that the concentation of glutathione and the activities of SOD, catalase and glutathione peroxidase were increased in the guinea pig heart following supplementation and it was concluded that high doses of vitamin C were able to increase the global antioxidant capacity of heart tissue in the guinea pig (Rojas *et al.*, 1994). Further evidence of the beneficial effect of vitamin C supplementation showed that large doses of vitamin C for 30 days reduced alcohol-induced oxidative stress and also increased antioxidant capacity in the liver of guinea pigs (Suresh *et al.*, 1999).

1.3.5 Vitamin E

Vitamin E is the main membrane-residing component of the cellular antioxidant defence system. Its existence was first recognised in 1922 when it was realised that a nutritional factor was required to prevent the reabsorption of the foetus in an adult rat (Evans & Bishop, 1922). Its importance in maintaining the integrity of nerve tissue was soon discovered and it was reported that chicks fed a vitamin deficient diet developed cerebellar encephalomalacia (Pappenheimer & Goettsch, 1931). It is now clear that vitamin E is an essential requirement in the diet of mammals. A deficiency in this vitamin can lead to many neuronal disease states including areflexia, peripheral neuropathy, decreased proprioception and cerebellar motor control dysfunction (Sokal, 1989).

Vitamin E is a fat-soluble antioxidant concentrated in the hydrophobic membranes of cells. There are a number of different componds that fall under the umbrella term of "vitamin E". These are α-, β-, γ-, and δ-tocopherol however it is believed that the active molecule is α-tocopherol (Sies, 1991). It is not highly

localised in the brain but brain tissue concentration is highly conserved. In a vitamin E depletion experiment, mice were fed a diet free of this vitamin. By the 6th week of this regime the vitamin E concentrations in the liver, plasma and spleen of these animals were severely depleted however by the 20th week there still was significant concentrations of the vitamin in brain tissue. It was reported that some areas of the brain were more prone to depletion than others with cerebellar regions losing their vitamin E concentrations more quickly than the cerebral hemispheres, medulla or pons (Vatassery *et al.*, 1984). Dietary vitamin E is taken up by the liver with lipoprotein and then resecreted into the plasma in a very low density lipoprotein (Arita *et al.*, 1997). However the mechanisms by which it is transported through the blood-brain barrier await elucidation.

Vitamin E supresses fatty acid oxidation by contributing a hydrogen atom from its phenolic hydroxyl group to lipid derived peroxyl radicals. These radicals are generated primarily through interaction with ROS. It prevents these energetic lipid peroxyl radicals from attacking the unsaturated fatty acids of adjacent membrane lipids (Burton *et al.*, 1983; McCay, 1985). This prevents lipid peroxidation and a damaging alteration in membrane fluidity. The resultant vitamin E radical is capable of being regenerated by vitamin C (Packer *et al.*, 1979; Doba *et al.*, 1985). The benefits of dietary supplementation with vitamin E are well established. In a transgenic mice model of amyotrophic lateral sclerosis, a disease state charachterised by an increase in ROS production and lipid peroxidation, dietary supplementation with vitamin E delayed onset of symptoms and slowed the progression of the disease state (Gurney *et al.*, 1996). The Iowa Women's Health Study found that a high dietary vitamin E intake decreased the risk of colon cancer, especially in individuals under 65 years of age (Bostick *et al.*, 1993) while a high dietary intake of vitamin E was also found to be protective against the occurrence of Parkinson's Disease (de Rijk *et al.*, 1997).

1.3.6 Glutathione (GSH)

This is a very abundant tri-peptide (γ-glutamyl-cysteine-glycine) present in all cells. It has a multi-functional role being involved in many biological processes such as the metabolism of sulphur-containing amino acids and the biosynthesis of leukotrienes and DNA. It acts both as an antioxidant that scavenges ROS directly and as a substrate for the metabolism of H_2O_2 by the enzyme glutathione peroxidase (Shivakumar *et al.*, 1995). Its role as an important antioxidant is emphasised by the observation that experimentally induced depletion of glutathione in cultured oligodendrocytes leads to toxic increases in intracellular hydroxyl radicals (Yonezawa *et al.*, 1996). Similarily inhibition of glutathione synthesis leads to rapid death of cultured rat cortical neurons; this effect can be inhibited by the addition of antioxidants to the cell medium (Gwag *et al.*, 1995).

1.4 ROS, the CNS and Disease

The CNS is particularly vulnerable to the actions of ROS. Neuronal membranes possess membrane lipids that contain high concentrations of polyunsaturated fatty acids (Rice-Evans & Burdon, 1993) which leaves them susceptible to oxidation. The brain also has low levels of antioxidants despite utilising 20% of available O_2 (Halliwell, 1996) implying that neurons have a limited capablity to respond to oxidative stress. Also excitatory amino acids under certain conditions are capable of generating excessive amounts of free radicals (Dugan *et al.*, 1994).

ROS can cause extensive cellular damage resulting in either necrotic or apoptotic death. Because they oxidise membrane lipids, membrane permeability can increase and this can lead to an inability to maintain calcium homeostasis (Orrenius *et al.*, 1989). They also can interact with proteins such as Na^+ -K^+ ATPase which may adversely effect the intracellular ionic concentrations of Na^+ and K^+. These actions can lead to rapid cell swelling and lysis or activate the intrinsic apoptotic machinery of a

cell leading to DNA fragmentation, cytoskeletal disruption, cell shrinkage and membrane blebbing. Oxidative injury has been implicated in a number of neurodegenerative conditions.

1.4.1 Parkinson's Disease

This disease is believed to be caused by a loss of dopaminergic neurons in the substantia nigra leading to a loss in fine control of muscle movement. It is associated with an increase in free iron concentration in that area, a decrease in the concentration of glutathione and an increase in lipid peroxidation (Owen *et al.*, 1996). It has been suggested that ROS are produced by the interaction of Fe^{2+} and catecholamines and that this production leads to the release and subsequent loss of glutathione from neuronal cells (Jenner, 1991). Monoamine oxidase also produces H_2O_2 in the course of dopamine metabolism and this agent, in the presence of Fe^{2+} , can cause the production of $OH^{\bullet}$ radicals. It is believed that a sustained and chronic oxidative stress leads to the loss of dopaminergic neurons in this disease. It is known that there is a decrease in the activity in complex I of the mitochondrial elctron transport chain in the tissue of patients suffer ing from this disease (Shapira *et al.*, 1990). It is not however known if this decrease in the functionality of complex I, which could lead to the abnormal generation of ROS, is a consequence or a cause of the illness. MPP+, the metabolite of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) inhibits complex I, increases $OH^{\bullet}$ radical production, induces neuronal loss in the substantia nigra and causes symptoms similar to Parkinson's disease (Langston *et al.*, 1987). This suggests that complex I activity may have a role in the aetiology of the disease.

1.4.2 Alzheimer's Disease

This disease is characterised by the presence of large numbers of neuritic plaques in cortical and hippocampal tissue containing predominately the neurotoxic

amyloid β-peptide. These plaques are generally surrounded by reactive astrocytes (Harris *et al.*, 1996). It is a progressive illness which leads to impaired energy metabolism, decreased ATP concentrations and eventual neuronal cell death (Markesbery, 1997). Incubation of rat cortical neurons with amyloid ß-fragment 25-35 leads to ROS mediated neuronal cell death. While the addition of physiological concentrations of ascorbate protects cultured hippocampal neurons from the toxicity associated with addition of amyloid β-peptide (Prehn *et al.*, 1996). This implies that the plaques may be mediating their toxic effects through the production of ROS. Amyloid β-peptide activates NF-κB, a neuronal early gene transcription regulator of numerous pro-inflammatory genes, and this activation appears to involve free radical production as its activation can be blocked by antioxidants astrocytes (Harris *et al.*, 1996). This peptide also prevents the uptake of glutamate by astrocytes and the damage to this uptake mechanism appears to be mediated by free radicals as the addition of the antioxidant TROLOX, an analogue of vitamin E, blocks this action in astrocytic cultures (Harris *et al.*, 1996). Also in this disease the nitrotyrosine concentration is increased in samples of tissue from affected areas and it is suggested that this indicates a reaction of $ONOO^-$ with tyrosine residues on proteins (Smith *et al.*, 1997). The glial factor S100-ß is overexpressed in these samples also. This protein causes the induction of astrocyte iNOS and $NO^\bullet$ mediated neuronal cell death in an astrocyte/neuronal co-culture system (Hu *et al.*, 1997). While ß-amyloid activates NOS in a substantia nigra/ neuroblastoma cell line (Le *et al.*, 1995).

1.4.3 Stroke

Stroke occurs when a blood vessel becomes blocked and deprives the neuronal area it supplies of O_2 and glucose. After only 2 minutes, concentrations of O_2 and glucose can fall to undetectable concentrations and neurons in the affected area suffer membrane degradation, an increase in plasma membrane permeability and increased

intracellular concentrations of Na^+ and Ca^{2+} (Choi *et al.*, 1996). Ischemia is believed to cause the uncoupling of mitochondrial electron transport and the activation of nitric oxide synthase and phospholipase A2. Ischemic infarction is significantly reduced in neuronal NOS knock-out mice (Huang *et al.*, 1994) but infarct volume is significantly increased in CuZnSOD and MnSOD knockout mice (Mikawa *et al.*, 1995; Mikawa *et al.*, 1996). These reports imply that the neuronal death that occurs may be due to the increased generation of $NO^{\bullet}$ and $O_2^{-\bullet}$.

1.5 Hippocampal Formation and Long-Term Potentiation

The hippocampal formation, which consists of the dentate gyrus, hippocampus and the subiculum lies at the floor of the lateral ventrical, beneath the cortical structures. The hippocampal formation is connected to other brain areas through 2 main pathways, the fornix and the perforant pathway. Through the fornix the hippocampal formation is connected to the septal area, the thalamus and the hypothalamus while the perforant pathway is connected with cortical association areas in the temporal lobe and specifically with the entorhinal cortex (Gilman & Newman, 1990). The primary component of the perforant path are the projections from the spiny stellate neurons of the entorhinal cortex which synapse with the dendrites of the granule cells of the dentate gyrus. The axons of these granule cells form the mossy fibres that contact the pyramidal cells of the CA3 region of the hippocampus. The axons from these cells in turn contact the dendrites in the stratum radiatum and lacunosum moleculare of the CA1 area of the hippocampus. These pathways together form the trisynaptic loop of the hippocampus (Bliss & Collinridge, 1993).

1.5.1 Long Term Potentiation (LTP)

How the brain stores information is still a controversial area of neuroscience research. Current opinion suggests that information is stored by alteration of neuronal

activity and more specifically by an alteration of synaptic activity. This alteration is believed to be by means of an enhanced efficiency in synaptic signalling through an increase in neurotransmitter release, enhanced responsiveness to that transmitter and an ability to fine tune communication between the post synaptic dendritic spine and pre-synaptic bouton.

In the 1940's it was proposed that a synapse linking 2 neurons is strengthened if both cells are active at the same time (Hebb, 1949). An early description of synaptic strengthening was made in the hippocampus of the anesthetised rabbit where brief trains of high frequency stimulation to the perforant path caused a sudden and sustained increase in the efficiency of synaptic transmission recorded in the dentate gyrus (Bliss & Lomo, 1973). This phenomenon was termed long-term potentiation (LTP) and is the best investigated model of activity dependent synaptic plasticity in the mammalian brain. It has been proposed as a putative model for learning and memory whereby events are recorded by the strengthening of specific synaptic pathways. In this thesis LTP is used as a model of functionality of the perforant path/dentate gyrus system. LTP is expressed as a persistent increase in the size of the synaptic component of the evoked response. It can be induced in both individual and populations of neurons. It possesses three characteristics; co-operativity, associativity, and input specificity.

Co-operativity.

The induction of LTP requires a "strong" stimulus. There is, in effect, a threshold of intensity below which attempts to induce LTP will not succeed. The pattern of stimulation contributes as significantly to LTP as the intensity of the stimulus (McNaughton *et al.*, 1978).

Associativity.

A "weak" stimulus can induce LTP if it occurs at the same time as a "strong" stimulus applied to a convergent but separate neuronal pathway (Levy & Steward, 1979).

Input specificity.

Only the pathway which receives the stimulus gives rise to LTP. Alternate neighbouring pathways are unaffected (Lynch *et al.*, 1977).

There are numerous glutamate receptor types. For the induction of LTP it is believed that activation of the N-methyl-D-aspartate (NMDA) receptor on the post-synaptic spine is crucial. This is coupled to a voltage operated calcium channel which is blocked by Mg^{2+} ions; it has been proposed that this block is due to a hyperpolarisation effect caused by enhanced Cl^- conductance induced by GABAergic neurons (Ascher & Nowak, 1988). Another ionotropic glutamate receptor is the R,S-α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid-sensitive receptor (AMPA) which is coupled to a Na^+ channel. To induce LTP, glutamate is released from synaptic vesicles into the synaptic cleft and binds with both the AMPA and NMDA receptors. Sustained glutamate release allows sufficient Na^+ ions to enter the cell to diminish the hyperpolarisation effect and to release the Mg^{2+} block on the NMDA-linked calcium channel. This allows an influx of calcium which is believed to be the key signal for the increase in synaptic efficiency (Bliss & Collinridge, 1993). However LTP in mossy fiber-CA3 synapses is NMDA independent and this evidence demonstrates that there may be a number of mechanisms that enhance synaptic signalling.

These events explain the 3 characteristics of LTP; co-operativity is the requirement of a sufficient threshold of stimulus to remove the Mg^{2+} block on the NMDA channel; associativity can be explained as the spreading depolarisation effect

caused by a separate strong stimulus that unblocks the NMDA channel in a convergent pathway undergoing weak stimulus; input specificity is the activation of pathway specific pre-synaptic terminals releasing glutamate to open the AMPA- and NMDA-coupled Na^+ and Ca^{2+} channels. The role of metabotropic glutamate receptors in the induction of LTP awaits elucidation but these receptors appear to be important as aminocyclopentane dicarboxylate (ACPD) a mGluR agonist, can augment tetanus induced potentiation (McGuinness *et al.*, 1991) and the mGluR antagonist 2-amino-3-phosphonobutanoate (AP3) can block the induction and maintenance phase of LTP in rat hippocampal slices (Izumi *et al.*, 1991) .

1.5.2 Mechanisms involved in LTP

The precise mechanisms that cause the increase in the synaptic response that characterises LTP are still controversial and the exact contribution of the pre- and post-synaptic elements awaits clarification. There is strong evidence that there is an increase in vesicular glutamate release from pre-synaptic terminals in the dentate gyrus after tetanus (Dolphin *et al.*, 1982; Bliss *et al.*, 1986; Errington *et al.*, 1987). In synaptosomes prepared from dentate gyrus following the induction of LTP *in vivo* there was an increase in depolarisation induced release of radiolabelled and endogenous glutamate (McGahon & Lynch, 1996; McGahon *et al.*, 1997). Also inhibition of LTP by electrophysiological or chemical means attenuates significantly this increase in the release of glutamate (Bliss *et al.*, 1986; Errington *et al.*, 1987; McGahon *et al.*, 1998; Kelly *et al.*, 1998).

There is an increase in the activity of phospholipase C presynaptically after the induction of LTP in perforant-path-granule cells synapses (Lynch *et al.*, 1991). This may, in part, account for the increase in the concentration of calcium observed in synaptosomes from potentiated dentate gyrus 45 minutes after the induction of LTP (Bliss *et al.*, 1990). PLC cleaves inositol triphosphate (IP_3) and diacylglycerol (DG)

from the lipid phosphatidylinositol biphosphate. IP_3 causes an increase in the release of calcium from intracellular stores and this may activate many biological processes some of which may be involved in LTP. For example, KN-62, an inhibitor of calcium-calmodulin-dependent kinase (CaCaMKII) inhibits LTP and the LTP-associated increase in phosphorylation of synapsin I, a protein believed to be critical for exocytosis (Nayak *et al.*, 1996). DG also acts as a mediator for numerous biological processes and is the endogenous activator of protein kinase C (PKC). A role for PKC activation is demonstrated by the finding that inhibitors of PKC block LTP. This is supported by the finding that B50/GAP43, a substrate for PKC, is increased after the induction of LTP while phosphorylation of this protein correlates with an increase in glutamate release (Dekker *et al.*, 1990).

During the maintenance phase of LTP there is a persistent and consistent increase in glutamate release and a change in the numbers and distribution of synaptic vesicles (Applegate *et al.*, 1987; Meshul & Hopkins, 1991). There is also an increase in newly synthesised proteins related to excytosis in the dentate gyrus including synaptophysin, synapsin and synaptotagmin (Mullany & Lynch, 1997; Lynch *et al.*, 1994).

Postsynaptically there is an increase in the area and number of dendritic spines with an increase in the proportion of large spines and branched spines (Fifkova & Van Harreveld,1977; Chang & Greenough, 1984; Lee *et al.*, 1980; Desmond & Levy, 1988; Dhanrajan *et al.*, 1997). There is also evidence for change in the number and in the functioning of glutamate receptors. There is an increase in the sensitivity of glutamate receptors to AMPA and quisqualate several hours post tetanus (Davies *et al.*, 1989). Extra-synaptic changes include a reduction in glutamate uptake in glia (Barbour *et al.*, 1989).

It is likely there is a combination of factors that allow LTP to be induced and maintained. It may be that different factors play more prominent roles in different

regions of the brain. For example there is no evidence of increased transmitter release in the Schaeffer collaterals-CA1 synapses of the rat hippocampus following tetanisation (Maeda *et al.*, 1997).

Post synaptically the signal transduction mechanisms that are required to enhance synaptic efficency have still to be elucidated but it appears that an increase in intracellular calcium concentration and an upregulation in the activity of a number of calcium-sensitive enzymes play a role in the induction and maintenance of LTP. Among the enzymes that show increased activation are calpain, calcieurin, phospholipases and protein kinases (Bliss & Collinridge, 1993). Intracellular injection of the calcium chelator EGTA blocks the induction of LTP demonstrating that the calcium increase initiated by the depolarisation and opening of the NMDA receptor associated channel is a key event in the induction of LTP (Lynch *et al.*, 1983). A persistent protein kinase activation in the maintenance phase of LTP has been noted and this was significantly attenuated by the selective inhibitor of protein kinase C, PKC(19-36)(Klann *et al.*, 1991). The maintenance phase of LTP was also inhibited by a blocker of the calcium-dependent enzyme, calpain (Oliver *et al.*, 1989). Protein synthesis appears to be required for the maintenance of LTP as ansiomycin, an agent that inhibits the translation of proteins from mRNA, reduces the duration of the maintenance of LTP to only 3-6 hours when applied at the time of tetanus (Krug *et al.*, 1984).

There appears also to be communication between the dendritic spine and pre-synaptic bouton in the form of retrograde messengers that function to co-ordinate the enhancement of synaptic strength by initiating the increase in transmitter release from the pre-synaptic terminal. One candidate retrograde messenger is arachidonic acid which has been shown to be released from cultured neurons in the presence of NMDA (Patel *et al.*, 1990) while an increase in its efflux and post synaptic availability following the induction of LTP has also been observed (Lynch *et al.*, 1991).

Furthermore, inhibitors of phospholipase A2, the enzyme responsible for releasing arachidonic acid from phosphotidylinositol, block the induction of LTP (Lynch *et al.*, 1988). Nitric oxide and carbon monoxide are other candidate retrograde messengers. It is known that perfusion of hippocampal slices with an inhibitor of nitric oxide synthase blocks the induction of LTP (Zhuo et al, 1998). However the pattern of stimulus appears to be important in determining the extent of inhibition of LTP using this methodology. One-train tetanus stimulation was completely blocked using the iNOS inhibitor while a four-train tetanus stimulation protocol induced LTP. It is speculated that NO may be released only under certain circumstances and during a specific pattern of stimulus. Inhibitors of heme oxygenase, the enzyme responsible for CO production, blocked LTP and this action was independent of the pattern of stimulus (Haley JE 1998). This has led to the hypothesis that constituitive production of CO is essential for potentiation to occur following high frequency stimulation. However, this hypothesis is complicated by the finding that mice that lack haem oxygenase-2 (HO-2), the major neuronal isoform of heme oxygenase, are capaple of LTP and have normal neural development. Furthermore, zinc protoporphrin IX, an inhibitor of heme oxygenase had nearly identical inhibitory effects on LTP in wild type and HO-2 mutant hippocampal slices (Poss et al., 1995). Clearly more investigation is required to find the precise role of this radical in LTP.

Clearly the ability of the perforant path/dentate gyrus to sustain LTP requires the efficient functioning of many intra- and extra-cellular components. In this thesis, LTP is used to assess the functional competency of the hippocampus and to test whether dietary supplementation can improve the functional capability of this brain area *in vivo*.

1.6 Cytokines

Cytokines are a heterogenous group of polypeptides that act as potent mediators of intracellular signalling. They interact with specific high affinity receptors that are generally membrane-associated. Both expression and activity of cytokines are increased in conditions of tissue stress, phases of acute growth (i.e. tissue repair), tissue dysregulation (i.e. chronic inflammatory states), infection and trauma. Cytokines have a broad range of actions and there is often an overlap in these actions between existing cytokines. Also a wide range of cells are capable of producing several of these mediators. The number of polypeptides included in the umbrella term "cytokine" range from the interleukins from 1 to 18, the interferons (IFN), tumor necrosis factors (TNF) and the transforming growth factors (TGF).

Cytokines have numerous biological effects in the CNS. Some of these factors are neurotrophic; for example IL-2, IL-6 and TGF-ß promote neurite outgrowth and promote survival among primary cultured neurons from various regions of the rat brain (Shimojo *et al.*, 1993; Hama *et al.*, 1989; Meier *et al.*, 1993). Cytokines such as TGF-α and IL-1ß can initiate glial cell migration and proliferation reflecting their roles in responding to injury and infection. Other effects include regulation of synaptic transmission and activity; for example IL-2 modulates KCl-dependent acetylcholine release from rat hippocampal slices (Araujo *et al.*, 1989) while IL-1ß can inhibit the KCl-stimulated release of glutamate from hippocampal synaptosomes (Murray *et al.*, 1997). Cytokines also regulate the expression and release of other cytokines; for example VIP increases IL-1α mRNA and protein expression in rat cortical astrocytes (Kushima & Hatanake, 1992). Behavioural activity can also be modulated; for example IFN-α can increase the duration of slow-wave sleep and macrophage inflammatory protein-1 (MIP-1) can induce anorexia (Birmanns *et al.*, 1990; Myers *et al.*, 1993).

1.6.1 Interleukin-1

Interleukin-1 is, in fact, a family of proteins consisting of IL-1α, IL-1β and IL-1ra (receptor antagonist). IL-1α and IL-1β share 25% homology and are separate gene products (Dinarello, 1991). IL-1ra is also a separate gene product but has two isoforms derived from post-translational processing. IL-1α and IL-1ß are believed to have identical actions in the periphery while IL-1ra serves as a specific endogenous receptor antagonist (Dinarello, 1996).

The active IL-1 agonist molecules are formed from precursors, termed Pro-IL-1α and Pro-IL-1ß, by enzymatic cleavage. Pro-IL-1α is an active molecule but is not believed to be secreted. Pro-IL-1ß is inactive and is cleaved by interleukin-1 converting enzyme (ICE) also called caspase 1 (Thornberry *et al.*, 1992). Recently it has been proposed that interferon-gamma inducing factor (IGIF) also termed IL-18, should be included as another member of the interleukin-1 family (Okamura *et al.*, 1995). This molecule is also cleaved by ICE and some investigators claim that IL-18 is that enzymes preferred substrate (Rano *et al.*, 1997).

All actions of IL-1 are mediated through a 80kDa Type I receptor which requires an accessory protein (AcP) to mediate signal transduction events. There is also a 68kDa Type II receptor which has a very short intracellular domain and is believed not to be involved in signal transduction (Bomsztyk *et al.*, 1989; Sims *et al.*, 1993; O'Neill *et al.*, 1996). Its suggested that its function is to bind excess extracellular IL-1 but it may have a physiological role in the brain as there have been reports that i.c.v. injection of the Type II receptor antibody in mice inhibits the febrile response (Luheshi *et al.*, 1993). There have also been reports of an IL-1 receptor related protein (IL-1RrP) which is homologous with known IL-1 receptors and it has been proposed that it may be the receptor for IL-18 (Torigoe *et al.*, 1997).

1.6.2 Actions of IL-1

IL-1ß is the main IL-1 agonist induced in the brain in response to either systemic insult, such as injury or infection, or local insult such as stroke (Rothwell & Luheshi, 1994). In an experimental model of stroke it was reported that IL-1ß mRNA expression increased within 15-30 minutes of invoked cerebral ischemia and protein concentrations within the brain were increased within 1 hour (Minami *et al.*, 1992). Immunocytochemical analysis has shown that microglia and meningeal macrophages are responsible for early expression of IL-1ß after ischemia or excitotoxic insult and that there is a delayed expression of the protein of between 24 hours to 7 days in astrocytes and invading immune cells (Rothwell, 1999). There is also a concomitant increase in the expression of ICE in microglia shortly after the induction of cerebellar ischemia (Bhat *et al.*, 1996). IL-1α is also elevated after cerebral ischemia but at lower concentrations and slightly after IL-1ß. IL-1ra is also increased following brain injury. Its concentration is increased slightly after that of IL-1ß and this increase is believed to be due to an increase expression of the protein by neurons (Rothwell, 1999).

Interocerebroventricular injection of IL-1ß into rats produces fever, anorexia, slow-wave sleep and alterations in the neuro-endocrine system. These actions are all believed to be mediated via the hypothalamus. There is also activation of glia, a modification of calcium homeostasis and the induction of various cytokines, growth factors and neurotrophins (Rothwell, 1997). IL-1ß also exacerbates the brain damage caused by cerebral ischemia (Relton & Relton, 1992) and traumatic injury (Loddick *et al.*, 1997) and in neuronal primary cultures it can enhance cell death in neurons already exposed to an apoptotic injury (Freilander *et al.*, 1996). Taken together this evidence indicates that application or cellular release of IL-1ß exacerbates the effects of injury *in vivo* and *in vitro*.

Conversely, the i.c.v. injection of IL-1ra at the time of induction of focal cerebral ischemia inhibits brain damage by as much as 70% (Relton & Rothwell,

1992) and the protein is neuroprotective when administered i.c.v. 30-60 minutes after the induction of cerebral ischemia. This indicates that IL-1ß may play a direct role in neurodegeneration as the i.c.v. injection of an antibody to IL-1ß also inhibits damage caused by cerebral ischemia (Yamasaki *et al.*, 1995). However *in vivo* and *in vitro* experiments have also shown that IL-1ß may have a protective role in the brain. It has been shown to induce the neurotrophin Nerve Growth Factor (NGF) (Spranger *et al.*, 1990), to inhibit calcium entry into neurons (Plata-Salaman *et al.*, 1991) and enhance the activity of the inhibitory neurotansmitter gamma-aminobutyric acid (GABA) and its receptor function (Coogan *et al.*, 1997; Miller *et al.*, 1997). Indeed there is growing evidence that IL-1ß may have a concentration-dependent biphasic action on the brain. Intracerebroventricular injection of 10 and 100 pg IL-1ß in the rat caused hyperalgesia to mechanical stimuli however higher doses (1 and 10 ng/rat) induced an analgesic effect (Yabuuchi *et al.*, 1996) and in rat cortical synaptosomes IL-1ß was found to have an inhibitory effect on calcium influx at 3.5 ng/ml and a stimulatory effect at a higher (100 ng/ml) concentration (Campbell & Lynch, 1998).

1.7 Apoptosis

Apoptosis is an important process in a wide variety of biological systems and it is crucially involved in normal cell turnover, embryonic development and the developement and maintenance of the immune system. However inappropriate apoptosis is believed to be involved in a number of neurodegenerative diseases including Alzheimer's disease, multiple sclerosis and ischemic brain damage. It is believed apoptosis is triggered in these conditions, at least in part, by oxidative stress. Apoptosis is charachterised by the condensation and fragmentation of nuclear chromatin, the compaction of cytoplasmic organelles, the dilatation of the endoplasmic reticulum, a decrease in cell volume and membrane blebbing which is believed to facilitate recognition and phagocytosis of an apoptotic cell by immune cells. The

precise mechanisms that occur during apoptosis have still to be elucidated (Aravind *et al.*, 1999). The biochemical processes that are involved were first elaborated in the nematode *Caenorhabditis elegans*. It was found that during the course of its normal development 131 cells out of 1090 cells die by apoptosis. Two genes *ced-3* and *ced-4* were found to be vital for this process while the *ced-9* gene prevented cell death. The CED-3 protein was discovered to share homology to Interleukin-1ß-converting enzyme (ICE) or caspase-1 and this protein was implicated in apoptosis in mammalian cells (Ellis *et al.*, 1991). Mammalian sensory neurons undergo apoptosis upon transfection with the active caspase-1 protein (Miura *et al.*, 1993) and Crem A, a cowpox viral protein that inhibits caspase-1 activity is capable of rescuing sensory neurons from NGF deprivation (Gagliardini *et al.*, 1994). Caspases are a family of 10 cysteine proteases that cleave protein, upon activation, after an aspartate residue. The precise role and interaction of caspases in apoptosis is still being investigated but it is known that they are synthetised as inactive pro-enzymes which are activated upon cleavage at specific aspartate cleavage sites. This group of proteases can be divided into 3 subfamilies; the ICE subfamily (caspase 1, 4, 5), the CPP32 subfamily (32kDa cysteine protease; caspase 3, 6, 7, 8, 9, 10) and the ICH-1 subfamily (Ice and Ced-3 homolgue; caspase 2) (Marks, 1999).

For the purpose of this thesis only caspase-1 and caspase-3 will be discussed. Caspase-1 is found in the cytosol of neurons in its inactive pro-enzyme form. The overexpression of murine caspase-1 gene induces apoptosis in murine fibroblasts. However mice deficient in caspase-1 develop normally, appear healthy and are fertile with no apparent abnormalities. This evidence suggests caspase-1 may not play a crucial role in apoptosis or that other proteases may compensate for its loss of function in these transgenic animals (Kiuda *et al.*, 1995; Li *et al.*, 1995). Caspase-3 in its active form is believed to be the key executioner in neuronal cells. It is responsible for the partial or total cleavage of many key proteins including Poly(ADP-ribose) polymerase

(PARP)(Lazebnik *et al.*, 1993), sterol regulatory binding protein (Wang *et al.*, 1995), actin (Kothakota *et al.*, 1997), fodrin (Janicke *et al.*, 1998) and ribonucleicproteins (Casciola-Rosen *et al.*, 1996). Caspase-3 deficient mice do not survive past 3 weeks and the brain development of these mice is abnormal with marked hyperplasia evident (Cohen, 1997).

1.7.1 Hierarchy of Caspase Involvement in Apoptosis

All caspases are cleaved at specific aspartate residues and it has been proposed that some caspases sequentially activate others (Martin & Green, 1995). Caspase 8 is believed to be an "initiator" which may be activated upon receptor activation or physiological stress. It is believed receptor-associated adaptor proteins facilitate the close association of certain caspases and this can promote autocatalysis of caspases (Boldin *et al.*, 1996). In neurons "apoptosis associated factors" or Apaf's are important and Apaf-1 may cluster caspases at intracellular sites and promote autoprocessing (Marks & Berg, 1999).

Caspase-8 is capable of activating "amplifier" proteases such as caspase-1 which then, in turn, are capable of activativing "effector" or "machinery" caspases such as caspase-3 which cleave cellular substrates such as PARP and initiate cell death. Also the activation of some caspases, such as caspase-6, results in their being able to cleave the caspase initially responsible for their formation thus contributing to the amplification effect (Cohen, 1997).

1.7.2 Cytochrome c

It was discovered that the protein Apaf-2 was cytochrome c, which is essential for cell survival while present in the mitochondria, but when present in the cytosol initiates cell death. Microinjection of cytochrome c into sympathetic ganglia is cytotoxic to these calls by a pathway that is sensitive to caspase inhibitors however

injection of this protein into cells lacking the functional caspase-3 gene is non-toxic (Zhivotovsky *et al.*, 1998; Liu *et al.*, 1996). It is believed that cytochrome c is required for the conversion of pro-caspase 3 to the active enzyme. This is thought to be due to its ability to interact with Apaf-1 which furthur enables interaction with caspase-9, transforming the caspase into its active form which then triggers a cascade of caspase activation and the initiation of cell death. Further studies in cell free systems show that cytochrome c can initiate processing of other caspases including caspase-2, 3, 6, 7, 8, and 9. This protein appears to be a key mediator in apoptosis (Slee *et al.*, 1999).

1.8 Aging

Two criteria which characterise aging are (a) the probability of death increases for an organism with time and (b) that an organism undergoes distinctive changes with time. One theory of aging asserts that it is the cumulative damage caused by ROS over a lifetime that gives rise to aging (Harman, 1981). There is some experimental evidence that supports this view. Transgenic Drosophilia that overexpress both CuZnSOD and catalase and thus increase their antioxidant capacity, live 34% longer than wild type controls (Orr & Sohal, 1994). Also the age-1 mutant of *C.elegans*, which possesses increased concentrations of SOD and catalase and, as a result, is more resistant to oxidative stress, lives twice as long as wild type (Vanfleteren & DaVreese, 1996; Larsen, 1993). It is important to acknowledge that these animal models are comprised wholly of post-mitotic cells and thus are more susceptible to cumulative oxidative damage. Mice knockouts for GPX1, SOD1 or SOD3 do not display characteristics of rapid aging despite having a compromised antioxidant defence system (Ho *et al.*, 1997; Melov *et al.*, 1998). An extension of the free radical theory of aging is that free radical production enhances the mutation rate of DNA and more specifically of mitochondrial DNA (mtDNA). It is known that the mutation rate for

mtDNA is 10-20 times greater than for nuclear DNA and it has been suggested that this may lead to a compromise of mitochondrial function, and specifically to a decrease in ATP synthesis and an increase in free radical production (Cortopassi *et al.*, 1990). Mitochondrial genomes of humans and rats accumulate deletion and/or rearrangement mutations with age (Gadaleta *et al.*, 1994; Cortopassi *et al.*, 1990). It is suggested that mtDNA mutations accumulate preferentially in post-mitotic tissues that are metabolically very active such as neurons. There is also evidence of an age-related decline in the electron transport chain activity in mice, rats and humans (Papa, 1996) and in the primary drive in the synthesis of ATP, the mitochondrial membrane potential (Hagen *et al.*, 1997). This evidence suggests that in post-mitotic cells such as neurons, oxidative damage to mitochondria may lead to further oxidative stress to the cell and enhance ROS mediated cellular damage.

1.8.1 Aging and the Neuronal Membrane

There are age-related changes in membrane composition in the brain and this is believed to contribute to functional deficits that occur with time. These changes are coupled with a decrease in membrane fluidity and this is believed to be due to a number of factors. There is an age-related increase in the cholesterol content in the aged brain and an increase in the cholesterol:phospholipid ratio in the hippocampus (Calderini *et al.*, 1983) and this is accompanied by an increase in sphingomyelin which can also adversely impact on membrane fluidity (Giusto *et al.*, 1992). Brain membranes are rich in phospholipids containing the long chain polyunsaturated fatty acids arachidonate and docodsahexanoate. In the aged brain there is a decrease in phospholipid metabolism and a decrease in the incorporation of fatty acids into phospholipids (Bazan, 1989; Fonlupt, 1994). The high polyunsaturated fatty acid content of brain lipid leaves neuronal membranes vulnerable to free radical attack and there has been an age-related increase in lipid peroxidation reported in the aged rat

hippocampus with a parallel decrease in the concentration of arachidonic acid in that tissue (Lynch *et al.*, 1994; McGahon *et al.*, 1997). Changes in the fluidity of a membrane are likely to have knock-on effects on the membrane-associated signal transduction mechanisms and, ultimately, on neuronal function.

1.8.2 Aging and Neuron Density

It was believed in the past that most neocortical and certain hippocampal regions lost 25-50% of their neurons with age but this data was derived from the measurement of neuronal density rather than the counting of actual cell numbers. More recently with advances in histology it has been realised that there is little to no neuron loss in these structures as a consequence of aging (Pakkenberg & Gundersen, 1997). In a key experiment proving this hypothesis aged and young rats were placed in a Morris water maze and trained in a spatial learning task before the total number of neurons in the principal layers of the dentate gyrus and hippocampus were quantified. Neuronal number was preserved in aged animals although spatial learning was significantly impaired (Rapp & Gallagher 1996; Rapp *et al.*, 1987). Despite the fact that neuronal numbers in the hippocampus are unaffected by age, there is a functional deficit associated with aging in both rodents and in non-human primates. The best characterised of these is the failure to maintain LTP (Flood & Coleman, 1988; Barnes & McNaughton, 1985). This implies that while there is no circuit degradation there are more subtle biochemical changes occurring which adversely affect function.

1.8.3 Aging and LTP

It is known that there are deficits in spatial information processing in aged animals (Rapp *et al.*, 1987) and it has been well characterised that aged rats have a reduced ability to maintain LTP (Barnes & McNaughton, 1985; Lynch & McGahon, 1997; Murray & Lynch, 1998). Reduced LTP in the CA1 region of the hippocampus

has been shown to be associated with an age-dependent impairment in spatial learning (Diana *et al.*, 1995) however it has yet to be shown conclusively that an impairment in spatial learning is paralleled by an impairment in LTP in any specific region of the hippocampus. Among the age-related changes which might contribute to compromised LTP are an increase in lipid peroxidation with age in the hippocampus (Lynch *et al.*, 1994), an age-related decrease in the release of glutamate in synaptosomes prepared from the dentate gyrus (Mullany & Lynch, 1997), an age-related decrease in calcium channel number and activity with age (Govoni *et al.*, 1985; Verkhratsky *et al.*, 1994) and a decrease in membrane calcium channel density (Moresco *et al.*, 1990). Other age-related neuronal changes include a decrease in the activity of the Ca^{2+} ATPase (Horn *et al.*, 1996), a decrease in the transport of Ca^{2+} across the mitochondrial membrane and an increase in the intramitochondrial Ca^{2+} concentration (Vitorica & Satrustegui, 1986). These effects may lead to mitochondrial dysfunction, a decrease in ATP synthesis and an increase in ROS production. There is also a general decline in protein synthesis in the whole brain (Goldspink, 1988) and in the entorhinal cortex where an age-related decline in the expression of synaptophysin, an important component in transmitter release, has also been reported (Mullany & Lynch, 1997; Saito *et al.*, 1994).

1.9 Stress

Stress can be described as a condition that disturbs physiological or psychological homeostasis. In mammals the limbic-hypothalamus-pituitary-adrenal system is the major component of the stress response. This system receives neuroendocrine, autonomic, emotional and cognitive inputs into the limbic and hypothalamic strucures and intergrates these inputs to elicit the appropriate magnitude and duration of response to the stressful stimulus.

The adrenal cortex synthesises 2 main classes of steroid: the corticosteroids and the androgens. The corticosteroids can be further divided into 2 classes, the mineralocorticoids which are primarily responsible for the maintenance of salt homeostasis and the glucocorticoids which are the main effectors of the stress response (Fuchs & Flugge, 1998). Glucocorticoids profoundly affect carbohydrate, protein and lipid metabolism and their key sites of action are the cardiovascular system, skeletal muscle and nervous system. One of their key functions is to increase the supply of glucose to cells and inhibit unnecessary physiological mechanisms that may utilise this extra glucose (Marginos *et al.*, 1997).

Stressful environmental conditions activate the HPA resulting in the secretion of corticotrophin-releasing hormone (CRH) by the neurons in the paraventricular nucleus of the hypothalamus. This hormone is released into the capillary bed of the median eminence and is carried by the portal blood flow to the anterior pituitary where it initiates the cleavage of proopiomelanocortin into adrenocoticotrophin (ACTH). This hormone is released into the circulation and travels to the adrenal cortex where it promotes the *de novo* synthesis and secretion of corticosteroids by stimulating the conversion of cholesterol to pregnenolone which is the precursor of all adrenal corticosteroids (Imura & Fukata, 1997). In humans, the main glucocorticoid is cortisol while its equivalent in the rat is corticosterone. Corticosteroids exert a negative feedback control on both the anterior pituitary and hypothalamus and thus the release of these hormones is very tightly controlled. In mammals there is a diurnal fluctuation of glucocorticoids in the body; in humans approximately 20-30mg of cortisol is secreted per day with high concentrations found in the morning with a gradual decline during the rest of the day (Reagan & McEwen, 1997). Corticosteroids act by modulating the rate of protein synthesis. They interact with specific receptors in the cytosol to form a steroid-receptor complex. This translocates to the nucleus and binds

to specific glucocorticoid responsive elements present in the promoter region of select genes. They generally promote the transcription of these genes (McEwen, 1992).

1.9.1 Cytokines, Stress and the CNS

Cytokines that are found in the brain may be either of peripheral or central origin. Those circulating in plasma are believed to enter the brain via the circumventricular organ and cross the BBB via a saturable bi-directional transport. Cytokines may also be released by activated T-lymphocytes that enter the CNS in response to injury or infection (Banks *et al.*, 1990). They are also produced centrally by neurons, astrocytes and microglia (De Cunha *et al.*, 1993). Cytokines are believed to be co-expressed by cells and co-localised with neurotransmitters, and neurotrophic factors. IL-1ß and NGF are co-localised throughout the adult rat brain (Rothwell, 1999). There is controversy as to whether cytokines are expressed constituitively in the CNS or expressed only in response to stress. Much of this controversy is due to the extremely low concentration of cytokines required to elicit a biological effect and a lack of sensitivity in current detection assay systems.

Pro-inflammatory cyokines, like IL-1ß, link the immune system with the endocrine system. It has been demonstrated that the exogenous administration of cytokines can induce activation of not only the HPA axis but also cause the inhibition of thyroid function, sexual function and alterations in carbohydrate and lipid metabolism. This communication between the immune and endocrine systems is bi-directional. Glucocorticoids selectively inhibit the transcription of the IL-1ß gene and decrease the stability of IL-1ß mRNA and are thus thought to have a negative regulatory effect on pro-inflammatory cytokines (Lee *et al.*, 1988).

Numerous cytokines activate the HPA axis when given systemically to rodents, rabbits or humans and these include IL-2, IL-6 and TNF-alpha (Naito *et al.*, 1989; Sharp *et al.*, 1989; Denicoff *et al.*, 1989; Fukata *et al.*, 1989). They act via the

hypothalamus to cause the secretion of CRH. There is evidence they may also increase the expression of proopiomelanocortin (Harbuz *et al.*, 1992). IL-1, IL-2, IL-6 and IFN-α also can act directly on the adrenal cortex to enhance the biosynthesis of glucocorticoids (Tominaga *et al.*, 1991).

IL-1ß stimulates the secretion of hypothalamic CRH and this effect is believed to be involved in the pyrogenic and thermogenic effects of this cytokine (Saplosky *et al.*, 1987; Rothwell & Luheshi, 1989). The hypothalamus contains IL-1 receptors and IL-1 immunoreactive neurons are heavily distributed in this region (Breder *et al.*, 1988; Lechan *et al.*, 1990; Katsuura *et al.*, 1988). However IL-6 synthetised via the increase in IL-1 and TNF-α is suggested to mediate the activation of the HPA (Fukata *et al.*, 1993; Perlstein *et al.*, 1993). In the CNS it is thought glial and neuronal cells release IL-1ß and TNF-α in response to injury or infection. This induces the synthesis and release of IL-6 which acts directly on hypothalamic neurons to induce the secretion of CRH.

1.9.2 Stress and LTP

Stress is known to induce an increase in circulating corticosteroids and to inhibit LTP in the hippocampus however glucocorticoids have been shown to have a biphasic effect on LTP with low concentrations enhancing LTP and high concentrations inhibiting LTP (Diamond *et al.*, 1994; Diamond *et al.*, 1992; Pavlides *et al.*, 1993; Diamone *et al.*, 1989). This effect is believed to be due to the high density in the hippocampus of the 2 types of glucocorticoid receptor. The type I receptor (Mineralocorticoid receptor (MR)) has a high affinity for glucocorticoid and receptor occupancy is nearly maximal at basal concentrations of corticosterone. The type II receptor (glucocorticoid receptor (GR)) has a lower affinity for corticosterone and binds the hormone only with the elevation in glucocorticoid concentrations caused by stress (McEwen *et al.*, 1986). The selective activation of type I receptor has been

shown to enhance LTP while type II receptor stimulation induces a long term synaptic depression and a failure to induce and maintain LTP (Pavilides *et al.*, 1994; Pavilides *et al.*, 1995). Also adrenalectomy impairs spatial memory performance in adult rats but consequent selective type I receptor activation restores performance whereas a combination of type I and type II receptor activation impairs performance (Vaher *et al.*, 1994).

The mechanism underlying type II receptor inhibition is unclear however it is known that glucocorticoids are capable of inhibiting the transport of glucose in the hippocampus. This effect is mediated by type II receptor activation and appears to involve the translocation of glucose transporters from the cell surface to intracellular locations and a decrease in the protein synthesis of glucose transporters (Horner *et al.*, 1990; Virgin *et al.*, 1991; Horner *et al.*, 1987). LTP is a highly energy-dependent process and the inhibition of glucose transport is speculated to cause a depletion of neuronal ATP stores. It is known that ATP depletion in hippocampal neurons activates ATP-dependent potassium channels and this results in a hyperpolarisation effect leading to a dampening of neuronal excitability (Ashcroft *et al.*, 1990; Spuler *et al.*, 1988).

1.10 Aging and Glucocorticoids

Long-term exposure to stress or glucocorticoids can cause atrophy of dendritic branches in pyramidal cells of the CA3 region of the hippocampus and also induces a reversible impairment of initial learning of a spatial memory task on an eight arm radial maze (Wooley *et al.*, 1990; Watanbe *et al.*, 1992; Luine *et al.*, 1994). In addition glucocorticoids increase the concentrations of extracellular glutamate in the hippocampus (Stein-Behrens *et al.*, 1994) while more persistent exposure to elevated corticosterone can result in irreversible hippocampal dysfunction (Reagan *et al.*, 1997). The effects of long-term glucocorticoid administration on the hippocampus mimics the

neuroanatomical changes and loss of function in hippocampal neurons of aged animals. This observation led to a development of a model for aging in the hippocampus which stated that the chronic elevation of glucocorticoids that occurs in aged animals leads to a chronic inhibition of glucose transport and a gradual disruption in metabolic homeostasis in hippocampal neurons (Landfield & Eldridge, 1994). This disruption impairs the ability of neurons to carry out metabolically expensive "house-keeping" tasks such as efficient glutamate uptake, intracellular calcium sequestration and oxidative damage repair. The overall effect of this is an impairment of neuronal efficiency in hippocampal neurons of the aged animal. Evidence supporting this model showed that rats which had a lifelong reduction in exposure to glucocorticoids had fewer age-related memory deficits (Landfield 1987). Further evidence came for studies which showed that while glucocorticoid hypersecretion has been observed in most aged rats, one subgroup of aged rats which do not demonstrate deficits in spatial learning did not display glucocorticoid hypersecretion (Issa *et al.*, 1990). Another study noted that aged rats fail to downregulate hippocampal glucocorticoid type II receptors in response to repeated stress and it was suggested that hippocampal neurons from these rats were vulnerable to the consequences of type II receptor activation as discussed previously. This inability to downregulate the type II receptor with age has been postulated to explain why middle-aged rats but not young rats had impaired spatial learning after being treated for 3 months with a high dose of corticosteroids and why mid-aged rats also exhibited impaired spatial learning after being exposed to 6 months of chronic stress (Bodnoff *et al.*, 1994).

1.11 Aim of this Study

The aim of this thesis is to:

(a) assess the efficacy of the antioxidant defence system in cortical tissue prepared from young and aged rats and to measure markers of oxidative stress in that

tissue. The effect of dietary supplementation with high doses of vitamin C and vitamin E will also be assessed.

(b) assess the efficacy of the antioxidant defence system in hippocampal tissue prepared from aged and young rats and to measure markers of oxidative stress. Also the role of IL-1ß in inducing oxidative stress will be examined *in vitro*.

(c) the effect of intracerebroventricular injection of IL-1ß on LTP, the antioxidant defence system and markers of oxidative stress will be assessed. The effect of dietary supplementation with high doses of vitamin C and vitamin E will also be appraised.

(d) the effect of isolation stress on LTP, the antioxidant defence system and markers of oxidative stress will be assessed. The effect of dietary supplementation with high doses of vitamin C and vitamin E will also be examined.

(e) the effect of short and long term incubation with IL-1ß on markers for apoptosis in cultured hippocampal neurons will be assessed.

Chapter 2

Materials and Methods

2.1 Materials.

The full names and addresses of the scources listed below are given in Appendix I

Materials	**Source**
Acetic Acid	Sigma
Acrylamide	Sigma
Aprotinin	Sigma
Arachidonic Acid (AA)	Sigma
Anti-rabbit IgG	Amersham
Anti-rat Cytochrome C Antibody	Santa Cruz
Ascorbic Acid (Diet)	Beeline Healthcare
Bio-Rad	Bio-Rad
Laboratories	
Bovine Serum Albumin	Sigma
Bromophenol Blue	Sigma
Butylated Hydroxytoluene (BHT)	Sigma
Calcium Chloride ($CaCl_2$)	Sigma
Catalase	Sigma
Copper Sulfate ($CuSO_4$)	Sigma
Corticosterone [^{125}I] RIA kit	IDS, Ltd.
Cumene Hydroperoxide	Sigma
DEVD peptide	Santa Cruz
2'7' dichlorofluorescein	Sigma
2'7' dichlorofluorescin diacetate	Molecular Probes
Diethylenetriaminepentaacetic Acid (DETAPAC)	Sigma
Dimethyl sulfoxide	Lennox
Dinitrophenylhydrazine	Sigma

Dithiothreitol (DTT)	Sigma
5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB)	Sigma
Docosahexanoic Acid	Sigma
DNAase	Sigma
dL-α-tocopheryl Acetate (diet)	Beeline Healthcare
DuoSet ELISA IL-1ß Kit	Genzyme
Enhanced Chemiluminescence Detection Kit (ECL)	Amersham
Ethanol	Sigma
Ethylenediamineteraacetic Acid (EDTA)	Sigma
1-Ethyl-3-(3-dimethylaminopropyl)carbo-diimide Hydrochloride (EDC)	Sigma
Ferrous Sulfate ($FeSO_4.7H_2O$)	Sigma
Glutamax	Sigma
Glutathione (GSH)	Sigma
Glutathione Reductase	Sigma
Glycine	Sigma
HEPES	Sigma
Hexane	Sigma
Hydrochloric Acid (HCl)	Sigma
Hydrogen Peroxide (H_2O_2)	Sigma
Leupeptin	Sigma
Magnesium Sulfate ($MgSO_4$)	Sigma
Malondialdehyde (MDA)	Sigma
Mercaptoethanol	Sigma
Methanol	Sigma
Neurobasal Medium (NBM)	Sigma
Nicotinamide Adenine Dinucleotide Phosphate (NADPH)	Sigma

Nitroblue Tetrazolium (NBT)	Sigma
2-Nitrophenylhydrazine Hydrochloride (NPH-HCL)	Sigma
Penicillin/Streptomycin	Sigma
Pepstatin	Sigma
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma
Poly-Lysine	Sigma
Potassium Chloride (KCl)	Sigma
Potassium Hydroxide (KOH)	Sigma
Potassium Phosphate (KH_2PO_4)	Sigma
Potassion Thiocyanate (KSCN)	Sigma
Sodium Azide (NaN_3)	Sigma
Sodium Chloride (NaCl)	Sigma
Sodium Carbonate (Na_2CO_3)	Sigma
Sodium Dodecylsulfate (SDS)	Sigma
Sodium Hydocarbonate ($NaHCO_3$)	Sigma
Sodium Hydroxide (NaOH)	Sigma
Soyabean Trypsin Inhibitor (STBI)	Sigma
Sulfuric Acid (H_2SO_4)	Sigma
Tetramethoxypropane (TMP)	Sigma
Thiourea	Sigma
Thiobarbituric Acid (TBA)	Sigma
Trichloroacetic Acid (TCA)	Sigma
Tris Acid	Sigma
Tris Base	Sigma
Trypsin	Sigma
Tween-20	Sigma
Xanthine	Sigma

Xanthine Oxidase	Sigma
YVAD Peptide	Santa Cruz

2.2 Animals

2.2.1 Housing of Animals

All adult animals used were male Wistar rats. All neonate animals (0-4 days old) were female Wistar rats. All "young" rats used were aged between 2 to 4 months, weighed between 250-350g and were an inbred strain supplied by the BioResources Unit of Trinity College. They were housed in groups of 6 unless stated otherwise. "Aged" rats were used in some of the studies; these were between 22 and 24 months old, weighed between 500 and 550g and were an inbred strain supplied by either the BioResources Unit of Trinity College or Charles River Laboratories, U.K. They were housed in pairs. All rats had free access to food (laboratory rat chow) and water unless stated otherwise. They were maintained under a 12 hr light/dark cycle and at a temperature between 22 and 23°C.

2.2.2 Induction of Stress

In the "stress" studies rats were aged between 2-4 months and obtained from the BioResources Unit, Trinity College. Initially, all rats were housed in groups of 6 for 5 days. Control (non-stressed) animals continued to be housed in groups of six and maintained under a 12 hr light/dark cycle at a temperature between 22 and 23°C. Experimental animals were stressed by social isolation. In this case rats were removed from the group cage and placed alone in a smaller cage for a period of 5 days before being sacrificed. All rats had free access to food (laboratory rat chow) and water unless stated otherwise.

2.2.3 Dietary Manipulation

Animals were randomly divided into subgroups. Subgroups of rats were fed an experimental diet (vitamin diet) of laboratory chow to which dL-α-tocopheryl acetate (vitamin E; 250 mg/rat/day; Beeline Healthcare, Ireland.) dissolved in corn oil was

added and given drinking water containing L-ascorbic acid (vitamin C; 250 mg/rat/day; Beeline Healthcare, Ireland.). The duration of dietary manipulation varied with experiment. Rats fed on the control diet (control diet) were given laboratory chow to which additional corn oil had been added to ensure isocaloric intake with the vitamin diet groups. Before the start of dietary manipulation daily food and water intake was measured for 5 days and during the period of dietary manipulation, rats were offered 95% of their average daily food and water intakes so that the full daily allowances of vitamins would be ingested.

2.3 **Induction of LTP *in vivo*.**

2.3.1 Preparation of Rats

Each rat was weighed before use and anaesthetised by injection of urethane (1.5 g/kg) intraperitoneally. The absence of the pedal reflex was used as an indicator of required depth of anesthesia. This usually occurred within 10 min after injection. The hair of the scalp was trimmed and the rat's head was positioned in a head holder within a stereotaxic frame. A midline incision was made with a scalpel and the skin pulled back to reveal the skull. The periosteum was scraped away to allow precise identification of lambda and bregma. Holes were drilled to allow the insertion of the recording and stimulating electrodes. The recording chamber consisted of a stereotaxic unit attached to the laboratory bench and surrounded by a Faraday cage to isolate it from interference from the external environment. All instruments in the cage were grounded to eliminate noise.

2.3.2 Electrode Implantation

A bipolar stimulating electrode (Clark Electromedical, UK) was placed 4.4mm lateral to lambda and a recording electrode (Clark Electromedical, UK) was placed 2.5mm lateral and 3.9mm posterior to bregma. The stimulating electrode was slowly

lowered ,to a depth of 2-3.5 mm, to the perforant path while the recording electrode was moved, to a depth of 3-3.5 mm, to the granule cell layer of the dentate gyrus. Evoked responses were detected by the recording electrode and displayed on a computer screen. The final position of both the stimulating and recording electrodes were adjusted to give a response between 1 and 2 mV in amplitude and stimuli were then delivered at 30 second intervals.

2.3.3 EPSP Recordings

The population field excitatory post-synaptic potential (field EPSP) was used as a measure of excitatory synaptic transmission in the hippocampus (see Diagram 2.1). EPSPs were achieved by passing a single square wave pulse of current at low frequency (0.033Hz, 0.1 sec., 2msec delay), generated by a constant current isolation unit, to the bipolar stimulating electrode. The evoked response was transmitted via a pre-amplifier (gain 40) to an analogue-to-digital converter (Maclab/2e, Analog Digital Instruments). This was a digitised system linked to an Apple Macintosh computer (Performa 200) which interfaced with the converter via a specifically written software package (Scope,Version 3.36). It was customized to control both the generation of the square wave pulses and recording of the evoked potentials. The field EPSPs were therefore displayed on-line and could be analysed at the time of recording or at a later date. The slope of the field EPSP was taken as the main indicator of excitatory synaptic transmission. Test shocks every 30 sec were delivered for a 10 min. control period to establish baseline recordings. This was followed by delivery of 3 trains of stimuli (250Hz for 200msec) at 30 sec intervals. Recording at test shock frequency then resumed for 40 min.

Diagram 2.1

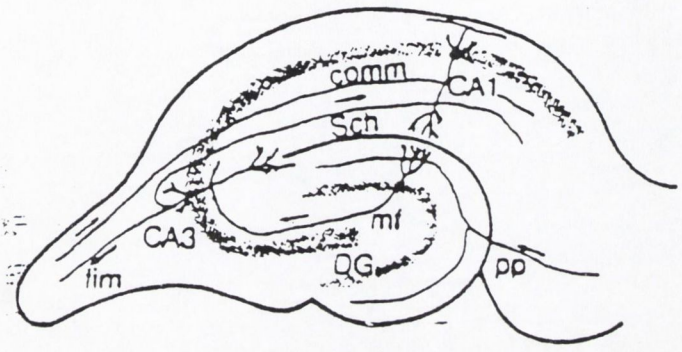
a ***Hippocampus***

Diagram of a traverse section through the hippocampus of the rat showing the main neuronal fields (granule cells of the dentate gyrus and the pyramidal cells of area CA3 and CA1) and the main excitatory afferent projections (pp=perforant path; mf=mossy fibers; Sch comm=Schaeffer collaterals)

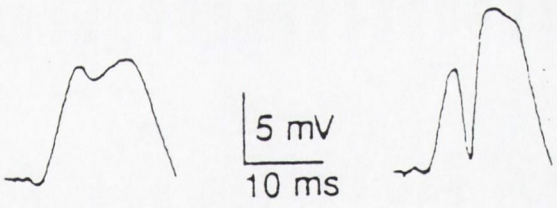
b ***Example of LTP***

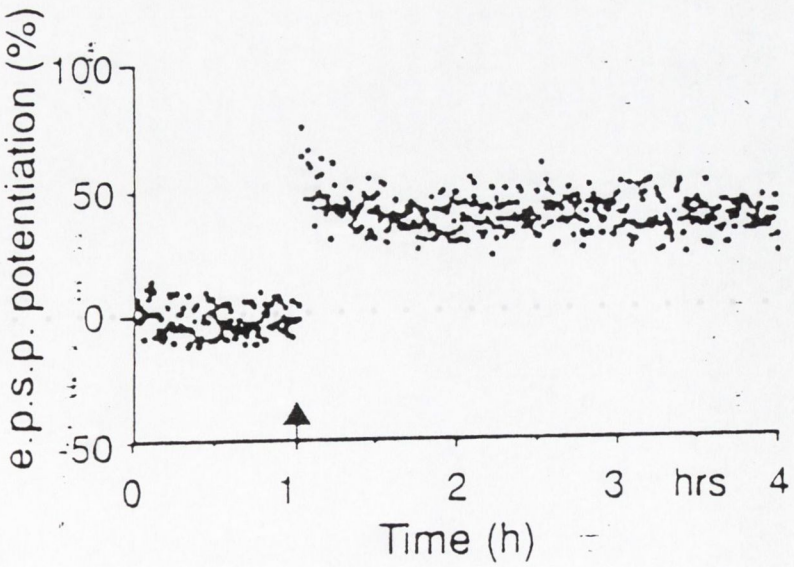
The graph plots the rising phase of the evoked response (population e.p.s.p) recorded from the cell body region in response to constant test stimuli, for 1 hr before and 3h followig a tetanus (50Hz, 200ms). Delivered at the time indicated by the arrow. Representative traces before and after the induction of LTP are illustrated above the graph

a


comm
CA1
Sch
mf
CA3
DG
pp
tim

b


5 mV
10 ms


e.p.s.p. potentiation (%)
100
50
0
-50
0
1
2
3
hrs
4
Time (h)

2.3.4 Intracerebroventricular Injection

In some experiments rats received i.c.v injections before electrophysiological recording began. In these experiments lamda and bregma were identified and a hole was drilled 2.5mm ventral to bregma and 0.5 mm lateral to the midline. 5μl of either 3.5 ng/ml of interleukin-1β (IL-1β) in a 0.9% saline solution or a 0.9% saline solution alone was injected into the ventricle using a Hamilton syringe.

2.4 **Preparation of Tissue.**

All animals were killed by cervical dislocation and their heads removed by decapitation. The brains were removed quickly and placed on a glass platform on an ice bath. The hippocampus and cortex were removed by dissection and portions of both were either homogenised or cross chopped to prepare tissue slices. A portion of tissue (cortex or hippocampus) was removed, quickly weighed to obtain the wet weight and placed in a Jencon's glass homogeniser with 1ml of ice cold Krebs solution (NaCl, 136 mM; KCl, 2.54 mM; KH_2PO_4 , 1.18 mM; $MgSO_4.7H_2O$, 1.18 mM; $NaHCO_3$, 16 mM; $CaCl_2$, 2mM; glucose 10 mM) or 5% trichloroacetic acid (TCA). The teflon glass pestle was inserted and plunged 15 times to break up the tissue. The homogenate was transferred to the appropriate microfuge tubes and frozen at -80°C. Portions of cortex and hippocampus were sliced and frozen according to set protocol (Haan & Bowen, 1981). Slices (approx. 350μm) were prepared by chopping the tissue bidirectionally using a McIlwain tissue chopper. The slices were rinsed once with ice-cold Krebs solution and allowed to settle. The slices were twice rinsed with Krebs solution containing 10% dimethylsulpoxide (Krebs/DMSO). A final aliquot of Krebs/DMSO (250μl) was added and the samples were frozen at -80°C. In the stress experiment aliquots of blood were obtained and frozen at -80°C for corticosterone determination.

2.4.1 Method of Cell Culture

All solutions were sterilised prior to use by filtering through a 2 micron filter and all procedures were carried out in a cell culture laminar flow hood. Coverslips were immersed in 70% alcohol overnight and allowed to dry in a cell culture hood. They were placed in a solution of poly-lysine (20μg/ml) and incubated for 20 min at 37°C. They were allowed dry before being placed in the individual wells of a 24 well plate. Rats (0-4 days old) were decapitated and the brains removed quickly and placed on a glass platform on an ice bath. The hippocampi were isolated and placed in a trypsin solution (10 ml; 0.25mg trypsin/ml Dulbecco's Modified Phosphate Buffered Saline (DM-PBS)) for 25 min at 37°C. A solution of trypsin inhibitor (TI; 10ml, 0.025 mg soyabean trypsin inhibitor (SBTI), DNAase (100μl), $MgSO_4$ (100μl)/ ml DM-PBS) was added and the contents allowed to settle. The supernatant was removed and 2 ml of concentrated TI solution (1mg SBTI, DNAase (100μl), $MgSO_4$ (100μl)/ml DM-PBS) was added. The solution was triturated using a sterile plastic pipette followed by a fire-polished glass pipette and filtered through a gauze into a sterile Falcon tube. The tube was centrifuged (17°C for 2 min at 3000 rpm) and the supernatant removed. Modified Neurobasal Medium (mNBM; 10mls, Fetal Calf Serum (10mls), Glutamax (1ml), Penicillin/Streptomycin(1ml))/100mls NBM) was added and the solution was triturated gently. Drops of the cell suspension were placed onto each coverslip and the plates were incubated for 4 hr at 37°C. mNBM (500μls) was added to each well.

After 3 days the medium was removed from the plates and replaced with mNBM. IL-1ß (3.5ng/ml) or H_2O was added and the plates were incubated for 48 hr. The mNBM solution was removed and replaced with lysis buffer (HEPES (25mM), $MgCl_2$ (5mM), DTT (5mM), EDTA (5mM), PMSF (2mM), leupeptin (10µg/ml) and pepstatin (10µg/ml), pH 7.4)). The plates were left at 4°C for 20 min before the solution was removed and centrifuged at 15,000 rpm for 15 min. The supernatant was removed and stored at -20°C. In some experiments plates were incubated for a further

24 hr before the addition of either H_2O (control), IL-1ß (3.5ng/ml), H_2O_2 (0.5mM) or a combination of IL-1ß (3.5ng/ml) and H_20_2 (0.5mM). After 1 hr the mNBM solution was removed and replaced with lysis buffer (HEPES (25mM), $MgCl_2$ (5mM), DTT (5mM), EDTA (5mM), PMSF (2mM), leupeptin (10μg/ml) and pepstatin (10μg/ml)). The plates were left at 4°C for 20 min before the solution was removed and centrifuged at 15,000 rpm for 15 min. The supernatant was removed and stored at -20°C.

2.5 **Analysis of Samples.**

2.5.1 Analysis of Protein Concentration

Protein concentration was assessed according to the method described by Bradford (Bradford, 1973). In brief homogenate or supernatant (5μl) was transferred to a microfuge tube containing H_2O (155μl) and the Biorad protein dye (40μl; Biorad Laboratories). Standards (6.25-100μg/ml) were prepared using bovine serum albumin (BSA). The standards (160μl) were also mixed with Biorad (40μl). Duplicate aliquots (90μl) of standard and sample were transferred to a 96 well plate and the absorbance read at 630 nm on a Sigma Diagnostics EIA Multiwell Reader. A standard curve was plotted and protein concentration determined.

2.5.2 Determination of Total Glutathione

Total glutathione was measured by the enzyme recycling method of Tietze with some modifications (Tietze, 1969). Samples of tissue homogenised in 5% TCA and frozen as previously described, were thawed and centrifuged at 15,000 rpm for 10 min at 4°C to obtain a pellet and supernatant. These samples were then kept on ice until required. Standards of glutathione (6.25-100 μM) and sample buffer (100mM potassium phosphate buffer, pH 7.5, containing 5mM EDTA) were prepared. Supernatant (30μl) or standard (30μl) was placed in a 1.5ml glass cuvette which was kept on ice. Sample buffer (450μl), glutathione reductase (100μl, 5 units/ml) and

10mM 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB; 50μl) were added to the cuvette. After incubating for 1 min 2.4mM NADPH (100μl) was added and the absorbance at 412 nm was monitored for 2 min using a colorimeter linked to a Maclab 2E Data Acquisition System connected to a Macintosh Performa 5200. A standard curve of change of absorbance (slope of trace) against GSH concentration was constructed. Results are expressed as μmol GSH per gram of tissue.

2.5.3 Determination of Glutathione Peroxidase (GPx) Activity.

Enzyme activity was measured using the coupled assay procedure of Lawrence and Burk (Lawrence & Burk, 1976). Solutions of NADPH were prepared in the range of 6.25-100μM and absorbance of these standards was recorded. A standard curve of absorbance versus NADPH concentration was constructed. Samples of tissue homogenised in Krebs solution and frozen as previously described, were thawed and centrifuged at 15,000 rpm for 10 min at 4°C to obtain a pellet and supernatant. These samples were then kept on ice until required. The reaction medium consisted of; 50mM potassium phosphate buffer (pH 7.0), 1mM EDTA (ethylene diaminetetraacetic acid), 1mM NaN_3 (sodium azide), 0.2mM NADPH, 1 unit of glutathione reductase, 1mM GSH (glutathione) and 1.5mM cumene hydroperoxide in a total volume of 1ml. All ingredients except sample supernatant and peroxide were combined at the beginning of each day. The sample supernatant (100μl) was added to 0.8ml of the above medium and allowed to incubate for 5 min at room temperature before the initiation of the reaction by the addition of 15mM cumene hydroperoxide solution (100μl). Absorbance at 340nm was recorded for 5 min using a spectrophotometer linked to a Maclab 2E Data Acquisition System connected to a Macintosh Performa 5200. The activity was calculated from the slope of the obtained trace and the results were translated, using the NADPH standard curve as a reference, to μmoles NADPH

oxidised per min per milligram of protein. Blank reactions with sample supernatant replaced by distilled water were subtracted from each value.

2.5.4 Determination of the Concentration of Vitamin C

Ascorbic acid concentrations were determined by a modification of the method of Omaye (Omaye *et al.*, 1979). Samples of tissue homogenised in 5% TCA and frozen as previously described were thawed and centrifuged at 15,000 rpm for 10 min at 4°C to obtain a pellet and supernatant. These samples were then kept on ice until required. A solution of 2,4-dinitrophenylhydrazine/thiourea/copper (DTC; 0.04g thiourea, 0.005g copper sulphate ($CuSO_4$) and 0.3g dinitrophenylhydrazine) was prepared and brought to a total volume of 10mls with 9N H_2SO_4 (2.43mls H_2SO_4 in 10mls dH_2O). Ascorbic acid standards (6.25-100 μg/ml) were also prepared in 5% TCA. Supernatant or standard (100μl) was mixed with DTC (20μl) and incubated for 3 hr in a waterbath. Ice-cold 65% H_2SO_4 (150μl) was added and mixed with each sample. This solution stood at room temperature for an additional 30 min before an aliquot (100μl) of each sample and standard was transferred to a 96 well plate. Duplicate samples were read at 545nm on a Sigma Diagnostics EIA Multiwell Reader. A standard curve of ascorbic acid concentration against absorbance was constructed and the results were expressed as μmoles of ascorbic acid per gram of tissue.

2.5.5 Determination of Superoxide Dismutase (SOD) Activity

Enzyme activity was determined by a modification of the method of Spitz and Oberley (Spitz & Oberley, 1989). Samples of tissue, homogenised in Kreb's solution and frozen as previously described, were thawed and centrifuged at 15,000 rpm for 10 min at 4°C to obtain a pellet and supernatant. These samples were then kept on ice until required. An assay mixture was prepared containing; 1.8mM xanthine, 2.24mM nitroblue tetrazolium (NBT), 40 units of catalase, xanthine oxidase (7μl/ml) and

1.33mM diethylenetriaminepentaacetic acid (DETAPAC) dissolved in 50mM potassium buffer (pH 7.8). An aliquot (900μl) of the above mixture was added to a 1.5ml microfuge tube. A series of dilutions were made from supernatant (1:2,1:5,1:10,1:20,1:50 and 1:100) and these were treated as individual samples. Sample (100μl) was added to the microfuge tube containing assay solution (900μl). Phosphate buffer (100μl) served as a blank. The absorbance was monitored at 560nm over 5 min on a spectrometer (Phillips Pye Unicam 8625 UV/VIS Spectrometer, Phillips, U.K.) linked to a Maclab 2E Data Acquisition System connected to a Macintosh Performa 5200. The rate of NBT reduction (slope of trace) was progressively inhibited with increasing concentration of protein. The data was plotted as percentage inhibition of NBT reduction (100-((sample slope/blank slope) *100)) vs protein concentration. One unit of activity was defined as that amount of protein necessary to decrease the rate of reduction of NBT by 50%. Results are expressed in units of SOD activity per milligram of protein.

2.5.6 Determination of Catalase Activity

Enzyme activity was determined by a modification of the method of Cohen (Cohen *et al.*, 1996). Samples of tissue, homogenised in Kreb's solution and frozen as previously described, were thawed and centrifuged at 15,000 rpm for 10 min at 4°C to obtain a pellet and supernatant. These samples were then kept on ice until required. The following reagents were prepared: 60mM H_2O_2 (diluted from stock 30% H_2O_2), 2.5M potassium thiocyanate (KSCN), 10mM ferrous sulfate ($FeSO_4.7H_2O$) and 0.6N H_2SO_4. The sample buffer was 10mM potassium phosphate (pH 7.0). All reagents were kept on ice except for the H_2SO_4 which was at room temperature.

Sample supernatant (100μl) was added to microfuge tubes containing phosphate buffer (800μl). An equal volume of water was added to controls. The reaction was initiated by the addition of 60mM H_2O_2 (100μl) followed by gentle mixing. At 2 and 10 min

intervals after the addition of the H_2O_2 duplicate aliquots (100μl) were removed and quenched by addition to a mixture of 0.6N H_2SO_4 (4 ml) and 10mM $FeSO_4$ (1ml) at room temperature. Colour was developed at room temperature by addition of 2.5M KSCN (400μl). Two aliquots (200μl) from each tube were transferred immediately to a 96 well plate. The plate was read at 492nm on a Sigma Diagnostics EIA Multiwell Reader. The results were expressed in terms of the first order reaction rate constant (k) and protein, as follows:

$$enzyme\ units = k/protein = [ln(A1/A2/t]/protein$$

where ln is the natural log, A1 and A2 are the observed mean absorbance at the two selected time points and t is the time diferential between the two points (8 min.). Results are expressed as enzyme units per milligram of protein.

2.5.7 Determination of Interleukin-1ß (IL-1ß) Concentration

The IL-1ß concentrations were assessed using the Genzyme Diagnostics ELISA (Enzyme linked Immunosorbent assay) IL-1β kit and a general ELISA protocol. A 96-well microtiter plate was coated with capture antibody (100μl, 1:250 dilution in a carbonate buffer (0.32g Na_2CO_3,0.58g $NaHCO_3$ in 100mls distilled H_2O) by incubating overnight at 4°C. The wells were washed with buffer (phosphate buffered saline (PBS; 11.5g Na_2HPO_4, 2.96g NaH_2PO_4 and 5.84g NaCl in 1L (pH7.4)) with 0.05% Tween-20). Blocking buffer (250μl, PBS ; pH 7.4 with 4% BSA) was added per well and incubated for 2 hr at 37°C. The wells were aspirated and washed in wash buffer before the addition of standards and sample homogenate (50μl). The standards were in the range of 62.5-1000 pg/ml of IL-1ß. Samples were incubated for 1 hr at 37°C and washed 3 times before the addition of diluted secondary antibody (100μl, 1:300 dilution with PBS, 1% BSA, 0.05% Tween-20). Samples were incubated for a further 1 hr at 37°C. Diluted detection reagent (100μl, 1:1000 dilution

in PBS with 1% BSA, 0.05% Tween-20) was added and the plate was incubated for 15 min at 37°C before further washing. Working TMB substrate solution (100μl) was added per well and the plate was incubated for 10 min at room temperature. 2N H_2SO_4 (100μl) was added to stop the reaction. The plate was read at 450nm on a Sigma Diagnostics EIA Multiwell Reader within 30 min. A standard curve was constructed plotting the standards against their absorption. Results are given as pg IL-1ß per milligram protein.

2.5.8 Determination of Lipid Peroxidation

Lipid peroxidation was determined by a modification of the method of Dexter (Dexter *et al.*,1990). Standards of tetramethoxypropane (TMP) ranging from 6.25-100 μM/L were prepared. Sample homogenate was thawed and an aliquot (30μl) was placed in a microfuge tube and stored on ice. An assay solution (3.95 ml acetic acid added to 15 ml of distilled H_2O; pH 3.5) was prepared and added to 0.213g sodium dodecylsulfate (SDS) and 0.158g of thiobarbituric acid (TBA). The resultant solution was brought to 50mls by the addition of distilled H_2O. Aliquots of this solution (570μl) were added to microfuge tubes containing the standards (30μl) or samples (30μl) and incubated at 90°C for 1 hr. Aliquots (200μl) from each tube were transferred to a 96-well plate and read at 545nm on a Sigma Diagnostics EIA Multiwell Reader. A standard curve of malondialdehyde (MDA) concentration against absorbance was constructed and results were expressed as nmoles of MDA per gram of tissue.

2.5.9 Determination of Vitamin E

Vitamin E concentration was determined according to a modification of the method of Vatassery (Vatassery *et al.*, 1993). Sample homogenate was thawed and stored on ice. Ethanol (150μl) containing 0.025% BHT (butylated hydroxytoluene)

and 25% ascorbic acid (70μl) were added to each sample and incubated in a water bath at 60°C for 2 min before the addition of 10% (w/v) KOH (135μl; potassium hydroxide). Incubation continued for 30 min at 60°C before placing samples on an ice bath. Hexane (540μl) containing 0.025% BHT was added and mixed thoroughly before centrifugation at 1500 rpm for 6 min. The top phase (hexane phase) was removed and evaporated under a stream of nitrogen. Samples were stored at -80°C until analysis. For analysis the samples were re-suspended in methanol (150μl) with 0.025% BHT. Standards containing 95% α-tocopherol were also prepared in methanol with 0.025% BHT and were in the range of 6.25-100 μg/ml. Samples were analysed by reverse phase liquid chromatography with 75% acetonitrile:25% methanol as mobile phase at a flow rate of 1.2 ml/min. The HPLC machine consisted of a SP8800 Ternary HPLC pump linked to an Ultrasphere ODS (5 microns) column. This in turn was connected to a UV detector (Spectra 100 Variable Wavelength Detector) attached to a computer (Dell Optiplex PC 5133L) to allow recording and analysis of data. Samples were placed in an autosampler (SpectraSYSTEM AS3000) and samples and standards (30μl) were automatically injected into the column. The α-tocopherol was detected using the detector reading at 298nm. Vitamin E was determined from the a standard curve prepared by plotting the area under the peak against the corresponding standard concentration. Results were expressed as nmoles vitamin E per gram of tissue.

2.5.10 Determination of Fatty Acid Concentrations

Aliquots of homogenate (200µl) were thawed and added to a chloroform: methanol (2:1 v/v, 1ml) solution. Fatty acids were extracted by vigorous shaking for 10 min followed by centrifugation at 1000 rpm for 5 min to separate the aqueous and non-aqueous phases. The aqueous layer was discarded and the chloroform layer was evaporated under nitrogen and resuspended in ethanol for analysis. Fatty acids were

analysed as their 2-nitrophenylhydrazine (NPH) derivatives by high pressure liquid chromotography (HPLC). Fatty acids were derivatised by adding 2-NPH-HCl solution (0.02M 2-nitro-phenyl hydrazine-hydrochloride in 40mM HCL-ethanol (3:1, v/v)) and EDC solution (1-ethyl-3-(3-dimethyllaminopropyl)carbo-diimide hydrochloride; 0.25M EDC in ethanol mixed in equal volumes with 3% ethanolic pyridine), and incubated at 60°C for 20 min. After addition of KOH (15% w/v in methanol:water, 80:20) samples were incubated at 60°C for 15 min and cooled in running water. n-Hexane (300μl) and phosphate buffer (400μl, 0.033M, pH 6.4) were added, samples were vortex-mixed for 30 sec, centrifuged for 5 min at 1500 rpm and the hexane phase removed and evaporated to dryness under nitrogen. The HPLC machine consisted of a SP8800 Ternary HPLC pump linked to an Microsorb C18 column. This in turn was connected to a UV detector (Spectra 100 Variable Wavelength Detector) attached to a computer (Dell Optiplex PC 5133L) to allow recording and analysis of data. Samples were placed in an autosampler (SpectraSYSTEM AS3000) and samples and standards (30μl) were automatically injected into the column.

The samples were resuspended in methanol, injected into the column (Microsorb C18) and fatty acid derivatives were separated in isocratic mode with a mobile phase of 85% methanol:15% water (maintained at pH 4.5 with HCl) and detected by UV spectroscopy at 230nm. Individual fatty acids were determined from a standard curve prepared by plotting the area under the peak against the corresponding standard concentration. Individual fatty acid concentrations were expressed as nmol/mg of tissue.

2.5.11 Determination of Interleukin1β Converting Enzyme (ICE) Activity in Tissue

Slices of hippocampus stored in Kreb's/DMSO were thawed and washed three times in fresh Krebs solution. The slices were homogenised in lysis buffer (400μl; 25mM Hepes, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10μg/ml

leupeptin, 10μg/ml pepstatin, pH 7.4) and put through 4 freeze thaw cycles. The samples were centrifuged for 20 min at 15,000rpm at 4°C. Supernatant (90μl) was removed and added to 100μM Interleukin-1β Converting Enzyme substrate (10μl; YVAD peptide, Santa Cruz, USA) and the solution was incubated for 1hr at 37°C. Incubation buffer (900μl; 100mM HEPES, 5mM DTT, pH 7.4)) was added to the solution and the samples were transferred to cuvettes and the emission of fluoresence at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC (6.25-100μM) were prepared and a standard curve of absorbance against concentration was plotted. Results are expressed as nmoles AFC/mg.

2.5.12 Determination of Caspase 3 Activity in Cell Culture

Cell lysate (90μl) was added to 100μM caspase 3 substrate (10μl) (DEVD peptide, SantaCruz, USA) and incubated for 1hr at 37°C. Incubation buffer (900μl; 100mM HEPES, 5mM DTT, pH 7.4) was added to the solution and the samples were transferred to cuvettes and the emission of fluoresence at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC (6.25-100μM) were prepared and a standard curve of absorbance against concentration was plotted. Results are expressed as nmoles AFC/mg.

2.5.13 Determination of ICE Activity in Cell Culture

Cell lysate (90μl) was added to 100μM ICE substrate (10μl) (YVAD peptide, Santa Cruz) and incubated for 1hr at 37°C. Incubation buffer (900μl; 100mM HEPES, 5mM DTT, pH 7.4) was added to the solution and the samples were transferred to cuvettes and the emission of fluoresence at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC (6.25-100μM) were prepared and a standard curve of absorbance against concentration was plotted. Results are expressed as nmoles AFC/mg.

2.5.14 Determination of Reactive Oxygen Species (ROS) Production

Slices of hippocampus stored in Krebs/DMSO were thawed and washed three times in fresh Krebs solution. Synaptosomes were prepared by homogenising in sucrose (0.32M) and centrifuging at 5000 rpm for 5 min at 4°C. The supernatant and "creamy layer" were removed to microfuge tubes and centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was discarded and ice-cold Tris buffer (1ml; 40mM, pH 7.4) added. DCFH-DA (10μl, 5μM) was added and the samples were incubated for 15 min at 37°C. Centrifugation for 8 min at 13,000g at 4°C followed before the removal of the supernatant. Tris buffer (1.5mls, 40mM) was added to the pellets and the samples were vortex-mixed before being placed in a 37°C water bath. The emission at 525nm was measured upon excitation at 488nm in a fluorescent spectrometer linked to a Macintosh Performa 5200 via a Maclab 2E Data Acquisition System. Results are expressed as nmoles DCF/mg.

2.5.15 Gel electrophoresis for the Determination of Cytochrome C

A 12% gel was cast (see Appendix II) between 2 gel plates and the glass plates were mounted on an electrophoresis unit (Sigma Techware, UK) using spring clamps. The upper and lower reservoirs were filled with electrode running buffer (Tris base; 25mM, glycine; 200mM, SDS; 17mM). All samples had their protein concentrations equalised with sample buffer (30μl; Tris-HCL; 0.5M, pH 6.8; glycerol; 10% (v/v); SDS, 10% (w/v); ß-mercaptoethanol 5% (v/v); bromophenol blue, 0.05% (w/v)). Samples were boiled for 5 min after the addition of sample buffer. Sample (10μl) was loaded into the wells. A 30mA current was applied to the gel apparatus and migration of the bromophenol blue was monitored. The current was switched off when the dye band reached the end of the gel (approx. 20 minutes).

2.5.16 Western Blotting for Cytochrome C

Gels were removed from the gel apparatus and placed on nitrocellulose blotting paper wetted by bathing in transfer buffer (Tris base 25mM; glycine, 192mM; methanol 20% (v/v) and SDS 0.05% (w/v); pH 8.3). Filter paper was placed over the gel and another piece was placed under the nitrocellulose paper to form a "sandwich". This was wetted in transfer buffer and placed on a blotter (Sigma Techware, UK). The plates of the blotter had been previously wetted with transfer buffer. These were positioned into place and a constant current was applied (250mA for 1.5 hr). The "sandwich" was removed from the blotter and the nitrocellulose paper was placed in blocking buffer (10% skimmed milk powder (w/v) in PBS-T (NaH_2PO_4, 80mM; NaH_2PO_4, 20mM; NaCl, 100mM containing 0.1% Tween-20)) overnight at 4°C. The nitrocellulose paper was washed in PBS-T 3 times for 15 min. A primary antibody (1:1000 dilution in 2% skimmed milk (w/v) in PBS-T; rabbit anti-rat polyclonal antibody against cytochrome C, Santa Cruz, USA)was added for 1 hr at room temperature. The nitrocellulose paper was washed in PBS-T 3 times for 15 min. A secondary antibody (1:2000 dilution in 2% skimmed milk (w/v) in PBS-T; sheep anti-rabbit polyclonal, Amersham Life Sciences, UK)) was added for 1 hour at room temperature. The nitrocellulose paper was washed in PBS-T 3 times for 25 min. ECL solution (ECL Detection Reagents mixed 1:1 (v/v), Amersham Life Sciences, UK) was added to the paper which was then placed between 2 transparent plastic sheets. Whilst in a dark room a photographic film was placed over the top plastic sheet so as to cover the nitrocellulose paper. These were placed in a cassette which prevented the entry of light and left overnight at 4°C. The film was developed on a Fuji Processor. The intensity of the bands that appeared were taken to be proportional to the concentration of cytochrome C present in the sample. The intensity of the bands were measured using a densitometer and the concentration of cytochrome C present was expressed in arbitary units.

2.5.17 Determination of Corticosterone Concentration in Plasma

Corticosterone was determined by radioimmunoassay (RIA) a technique that is based on the ability of a limited quantity of antibody to bind to a fixed amount of radiolaballed antigen. The percentage of bound radiolabelled antigen decreases as a function of the increasing concentration of unlabelled antigen in the test sample. Separation of the bound and free labelled antigen is accomplished by the addition of a second antibody directed against the primary antibody. The quantity of unlabelled antigen in an unknown sample is then determined by comparing the radioactivity of the percipitate, after centrifugation and decantation, with values established using known standards in the same assay system. Corticosterone was determined using an Immunodiagnostic System Gamma-B ^{125}I Corticosterone kit. Standards were prepared by serial dilution from a 1000ng/ml stock . Standard and samples (100μl) were added to 12*75mm RIA tubes and ^{125}I-corticosterone was added followed by corticosterone anti-serum to all tubes except Total Counts (TC) and Non Specific Binding Tubes (NSB) (100μl; rabbit anti-corticosterone antiserum). All tubes were incubated overnight at 4°C before secondary antibody was added (100μl; goat anti-rabbit globulin) and all tubes vortexed. Saline (0.9% NaCl) was added (1ml) before centrifugation at 5000rpm for 15 min. Supernatant was decanted and radioactivity counted in a Spectron Gamma Counter. The average counts (cpm) were determined and the average NSB counts subtracted from the average Maximun Bound (Bo), standards and unknown samples. This was multiplied by 100 to yield the % B/Bo for each sample

$$\%\ B/Bo = \frac{cpm\ (std,\ or\ unknown) - cpm\ NSB}{(cpm\ Bo - cpm\ NSB)}$$

A semilogarithmic graph of %B/Bo v standard conc was plotted and unknowns determined. Results are expressed as mg of corticosterone/ml.

Chapter 3

Long Term Dietary Supplementation with Vitamin C and Vitamin E Reverses Age-Related Changes in the Rat Cortex.

3.1 **Introduction.**

Reactive oxygen species (ROS), generated primarily through oxidative phosphorylation but also through other pathways (e.g via the mixed function oxidases of the cytochrome P450 system present in the endoplasmic recticulum), are toxic to the cell. To counter these molecules an elaborate defence system has evolved comprising enzymatic and non-enzymatic components.. It is recognised that there is an increase in lipid peroxidation in neuronal tissue of aged animals (Dexter *et al.,* 1989; Rikans & Moore, 1998) and this is indicative of oxidative stress i.e. there is an imbalance between pro and antioxidant forces within the cellular environment. The aim of the study which follows is to describe the age-related changes in the antioxidant defence system of the rat cortex and to measure lipid peroxidation a marker for oxidative stress within that tissue. In addition, the effect of dietary supplementation with antioxidant vitamins on these parameters will be investigated. A furthur aim will be to measure the effect of IL-1ß on lipid peroxidation in the cortex and to measure its concentration in cortical tissue prepared from aged rats.

3.2 **Methods.**

3.2.1 Animals for *in vitro* Experiment

Male adult Wistar rats were housed in groups of 6 and had free access to laboratory chow and water. They were killed by cervical dislocation and their cortex was removed.

3.2.2 Animals and Dietary Supplementation

Young (2-4 months) and aged (22-24 months) male Wistar rats were randomly divided into subgroups (8 young or 12 aged rats in each subgroup). The young rats were housed 4 per cage while the aged rats were housed in pairs. One aged and one young subgroup were fed for 12 weeks on normal laboratory chow supplemented with

a daily dose of dl-α-tocopheryl acetate (250mg/rat/day; 50% Type SD Vitamin E, Beeline Healthcare, Dublin) dissolved in corn oil. Each gram of the dry powder product contained 500mg of dl-α-tocopheryl acetate. Vitamin C (250 mg/rat/day; Beeline Healthcare, Dublin) was added to the drinking water supplied to the cages. The vitamin C product consisted of approximately 97% L-ascorbic acid. The other aged and young subgroups were fed for the same 12 week period on normal laboratory chow with added corn oil to ensure isocaloric intake. The drinking water to these cages was not supplemented with vitamin C. Daily food and water intake was monitored over a 2 week period before the start of dietary supplementation. During the 12 week dietary phase animals were given 95% of their average daily food intake and 100% of their average water intake. Rats were weighed before, periodically during and after the 12 week experiment. General heath and behaviour was monitored daily. The vitamin supplemented diet will be referred to hereafter as the "test diet".

3.2.3 Tissue Storage

Cortical tissue was either; (a) homogenised in Krebs solution and stored at -80°C, (b) chopped into slices placed in a 10% Krebs/DMSO solution and stored at -80°C or (c) homogenised in a 5% TCA solution and stored at -80°C (for more details see section 2.4). For *in vitro* experiments the cortex was sliced and placed into microfuge tubes containing oxygenated Krebs solution and the individual experiments proceeded as described below. After the appropriate incubation, tissue was homogenised in Krebs solution and stored at -80°C.

3.2.4 Analysis of SOD Activity

This method is described in detail in section 2.5.5. Homogenised tissue (see 3.2.3) was thawed and supernatant obtained by centrifugation. The supernatant was serially diluted in phosphate buffer and an aliquot from each dilution was added

to assay buffer. The absorbance at 560nm was monitored for 5 min. The rate of NBT reduction (the slope of trace with time) was progressively inhibited with increasing concentration of protein. The data was plotted as protein concentration versus percentage inhibition of NBT reduction. One unit of activity was defined as that amount of protein necessary to decrease the rate of reduction of NBT by 50%. Results are expressed in units of SOD activity per milligram of protein.

3.2.5 Analysis of GPx Activity

This method is described in detail in section 2.5.3. A standard curve of absorbance versus NADPH concentration was constructed. Homogenised tissue (see 3.2.3) was thawed and supernatant obtained by centrifugation. Supernatant was added to assay mixture and allowed to incubate for 5 min at room temperature before the initiation of the reaction by the addition of 15mM cumene hydroperoxide solution. Absorbance at 340nm was recorded for 5 min using a spectrophotometer. The activity was calculated from the slope of the obtained trace and the results were translated, using the NADPH standard curve as a reference, to μmoles NADPH oxidised per min per milligram of protein. Blank reactions with sample supernatant replaced by distilled water were subtracted from each value.

3.2.6 Analysis of Catalase Activity

This method is described in detail in section 2.5.6. Homogenised tissue (see 3.2.3) was thawed and supernatant obtained by centrifugation. Sample supernatant was added to microfuge tubes containing phosphate buffer. An equal volume of water was added to controls. The reaction was initiated by the addition of 60mM H_2O_2 followed by gentle mixing. At 2 and 10 min duplicate aliquots were removed and quenched by addition to a mixture of 0.6N H_2SO_4 and 10mM $FeSO_4$ at room temperature. Colour was developed at room temperature by addition of 2.5M KSCN.

Two aliquots from each tube were transferred immediately to a 96 well plate and read at 492nm on a Sigma Diagnostics EIA Multiwell Reader. The results were expressed in terms of the first order reaction rate constant (k) and protein.

3.2.7 Analysis of the Concentration of Vitamin C

This method is described in detail in section 2.5.4. Tissue stored in 5% TCA solution (see 3.2.3) was thawed and supernatant obtained by centrifugation. Ascorbic acid standards were prepared in 5% TCA. Supernatant or standard was mixed with assay buffer and incubated for 3 hr in a waterbath. Ice-cold 65% H_2SO_4 was added and mixed with each sample. This solution stood at room temperature for an additional 30 min before an aliquot of each sample and standard was transferred to a 96 well plate. Duplicate samples were read at 545nm on a Sigma Diagnostics EIA Multiwell Reader. A standard curve of ascorbic acid concentration against absorbance was constructed and the results were expressed as μmoles of ascorbic acid per gram of tissue.

3.2.8 Analysis of GSH Concentration

This method is described in detail in section 2.5.2. Tissue stored in 5% TCA solution (see 3.2.3) was thawed and supernatant obtained by centrifugation. GSH standards were prepared in 5% TCA. Supernatant or standard were placed in a 1.5ml glass cuvette which was kept on ice. Sample buffer was added to the cuvette. After incubating for 1 min 2.4mM NADPH was added and the absorbance at 412 nm was monitored for 2 min using a colorimeter. A standard curve of change of absorbance (slope of trace) against GSH concentration was constructed. Results are expressed as μmol GSH per gram of tissue.

3.2.9 Analysis of the Concentration of Vitamin E

This method is described in detail in section 2.5.9 of chapter 2. Homogenate was thawed (see 3.2.3) and stored on ice. The tissue was saponified by boiling and its lipid soluble elements extracted by washing in hexane. The hexane phase was removed and evaporated under a stream of nitrogen. Samples were stored at -80°C until analysis. Standards containing 95% α-tocopherol were also prepared. Samples were analysed by reverse phase liquid chromatography. Samples and standards were placed in an autosampler and aliquots were automatically injected into the HPLC column. α-Tocopherol was detected at 298nm using a SpectraPhysics HPLC. Vitamin E was determined from the a standard curve prepared by plotting the area under the peak against standard concentration. Results were expressed as nmoles vitamin E per gram of tissue.

3.2.10 Analysis of Lipid Peroxidation

This method is described in detail in section 2.5.8. Standards of tetramethoxypropane (TMP) were prepared. Homogenate was thawed (see 3.2.3) and an aliquot was placed in a microfuge tube. Aliquots of assay solution were added to microfuge tubes containing the standards or samples and incubated. Aliquots from each tube were transferred to a 96-well plate and read at 545nm on a Sigma Diagnostics EIA Multiwell Reader. A standard curve of malondialdehyde (MDA) concentration against absorbance was constructed and results were expressed as nmoles of MDA per gram of tissue.

3.2.11 Analysis of IL-1ß Concentration

This method is described in detail in section 2.5.7. Homogenate was thawed (see 3.2.3) and stored on ice. A 96-well microtiter plate was coated with capture antibody. Blocking buffer was added and the plate was incubated. The wells were aspirated and washed before the addition of the diluted standards (obtained from the

ELISA kit) and sample homogenate. Samples were incubated for 1 hr and the plates washed before the addition of diluted secondary antibody for a further 1 hr. Diluted detection reagent (obtained from the ELISA kit) was added and the plate was incubated for 15 min and washed. Working TMB substrate solution was added per well. 2N H_2SO_4 was added to stop the reaction. The plate was read at 450nm on a Sigma Diagnostics EIA Multiwell Reader within 30 min. A standard curve was constructed plotting the standards against absorption. Results are given as pg IL-1ß per gram of tissue.

3.2.12 *In vitro* Incubation of Cortical Slices with IL-1ß

The cortex was sliced and placed into microfuge tubes containing oxygenated Krebs solution. These tubes were then placed in a water bath at 37°C. IL-1ß (3.5ng/ml) or a water control was added to the microfuge tubes and the tissue was incubated for 30 min. After the incubation period the tissue was homogenised and frozen at -80°C.

3.3 **Results**

3.3.1 **Effect of Diet on SOD Activity**

Figure 3.1 indicates that there was a significant increase in the activity of SOD in the cortical supernatant of aged rats fed the control diet compared with the activity of the enzyme in the cortical supernatant of young rats (*$p<0.05$; Student's t-test). This increase in SOD activity in cortical supernatant was not seen in aged rats fed the test diet, but was similar to the activity of the enzyme in the cortical supernatant of young rats.

3.3.2 **Effect of Diet on GPx Activity**

Figure 3.2 indicates that there was no change in the activity of GPx in cortical supernatant of aged rats fed the control diet compared with the activity of the enzyme in the cortical supernatant of young rats. However there was a significant reduction in the activity of the enzyme in the cortical supernatant of rats fed the test diet compared with the activity of the enzyme in the cortical supernatant of aged rats fed the test diet (*$p<0.05$; Student's t-test).

3.3.3 **Effect of Diet on Catalase Activity**

Figure 3.3 indicates that there was no significant difference in the activity of the enzyme in cortical supernatant between young and aged rats.

3.3.4 **Effect of Diet on Vitamin C Concentration**

Figure 3.4 indicates that there was a significant reduction in the concentration of vitamin C in cortical tissue from aged rats fed the control diet compared with tissue of young rats (*$p<0.05$; Student's t-test). This significant reduction was not observed in cortical tissue of aged rats on the test diet compared with tissue of young rats.

3.3.5 **Effect of Diet on GSH Concentration**

Figure 3.5 indicates that there was no change in the concentration of GSH between any of the groups examined.

3.3.6 **Effect of Diet on Vitamin E Concentration**

Figure 3.6 indicates that there was a significant reduction in the concentration of vitamin E in aged animals fed the control diet compared with tissue from animals (*$p<0.05$; Student's t-test). This reduction in the concentration of vitamin E was reversed in the tissue of aged rats fed the test diet compared with tissue from aged rats fed the control diet (*$p<0.05$; Student's t-test).

3.3.7 **Effect of Diet on Lipid Peroxidation**

Figure 3.7 indicates that there was a significant increase in the extent of lipid peroxidation in the tissue of aged rats fed the control diet compared with the tissue of young rats (*$p<0.05$; Students t-test). The extent of lipid peroxidation was significantly attenuated in the tissue of aged rats fed the test diet compared with the tissue of aged rats fed the control diet (+<0.05; Student's t-test).

3.3.8 **Effect of Diet on IL-1ß Concentration**

Figure 3.8 indicates that there was a significant increase in the concentration of IL-1ß in the tissue of aged rats fed the control diet compared with the tissue of young animals (*$p<0.05$; Student's t-test). This increase in the concentration of IL-1ß was significantly attenuated in the tissue of aged rats fed the test diet compared with aged rats fed the control diet.

3.3.9 **Effect of IL-1ß on Lipid Peroxidation**

Figure 3.9 indicates that there was a significant increase in lipid peroxidation in tissue incubated with 3.5 ng/ml IL-1ß for 30 min compared with tissue incubated with a control solution.

Figure 3.1 Dietary supplementation attenuates the age-related increase in SOD activity in cortical tissue.

SOD activity was measured in cortical supernatant prepared from young and aged rats. There was a significant increase in SOD activity in the aged group (n=12) fed the control diet compared with tissue from the pooled young group (n=14). This increase in activity was not observed in aged animals fed the test diet (n=8) compared with tissue from the young rats. Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test). Results are expressed as enzyme units (U)/mg protein.

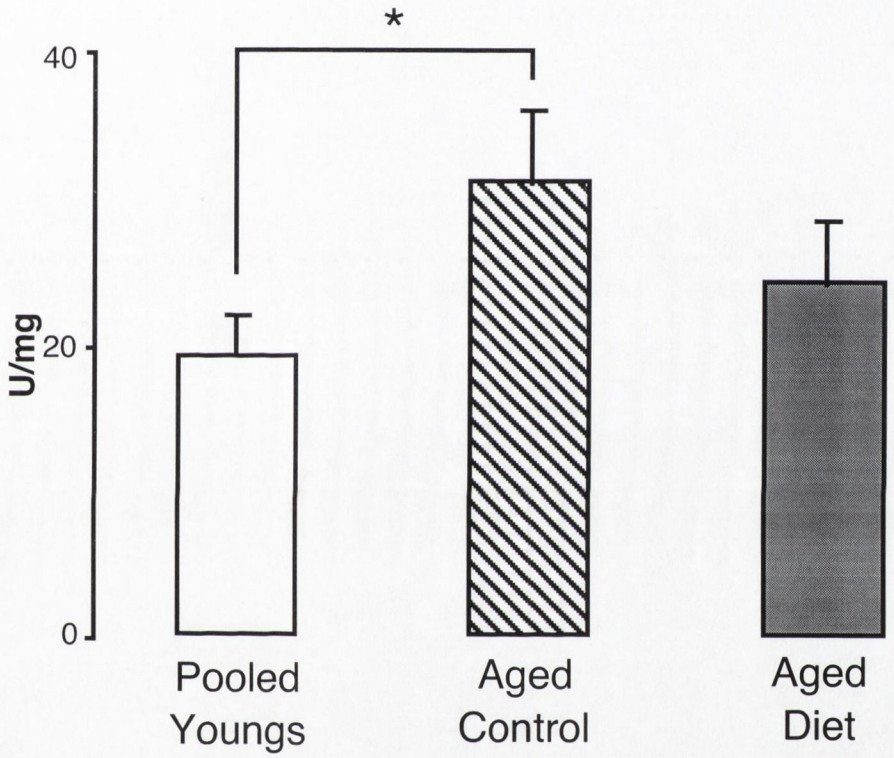

*
40
U/mg
20
0
Pooled
Youngs
Aged
Control
Aged
Diet

Figure 3.2 Dietary supplementation causes a significant reduction in the activity of GPx in aged cortical tissue.

There was no change in the activity of GPx in supernatant prepared from cortical tissue from the aged group of rats fed the control diet (n=10) compared with supernatant from the pooled young group (n=16). There was a significant decrease in activity of GPx in supernatant prepared from cortical tissue of rats fed the test diet (n=8) compared with supernatant from aged rats fed the control diet. Histograms represent means and bars represent standard errors. (*$p<0.05$; Student's t-test). Results are expressed as μM NADPH oxidised/min/mg protein.

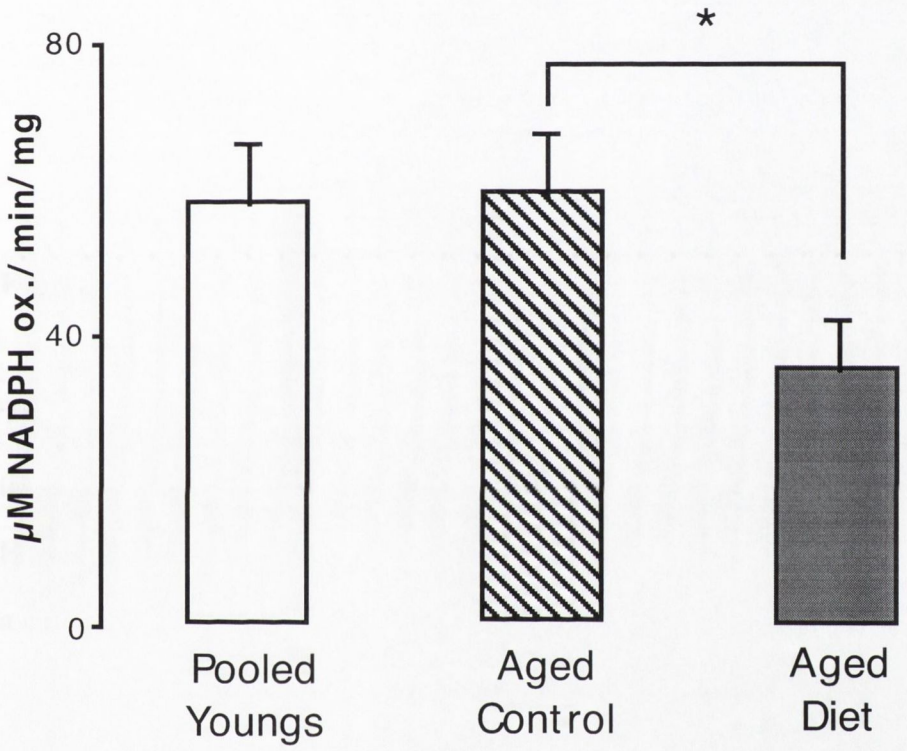

*
80
40
0
μM NADPH ox./ min/ mg
Pooled Youngs
Aged Control
Aged Diet

Figure 3.3 There is no age-related change in the activity of catalase in cortical tissue.

There was no change in the activity of catalase in supernatant prepared from cortical tissue from aged rats (n=6) compared with supernatant from young rats (n=6). Histograms represent means and bars represent standard errors. Results are expressed as a function of the reaction rate (k)/mg protein.

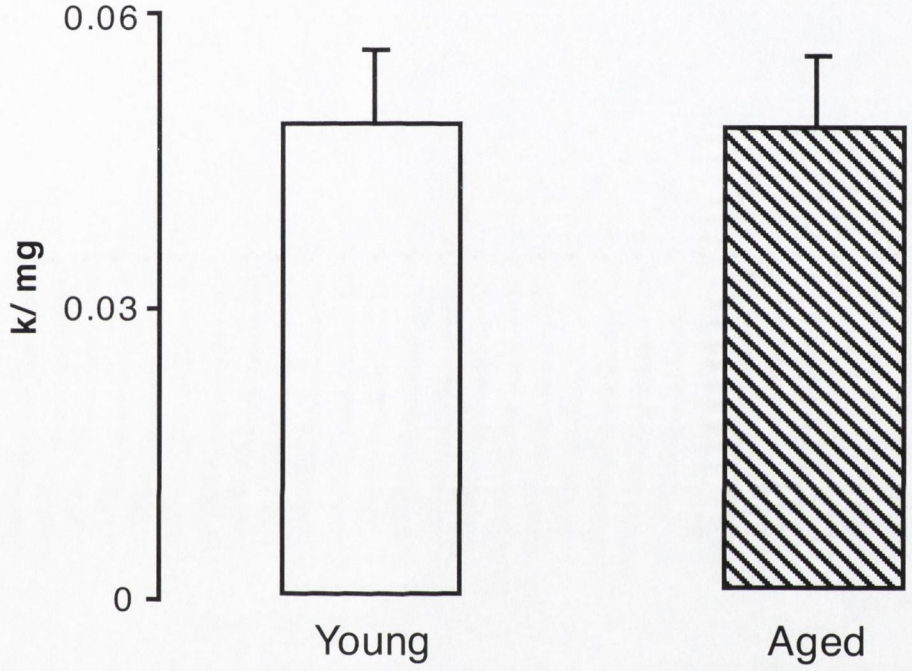

0.06
0.03
0
k/ mg
Young
Aged

Figure 3.4 Dietary supplementation attenuates the age-related decrease in vitamin C concentrations in cortical tissue.

There was a significant decrease in the concentration of vitamin C in cortical tissue from aged animals fed the control diet (n=10) compared with the pooled young group (n=14). This significant decrease in vitamin C concentration was not observed in cortical tissue from aged rats fed the test diet (n=7) compared with tissue from the pooled young group. Histograms represent means and bars represent standard errors. (*$p<0.05$; Student's t-test). Results are expressed as μmoles vitamin C/g tissue.

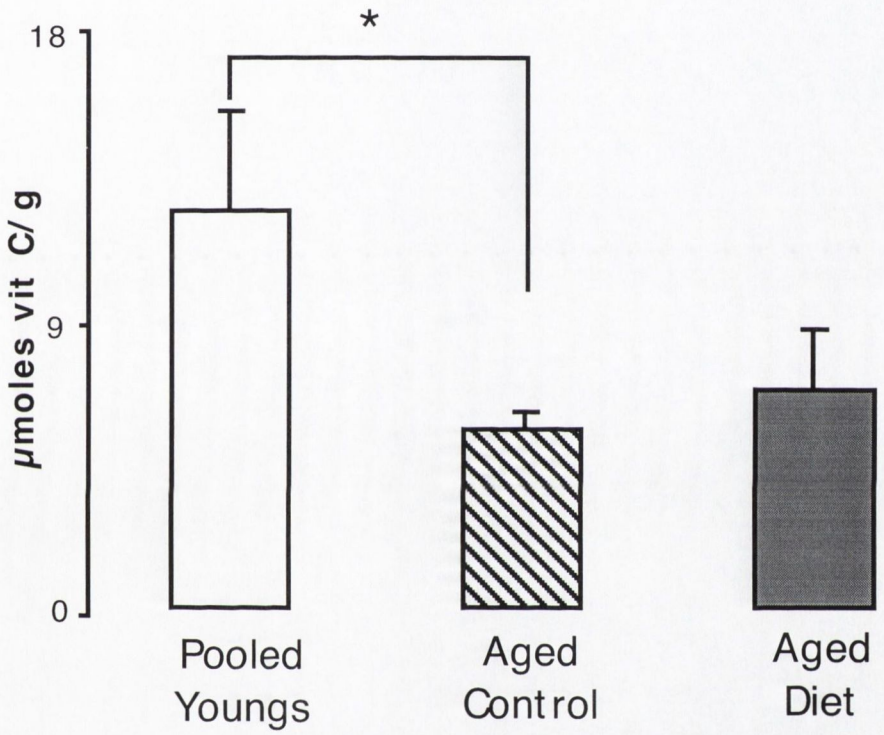

*
18
9
0
µmoles vit C/ g
Pooled
Youngs
Aged
Control
Aged
Diet

Figure 3.5 The concentration of GSH in cortical tissue is unaffected by age or dietary supplementation.

There was no significant differences in the concentration of GSH in cortical tissue from the pooled young group (n=15), the aged group fed the control diet (n=10) or the aged group fed the test diet (n=6). Histograms represent means and bars represent standard errors. Results are expressed as μmoles GSH/g tissue.

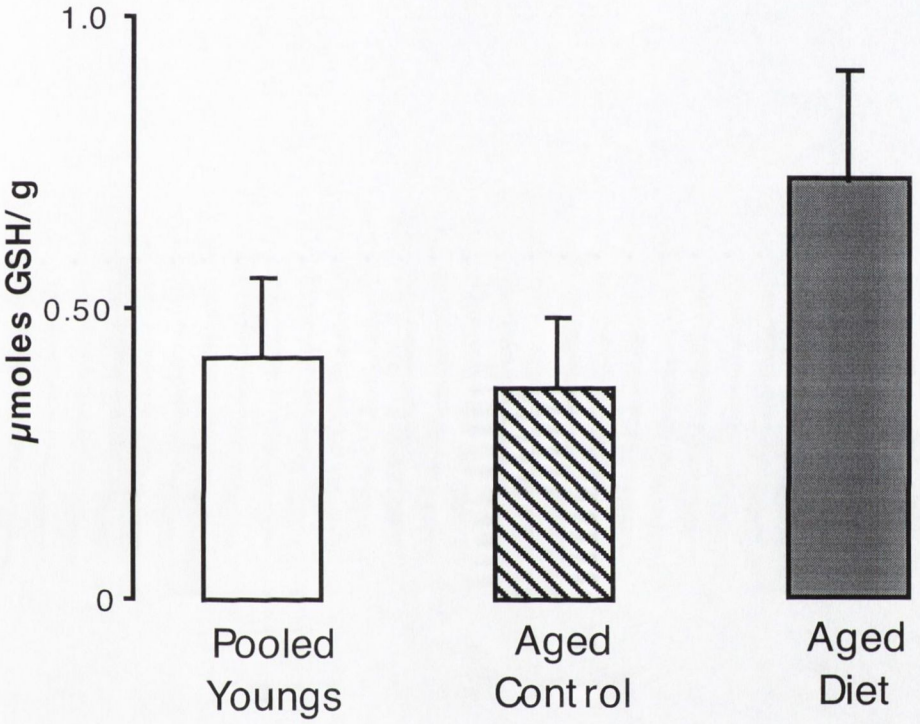

1.0
0.50
0
μmoles GSH/ g
Pooled
Youngs
Aged
Control
Aged
Diet

Figure 3.6 Dietary supplementation reverses the age-related decrease in the concentration of vitamin E.

There was a significant decrease in the concentration of vitamin E in cortical tissue from aged animals fed the control diet (n=11) compared with tissue from the pooled young group (n=9). This decrease was reversed as there was a significant increase in the concentration of vitamin E in cortical tissue from aged animals fed the test diet (n=8) compared with tissue from aged animals fed the control diet. Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as nmoles of vitamin E/g tissue.

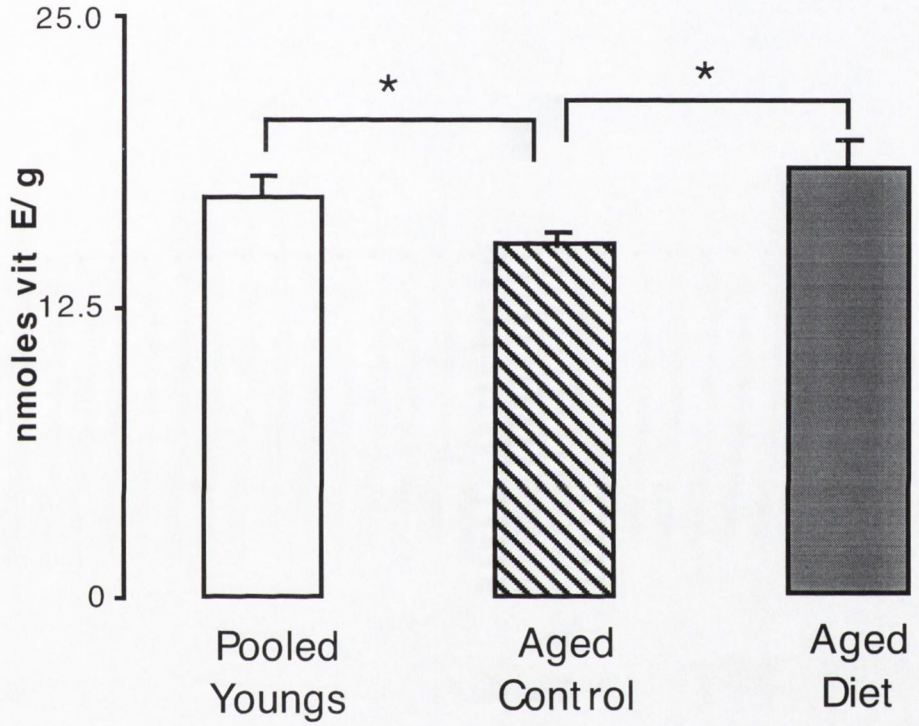

25.0
12.5
0
nmoles vit E/g
*
*
Pooled Youngs
Aged Control
Aged Diet

Figure 3.7 Dietary supplementation reverses the age-related increase of lipid peroxidation in cortical tissue.

There was a significant increase in lipid peroxidation in cortical tissue from aged animals fed the control diet (n=9) compared with tissue from the pooled young group (n=10). There was a significant decrease in lipid peroxidation in cortical tissue from aged animals fed the test diet (n=9) compared with tissue from aged animals fed the control diet. Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as nmoles of MDA/g tissue.

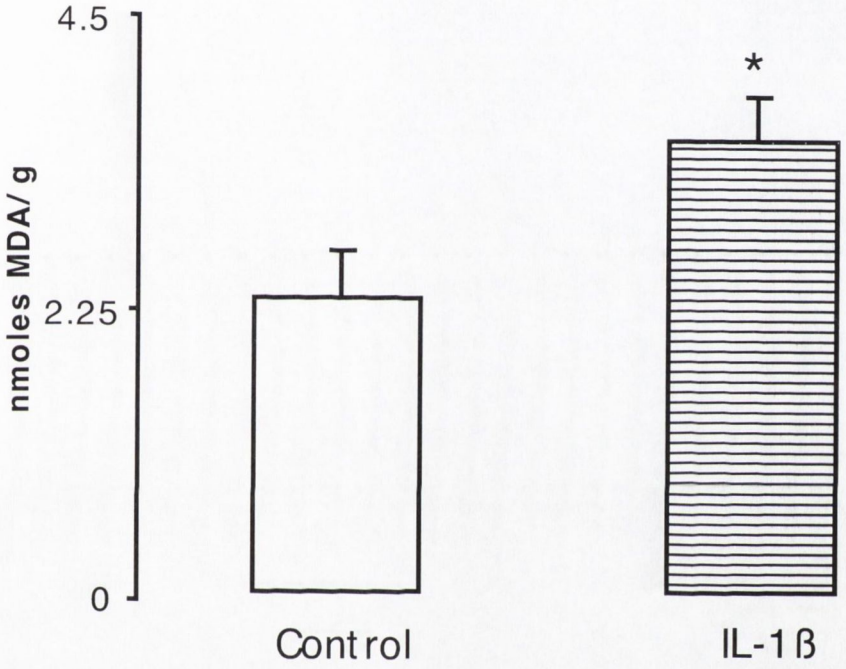

4.5
2.25
0
nmoles MDA/ g
*
Control
IL-1ß

Figure 3.8 Dietary supplementation reverses the age-related increase in the concentration of IL-1ß in cortical tissue.

There was a significant increase in the concentration of IL-1ß in cortical tissue from aged animals fed the control diet (n=12) compared with tissue from the pooled young group (n=14). However there was a significant decrease in IL-1ß in cortical tissue from aged animals fed the test diet (n=8) compared with tissue from aged animals fed the control diet. Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as pg IL-1ß/g of tissue.

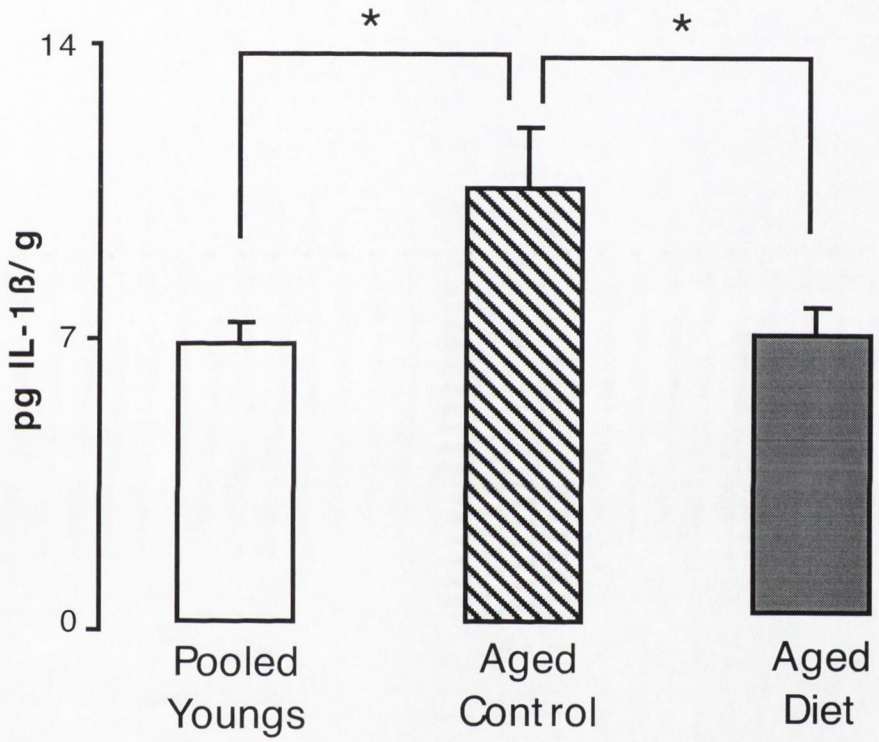
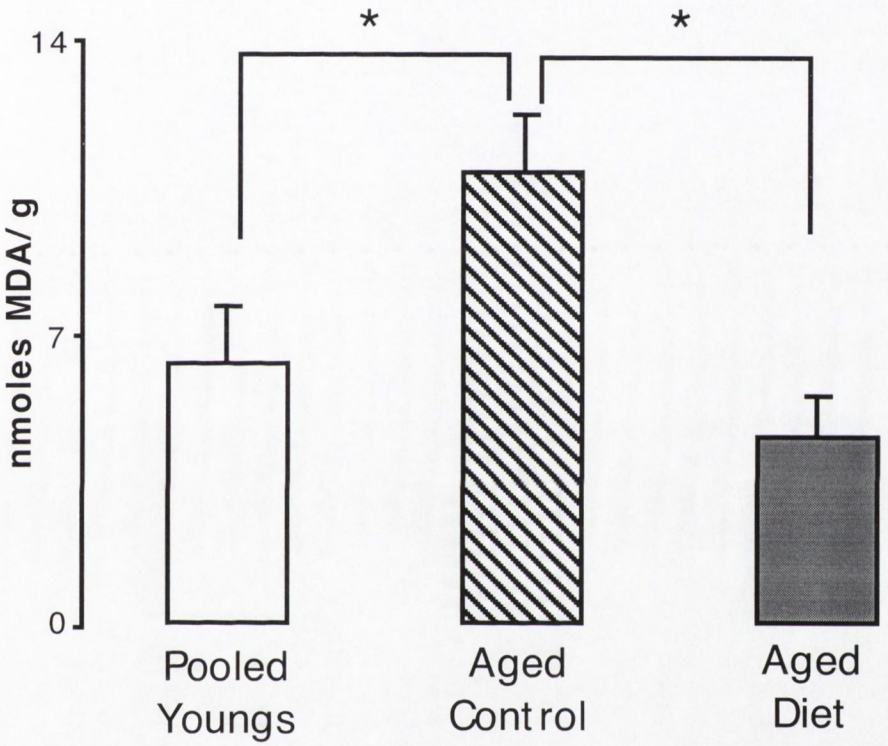

*
*
14
7
0
pg IL-1 ß/ g
Pooled
Youngs
Aged
Control
Aged
Diet

Figure 3.9 IL-1ß causes an increase in lipid peroxidation in cortical tissue *in vitro*.

There was a significant increase in lipid peroxidation in cortical slices prepared from young rats incubated with 3.5 ng/ml of IL-1ß (n=6) for 30 minutes compared with cortical slices incubated with a control solution (n=6). Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as nmoles of MDA/g tissue.


*
*
14
7
0
nmoles MDA/ g
Pooled
Youngs
Aged
Control
Aged
Diet

3.4 **Discussion**

It has been shown that there is an age-related increase in ROS in the cortex (Martin, Pers Comm) and hippocampus (Murray & Lynch, 1998) of male rats and this increase is coupled with an increase in lipid peroxidation in these areas. This implies that neuronal tissue from aged rats is under oxidative stress and the aim of this study was to assess age-related changes in the antioxidative defence system in the cortex of the rat. Is the increase in lipid peroxidation due to a compromised antioxidant defence system? A further aim was to assess the impact on aged cortical tissue of long term dietary supplementation with vitamin C and vitamin E.

The data showed that there was a significant increase in SOD activity, but not in the activities of catalase or glutathione peroxidase, in cortical tissue prepared from aged rats compared to the activity of these enzymes in cortical tissue from young animals. This increase in SOD activity was not seen in the cortical tissue prepared from aged rats fed the test diet. SOD is crucial in the dismutation of $O_2^{\bullet-}$ to H_2O_2 (Halliwell, 1992) and therefore an increase in its activity could imply that there is an increase in the generation of H_2O_2. Not only is H_2O_2 reactive but it is also capable of diffusing through cellular membranes, potentially creating an oxidative stress removed from the site of its generation (Halliwell, 1992). In the presence of transition metals it can undergo a Fenton reaction and generate the highly toxic $OH^{\bullet}$ radical (Aruoma *et al.*, 1989). Evidence of oxidative stress and in particular $OH^{\bullet}$ radical stress was found in the aged gerbil hippocampus and this was accompanied by an increase in lipid peroxidation (Zhang *et al.*, 1993). Studies with transgenic mice with an increased expression of CuZnSOD have shown that there is a long-term oxidative stress generated in muscle and brain tissue of these animals accompanied by an enhanced production of the $OH^{\bullet}$ radical (Peled-Kamar *et al.*, 1997). Further evidence of an age-related increase in SOD activity was obtained from studies using senescence accelerated mice in which an increase in SOD activity was coupled with an increase in

lipid peroxidation (Liu & Mori, 1993). While enhanced SOD activity was also found in the aged rat brain using conventional biochemical assays and electron spin resonance spectrometry (Scarpa *et al.*, 1987; Hiramatsu *et al.*, 1992). In addition, enzyme activity and mRNA concentrations for MnSOD also showed a linear increase with age in the brains of mice. In agreement with the data from this chapter there was no concomitant increase in the activity GPx (Hiramatsu *et al.*, 1992; de Haan *et al.*, 1992).

Glutathione peroxidase and catalase activity did not alter in the cortices of young and aged rats. This finding is in agreement with results found in other studies. For example, there was no change in the activity of GPx, the most important enzyme responsible for H_2O_2 hydrolysis, in the cortex, striatum, hypothalamus and hippocampus of rats aged 4, 15 or 27 months (Vertechy *et al.*, 1993; Geremia *et al.*, 1990). An increase in the ratio between CuZnSOD and GPx activity was observed in the aged mouse brain and this was accompanied by an increase in lipid peroxidation within this tissue. It was noted that in organs, such as the lung, where the CuZnSOD to GPx or catalase ratios were maintained during aging, no increase in lipid peroxidation was detected (Cristiano *et al.*, 1995). In cell lines that had been transfected with the gene for CuZnSOD and had an elevation in the ratio of Cu-Zn SOD activity to GPx activity, higher concentrations of H_2O_2 were produced and markers for cellular senesence were evident. While in CuZnSOD transfected cells with an unaltered enzyme activity ratio there was no increase in H_2O_2 production and no evidence of cellular senescence (de Haan *et al.*, 1996). It seems reasonable to suggest from the data presented in this chapter that a gradual oxidative stress may be created in the rat cortex through an imbalance in the activities of the enzymes of the antioxidant defence system. This imbalance may be responsible for the increase in ROS production observed in aged neuronal tissue. Interestingly, there was a significant reduction in the activity of GPx in the cortical tissue of rats fed the high

antioxidant diet. The reasons for this are unclear but may be due to a downregulation of its activity as age-related oxidative stress is relieved by dietary supplementation with the antioxidant vitamins.

However the reported increase in SOD activity in aged neuronal tissue is still a matter of controversy. Other reports show no difference in the activity of SOD in aged compared to young whole rat brain homogenate (Barja de Quiroga *et al.*, 1990) or else a decrease in SOD activity and in the concentration of SOD mRNA in whole brain homogenate (Rao *et al.*, 1990). There was no change observed in the activity of SOD from whole brain homogenate prepared from aged Sprauge-Dawley rats while SOD activity was found to decrease significantly in whole brain preparations derived from Fischer 344 rats (Kellogg & Fridovich, 1976; Rao *et al.*, 1990). Clearly there are strain differences in the age-related activity profile of this enzyme and in addition, the activity of this enzyme and indeed the activities of other enzymes of the antioxidant defence system appear to vary according to brain region. For example, in one study an age-related increase in SOD activity was observed in the parietal cortex and mesencephalon while in the caudate nucleus SOD activity did not change (Ciriolo *et al.*, 1991). In a second study the specific enzyme activities of SOD and GPx differed with age according to the regions examined in male Wistar rats; these areas included the parieto-temporal cortex, caudate-putamen, substantia nigra and thalamus (Benzi *et al.*, 1988). This implies that studies performed on whole brain homogenate may mask brain regional differences in the activities of these enzymes. Another study concluded that age-related changes in the activities of the antioxidative enzymes are not only region specific but also sex selective in the rat. This report found SOD activity to be markedly increased in the substantia nigra, striatum and hippocampus of aged male rats but lower in the cerebellum, while the activities of GPx and catalase were unaffected. These trends however were not seen in the aged female rat with only a modest increase seen in SOD activity in most brain areas (Carrillo *et al.*, 1992).

Vitamin C concentrations were significantly reduced in the cortical tissue of aged rats fed the control diet and the test diet did not reverse this age-related decline. Electrochemical detection from the nucleus accumbens in 3, 6 and 18 month old rats revealed a similar age-related decrease in the basal extracellular vitamin C concentration in this area (Svensson *et al.*, 1993). The decrease in vitamin C concentrations in the cortex is likely to further contribute to the oxidative load in the brain as not only is this vitamin a potent antioxidant, it also recycles the tocopherol radical, generated through the action of vitamin E on ROS, to the non-radical molecule (Yu, 1996). It is known that this age-related decrease in vitamin C concentration is not confined to neuronal tissue. Aged rat hepatocytes had 54% decrease in vitamin C concentration compared with liver cells from young animals and they also exhibited a significantly decreased vitamin C recycling capacity in response to oxidative stress (Lykkesfeldt *et al.*, 1998). The reason for the lack of change in vitamin C concentration following long term vitamin supplementation is not known, but several possibilities exist. There may be an age-related downregulation of vitamin C transporters in the gastrointestinal lining, along the blood-brain barrier or on the cortical neurons themselves.

Glutathione concentrations were unchanged in aged compared to young cortical samples in the study. This result agrees with data obtained from liver, heart and brain of aged Fischer rats (Rikans *et al.*, 1988) however GSH concentration was found to be lower in the cortex, cerebellum, striatum, thalamus and hippocampus of aged Sprague Dawley rats with a parallel increase in lipid peroxidation in these areas (Ravindranath *et al.*, 1989). The increase in oxidative stress experienced by the aged brain may be exacerbated by the lack of a compensatory increase in the concentration of this important antioxidant and this may contribute to the gradual increase in lipid peroxidation over time observed with aging. Dietary supplementation did not alter the concentration of GSH in the young or aged group.

Vitamin E concentrations were significantly reduced in cortical tissue prepared from aged rats fed the control diet compared to those in young rats. This vitamin resides in the hydrophobic interior of cellular membranes and breaks the lipid peroxidation cycle generated by ROS and lipid peroxyl radicals (Vatassery, 1992). This age-related decrease in vitamin E concentrations may therefore contribute to the age-related increase in lipid peroxidation. This view is in agreement with the findings of other studies. Lipid peroxidation in the brain region of rats fed a vitamin E deficient diet for 9 months were significantly higher than in age matched controls (Noda *et al.*, 1982). Another study reported that expression of mutant human CuZnSOD in mice, as a model of amyotrophic lateral sclerosis (ALS), caused depletion of vitamin E in the central nervous system and an increase in neuronal oxidative stress. Dietary supplementation with vitamin E slows the progression of the disease in this animal model (Gurney *et al.*, 1996). Other reports have shown that vitamin E administration prevents oxidative injury to neuronal synapses caused by the exposure of young rats to 100% O_2 for 48hrs (Urano *et al.*, 1997) which further highlights the important protective effect of vitamin E on neuronal tissue. This is also demonstrated by the reverse experiment where an increase in lipid peroxidation was observed in neuronal tissues from vitamin E-deficient young rats: the cortex of these animals was shown to be particularly vulnerable to *in vitro* oxidative stress (MacEvilly *et al.*, 1996). The data presented in this chapter indicate that dietary supplementation with high doses of vitamin E can reverse this age-related decrease in the concentration of vitamin E in the cortex. Dietary vitamin E supplementation requires a significant amount of time to alter vitamin E concentrations in the brain. Following administration with a single oral dose of radioactive vitamin E to the rat it was found that high radioactivity was recorded in the adrenal cortex, spleen and liver, moderate activity noted in the myocardium, lung and gastric mucosa and very low activity recorded in the brain (Fand & McNally, 1981). This indicates that there is a slow uptake mechanism for this

molecule in the central nervous system. Further evidence of this is provided in another study where 4 week old male rats were fed high concentrations of vitamin E for 4 months. A 4 fold increase in vitamin E concentration was found in the liver, 2.2 fold increase in adipose tissue and serum but only 1.4 fold increase in concentration of the vitamin in the cerebrum, cerebellum and striatum (Vatassery *et al.*, 1988). It is important to note that the small but significant increase in vitamin E concentrations in the aged cortex of rats fed the test diet matched a corresponding decrease in lipid peroxidation.

Aging increases oxidative stress in neuronal tissue. Lipid peroxidation was increased in the cortices of aged rats fed the control diet. Other groups have reported an enhanced lipid peroxidation in the frontal cortex, hippocampus, caudate-putamen and substantia nigra of aged rats (Mizuno & Ohta, 1986;Santiago & Mori, 1993) This reflects the results of many studies which have shown an age-related increase in lipid peroxidation in mammalian brain (Rikans *et al.*, 1997). The data presented in this chapter indicate that the increase in lipid peroxidation parallels the age-related changes to the antioxidant defence system; in particular the alteration in the activity of SOD and the decrease in the concentrations of vitamin C and vitamin E observed in the cortex. The age-related increase in lipid peroxidation was attenuated in the aged rats fed the test diet and mirrored a parallel increase in the concentration of vitamin E and a reduction in the activity of SOD. This indicates that the aged cortex experiences a continual oxidative stress, possibly as a result of a compromised antioxidant defence system, and that this stress can be reduced by the addition of antioxidants to the diet. This result confirms earlier data which showed decreased lipid peroxidation in guinea pig heart and an increase in total antioxidant capacity following vitamin E supplementation (Rojas *et al.*, 1996). Lipid peroxidation was also observed to be reduced in rat kidney following dietary supplementation with a variety of antioxidants including vitamin E and vitamin C (Knudsen *et al.*, 1996).

An increase in lipid peroxidation in tissue is one consequence of an increase in ROS production within that tissue. The data in this chapter showed that incubation of cortical tissue in the presence of IL-1ß *in vitro* caused a significant increase in lipid peroxidation. This implies that this cytokine may trigger an increase in ROS production in aged neuronal tissue. IL-1ß has been shown to induce an increase in ROS production in the hippocampus (Murray & Lynch, 1998). The concentration of IL-1ß was significantly higher in the cortices of aged rats on the control diet. This result was attenuated in rats fed on the test diet. IL-1ß is the predominant IL-1 isoform in the brain. While its concentration is generally low its expression can be increased in response to brain injury or disease states. It can be synthesised by glia and neurons and also by macrophages that may cross the blood brain barrier (Rothwell & Luheshi, 1996). IL-1ß may increase ROS production by initiating an upregulation in the activity of SOD since IL-1ß was shown to increase Mn-SOD gene expression in a time dependent manner in cultured rat hepatocytes (Antras-Ferry *et al.*, 1997). The cytokine was shown to be a strong but slow inducer of this gene. While other studies have shown that IL-1β increased the activities of both the Mn and CuZnSOD isoforms in rat pancreatic islets with a 10-fold increase in MnSOD mRNA content after a 3 hour incubation with the cytokine (Borg *et al.*, 1992). Dietary manipulation with vitamin C and vitamin E reversed the age-related increase in IL-1ß. This suggests that the increase in IL-1ß may be a consequence of an increase in ROS; this is consistent with the view that IL-1ß is increased in response to stress, including oxidative stress. A casual relationship between an increase in IL-1ß concentration and an increase in SOD activity is suggested by the age-related increase in both parameters. Similarily a casual relationship may be suggested between IL-1ß and lipid peroxidation.

It may be hypothesised that a chronic increase of this cytokine in the aged cortex may be responsible for the age-related increase in SOD activity observed in this and previous studies. To hypothesise further, the age-related decrease in the

concentrations of vitamin C and vitamin E in the cortex may result in the generation of an oxidative stress and subsequent neuronal damage. This may lead to the release of IL-1β from neuronal tissue in response to this stress and this, in turn, may trigger a chronic increase in the activity of SOD. Further oxidative stress is generated and a destructive positive feedback loop is created.

The findings presented here suggest that dietary supplementation with antioxidant vitamins significantly decreased oxidative stress in the aged cortex. The data showed that there is an age-related increase in SOD activity and an age-related decrease in the concentration of vitamin E and vitamin C in aged rats fed the control diet. This resulted in a significant oxidative stress in this tissue as assessed by lipid peroxidation. Vitamin supplementation reversed these age-related changes. While the evidence remains tentative and requires further investigation, it is possible that IL-1β may play a role in inducing this age-related oxidative stress, perhaps by increasing the activity of SOD. It is noteworthy that the age-related increase in IL-1β is reversed by antioxidant vitamin supplementation.

Chapter 4

Analysis of Age-Related Changes in the Rat Hippocampus.

4.1 **Introduction**

In the previous chapter it has been shown that there is an age-related decline in the antioxidant defence system of cortical tissue but an increase in the activity of SOD, a major component of that system. An increase in lipid peroxidation and an increase in the concentration of IL-1ß were also observed. In the aged rat hippocampus it is known there is an impairment in maintaining LTP and that this impairment can be overcome by the long-term dietary supplementation of aged rats with high doses of vitamin C and vitamin E (Murray & Lynch, 1998). Could the functional impairment seen in the hippocampus be due to changes in antioxidant defence in hippocampal tissue? The aims of this study will be;(a) to compare the antioxidant defence systems in hippocampal tissue prepared from aged and young rats; (b) to analyse the effects of IL-1ß and H_2O_2 on ROS production and SOD activity in hippocampal tissue *in vitro* and (c) to analyse the effect of H_2O_2 on IL-1ß concentration in hippocampal tissue *in vitro*.

4.2 **Methods**

4.2.1 Animals for *in vitro* Experiments

Male adult Wistar rats were housed in groups of 6 and had free access to laboratory chow and water. They were killed by cervical dislocation and their hippocampii were removed.

4.2.2 Aged and Young Animals

All rats were male Wistars. Young rats were housed 4 per cage while the aged rats were housed in pairs. All rats had free access to laboratory chow and water.

4.2.3 Tissue Storage

Hippocampal tissue was either (a) homogenised in Krebs solution and frozen at -80°C, (b) chopped into slices placed in a 10% Krebs/DMSO solution and frozen at -80°C or (c) homogenised in a 5% TCA solution and frozen at -80°C (for more details see section 2.4). For *in vitro* experiments the hippocampus was sliced and placed into microfuge tubes containing oxygenated Krebs solution and incubated at 37°C for 30 min. After the *in vitro* incubation period, the tissue was homogenised and frozen at -80°C (see section 2.4).

4.2.4 Analysis of SOD Activity

This method is described in detail in section 2.5.5. Homogenised tissue (see 4.2.3) was thawed, supernatant obtained and serially diluted in phosphate buffer. An aliquot from each dilution was added to assay buffer. The rate of NBT reduction was assessed at 560nm for 5 min. Results are expressed in units of SOD activity per milligram of protein.

4.2.5 Analysis of GPx Activity

This method is described in detail in section 2.5.3. A standard curve of absorbance versus NADPH concentration was constructed. Homogenised tissue (see 4.2.3) was thawed, supernatant obtained and added to assay mixture. The reaction was initiated by the addition of 15mM cumene hydroperoxide solution. Absorbance at 340nm was recorded for 5 min using a spectrophotometer. Results were expressed as μmoles NADPH oxidised per min per milligram of protein.

4.2.6 Analysis of Catalase Activity

This method is described in detail in section 2.5.6. Homogenised tissue (see 4.2.3) was thawed, supernatant obtained and added to microfuge tubes containing phosphate buffer. The reaction was initiated by the addition of 60mM H_2O_2. At 2 and

10 min duplicate aliquots were removed and quenched by addition to a mixture of 0.6N H_2SO_4 and 10mM $FeSO_4$ at room temperature. Colour was developed at room temperature by addition of 2.5M KSCN. Two aliquots from each tube were transferred immediately to a 96 well plate and read at 492nm. The results were expressed in terms of the first order reaction rate constant (k) and protein.

4.2.7 Analysis of the Concentration of Vitamin C

This method is described in detail in section 2.5.4. Tissue stored in 5% TCA solution (see 4.2.3) was thawed, supernatant obtained and mixed with assay buffer and incubated for 3 hr in a waterbath. Ice-cold 65% H_2SO_4 was added and mixed with each sample or standard. Samples and standards were read at 545nm and the results were expressed as μmoles of ascorbic acid per gram of tissue.

4.2.8 Analysis of GSH Concentration

This method is described in detail in section 2.5.2. Tissue stored in 5% TCA solution (see 4.2.3) and supernatant. Supernatant or standard was added to sample buffer. After incubating for 1 min 2.4mM NADPH was added and the absorbance at 412 nm was monitored for 2 min using a colorimeter. Results are expressed as μmol GSH per gram of tissue.

4.2.9 Analysis of the Concentration of Vitamin E

This method is described in detail in section 2.5.9. Homogenate was thawed, saponified by boiling and its lipid soluble elements extracted by washing in hexane. The hexane phase was removed and evaporated under a stream of nitrogen. Samples and standards were analysed by reverse phase liquid chromatography with 75% acetonitrile:25% methanol as mobile phase at a flow rate of 1.2 ml/min. Samples and standards were placed in an autosampler and α-tocopherol was detected at 298nm

using a SpectraPhysics HPLC. Results were expressed as nmoles vitamin E per gram of tissue.

4.2.10 Analysis of Lipid Peroxidation

This method is described in detail in section 2.5.8. Homogenate was thawed (see 4.2.3) and an aliquot was placed in a microfuge tube. Aliquots of assay solution were added to microfuge tubes containing the standards or samples and incubated. Aliquots from each tube were transferred to a 96-well plate and read at 545nm. Results were expressed as nmoles of MDA per gram of tissue.

4.2.11 Analysis of IL-1ß Concentration

This method is described in detail in section 2.5.7. Homogenate was thawed (see 4.2.3) and stored on ice. A 96-well microtiter plate was coated with capture antibody before blocking buffer was added per well and incubated. Standards and sample homogenate were incubated for 1 hr before the addition of diluted secondary antibody for a further 1 hr. Diluted detection reagent was added and the plate was incubated for 15 min and washed. Working TMB substrate solution was added. 2N H_2SO_4 was used to stop the reaction. The plate was read at 450nm. Results are given as pg IL-1ß per milligram protein.

4.2.12 Determination of Fatty Acid Concentrations

This method is described in detail in section 2.5.10. Aliquots of homogenate or fatty acid standards were thawed and washed in organic solvents to extract the organic phase of the sample. This phase was evaporated under nitrogen and resuspended in ethanol for analysis by high pressure liquid chromatography (HPLC). Fatty acids were first derivatised and then washed in a mixture of hexane and phosphate buffer. The hexane phase removed and evaporated to dryness under nitrogen. The samples and

standards were resuspended in methanol and injected into the HPLC column and detected by UV spectroscopy at 230nm. Individual fatty acid concentrations were expressed as nmol/mg of tissue.

4.2.13 Determination of Reactive Oxygen Species Production

This method is described in detail in section 2.5.14. Slices of hippocampus stored in Krebs/DMSO were thawed and washed three times in fresh Krebs solution. Synaptosomes were prepared and Tris buffer added before being incubated in the presence of DCFH-DA. Samples were centrifuged and the supernatant was removed. Tris was added to the pellets, the samples were vortex-mixed, placed water bath and fluorescence was measured at 525nm emission upon excitation at 488nm. Result are expressed as nmoles DCF/mg.

4.2.14 *In vitro* Incubation of Hippocampal Slices with IL-1ß or H_2O_2

Hippocampal slices were incubated in oxygenated Krebs solution placed in a water bath at 37°C. IL-1ß (3.5ng/ml), H_2O_2 (5mM) or a water control was added and the samples were incubated for 30 min. Tissue was homogenised and frozen at -80°C and later analysed for ROS production as described above.

4.3 **Results**

4.3.1 **Effect of Age on the Enzymatic Component of the Antioxidant Defence System**

Figure 4.1 indicates that there was a significant increase in the activity of SOD in the hippocampal tissue of aged rats compared with the activity of the enzyme in the hippocampal tissue of young rats (*$p<0.05$; Student's t-test). There was no age-related change in the activity of GPx. While there was a significant decrease in the activity of catalase in hippocampal tissue of aged, compared with young, rats

4.3.2 **Effect of Age on the Non-Enzymatic Component of the Antioxidant Defence System**

Figure 4.2 indicates that there was a significant reduction in the concentration of vitamin C in hippocampal tissue prepared from aged rats compared with tissue of young rats (*$p<0.05$; Student's t-test). GSH and vitamin E concentrations in the hippocampus were unaffected by age.

4.3.3 **Effect of Age on Lipid Peroxidation**

Figure 4.3 indicates that there was a significant increase in lipid peroxidation in hippocampal tissue of aged rats compared with tissue of young rats (*$p<0.05$; Students t-test).

4.3.4 **Effect of Age on Arachidonic Acid Concentration**

Figure 4.4 indicates that there was a significant decrease in the concentration of arachidonic acid in the tissue prepared from aged rats compared with the tissue of young rats (*$p<0.05$; Students t-test).

4.3.5 **Effect of Age on IL-1ß Concentration**

Figure 4.5 indicates that there was a significant increase in the concentration of IL-1ß in the tissue of aged rats compared with the tissue of young animals (*$p<0.05$; Student's t-test).

4.3.6 **Effect of IL-1ß on ROS Production**

Figure 4.6 indicates that there was a significant increase in the production of ROS in hippocampal tissue incubated with 3.5 ng/ml IL-1ß for 30 min compared with tissue incubated with a water control. There was also an increase in ROS production

with incubation of hippocampal tissue with H_2O_2 (5mM) though this did not reach statistical significance. (*p<0.05; Student's t-test).

4.3.7 **Effect of IL-1ß on SOD Activity**

Figure 4.7 indicates that there was a significant increase in activity of SOD in hippocampal tissue incubated with 3.5 ng/ml IL-1ß for 30 min compared with tissue incubated with a water control. There was also an increase in SOD activity with incubation of hippocampal tissue with H_2O_2 (5mM) though this did not reach statistical significance. (*p<0.05; Student's t-test).

4.3.8 **Effect of H_2O_2 on IL-1ß Concentration**

Figure 4.8 indicates that there was an increase in the concentration of IL-1ß in the hippocampus after incubation with H_2O_2 (5mM) though this did not reach statistical significance.

Figure 4.1 Age-related changes in the enzymatic component of the antioxidant defence system.

SOD activity was measured in hippocampal tissue prepared from young and aged rats. There was a significant increase in SOD activity in the aged group (n=7) compared with tissue from the young group (n=7). Results are expressed as enzyme units (U)/mg protein.

There was no change in the activity of GPx in hippocampal tissue prepared from the aged group of rats (n=7) compared with that in the young group (n=8). Results are expressed as μM NADPH oxidised/min/mg protein.

There was a significant decrease in the activity of catalase in hippocampal tissue from aged rats (n=7) compared with that from young rats (n=7). Results are expressed as a function of the reaction rate (k)/mg protein.

Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test).

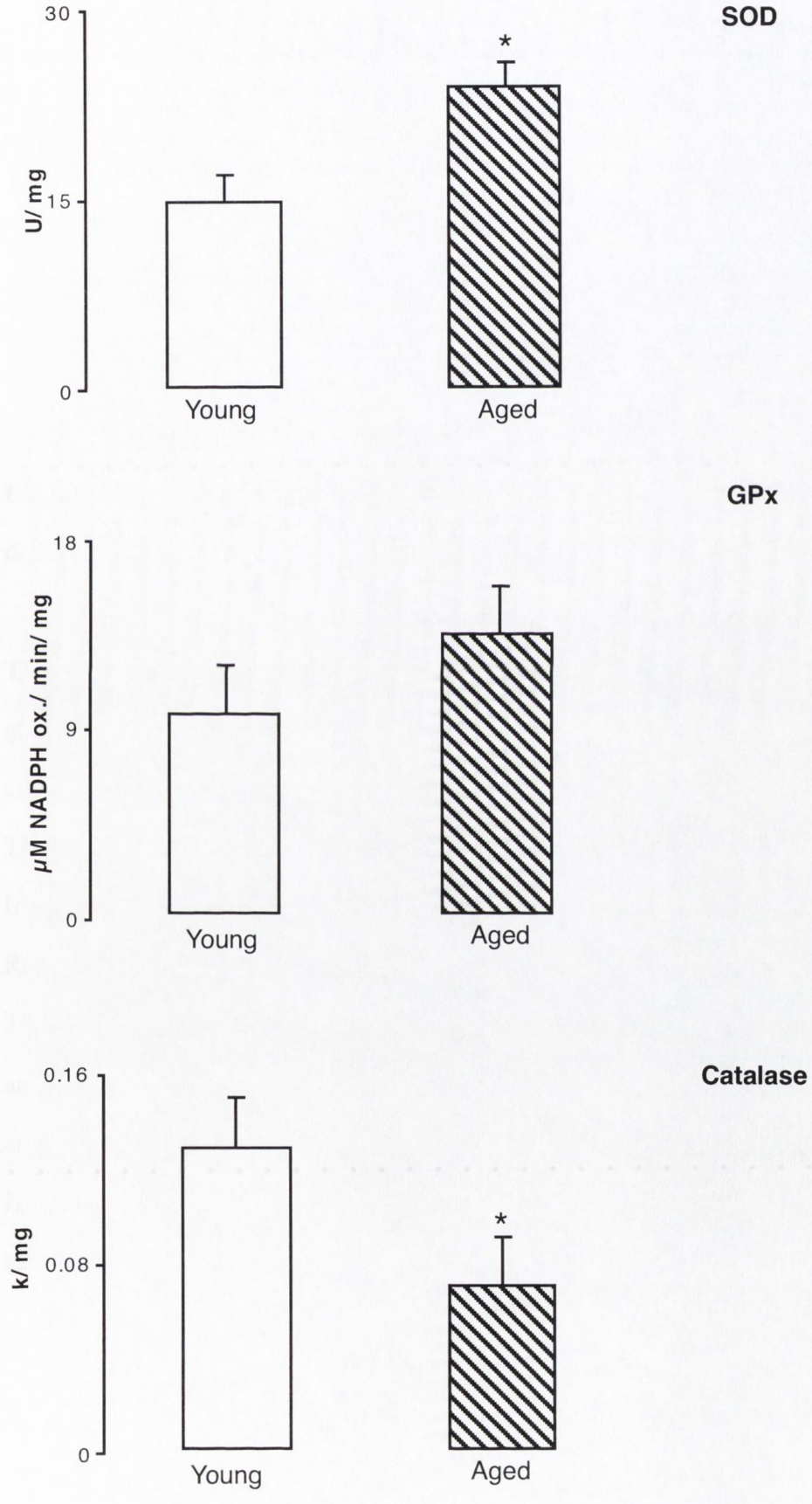

SOD
30
15
0
U/ mg
*
Young
Aged
GPx
18
9
0
µM NADPH ox./ min/ mg
Young
Aged
Catalase
0.16
0.08
0
k/ mg
*
Young
Aged

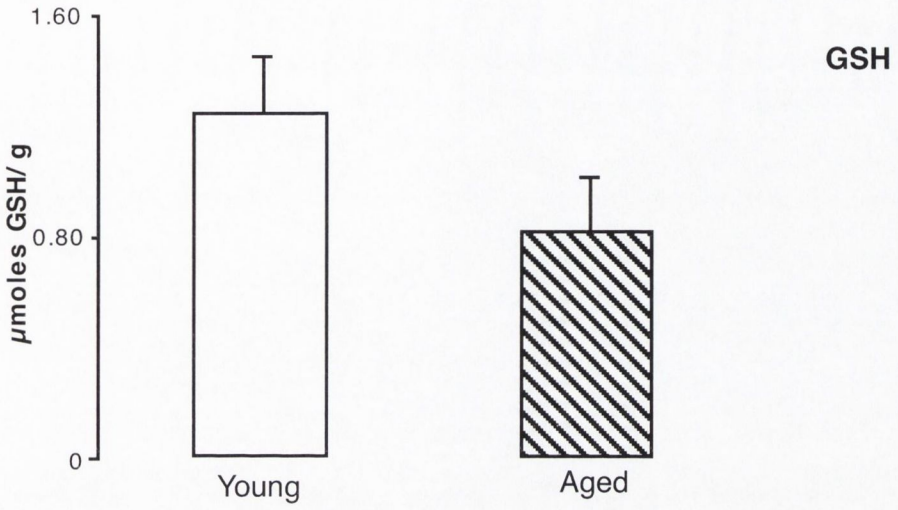
Figure 4.2 Age-related changes in the non-enzymatic component of the antioxidant defence system.

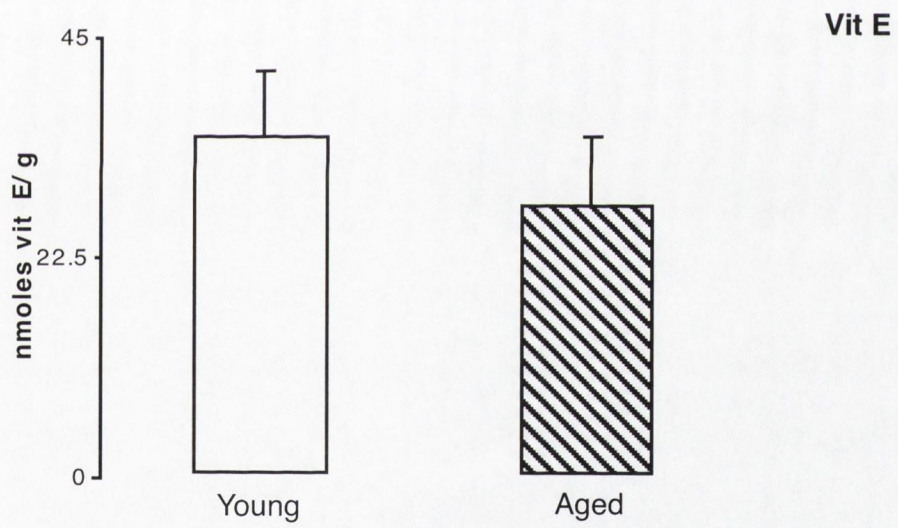
There was no change in the concentration of GSH in hippocampal tissue from the aged group of rats (n=7) compared with tissue from the young group (n=8). Results are expressed as μmoles GSH/g tissue.

There was a significant decrease in the concentration of vitamin C in hippocampal tissue from the aged group of rats (n=6) compared with tissue from the young group (n=7). Results are expressed as μmoles of vitamin C/g tissue.

There was no change in the concentration of vitamin E in hippocampal tissue from aged animals (n=8) compared with tissue from the young group (n=7). Results are expressed as nmoles of vitamin E/ g tissue.

Histograms represent means and bars represent standard errors. (*$p<0.05$; Student's t-test).


GSH
1.60
0.80
0
µmoles GSH/ g
Young
Aged


Vit E
45
22.5
0
nmoles vit E/ g
Young
Aged

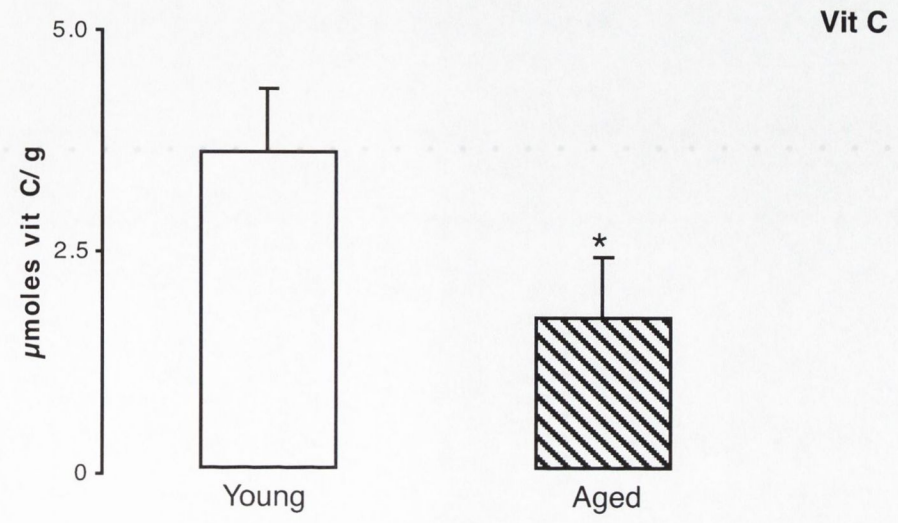

Vit C
5.0
2.5
0
µmoles vit C/ g
*
Young
Aged

Figure 4.3 Lipid peroxidation is increased with age in hippocampal tissue.

There was a significant increase in lipid peroxidation in hippocampal tissue from aged animals (n=5) compared with tissue from the young group (n=5). Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as nmoles of MDA/ g tissue.

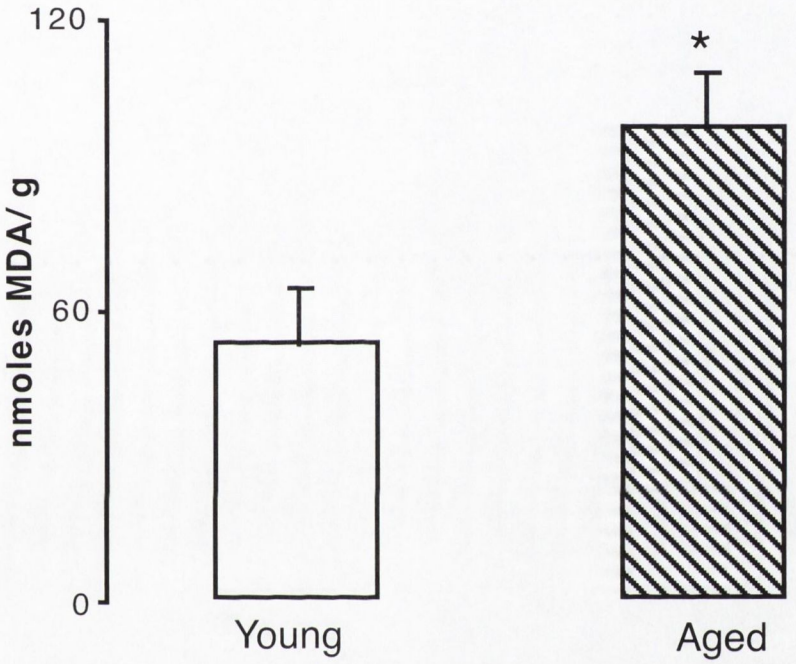

120
60
0
nmoles MDA/ g
*
Young
Aged

Figure 4.4 Arachidonic acid concentration is decreased with age in hippocampal tissue.

There was a significant decrease in the concentration of arachidonic acid in hippocampal tissue from aged animals (n=8) compared with the young group (n=8). Histograms represent means and bars represent standard errors. (*$p<0.05$; Student's t-test). Results are expressed as nmoles arachidonic acid (AA)/mg tissue.

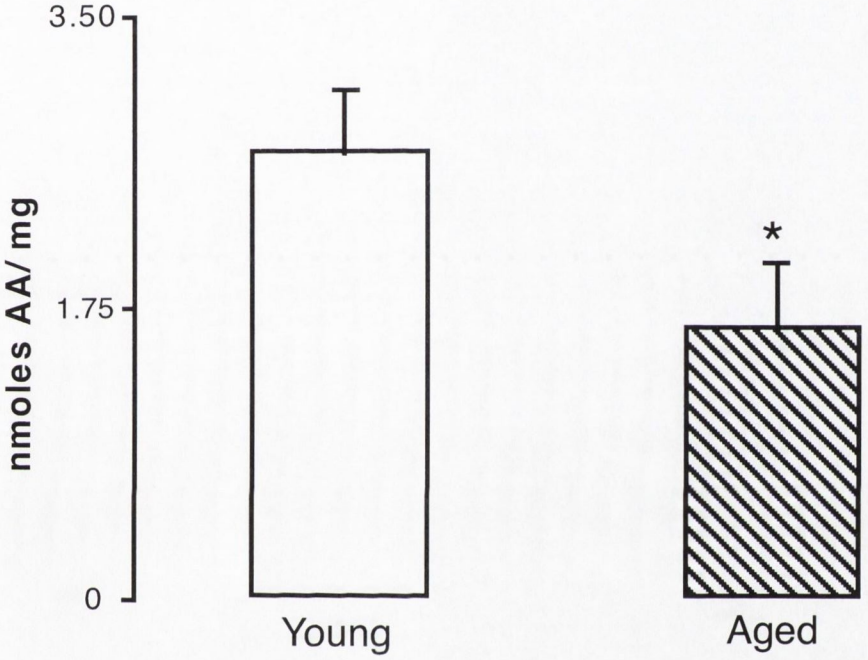

3.50
1.75
0
nmoles AA/ mg
*
Young
Aged

Figure 4.5 IL-1ß concentration is increased with age in hippocampal tissue.

There was a significant increase in the concentration of IL-1ß in hippocampal tissue from aged animals (n=6) compared with tissue from the young group (n=6). Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as pg IL-1ß/ g of tissue.

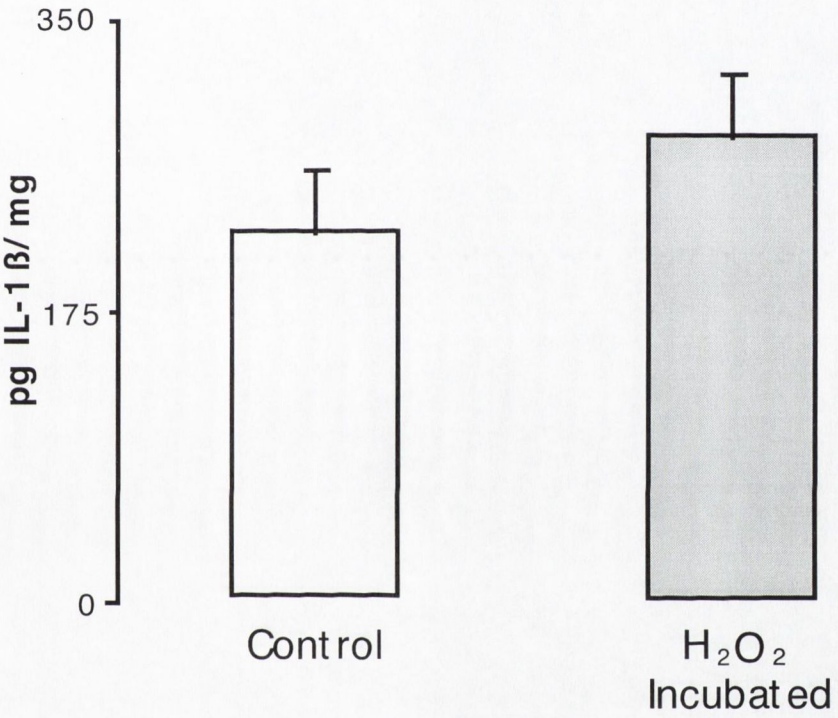

350
175
0
pg IL-1ß/ mg
Control
H2O2
Incubated

Figure 4.6 IL-1ß causes a significant increase in ROS production in hippocampal tissue *in vitro*.

IL-1ß (3.5 ng/ml) significantly increased ROS production in hippocampal slices prepared from young rats. There was also an increase in ROS production following H_2O_2 incubation (5mM, 30 minutes), though this did not reach statistical significance. Histograms represent means of 6 observations and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as nmoles of DCF/ mg tissue.

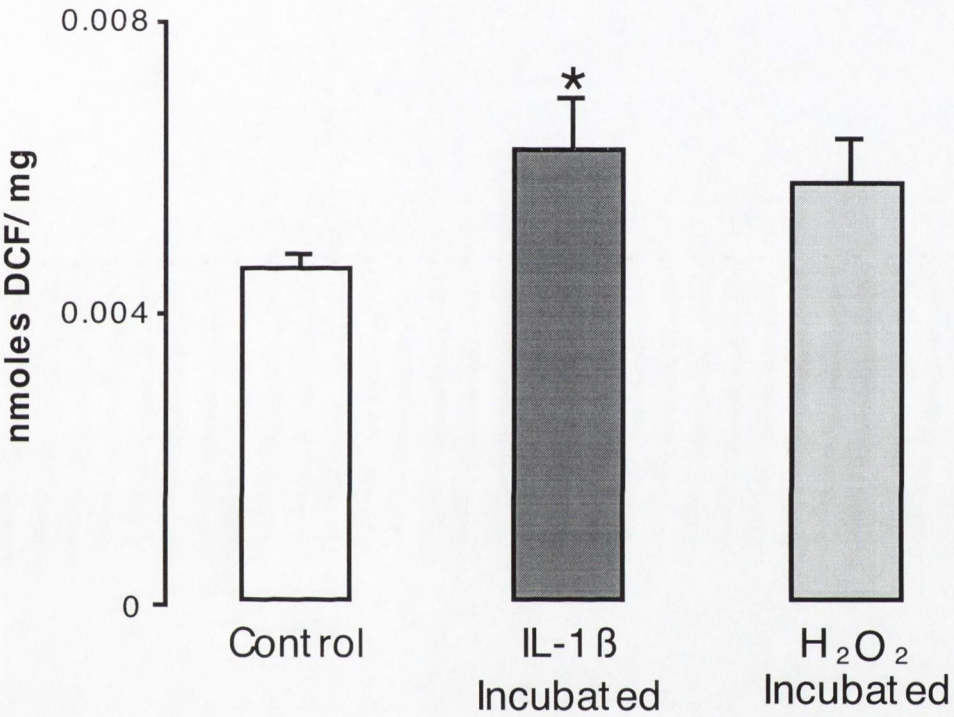

0.008
0.004
0
nmoles DCF/ mg
*
Control
IL-1 ß
Incubated
H2O2
Incubated

Figure 4.7 IL-1ß causes a significant increase in the activity of SOD in hippocampal tissue *in vitro*.

There was a significant increase in total SOD activity in hippocampal slices prepared from young rats incubated with IL-1ß (3.5 ng/ml; n=6) for 30 min compared with control (n=6). There was also an increase (which did not reach statistical significance) in SOD activity following incubation of hippocampal tissue with H_2O_2 (5mM, 30 min). Histograms represent means and bars represent standard errors. (*$p<0.05$;Student's t-test). Results are expressed as enzyme units (U)/mg protein.

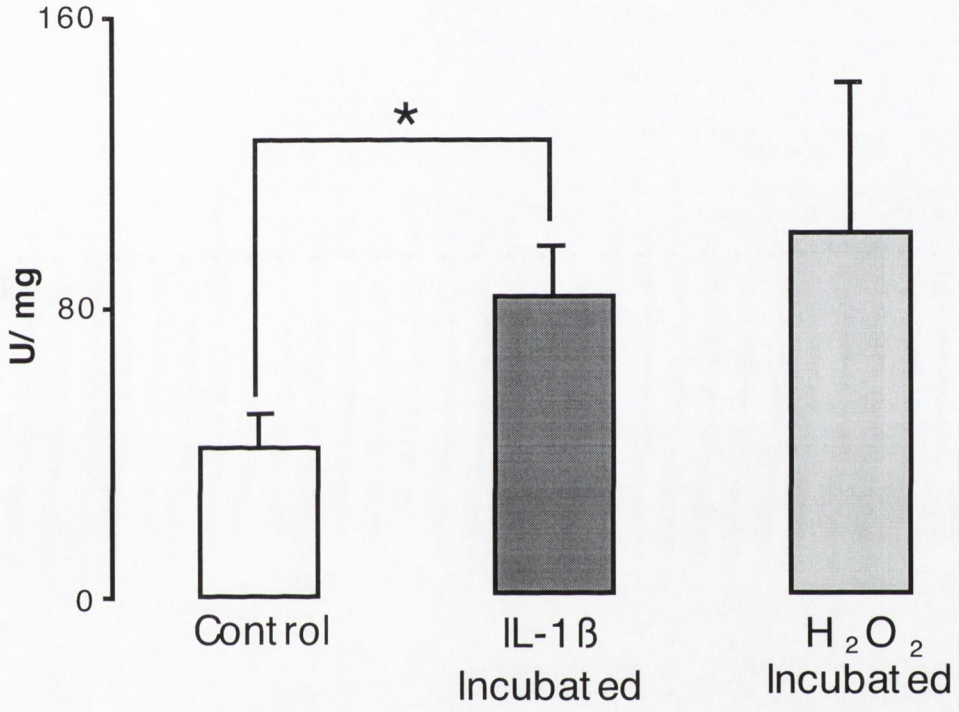
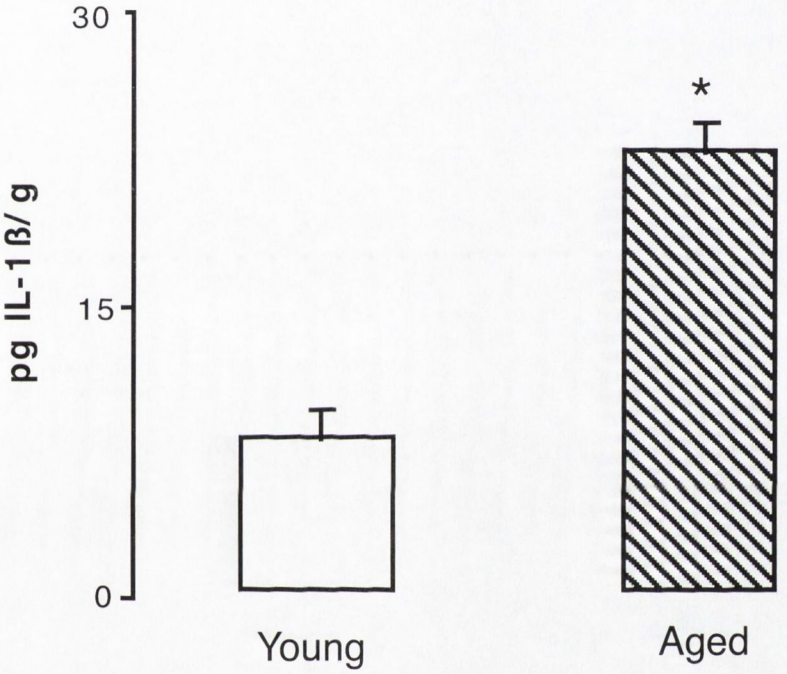

160
80
0
U/ mg
*
Control
IL-1ß
Incubated
H 2O 2
Incubated

Figure 4.8 H_2O_2 increases the concentration of IL-1ß in hippocampal tissue *in vitro*.

There was an increase (which did not reach statistical significance) in the concentration of IL-1ß in hippocampal slices prepared from young rats incubated with H_2O_2 (5mM, 30 minutes; n=6). Histograms represent means and bars represent standard errors. (*,+$p<0.05$;Student's t-test). Results are expressed as pg IL-1ß/ mg.


30
15
0
pg IL-1ß/ g
*
Young
Aged

4.4 **Discussion**

The aim of the first part of this study was to assess whether there was an age-related alteration in the antioxidant defence system of the hippocampus. The aim of the second part of the study was to assess a possible relationship between IL-1ß and ROS production in hippocampal tissue, using *in vitro* analysis.

The data presented demonstrated that the activity of SOD was increased in aged hippocampal tissue compared with young while the activities of GPx and catalase showed no change or decreased respectively. An age-related increase in SOD activity with no parallel increase in GPx activity was also noted in the hippocampus of Fischer 344 rat (Carrillo *et al.*, 1992). This result, similar to the findings in the cortex (chapter 3), implies an alteration in the balance of the enzymatic portion of the antioxidant system. This may result in the generation of an oxidative stress in the aged hippocampal tissue due to the generation of excessive concentrations of H_2O_2. As discussed in the previous chapter there appears to be a relationship between an increase in SOD activity and an increase in oxidative stress. The data obtained from hippocampus also show that the non-enzymatic portion of the antioxidant defence system was compromised and this may further contribute to the oxidative stress of the aged hippocampus. Interestingly the result differed from those obtained in the cortex. In the hippocampus there was a decrease in the concentration of vitamin C while no significant changes in GSH or vitamin E concentration were observed. However vitamin E concentration was found to be significantly decreased in a previous study which measured the concentration of the molecule in the dentate gyrus (Murray & Lynch, 1998). As mentioned in the previous chapter relating to different regional profiles observed in the activities of the antioxidant enzymes, it may be that there are regional differences in the concentration of antioxidants in the brain as well and therefore specific brain regions may be particularly vulnerable to oxidative stress. It has been reported that there was regional susceptibility to oxidative stress in the brain

tissue of rats that were chronically treated with ethanol for 12 days. There was a significant decrease in GSH concentrations, which was used as a marker for oxidative stress, in the striatum and cerebellum while the cortical concentrations of this molecule were unaffected (Bondy & Guo, 1995). Similarily during ischemia it was noted that there was a regional pattern to vitamin C loss in the rat brain, which was used as a indicator of oxidative stress, with the hippocampus losing significantly greater concentrations than the prefrontal cortex. A significant gender difference to vitamin C loss was recorded with the male rat brain appearing to be more sensitive to oxidative stress than the female brain (Ferris *et al.*, 1995).

There was an age-related increase in lipid peroxidation in hippocampal tissue in agreement with previous studies which have observed a similar increase in lipid peroxidation in the hippocampus of the aged brain (Mizuno & Ohta, 1986; Rikans *et al.*, 1997). This suggests that an oxidative stress is present in aged rat hippocampal tissue and that this stress is unable to be neutralised by cellular antioxidant defences. The net effect of this is oxidative damage to the neuronal membranes of the hippocampus. Contributory factors in this age-related oxidative stress are likely to include the increase in the activity of SOD without concomitant increases in GPx or catalase activity and the decrease in the concentration of vitamin C without a concomitant, compensatory increase in the concentrations of GSH or vitamin E.

In parallel with the increase in lipid peroxidation there was a decrease in arachidonic acid concentrations in the aged hippocampus. Polyunsaturated fatty acids, such as arachidonic acid are important in maintaining membrane fluidity but are a target for ROS. Evidence that lipid peroxidation influences membrane rigidity has been obtained from a number of studies. For example, unilamella liposomes were peroxidised with a Fe^{2+}/ascorbic acid mixture before being incorporated into rat liver microsomal membranes. It was observed that the peroxidised liposomes induced substantial membrane rigidity and that membrane fluidity could be readily modulated

by the lipid peroxidation of membrane phospholipids (Choe *et al.*, 1995). Similarily it was found that an increase in lipid peroxidation in platelets prepared from aged rats was correlated with a decrease in membrane fluidity (Hossain *et al.*, 1999). Furthermore, it was noted that membrane fluidity in rat cortical preparations was decreased after peroxidisation and this caused an alteration in muscarinic receptor binding in these peroxidised membranes (Ghosh *et al.*, 1993). Clearly age-related peroxidation in brain regions can alter neuronal membrane fluidity and this effect will influence the protein structures embedded within those membranes.

It is known in the hippocampus that there is an age-related impairment in LTP. This impairment in LTP may be related to the increase in production of ROS, the compromised antioxidant defence system and the age-related increase in lipid peroxidation. This suggestion is consistent with 2 findings. First, there was an impairment of long-term potentiation in hippocampal slices obtained from rats fed with a vitamin E deficient diet for 3 months (Xie & Sastry, 1995). Second, it has been shown that dietary supplementation with high doses of vitamins C and E can reverse the age-related impairment of LTP, the increase in lipid peroxidation and the decrease in arachidonic acid seen in the dentate gyrus region of the rat hippocampus (Murray & Lynch, 1998). This implies that it is the deterioration in the antioxidant defences that may be primarily responsible for the inability to maintain LTP. This process relies on many signal transduction events both pre- and post- synaptically to enable the strenghtening of synaptic transmission to occur following high frequency stimulation (Bliss & Collinridge, 1993). Oxidative alterations to proteins or membranes due to enhanced oxidative stress in aged tissue may compromise the functionality of neurons and impede their ability to alter synaptic efficiency. It is important to note that oxidative stress can also influence the structure and function of cellular proteins. Aged gerbil brains were found to have a significantly higher concentration of oxidised protein compared with that of young animals and treatment with the antioxidant, N-

tert-butyl-alpha-phenylnitrone, markedly reduced the concentration of oxidised protein. Furthermore pre-treatment of aged gerbils with this compound for 2 weeks significantly improved their temporal and spatial memory compared with age-matched controls (Carney *et al.*, 1991). These studies imply that age-related oxidative stress within brain regions can significantly impact on the functioning of these areas and the removal of oxidative stress by dietary antioxidant supplementation can enhance the efficiency of aged tissue.

There was an increase in IL-1ß observed in hippocampal tissue of aged rats. As in the cortex, this increase in the expression of this cytokine may be a response to the increase in oxidative stress and the consequent oxidative damage to hippocampal tissue. In the previous chapter it was speculated that IL-1ß may contribute significantly to the oxidative stress experienced by aged neuronal tissue through its actions on the activity of SOD. IL-1ß and other inflammatory cytokines IL-6 and TNF-α, induced a significant increase in mRNA concentrations of Mn-SOD in cells derived from the corpus luteum of rats (Sugino *et al.*, 1998). It has been observed that IL-1ß induces MnSOD in rat neuronal and glial cells and treatment with lipopolysaccharide for 3 days, an agent known to induce the release of IL-1ß from neuronal tissue, elicited a 4-fold increase in the concentrations of Mn-SOD in cultured mouse astrocytes, while incubation with IL-1α also significantly effected the concentrations of this enzyme (Kifle *et al.*, 1996; Mokuno *et al.*, 1994). In this chapter the interaction between IL-1ß and SOD was investigated further in a series of *in vitro* studies. The findings from these experiments showed that incubation of hippocampal tissue with IL-1ß increased the production of ROS within the tissue. It has been previously reported that IL-1ß increased lipid peroxidation in hippocampal tissue prepared from 4 month old rats and that this effect was inhibited by vitamin E (Murray & Lynch, 1999). The data from this chapter showed that incubation of young hippocampal tissue with IL-1ß increased the activity of SOD. In another study it was

noted that the incubation of cortical tissue with a SOD inhibitor and IL-1ß *in vitro* reverses the increase in ROS production observed and the increase in the activity of the enzyme (Martin, Pers Comm). This implies that the increase in SOD activity may play a vital role in the chronic oxidative stress observed in neuronal tissue. This also strengthens the proposal that within the aged hippocampus there is a destructive positive feedback loop in operation.

Neuronal oxidative stress is generated in aged tissue perhaps by the decrease in the concentration of the non-enzymatic antioxidants. This causes tissue damage and initiates the release of IL-1ß which increases ROS production, perhaps by increasing the activity of SOD, which may then further enhance IL-1ß production through further oxidative damage to neuronal tissue. In support of this hypothesis, the data show that incubation of hippocampal tissue in the presence of 5mM H_2O_2 caused an increase in IL-1ß concentrations. It was previously shown that 4-hydroxy-2,3-noneal, a major derivative of membrane lipid oxidation induced both TGF-ß1 mRNA and TGF-ß1 protein in murine macrophages implying that enhanced oxidation can initiate the release of cytokines (Leonarduzzi *et al.*, 1997). After the focal application of kainate, a convulsant agent believed to cause an increase in the production of ROS, to the rat hippocampus, IL-1ß concentration was shown to be dramatically increased after 24 hours. The concentration of this cytokine was also increased in response to NMDA-receptor mediated excitotoxicity in the rat parietal cortex and striatum (Pearson *et al.*, 1999). Also cerebral ischemia induced by carotid occlusion also increased IL-1ß mRNA concentrations in the cortex and the hippocampus; the change was observed 2 hours after reperfusion and was maximally increased in the hippocampus after 8 hours (Sairnen *et al.*, 1997). Clearly neuronal tissue responds to damage in its environment and does this by the release of inflammatory cytokines that include IL-1ß. The increase in the activity of SOD, initiated by IL-1ß, may be a cellular response to an increase in ROS production caused by cellular damage. However in the aged brain,

this response exacerbates the oxidative stress on neuronal tissue and elicits further release of the cytokine. The net effect of this is an increase in lipid and protein oxidation and, possibly the reduced ability of that brain area to function efficiently.

The scheme shown in diagram 4.1 is supported by the observed data. The compromised antioxidant defence observed in hippocampal preparations from aged rats was accompanied by an increaseed ROS production which resulted in a consequent increase in IL-1ß. The evidence suggests that this increases SOD activity, further increasing ROS production and completing a positive feedback loop. Evidence from this and previous studies support the view that these changes contribute to the impairment in LTP observed in aged rats (see Diagram 4.1).

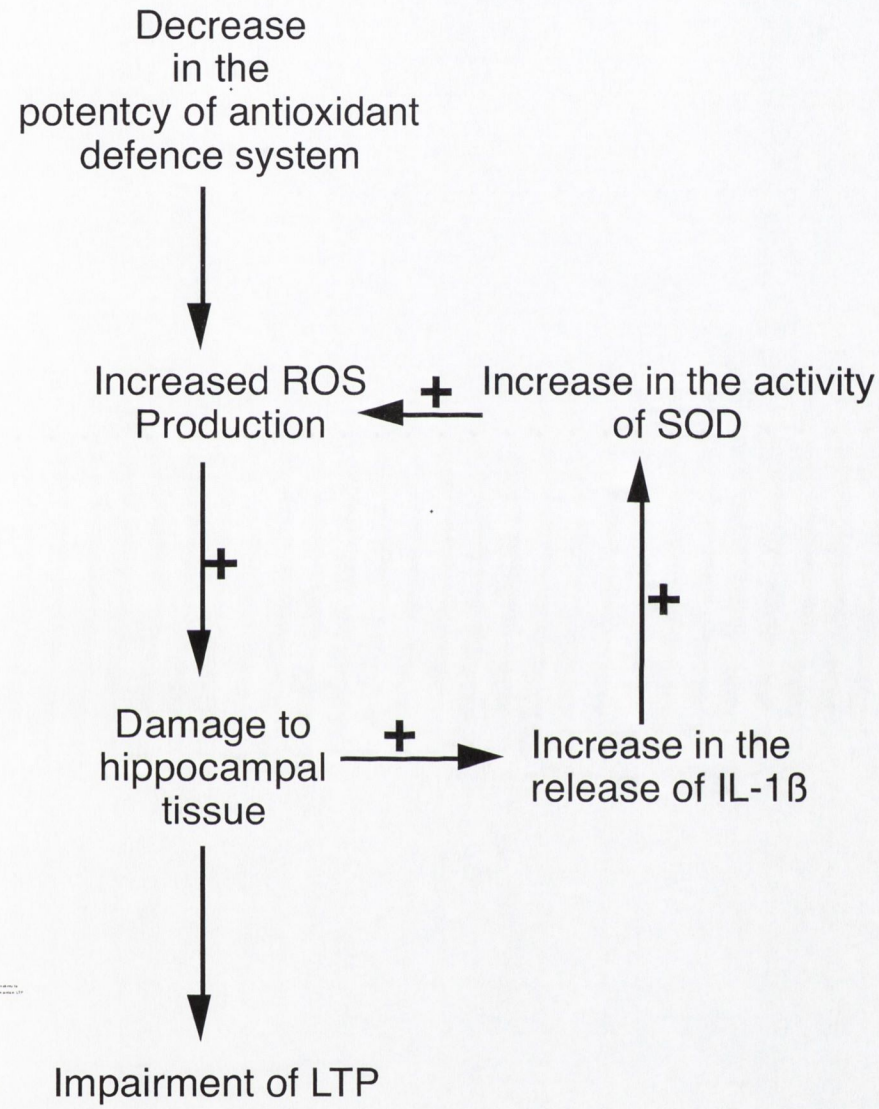


Diagram 4.1: A destructive loop is initiated in aged hippocampal tissue by an increase in oxidative stress that damages tissue and may lead to the an increase in IL-1ß concentration and a resultant increase in SOD activity. This in turn may enhance the oxidative stress

Chapter 5

Short Term Dietary Supplementation Reverses IL-1ß-Mediated Neuronal Changes.

5.1 **Introduction**

Data from this and previous studies (Murray & Lynch, 1998; Auerbach & Segal, 1997) implied that an increase in ROS production was related to the inability of aged animals to maintain LTP. In the data presented in previous chapters, a link between an increase in the production of ROS in the cortex and hippocampus and an increase in the concentration of IL-1ß in those areas has been suggested. The aim of this series of experiments will be to clarify and confirm this link by injecting IL-1ß directly into the ventricular cavity of anesthetised rats and assessing (a) ability to maintain LTP (b) changes in the antioxidant defence system of the hippocampii and (c) the oxidative stress status of that tissue as measured by ROS, lipid peroxidation and polyunsaturated fatty acid concentration. The efficacy of supplementing a subgroup of rats with high concentrations of vitamin C and vitamin E will also be determined.

5.2 **Methods**

5.2.1 Animals and Dietary Supplementation

Young (2-4 months) male Wistar rats were randomly divided into subgroups (6 rats in each subgroup). They were housed 6 per cage. 2 subgroups were fed for 5 days with normal laboratory chow supplemented with a daily dose of dl-α-tocopheryl acetate (250mg/rat/day; 50% Type SD Vitamin E, Beeline Healthcare, Dublin) dissolved in corn oil. Each gram of the dry powder product contained 500mg of dl-α-tocopheryl acetate. Vitamin C (250 mg/rat/day; Beeline Healthcare, Dublin) was added to the water supplied to the cages. The vitamin C product consisted of approximately 97% L-ascorbic acid. The other subgroups were fed for the same 5 day period on normal laboratory chow with added corn oil to ensure an isocaloric diet. The water to these cages was not supplemented. Daily food and water intake was monitored over a 5 day period before the start of dietary supplementation. During the 5 day dietary phase animals were given 95% of their average daily food intake and

100% of their average water intake. Rats were weighed before and after the 5 day experiment. General heath and behaviour was monitored daily. The vitamin supplemented diet will be referred to as the "test diet".

5.2.2 Intracerebroventricular (i.c.v.) Injection

Each rat was weighed and anaesthetised by injection of urethane (1.5 g/kg) intraperitoneally. The head was positioned in a head holder within a stereotaxic frame. A midline incision was made with a scalpel and the skin pulled back to reveal the skull. Lamda and bregma were identified and a hole was drilled 2.5mm ventral to bregma and 0.5 mm lateral to the midline. 5μl of either 3.5 ng/ml of interleukin-1ß (IL-1ß) in a 0.9% saline solution or a 0.9% saline solution alone was injected into the ventricle using a Hamilton syringe.

5.2.3 Induction of LTP *in vivo*

Following i.c.v injection, a window of skull was removed to allow the insertion of the stimulating and recording electrodes (for more details see section 2.3) 4.4mm lateral to lambda and 2.5mm lateral to and 3.9 mm posterior to bregma respectively. The stimulating and recording electrodes were slowly lowered to a depth of 2-3.5 mm to maximise the responses (1-2mV amplitude) and displayed on a computer screen. Stimuli were delivered at 30 second intervals and the slopes of the field EPSP were recorded for a 10 min period before and 40 min after delivery of 3 trains of stimuli (250Hz for 200msec) at 30 sec intervals.

5.2.4 Tissue Storage

At the end of the LTP recording period individual animals were killed by cervical dislocation and their hippocampii removed. Hippocampal tissue was either (a) homogenised in Krebs solution and frozen at -80°C, (b) chopped into slices placed

in a 10% Krebs/DMSO solution and frozen at -80°C or (c) homogenised in a 5% TCA solution and frozen at -80°C (for more details see section 2.4).

5.2.5 Analysis of SOD Activity

This method is described in detail in section 2.5.5. Homogenised tissue (see 5.2.4) was thawed, supernatant obtained and serially diluted in phosphate buffer. An aliquot from each dilution was added to assay buffer. The rate of NBT reduction was assessed at 560nm for 5 min. Results are expressed in units of SOD activity per milligram of protein.

5.2.6 Analysis of GPx Activity

This method is described in detail in section 2.5.3. A standard curve of absorbance versus NADPH concentration was constructed. Homogenised tissue (see 5.2.4) was thawed, supernatant obtained and added to assay mixture. The reaction was initiated by the addition of cumene hydroperoxide solution. Absorbance at 340nm was recorded for 5 min using a spectrophotometer. Results were expressed as μmoles NADPH oxidised per min per milligram of protein.

5.2.7 Analysis of Catalase Activity

This method is described in detail in section 2.5.6. Homogenised tissue (see 5.2.4) was thawed, supernatant obtained and added to microfuge tubes containing phosphate buffer. The reaction was initiated by the addition of H_2O_2 and after 2 and 10 min aliquots were removed and quenched. Colour was developed at room temperature by addition of KSCN. Two aliquots from each tube were transferred immediately to a well plate and read at 492nm. The results were expressed in terms of the first order reaction rate constant (k) and protein.

5.2.8 Analysis of the Concentration of Vitamin C

This method is described in detail in section 2.5.4. Tissue stored in 5% TCA solution (see 5.2.4) was thawed, supernatant obtained and mixed with assay buffer and incubated for 3 hr in a waterbath. Ice-cold 65% H_2SO_4 was added and mixed with each sample or standard. Samples and standards were read at 545nm and the results were expressed as μmoles of ascorbic acid per gram of tissue.

5.2.9 Analysis of GSH Concentration

This method is described in detail in section 2.5.2. Tissue stored in 5% TCA solution was thawed (see 5.2.4) and supernatant obtained. Supernatant or standard was added to sample buffer. After incubating for 1 min NADPH was added and the absorbance at 412 nm was monitored for 2 min using a colorimeter. Results are expressed as μmol GSH per gram of tissue.

5.2.10 Analysis of the Concentration of Vitamin E

This method is described in detail in section 2.5.9. Homogenate was thawed, saponified by boiling and its lipid soluble elements extracted by washing in hexane. The hexane phase was removed and evaporated under a stream of nitrogen.. Samples and standards were analysed by reverse phase liquid chromatography with 75% acetonitrile:25% methanol as mobile phase at a flow rate of 1.2 ml/min. Samples and standards were placed in an autosampler and α-tocopherol was detected at 298nm using a SpectraPhysics HPLC. Results were expressed as nmoles vitamin E per gram of tissue.

5.2.11 Analysis of Lipid Peroxidation

This method is described in detail in section 2.5.8. Homogenate was thawed (see 5.2.4) and an aliquot was placed in a microfuge tube. Aliquots of assay solution

were added to microfuge tubes containing the standard or samples and incubated at 90°C for 1 hr. Aliquots from each tube were transferred to a 96-well plate and read at 545nm. Results were expressed as nmoles of MDA per gram of tissue.

5.2.12 Determination of Fatty Acid Concentrations

This method is described in detail in section 2.5.10. Aliquots of homogenate or fatty acid standards were thawed and washed in organic solvents to extract the organic phase of the sample. This phase was evaporated under nitrogen and resuspended in ethanol for analysis by high pressure liquid chromatography (HPLC). Fatty acids were first derivatised, extracted into hexane and evaporated to dryness under nitrogen. The samples and standards were resuspended in methanol and injected into the HPLC column and detected by UV spectroscopy at 230nm. Individual fatty acid concentrations were expressed as nmoles/mg.

5.2.13 Determination of Reactive Oxygen Species Production

This method is described in detail in section 2.5.14. Synaptosomes were prepared, added to Tris buffer and incubated for 15 min at 37°C in the presence of DCFH-DA. Samples were centrifuged, the pellets were resuspended, vortex-mixed, incubated in a 37°C water bath and fluorescence was measured at 525nm emission upon excitation at 488nm. Resulta are expressed as nmoles DCF/mg.

5.3 **Results**

5.3.1 **Effect of IL-1ß Treatment and Diet on LTP**

Figure 5.1 shows that delivery of a high frequency train of stimuli to the perforant path resulted in an immediate increase in the slope of the epsp in all subgroups of rats and there was no effect on the epsp slope or the amplitude of the response by the test diet. Analysis of individual results showed that all rats injected

i.c.v. with saline sustained LTP for the duration of this experiment. Mean epsp slope was expressed as percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation.

In rats fed on control diet mean percentage change in the mean population epsp slope (± SEM) in the 2 min immediately after tetanic stimulation were 143.16 ± 5.46 and 127.07 ± 2.01 in saline treated and IL-1ß treated rats respectively. The corresponding values in the last 5 min of the experiment were 120.1 ± 1.25 and 107.48 ± 1.58.

In rats fed on the test diet mean percentage change in the mean population epsp slope (± SEM) in the 2 min immediately after tetanic stimulation were 140.96 ± 3.79 and 119.25 ± 3.52 in saline treated and IL-1ß-treated rats respectively. The corresponding values in the last 5 min of the experiment were 122.71 ± 9.65 and 125.85 ± 2.38.

Analysis of the data show that the maintenace of LTP was significantly decreased in the last 5 min of the experiment in IL-1ß treated rats fed the control compared with the saline group fed the control diet. ($p<0.01$; Student's t-test). This failure to maintain LTP was not seen in IL-1ß treated rats fed the test diet.

5.3.2 **Effect of IL-1ß Treatment and Diet on ROS production**

Figure 5.2 indicates that there was an increase in ROS production in hippocampal tissue of IL-1ß-treated rats fed the control diet compared with the hippocampal tissue of both groups of saline-treated rats. This increase in ROS production, which failed to reach statistical significance, was attenuated in IL-1ß-treated rats fed the test diet.

5.3.3 **Effect of IL-1ß Treatment and Diet on SOD Activity**

Figure 5.3 indicates that there was a significant increase in the activity of SOD in hippocampal tissue prepared from IL-1ß-treated rats fed the control diet compared with hippocampal tissue from both groups of saline-treated rats. There was also a significant increase in the activity of the enzyme in the hippocampal tissue of IL-1ß-treated rats fed the test diet when compared with both groups of saline-treated rats. (*$p<0.05$; Student's t-test).

5.3.4 **Effect of IL-1ß Treatment and Diet on GPx Activity**

Figure 5.4 indicates that the activity of GPx was similar in hippocampal tissue prepared from both groups of saline-treated rats and IL-1ß-treated rats fed the control and test diets.

5.3.5 **Effect of IL-1ß Treatment and Diet on Catalase Activity**

Figure 5.5 indicates that the activity of catalase was similar in hippocampal tissue prepared from both groups of saline-treated rats and IL-1ß-treated rats fed the control and test diets.

5.3.6 **Effect of IL-1ß Treatment and Diet on GSH Concentration**

Figure 5.6 indicates that the GSH concentration was similar in hippocampal tissue prepared from both groups of saline-treated rats and IL-1ß-treated rats fed the control and test diets.

5.3.7 **Effect of IL-1ß Treatment and Diet on Vitamin E Concentration**

Figure 5.7 indicates that the vitamin E concentration was similar in hippocampal tissue prepared from both groups of saline-treated rats and IL-1ß-treated rats fed the control and test diets. Data from IL-1ß-treated and saline-treated rats were pooled.

5.3.8 **Effect of IL-1ß Treatment and Diet on Vitamin C Concentration**

Figure 5.8 indicates that there was a significant increase in the concentration of vitamin C in the tissue of rats fed the control diet compared with the tissue of rats fed the test diet. (*$p<0.05$; Students t-test). Data from IL-1ß-treated and saline-treated rats were pooled.

5.3.9 **Effect of IL-1ß Treatment and Diet on Lipid Peroxidation**

Figure 5.9 indicates that there was a significant increase in lipid peroxidation in the tissue of IL-1ß-treated rats fed the control diet compared with the hippocampal tissue of the pooled group of saline-treated rats. (*$p<0.05$; Student's t-test). This increase in lipid peroxidation was significantly attenuated in the tissue of IL-1ß-treated rats fed the test diet.

5.3.10 **Effect of IL-1ß Treatment and Diet on Arachidonic Acid Concentration**

Figure 5.10 indicates that arachidonic acid concentration was similar in hippocampal tissue prepared from both groups of saline-treated rats and IL-1ß-treated rats fed the control and test diets.

5.3.11 **Effect of IL-1ß Treatment and Diet on Docosahexanoic Acid Concentration**

Figure 5.11 indicates that the docosahexanoic acid concentration was similar in hippocampal tissue prepared from both groups of saline-treated rats and IL-1ß-treated rats fed the control and test diets.

Figure 5.1 The IL-1ß induced impairment in LTP is reversed by dietary supplementation.

Rats were anaesthetised by intraperitoneal injection of urethane. Test shocks were given at a rate of 1/30 seconds and were delivered for 10 min prior to tetanic stimulation. LTP was induced by delivery of 3 high frequency trains of stimuli before stimulation at test shock frequency resumed and recordings continued for 45 min.

Mean percentage epsp slope in the dentate gyrus evoked by test stimuli delivered to the perforant path at 30 sec intervals before and after tetanic stimulation (arrow) in rats either injected i.c.v. with saline (open symbols) or IL-1ß (closed symbols) and fed either a control or test diet. Mean epsp slope for each group is expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation and the data is presented as the mean change with time. Values on the 5 min pre-tetanus were normalised to 100%.

Analysis of the data show that the maintenace of LTP was significantly decreased in the last 5 min of the experiment in IL-1ß treated rats fed the control compared with the saline group fed the control diet (120.1 ± 1.25 and 107.48 ± 1.58 in saline and IL-1ß –treated rats respectively)($p<0.01$;Student's t-test).

This failure to maintain LTP was not seen in IL-1ß treated rats fed the test diet (122.71 ± 9.65 and 125.85 ± 2.38 in saline and IL-1ß –treated rats respectively)

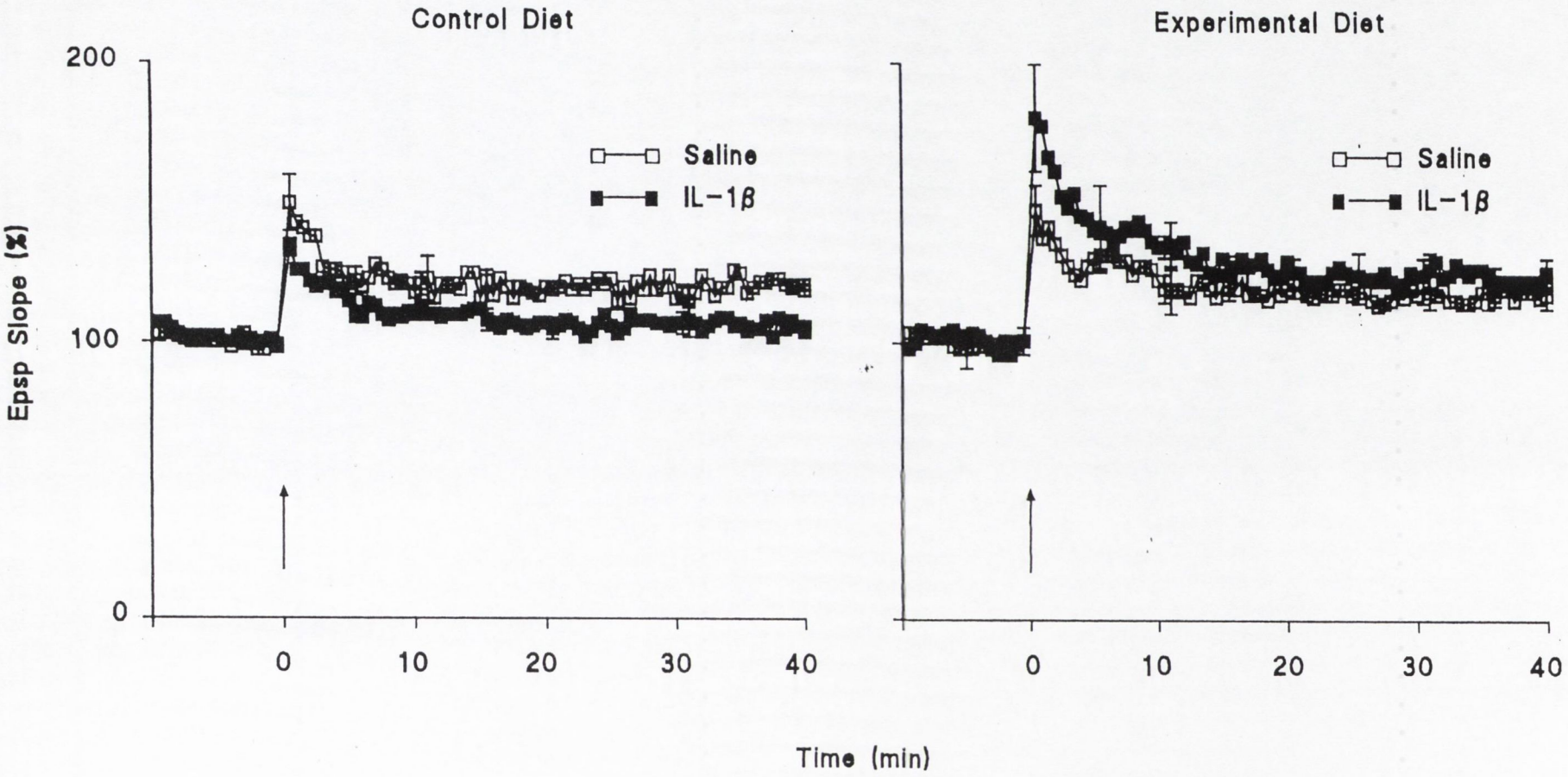

Control Diet
Experimental Diet
Saline
IL-1β
Saline
IL-1β
Epsp Slope (%)
200
100
0
0
10
20
30
40
0
10
20
30
40
Time (min)

Figure 5.2 Dietary supplementation reverses the increase in ROS production in the hippocampus caused by the i.c.v injection of IL-1ß

ROS production was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was an increase in ROS production, though not statistically significant, in the group (n=6) fed the control diet and injected i.c.v. with IL-1ß compared with tissue from the pooled group (n=8) injected i.c.v. with the saline control. This increase was not observed in animals fed the test diet (n=4) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test). Results are expressed as nmoles DCF/mg protein.

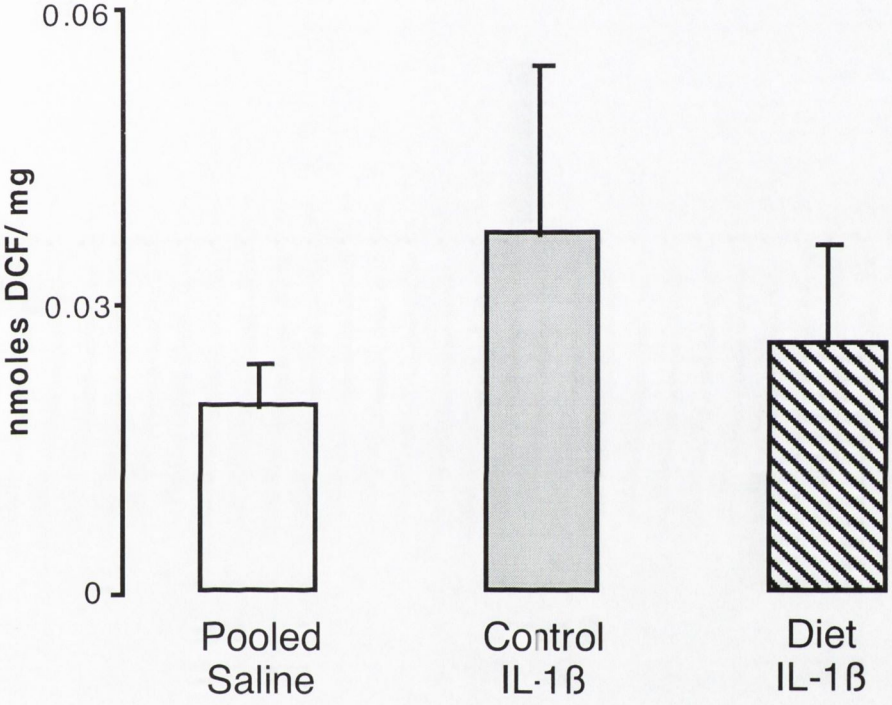

0.06
0.03
0
nmoles DCF/ mg
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.3 Dietary supplementation does not reverse the IL-1ß induced increase in SOD activity in the hippocampus.

SOD activity was measured in hippocampal supernatant prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was an increase in SOD activity in the group (n=5) fed the control diet and injected i.c.v. with IL-1ß compared with tissue from the pooled group (n=11) injected i.c.v. with the saline control. This significant increase was also observed in animals fed the test diet (n=5) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test). Results are expressed as enzyme units (U)/mg protein.

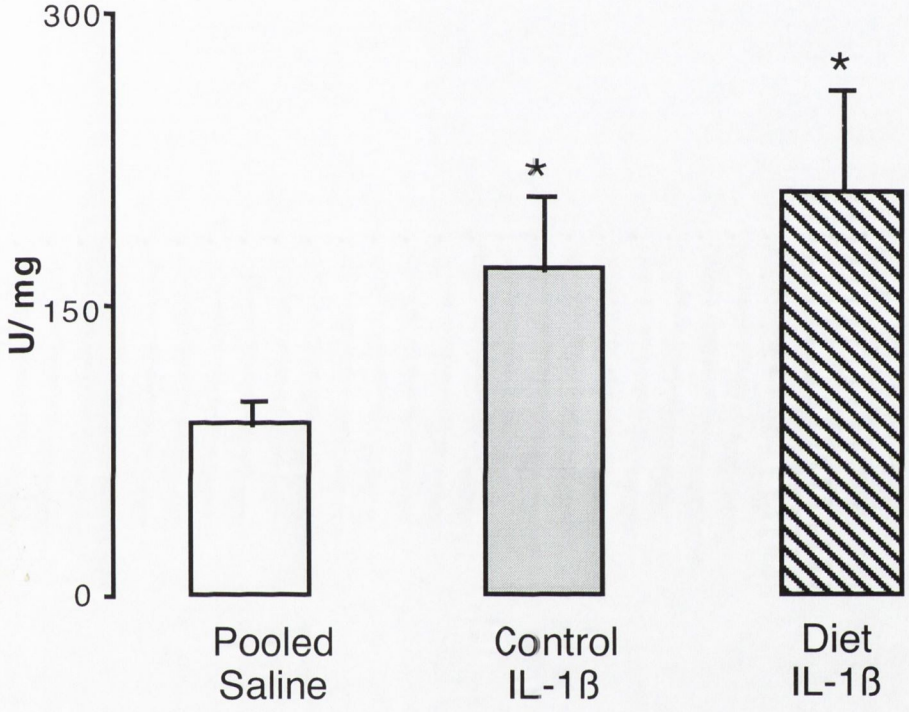

300
150
0
U/ mg
*
*
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.4 There is no IL-1ß or dietary induced change in the activity of GPx in the hippocampus.

GPx activity was measured in hippocampal supernatant prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was an no change in GPx activity between the pooled group (n=10) injected i.c.v. with the saline control, the group (n=5) fed the control diet and injected i.c.v. with IL-1ß or in animals fed the test diet (n=6) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. Results are expressed as μmoles NADPH oxidised/min/mg protein.

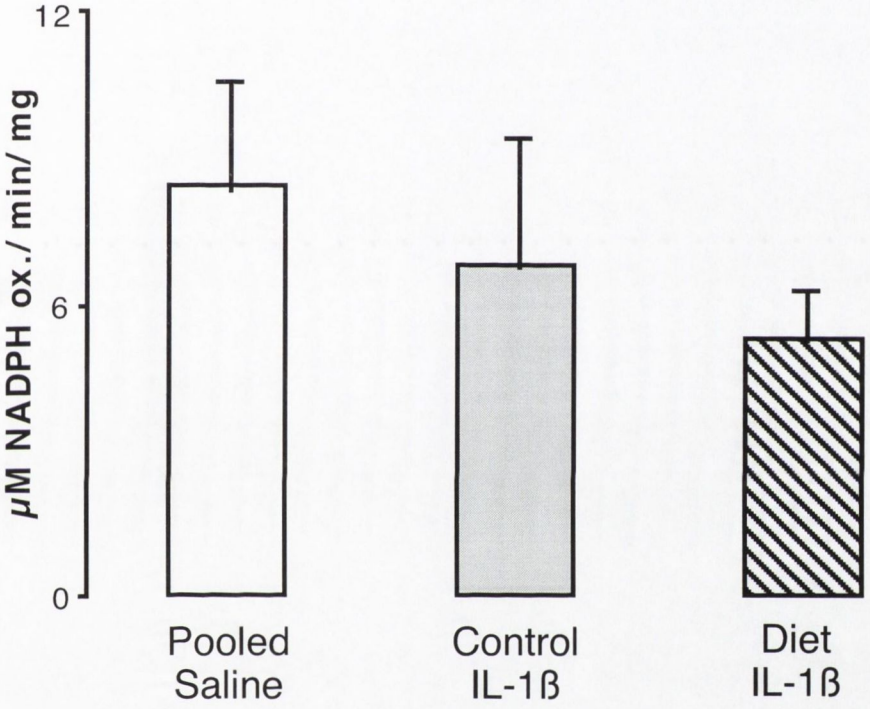

12
6
0
μM NADPH ox./ min/ mg
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.5 There is no IL-1ß or dietary induced change in the activity of catalase in the hippocampus.

Catalase activity was measured in hippocampal supernatant prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was no change in catalase activity between the pooled group (n=10) injected i.c.v. with the saline control, the group (n=5) fed the control diet and injected i.c.v. with IL-1ß or in animals fed the test diet (n=4) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. Results are expressed as a function of the reaction rate (k)/mg protein.

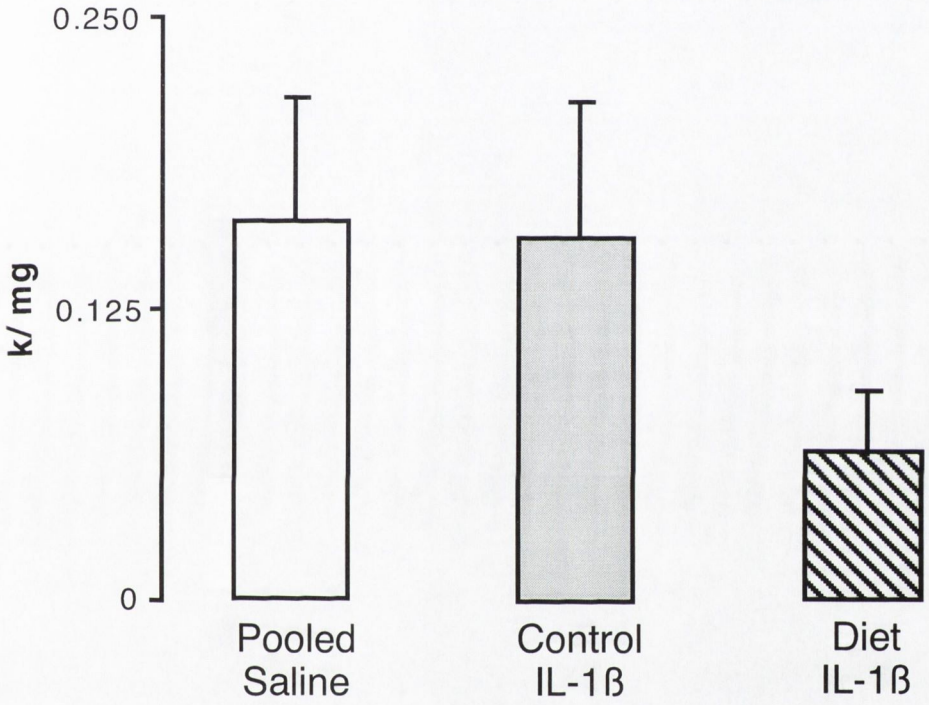

0.250
0.125
0
k/ mg
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.6 There is no IL-1ß or dietary induced change in the concentration of GSH in the hippocampus.

The concentration of GSH was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was no change in GSH concentration between the pooled group (n=10) injected i.c.v. with the saline control, the group (n=5) fed the control diet and injected i.c.v. with IL-1ß or in animals fed the test diet (n=6) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. Results are expressed as μmoles GSH/g tissue.

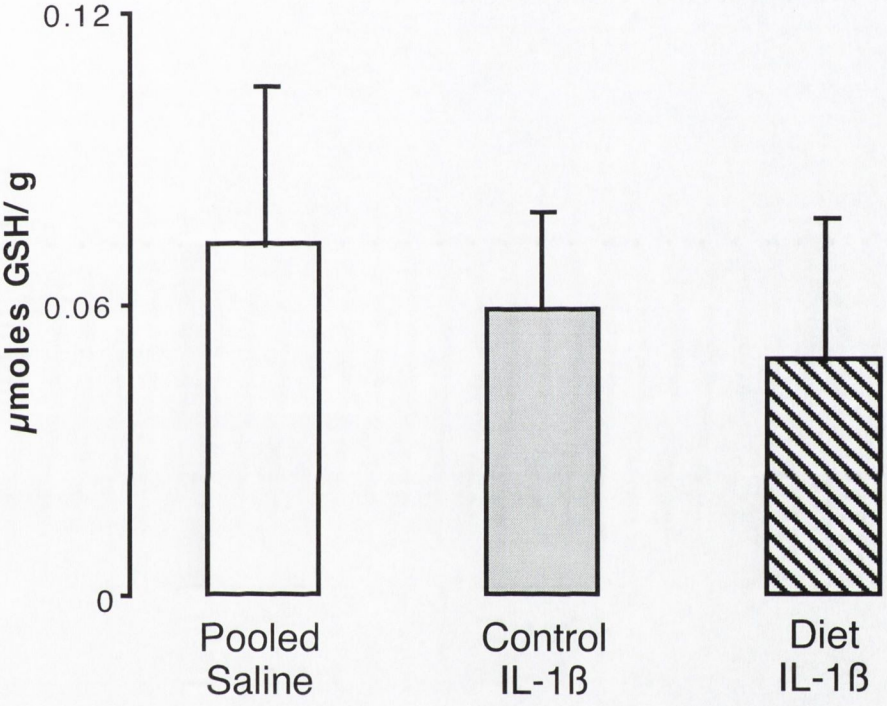

0.12
0.06
0
µmoles GSH/ g
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.7 Short term dietary supplementation does not increase the concentration of vitamin E in the hippocampus.

The concentration of vitamin E was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline and all the animals injected i.c.v with IL-1ß were pooled as there was no statistical difference between their respective results. There was no change in vitamin E concentration between the pooled group (n=10) fed the control diet and the pooled group (n=9) fed the test diet. Histograms represent means and bars represent standard errors. Results are expressed as nmoles vitamin E/g tissue.

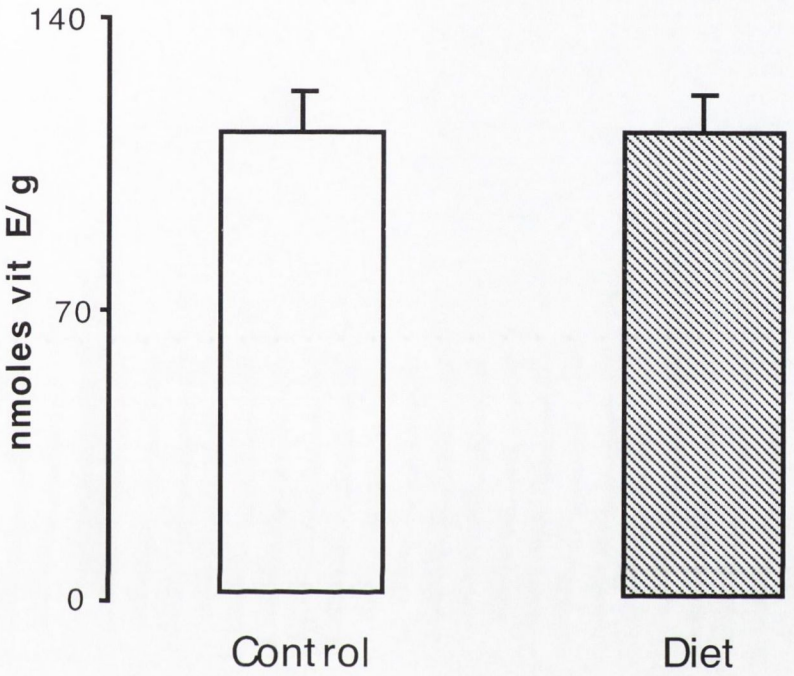

140
70
0
nmoles vit E/ g
Control
Diet

Figure 5.8 Short term dietary supplementation increases the concentration of vitamin C in the hippocampus.

The concentration of vitamin C was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline and all the animals injected i.c.v with IL-1ß were pooled as there was no statistical difference between their respective results. There was a significant increase in vitamin C concentration between the pooled group (n=10) fed the control diet and the pooled group (n=9) fed the test diet. Histograms represent means and bars represent standard errors. Results are expressed as μmoles vitamin C/g tissue.

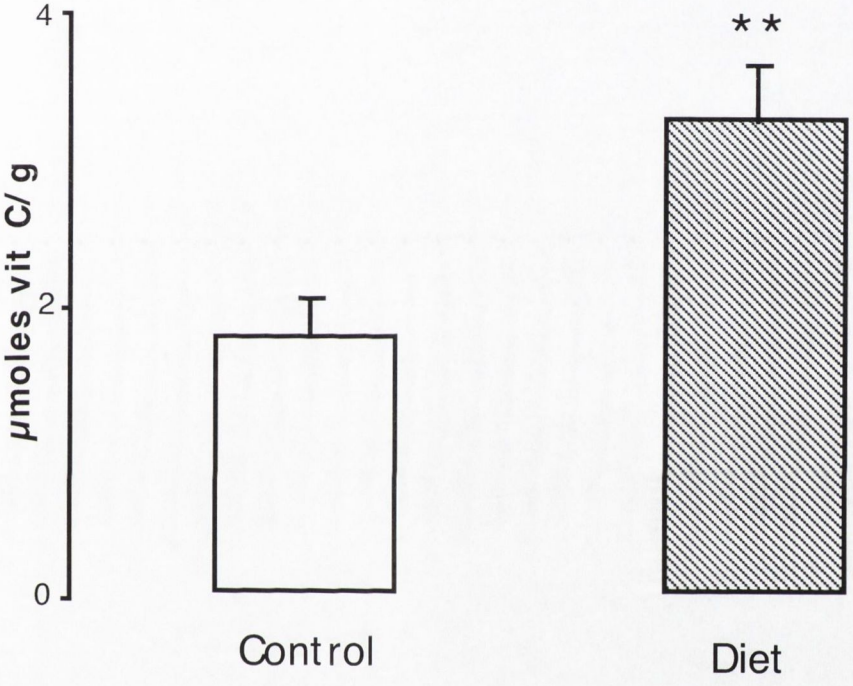

4
2
0
µmoles vit C/ g
* *
Control
Diet

Figure 5.9 Dietary supplementation reverses the increase in lipid peroxidation in the hippocampus caused by the i.c.v. injection of IL-1ß.

Lipid peroxidation was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was a significant increase in lipid peroxidation in the group (n=5) fed the control diet and injected i.c.v. with IL-1ß compared with tissue from the pooled group (n=11) injected i.c.v. with the saline control. This increase was not observed in animals fed the test diet (n=5) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test). Results are expressed as nmoles MDA/g tissue.

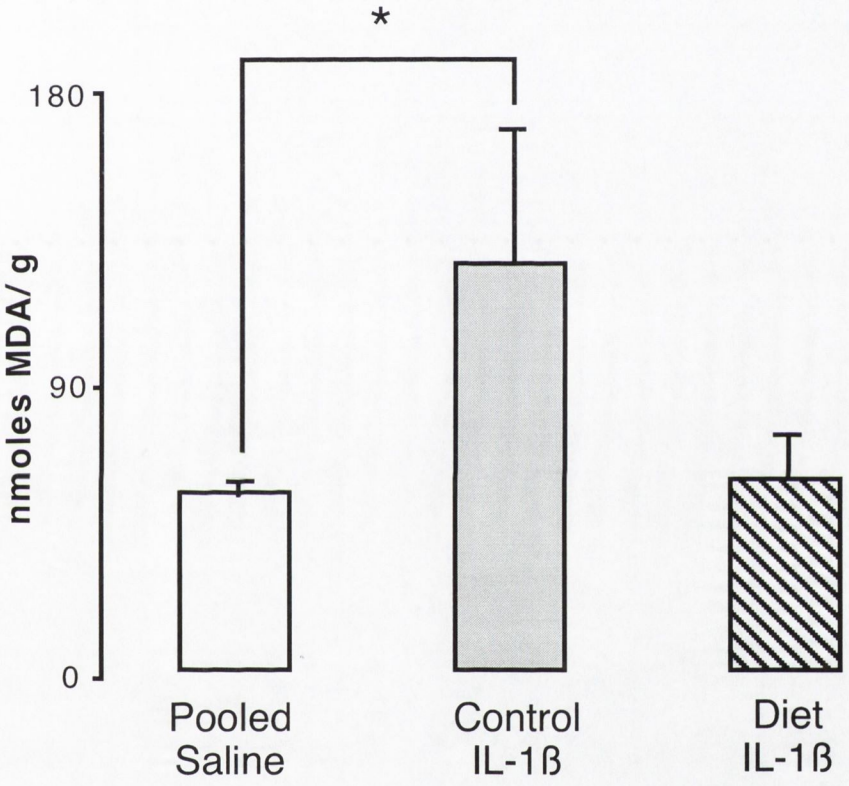

*
180
90
0
nmoles MDA/ g
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.10 There is no IL-1ß or dietary induced change in the concentration of arachidonic acid in the hippocampus.

The concentration of arachidonic acid was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was no change in arachidonic acid concentration between the pooled group (n=12) injected i.c.v. with the saline control, the group (n=6) fed the control diet and injected i.c.v. with IL-1ß or in animals fed the test diet (n=4) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. Results are expressed as nmoles of arachidonic acid (AA)/mg.

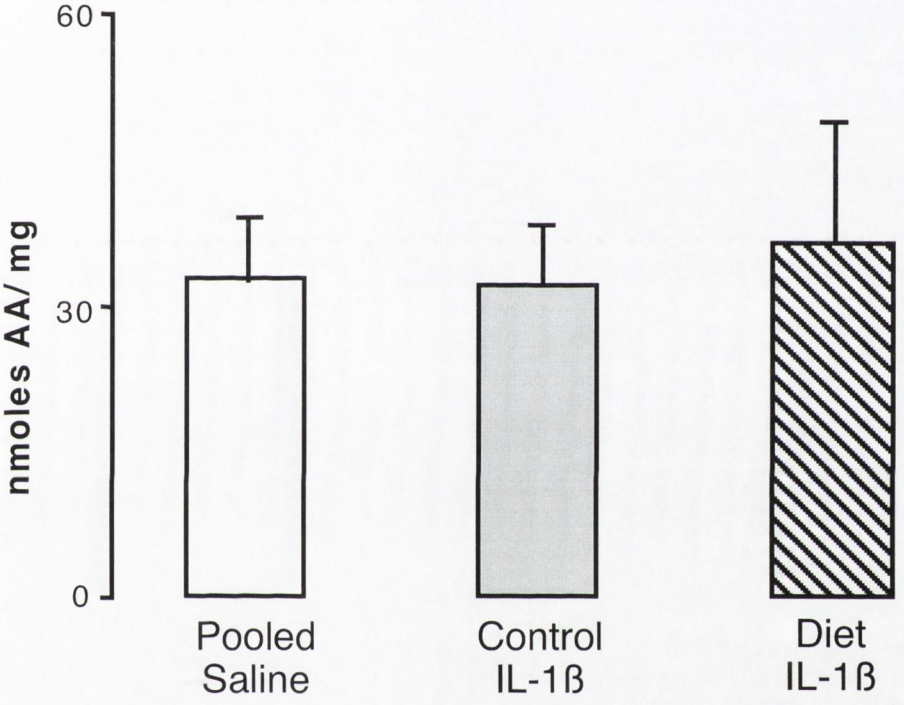
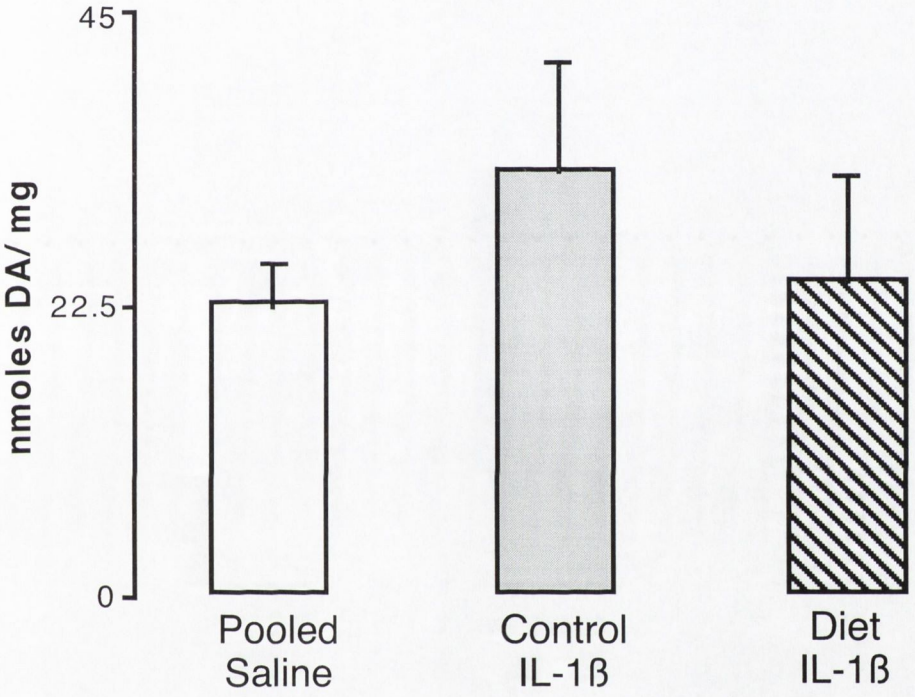

60
30
0
nmoles AA/ mg
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

Figure 5.11 There is no IL-1ß or dietary induced change in the concentration of docosahexanoic acid in the hippocampus.

The concentration of docosahexanoic acid was measured in hippocampal tissue prepared from rats fed a control or test diet and injected with either IL-1ß (3.5 ng/ml) or a saline control. The results for all the animals injected i.c.v. with saline were pooled as there was no statistical difference between their results. There was no change in docosahexanoic acid concentration between the pooled group (n=12) injected i.c.v. with the saline control, the group (n=6) fed the control diet and injected i.c.v. with IL-1ß or in animals fed the test diet (n=4) and injected i.c.v with IL-1ß. Histograms represent means and bars represent standard errors. Results are expressed as nmoles docosahexanoic acid (DA)/mg.


45
22.5
0
nmoles DA/ mg
Pooled
Saline
Control
IL-1ß
Diet
IL-1ß

5.4 **Discussion**

The aim of this study was to assess the effect of intracerbroventricular (i.c.v.) injection of IL-1ß on LTP, the antioxidant defence system, lipid peroxidation and ROS production in the hippocampus. The effect of short term dietary supplementation with vitamins C and E prior to i. c.v. injection of IL-1ß was also assessed.

LTP was impaired in the dentate gyrus of rats injected i.c.v. with IL-1ß and fed the control diet. Previous studies have shown that IL-1ß impairs LTP both *in vitro* and *in vivo*. IL-1ß inhibited LTP in the CA1 region of rat hippocampal slices (Bellinger *et al.*, 1993) and IL-1ß pre-treatment blocked the induction of long-term potentiation in the dentate gyrus *in vitro* while co-application of IL-1ra attenuated this inhibitory effect (Cunningham *et al.*, 1996; Coogan & O'Conner, 1997). In short term incomplete ischemia, experiments which do not cause the neuronal death associated with complete ischemia, there was an impairment of LTP in the dentate gyrus and CA1 region of the rat hippocampus. This inhibition of LTP was blocked by an IL-1ß tri-peptide antagonist (Lys-D-Pro-Thr) implicating the cytokine in the observed neuronal impairment (Yoshioka *et al.*, 1999). The effect of IL-1ß does not appear to be species specific as the cytokine also inhibited LTP in the CA3 region of mouse hippocampal slices (Katsuki *et al.*, 1990). However one study reported that IL-1ß was required for the successful maintenance of LTP; the data indicated that both *in vivo* and *in vitro* an increase in IL-1ß gene expression was observed during LTP in the rat dentate gyrus. This effect was long lasting and specific to potentiation and could be blocked by antagonists of the NMDA receptor. The study also showed that IL-1ra reversibly impaired the maintenance phase of LTP (Schneider *et al.*, 1998). It may be that this effect of IL-1ß is exerted when it is present in very low concentrations, below those that induce inflammatory effects. However this study measured IL-1ß gene expression rather than IL-1ß protein concentration in the dentate gyrus. Clearly large

concentrations of IL-1ß interfere with the increase in synaptic efficiency caused by tetanic stimulation to regions of the hippocampus.

The impairment in LTP caused by the i.c.v. injection of IL-1ß was reversed in the hippocampus of rats fed the test diet. This would imply that the impairment in LTP observed is due to the generation of an oxidative stress within the hippocampus. In the previous chapter it was suggested that IL-1ß may act as a trigger or mediator for oxidative stress within neuronal tissue and that this may adversely effect the functional efficiency of that tissue. The data from this chapter indicate that the i.c.v. injection of IL-1ß increased ROS production within the hippocampus of rats fed the control diet. This implies that the IL-1ß-treated neuronal tissue was under oxidative stress. In hepatic ischemia/reperfusion injury experiments it was demonstrated that ROS production was increased markedly and this was related to an increase in injured hepatic cells. However pre-treatment with IL-1ra, to block IL-1 mediated actions, significantly decreased ROS production and induced a marked decrease in the number of injured cells. This would imply that IL-1ß may not only be released as a *consequence* of an increase in oxidative stress in tissue but it may act as a central trigger to initiate the increase in ROS production (Shirasugi *et al.*, 1997). In previous studies it was shown that IL-1ß increased the production of ROS and lipid peroxidation *in vitro* in the hippocampus of 4 month old rats (Murray & Lynch, 1999). An increase in ROS production would inhibit the processes required to initiate and maintain LTP. For example it was found that H_2O_2 alters neuronal excitability in the CA1 region of the guinea pig hippocampus causing a reduction in excitatory post synaptic potentials (Pellmar, 1987). Low concentrations of H_2O_2 were shown to cause the loss of a non-NMDA associated potentiation in the CA1 region of the rat hippocampus (Auerbach & Segal, 1997). It was noted that the aged rat hippocampus was unable to produce non-NMDA associated potentiation and also possessed a higher concentration of H_2O_2 than hippocampal tissue of young rats, however this type of

LTP could be recovered in aged hippocampal slices with the addition of catalase implying that the excessive production of H_2O_2 was mediating the neuronal impairment (Auerbach & Segal, 1997). Another study demonstrated that an $O_2^{-\bullet}$ generator, dihydroxyfumarate (DHF) impaired neuronal excitability and increased lipid peroxidation in rat hippocampal slices (Pellmar & Lepinski, 1992). These studies indicate that a significant oxidative stress can impair the functionality of the hippocampus.

There may be some requirement for a basal formation of ROS in neuronal function. Data supporting this view showed that incubation of manganese porphyrin compounds, which specifically scavenge $O_2^{-\bullet}$, with hippocampal slices prevented the induction of LTP (Klann *et al.*, 1998). Furthermore it was observed that $O_2^{-\bullet}$ generation in conjunction with $NO^{\bullet}$ production was required for the successful induction of LTP in the CA1 region of rat hippocampal slices (Klann *et al.*, 1998).

There was a significant increase in SOD activity in both dietary groups of IL-1ß-injected animals. Since there was no concomitant increase in the activities of catalase or GPx in any of the groups, it might be predicted that an increase in SOD activity could lead to an increased production of H_2O_2. This increase in SOD activity may, in part, be responsible for the increase in lipid peroxidation seen in the animals injected i.c.v. with IL-1ß and fed the control diet. Previous studies support a role for enhanced SOD activity mediating an impairment in neuronal excitability; DHF a $O_2^{-\bullet}$ generator, impaired neuronal excitability and increased lipid peroxidation in rat hippocampal slices. The exogenous application of SOD to these slices exacerbated the DHF-induced electrophysiological impairment and lipid peroxidation by increasing the concentration of H_2O_2 in the tissue. Only a SOD plus catalase treatment attenuated the neuronal signalling impairment and membrane oxidation effects of the compound. (Pellmar & Lepinski, 1992). Further evidence is derived from studies which reported an impairment in LTP in transgenic mice that overexpress the gene for CuZnSOD,

these mice were also deficient in spatial memory. However LTP could be induced in hippocampal slices from these animals after treatment with catalase or the antioxidant N-t-butyl-phenylnitrone. It was proposed that enhanced CuZnSOD activation leads to the excessive formation of H_2O_2 in the neuronal tissue of these mice and this is the main cause of the observed cognitive deficits (Gahtan *et al.*, 1998). In the previous chapter it was shown that there was an increase in ROS production and in SOD activity upon incubation of hippocampal tissue with IL-1ß *in vitro*. The data from this chapter indicates that IL-1ß increases SOD activity *in vivo* and it may be predicted that one consequence of this is an increase in the production of ROS. However IL-1ß has been shown to increase ROS production by other mechanisms. For example, in vascular smooth muscle cells IL-1ß directly stimulated $NO^{\bullet}$ production and also enhanced the production of $ONOO^{-}$ through an IL-1ß mediated upregulation of inducible nitric oxide synthase (iNOS) (Boota *et al.*, 1996). In myenteric plexus neurons IL-1ß pre-treatment resulted in the increased expression of iNOS and MnSOD protein implying that enhanced production of $NO^{\bullet}$ as well as H_2O_2 may contribute to IL-1ß mediated neuronal oxidative stress (Valentine *et al.*, 1996).

In this study IL-1ß-treated rats fed the control diet showed an increase in lipid peroxidation although there was no change in the concentrations of arachidonic acid or docosahexanoic acid in hippocampal tissue. It is therefore proposed that short-term oxidative stress does not impact on the polyunsaturated fatty acid concentration of neuronal tissue. This indicates that polyunsaturated fatty acids were not the target molecules for lipid peroxidation in this study. This contrasts with previous studies; for example polyunsaturated fatty acid concentration was decreased in the hippocampus of aged rats in parallel to an increase in ROS production and an increase in lipid peroxidation (Murray & Lynch, 1998). The difference in observations may be due to the length of time tissue was exposed to oxidative stress. In the present study the tissue was exposed to IL-1ß for 90 minutes , in contrast it is likely that an age-related

increase in IL-1ß concentration in the cortex and hippocampus of the rat develops over a much longer time frame therefore exposing tissue to oxidative stress over a prolonged period. Another study observed that short term oxidative stress increased cell membrane vulnerability to lipid peroxidation without altering the polyunsaturated fatty acid content of the tissue. It was reported in rabbit aortic endothelial cells, which underwent short-term ischemia and reperfusion, that the incorporation of arachidonic acid into the membranes of cells was less inhibited than that of oleic or linoleic acids. This resulted in a greater polyunsaturated fatty acid concentration in the membranes and an increased susceptibility to lipid peroxidation (McLeod & Sevanian, 1997).

The test diet succeeded in significantly increasing the concentration of vitamin C in the hippocampal tissue of rats being fed on the supplemented diet. It is proposed that the increase in the concentration of vitamin C in the hippocampus was sufficient to attenuate the increase in lipid peroxidation. Other reports observed that large doses of vitamin C attenuated alcohol related oxidative stress as measured by lipid peroxidation in the guinea pig liver while another report showed that vitamin C supplementation increased the global antioxidant capacity of the heart (Suresh *et al.*, 1999; Rojas *et al.*, 1994). This finding is supported by the finding that in rabbit aortic endothelial cells which underwent ischemia and reperfusion there was a reduction in lipid peroxidation while incubated in the presence of vitamin C (McLeod & Sevanian, 1997).

There was no change in vitamin E concentration between the diet groups. This was not surprising since it has been shown that it takes some months before an increase in dietary vitamin E intake elevates concentrations of this molecule in the CNS (Vatassery *et al.*, 1988). GSH concentrations were also unchanged by diet.

The hypothesis put forward in this chapter is that IL-1ß increases oxidative stress on neurons in the dentate gyrus and this effect impairs cell function and the ability to maintain LTP. In support of this hypothesis is the finding that IL-1ß injected

animals fed the control diet had an impairment in LTP, an increase in ROS production and an increase in lipid peroxidation in hippocampal tissue. IL-1ß treated animals also had increased SOD activity without parallel increases in the activities of GPx or catalase. Dietary supplementation with antioxidant vitamins increased the concentration of vitamin C in the hippocampus, reversed the IL-1ß induced increase in lipid peroxidation and restored the ability to maintain LTP in IL-1ß injected animals. It is proposed that increased activity of SOD is, in part, responsible for the increase in the production of ROS and this increase disturbs cell function and interferes with the ability to sustain LTP. Increasing the antioxidant capacity of the hippocampus by dietary supplementation reduces the deleterious effects of increased ROS production, such as lipid peroxidation, and prevents impairment of neuronal function. It was found that dietary supplementation of aged animals with vitamin E and vitamin C decreased oxidative stress within hippocampal tissue, reduced concentrations of IL-1ß in that tissue and restored the ability to maintain LTP in the dentate gyrus (Murray & Lynch, 1998). This implies that the damage caused by the aging process may be mediated partly by oxidative stress and that the functional efficiency of aged tissue may be increased by dietary supplementation with large doses of antioxidants. The results from this chapter directly implicate IL-1ß as a key trigger in the initiation of oxidative stress in hippocampal tissue and the consequent reduction of functional efficiency within that tissue.

Chapter 6

Short Term Dietary Supplementation Reverses Stress-Mediated Neuronal Changes.

6.1 **Introduction**

In the previous experiments it was observed that an increase in the concentration of IL-1ß in the hippocampus was associated with an increase in the production of ROS, possibly through an increase in the activity of SOD. It has also been observed that dietary supplementation with high doses of the vitamins C and E attenuates increased ROS production. Isolation stress has been shown to increase the concentration of IL-1ß in the dentate gyrus and to impair LTP in rats (Murray & Lynch, 1998). Is IL-1ß responsible for the neuronal deficit observed in this stress paradigm and will vitamin supplementation reverse this deficit? The aim of this series of experiments will be to examine the effect of short term isolation stress and dietary supplementation on (a) the ability of animals to maintain LTP, (b) components of the antioxidant defence system, (c) ROS production and (d) markers of oxidative stress.

6.2 **Methods**

6.2.1 Induction of Stress

Young (2-4 months) male Wistar rats were housed in groups of 6 for 5 days. Experimental animals were stressed by social isolation i.e. rats were removed from the group cage and placed alone in a smaller cage for a period of 5 days before being sacrificed. Control (non-stressed) animals continued to be housed in groups of six. All animals were maintained under a 12 hr light/dark cycle at a temperature between 22 and 23°C. During this period all animals underwent dietary supplementation (see section 6.2.2). All rats had free access to their supplemented food and water.

6.2.2 Animals and Dietary Supplementation

Rats were randomly divided into subgroups (6 rats in each subgroup). They were housed 6 per cage or in isolation (see section 6.2.1). 2 subgroups were fed for 5 days with normal laboratory chow supplemented with a daily dose of dl-α-tocopheryl

acetate (250mg/rat/day; 50% Type SD Vitamin E, Beeline Healthcare, Dublin) dissolved in corn oil. Each gram of the dry powder product contained 500mg of dl-α-tocopheryl acetate. Vitamin C (250 mg/rat/day; Beeline Healthcare, Dublin) was added to the water supplied to the cages. The vitamin C product consisted of approximately 97% L-ascorbic acid. The other subgroups were fed for the same 5 day period on normal laboratory chow with added corn oil to ensure an isocaloric diet. The water to these cages was not supplemented. Daily food and water intake was monitored over a 5 day period before the start of dietary supplementation. During the 5 day dietary phase animals were given 95% of their average daily food intake and 100% of their average water intake. Rats were weighed before and after the 5 day experiment. General heath and behaviour was monitored daily. The vitamin supplemented diet will be referred to as the "test diet".

6.2.3 Induction of LTP *in vivo*

Each rat was weighed before use and anaesthetised by injection of urethane (1.5 g/kg) intraperitoneally. The head was positioned in a head holder within a stereotaxic frame (for more details see 2.3.2). A window of skull was removed to allow the insertion of the stimulating and recording electrodes (for more details see section 2.3) 4.4mm lateral to lambda and 2.5mm lateral to and 3.9 mm posterior to bregma respectively. The stimulating and recording electrodes were slowly lowered to a depth of 2-3.5 mm to maximise the responses (1-2mV amplitude) and displayed on a computer screen. Stimuli were delivered at 30 second intervals and the slopes of the field EPSP were recorded for a 10 min period before and 40 min after delivery of 3 trains of stimuli (250Hz for 200msec) at 30 sec intervals.

6.2.4 Tissue Storage

At the end of an LTP recording period individual animals were killed by cervical dislocation and their hippocampii were removed. Hippocampal tissue was either (a) homogenised in Krebs solution and frozen at -80°C, (b) chopped into slices placed in a 10% Krebs/DMSO solution and frozen at -80°C or (c) homogenised in a 5% TCA solution and frozen at -80°C (for more details see section 2.4). Blood samples were collected for analysis of plasma corticosterone concentration.

6.2.5 Analysis of SOD Activity

This method is described in detail in section 2.5.5. Homogenised tissue (see 6.2.4) was thawed, supernatant obtained and serially diluted in phosphate buffer. An aliquot from each dilution was added to assay buffer. The rate of NBT reduction was assessed at 560nm for 5 min. Results are expressed in units of SOD activity per milligram of protein.

6.2.6 Analysis of GPx Activity

This method is described in detail in section 2.5.3. A standard curve of absorbance versus NADPH concentration was constructed. Homogenised tissue (see 6.2.4) was thawed, supernatant obtained and added to assay medium. The reaction was initiated by the addition of cumene hydroperoxide solution. Absorbance at 340nm was recorded for 5 min using a spectrophotometer. Results were expressed as μmoles NADPH oxidised per min per milligram of protein.

6.2.7 Analysis of Catalase Activity

This method is described in detail in section 2.5.6. Homogenised tissue (see 6.2.4) was thawed, supernatant obtained and added to microfuge tubes containing phosphate buffer. The reaction was initiated by the addition of H_2O_2 and after 2 and 10 min aliquots were removed and quenched. Colour was developed at room

temperature by addition of KSCN. Two aliquots from each tube were transferred immediately to a well plate and read at 492nm. The results were expressed in terms of the first order reaction rate constant (k) and protein.

6.2.8 Analysis of the Concentration of Vitamin C

This method is described in detail in section 2.5.4. Tissue stored in 5% TCA solution (see 6.2.4) was thawed, supernatant obtained and mixed with assay buffer and incubated for 3 hr in a waterbath. Ice-cold 65% H_2SO_4 was added and mixed with each sample or standard. Samples and standards were read at 545nm and the results were expressed as μmoles of ascorbic acid per gram of tissue.

6.2.9 Analysis of GSH Concentration

This method is described in detail in section 2.5.2. Tissue stored in 5% TCA solution (see 6.2.4) was thawed and supernatant obtained. Supernatant or standard was added to sample buffer. After incubating for 1 min NADPH was added and the absorbance at 412 nm was monitored for 2 min using a colorimeter. Results are expressed as μmol GSH per gram of tissue.

6.2.10 Analysis of the Concentration of Vitamin E

This method is described in detail in section 2.5.9. Homogenate was thawed, saponified by boiling and its lipid soluble elements extracted by washing in hexane. The hexane phase was removed and evaporated under a stream of nitrogen.. Samples and standards were analysed by reverse phase liquid chromatography with 75% acetonitrile:25% methanol as mobile phase at a flow rate of 1.2 ml/min. Samples and standards were placed in an autosampler and α-tocopherol was detected at 298nm using a SpectraPhysics HPLC. Results were expressed as nmoles vitamin E per gram of tissue.

6.2.11 Analysis of Lipid Peroxidation

This method is described in detail in section 2.5.8. Homogenate was thawed (see 6.2.4) and an aliquot was placed in a microfuge tube. Aliquots of assay solution were added to microfuge tubes containing the standard or samples and incubated at 90°C for 1 hr. Aliquots from each tube were transferred to a 96-well plate and read at 545nm. Results were expressed as nmoles of MDA per gram of tissue.

6.2.12 Determination of Fatty Acid Concentrations

This method is described in detail in section 2.5.10. Aliquots of homogenate or fatty acid standards were thawed and washed in organic solvents to extract the organic phase. This phase was evaporated under nitrogen and resuspended in ethanol for analysis by high pressure liquid chromatography (HPLC). Fatty acids were first derivatised, extracted into hexane and evaporated to dryness under nitrogen. The samples and standards were resuspended in methanol and injected into the HPLC column and detected by UV spectroscopy at 230nm. Individual fatty acid concentrations were expressed as nmol/mg of tissue.

6.2.13 Determination of Reactive Oxygen Species Production

This method is described in detail in section 2.5.14 of chapter 2. Synaptosomes were prepared, added to Tris buffer and incubated for 15 min at 37°C in the presence of DCFH-DA. Samples were centrifuged, the pellets resuspended, vortex-mixed, and incubated in a 37°C water bath and fluorescence was measured at 525nm emission upon excitation at 488nm. Results are expressed as nmoles DCF/mg.

6.2.14 Analysis of IL-1ß Concentration

This method is described in detail in section 2.5.7. Homogenate was thawed (see 6.2.4) and stored on ice. A 96-well microtiter plate was coated with capture antibody by incubating overnight at 4°C. Blocking buffer was added and incubated for 2 hr at 37°C. The wells were aspirated and washed before the addition of the diluted standards and sample homogenate. Plates were incubated and washed before the addition of diluted secondary antibody. Diluted detection reagent was added and the plate was incubated for 15 min and washed. Working TMB substrate solution was added prior to the addition of H_2SO_4 to stop the reaction. The plate was read at 450nm. A standard curve was constructed plotting the standards against absorption. Results are given as pg IL-1ß per milligram protein.

6.2.15 Analysis of Corticosterone

This method is described in detail in section 2.5.17. Corticosterone was determined using an Immunodiagnostic System Gamma-B ^{125}I Corticosterone kit. ^{125}I-corticosterone was added to standards and samples followed by corticosterone anti-serum. All tubes were incubated overnight at 4°C before secondary antibody was added. Saline (0.9% NaCl) was added before centrifugation at 5000rpm for 15 min. Supernatant was decanted and radioactivity counted in a Spectron Gamma Counter. A semi-logarithmic curve of % B/Bo v standards was plotted and unknowns determined. Results are expressed as ng corticosterone/ ml

6.3 **Results**

6.3.1 **Effect of Stress and Diet on LTP**

Figure 6.1 shows that delivery of a high frequency train of stimuli to the perforant path resulted in an immediate increase in the slope of the epsp in all subgroups of rats and there was no effect on the epsp slope or the amplitude of the response by the test diet. Analysis of individual results showed that all unstressed rats

sustained LTP for the duration of this experiment. Mean epsp slope was expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation.

In rats fed on control diet mean percentage changes in the mean population epsp slope (± SEM) in the 2 min immediately after tetanic stimulation were 152.05 ± 7.79 and 117.13 ± 4.75 in non-stressed and stressed rats respectively. The corresponding values in the last 5 min of the experiment were 136.5 ± 2.86 and 97.81 ± 1.22

In rats fed on the test diet mean percentage changes in the mean population epsp slope (± SEM) in the 2 min immediately after tetanic stimulation were 185.35 ± 24.05 and 157.16 ± 9.28 in unstressed and stressed rats respectively. The corresponding values in the last 5 min of the experiment were 149.55 ± 4.56 and 125.14 ± 2.06.

Analysis of the data show that the maintenance of LTP was significantly decreased in the last 5 min of the experiment in stressed rats fed the control compared with the unstressed group fed the control diet. ($p<0.01$;Student's t-test). LTP was partially restored in stressed rats fed the test diet.

6.3.2 **Effect of Stress and Diet on ROS Production**

Figure 6.2 indicates that there was an increase in ROS production in hippocampal tissue of stressed rats fed the control diet compared with the hippocampal tissue of the pooled unstressed rats fed both diets. This increase in ROS production, which failed to reach statistical significance, was attenuated in stressed rats fed the test diet.

6.3.3 **Effect of Stress and Diet on SOD Activity**

Figure 6.3 indicates that there was a significant increase in the activity of SOD in the hippocampal tissue of stressed rats fed the control diet compared with the activity of the enzyme in the hippocampal tissue of the pooled group of unstressed rats fed both diets (*$p<0.05$; Student's t-test). This significant increase in SOD activity in hippocampal supernatant was not seen in stressed rats fed the test diet.

6.3.4 **Effect of Stress and Diet on GPx Activity**

Figure 6.4 indicates that GPx activity was similar in the hippocampal tissue of the pooled group of unstressed rats fed both diets, the stressed rats fed the control diet and the stressed rats fed the test diet.

6.3.5 **Effect of Stress and Diet on Catalase Activity**

Figure 6.5 indicates that catalase activity was similar in the hippocampal tissue of stressed rats fed the control diet compared with the activity of the enzyme in the hippocampal tissue of the pooled groups of unstressed rats fed both diets. However there was a significant decrease in catalase activity in the hippocampal tissue of stressed rats fed the test diet compared with the activity of the enzyme in the hippocampal tissue from the stressed rats on the control diet. (*$p<0.05$; Student's t-test).

6.3.6 **Effect of Stress and Diet on Vitamin C Concentration**

Figure 6.6 indicates that there was a significant increase in the concentration of vitamin C in the tissue of rats fed the control diet compared with the tissue of rats fed the test diet. (*$p<0.05$; Students t-test). For both groups, data from stressed and unstressed rats were pooled.

6.3.7 **Effect of Stress and Diet on GSH Concentration**

Figure 6.7 indicates that there was a significant decrease in concentration of GSH in hippocampal tissue of stressed rats fed the control diet compared with hippocampal tissue of the pooled groups of unstressed rats fed both diets (*$p<0.05$; Student's t-test). This significant decrease in GSH concentration in hippocampal tissue was not seen in stressed rats fed the test diet.

6.3.8 **Effect of Stress and Diet on Vitamin E Concentration**

Figure 6.8 indicates that vitamin E concentration was similar in the hippocampal tissue of the pooled group of unstressed rats fed both diets, the stressed rats fed the control diet and the stressed rats fed the test diet.

6.3.9 **Effect of Stress and Diet on IL-1ß Concentration**

Figure 6.9 indicates that there was an increase in the concentration of IL-1ß in the tissue of stressed rats fed the control diet compared with the hippocampal tissue of the pooled group of unstressed rats fed both diets. This increase in the concentration of IL-1ß, which did not reach statistical significance, was significantly attenuated in the tissue of stressed rats fed the test diet compared with stressed rats fed the control diet. (*$p<0.05$; Student's t-test).

6.3.10 **Effect of Stress and Diet on Lipid Peroxidation**

Figure 6.10 indicates that there was no change in the extent of lipid peroxidation in hippocampal tissue from either; the pooled group of unstressed rats fed both diets, the stressed rats fed the control diet or the stressed rats fed the test diet.

6.3.11 **Effect of Stress and Diet on Arachidonic Acid Concentrations**

Figure 6.11 indicates that arachidonic acid concentration was similar in hippocampal tissue from the pooled group of unstressed rats fed both diets, the stressed rats fed the control diet and the stressed rats fed the test diet.

6.3.12 **Effect of Stress on Corticosterone Concentration in Plasma**

Figure 6.12 indicates that corticosterone concentration was significantly increased in the plasma of stressed rats compared with unstressed rats. (*$p<0.05$; Student's t-test).

Figure 6.1 The stress-induced impairment in LTP is reversed by dietary supplementation.

Rats were anaesthetised by intraperitoneal injection of urethane. Test shocks were given at a rate of 1/30 seconds and were delivered for 10 min prior to tetanic stimulation. LTP was induced by delivery of 3 high frequency trains of stimuli before stimulation at test shock frequency resumed and recordings continued for 45 min.

Mean percentage epsp slope in the dentate gyrus evoked by test stimuli delivered to the perforant path at 30 sec intervals before and after tetanic stimulation (arrow) in rats either unstressed (open symbols) or stressed (closed symbols) and fed either a control or test diet. Mean epsp slope for each group is expressed as a percentage of the mean slope recorded in the 5 min immediately prior to tetanic stimulation and the data is presented as the mean change with time. Values on the 5 min pre-tetanus were normalised to 100%. Isolation stress impaired the induction (152.05 ± 7.79 and 117.13 ± 4.75 in unstressed and stressed rats respectively) and maintenance phase (136.5 ± 2.86 and 97.81 ± 1.22 in unstressed and stressed rats respectively) of LTP compared with unstressed controls ($p<0.01$;Student's t-test). LTP was partially restored in stressed animals (149.55 ± 4.56 and 125.14 ± 2.06 in unstressed and stressed rats respectively) fed the test diet.

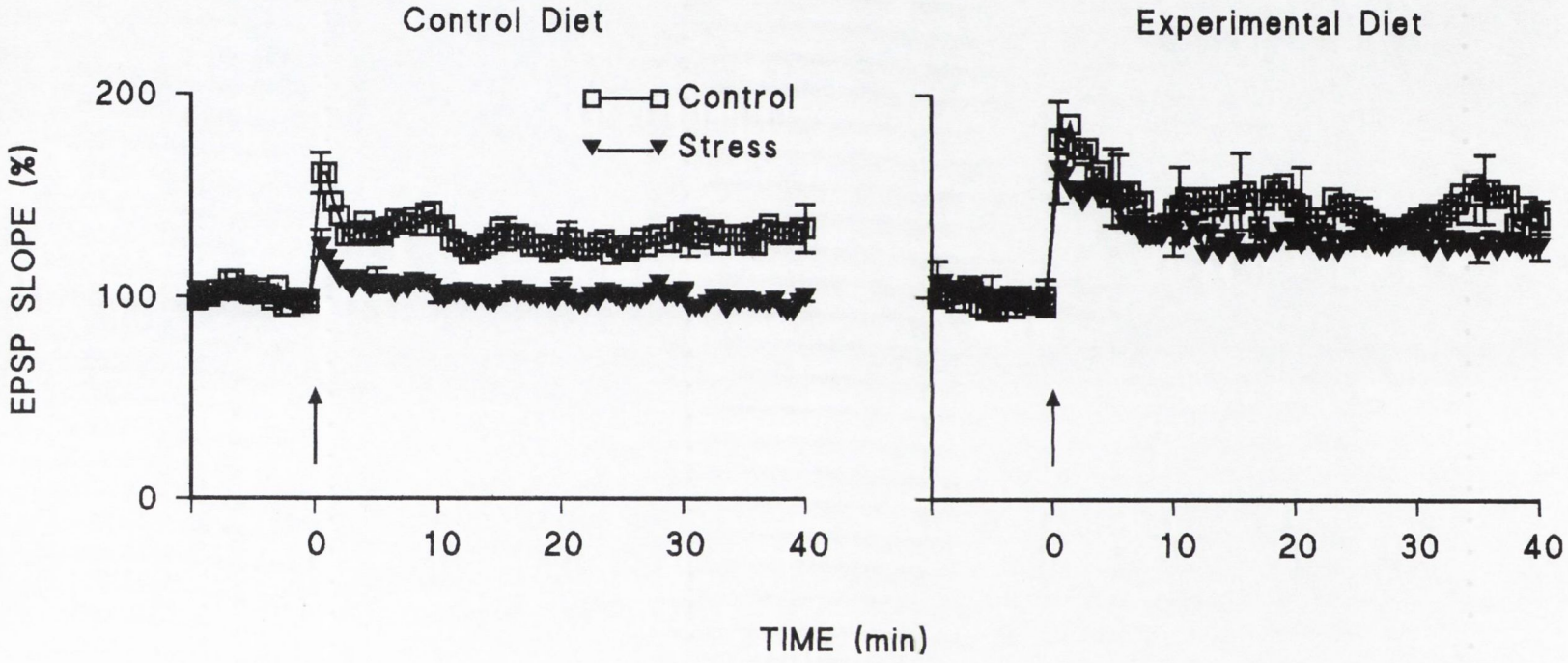

Control Diet
Experimental Diet
Control
Stress
200
100
0
EPSP SLOPE (%)
0
10
20
30
40
TIME (min)

Figure 6.2 Stress increases ROS production in the hippocampus.

ROS production was measured in hippocampal synaptosomes prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was an increase, which did not reach statistically significance, in ROS production in the stressed group (n=5) fed the control diet compared with tissue from the unstressed pooled group fed both diets (n=11). This increase was attenuated in stressed animals fed the test diet (n=5). Histograms represent means and bars represent standard errors. Results are expressed as nmoles DCF/mg tissue

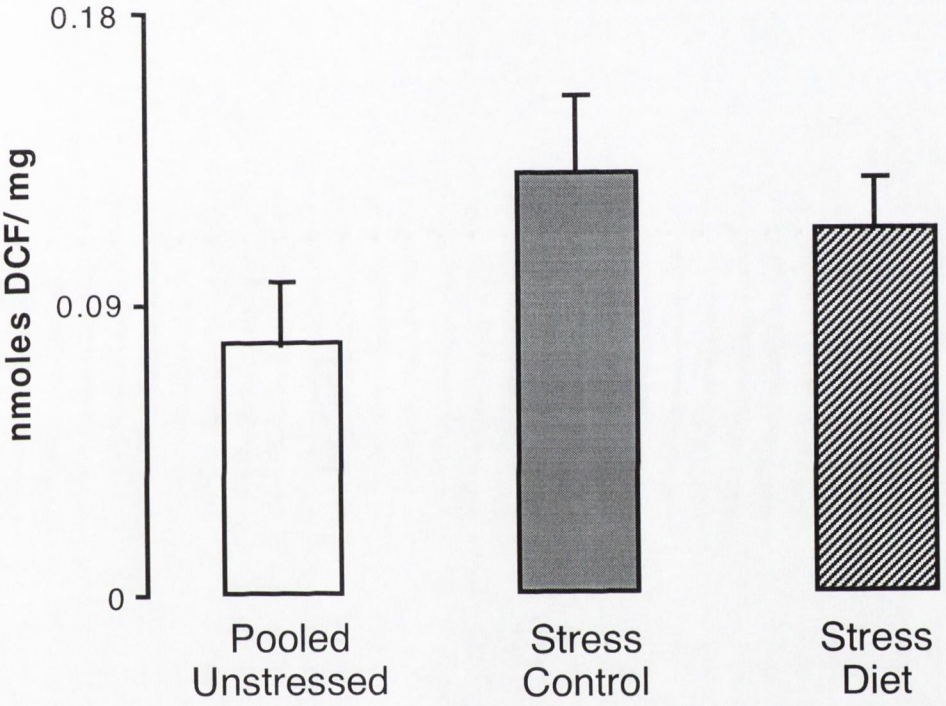

0.18
0.09
0
nmoles DCF/ mg
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.3 Dietary supplementation reverses the stress induced increase in SOD activity in the hippocampus.

SOD activity was measured in hippocampal supernatant prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was an increase in SOD activity in the stressed group (n=5) fed the control diet compared with tissue from the unstressed pooled group fed both diets (n=11). This significant increase was significantly attenuated in stressed animals fed the test diet (n=5). Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test). Results are expressed as enzyme units (U)/mg protein.

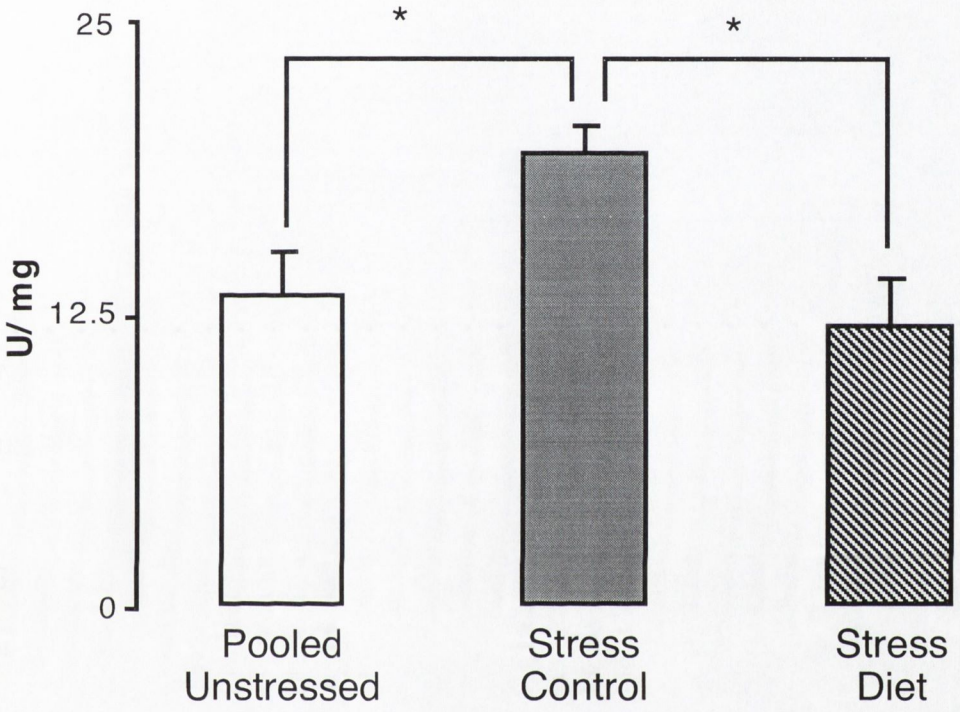

25
12.5
0
U/ mg
*
*
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.4 There is no stress or dietary related change in the activity of GPx in the hippocampus.

GPx activity was measured in hippocampal supernatant prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was no change in GPx activity between the unstressed pooled group fed both diets (n=11), the stressed group (n=5) fed the control diet or in stressed animals fed the test diet (n=5). Histograms represent means and bars represent standard errors. Results are expressed as μmoles NADPH oxidised/min/mg protein.

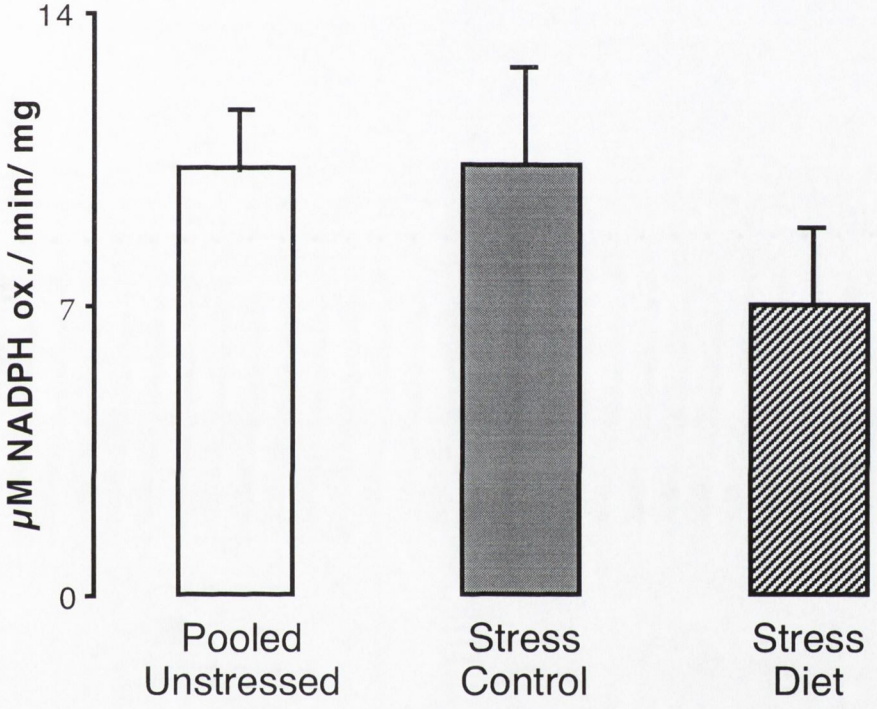

14
7
0
μM NADPH ox./ min/ mg
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.5 Dietary supplementation decreases catalase activity in the stressed hippocampus.

Catalase activity was measured in hippocampal supernatant prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was no change in catalase activity in the stressed group (n=5) fed the control diet compared with tissue from the unstressed pooled group fed both diets (n=11). There was, however, a significant decrease in the activity of the enzyme in hippocampal supernatant from stressed rats fed the test diet compared with samples from stressed rats fed the control diet. Histograms represent means and bars represent standard errors. Results are expressed as a function of the reaction rate (k)/mg protein. (*$p<0.05$; Student t-test).

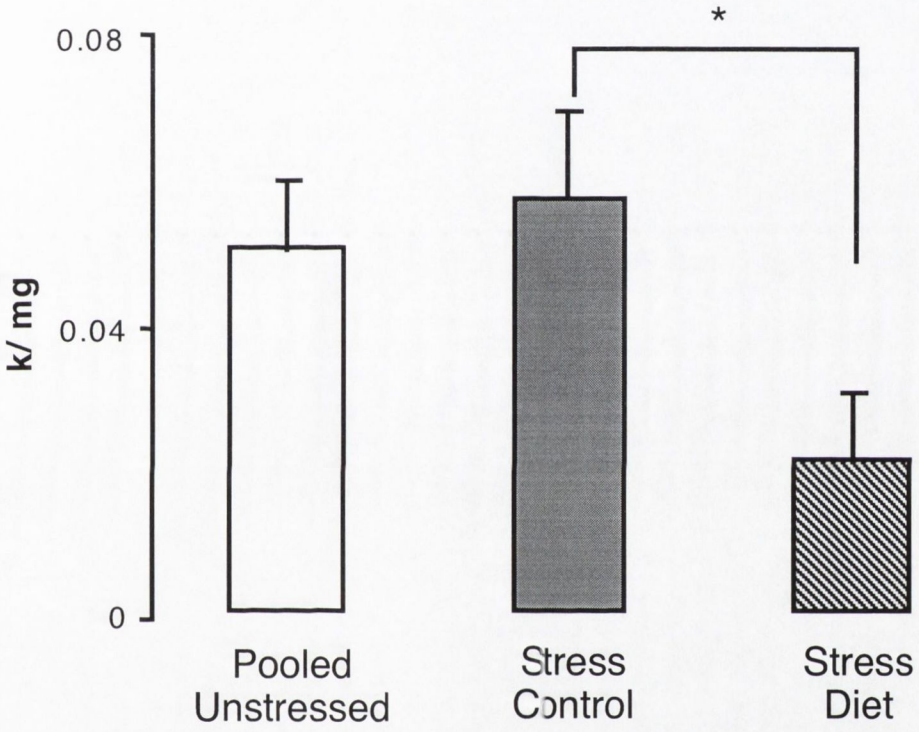

*
0.08
0.04
0
k/ mg
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.6 Dietary supplementation reverses the stress related change in the concentration of GSH in the hippocampus.

GSH concentration was measured in hippocampal tissue prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was a significant decrease in GSH concentration between the unstressed pooled group fed both diets (n=11) and the stressed group (n=5) fed the control diet. However this decrease was not observed in stressed animals fed the test diet (n=5). Histograms represent means and bars represent standard errors. Results are expressed as μmoles GSH /g tissue.

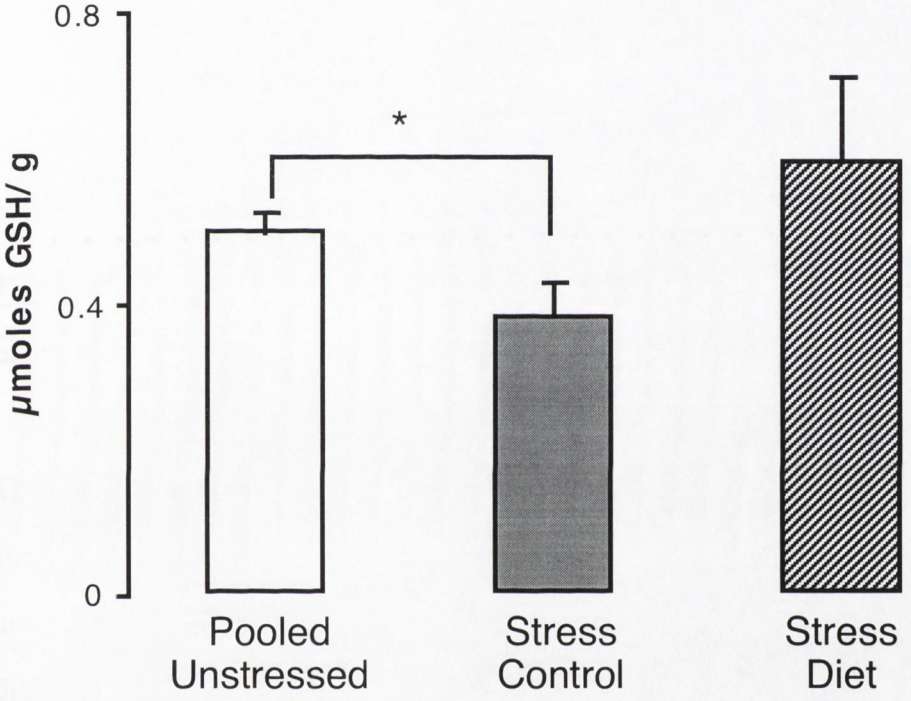

0.8
0.4
0
µmoles GSH/ g
*
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.7 Short term dietary supplementation does not increase the concentration of vitamin E in the hippocampus.

The concentration of vitamin E was measured in hippocampal tissue prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals and all the stressed animals were pooled as there was no statistical difference between their respective results. There was no change in vitamin E concentration between the pooled group (n=10) fed the control diet and the pooled group (n=9) fed the test diet. Histograms represent means and bars represent standard errors. Results are expressed as nmoles vitamin E/g tissue.

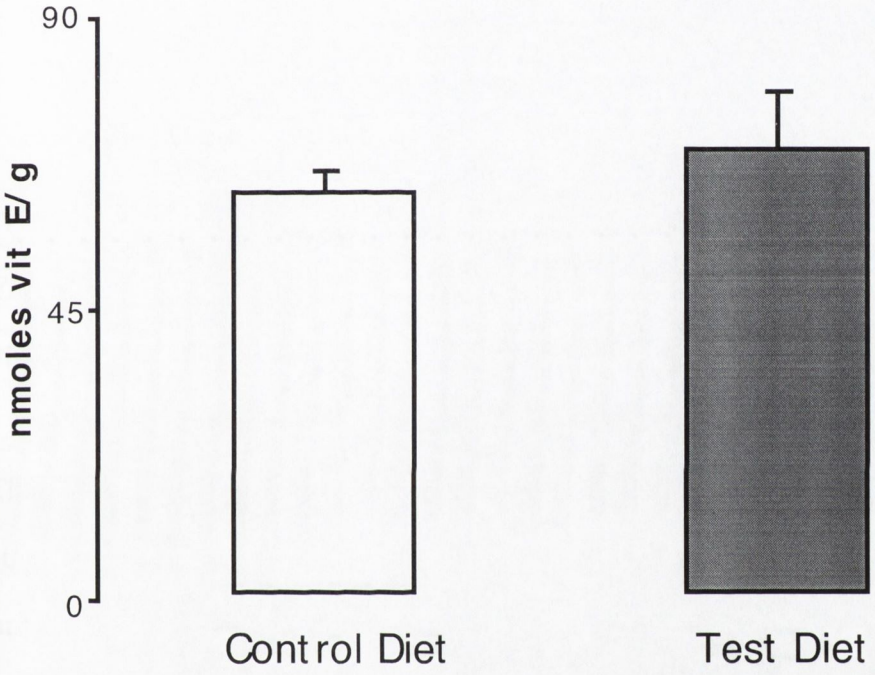

90
45
0
nmoles vit E/ g
Control Diet
Test Diet

Figure 6.8 Short term dietary supplementation increases the concentration of vitamin C in the hippocampus.

The concentration of vitamin C was measured in hippocampal tissue prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals and all the stressed animals were pooled as there was no statistical difference between their respective results. There was no change in vitamin C concentration between the pooled group (n=10) fed the control diet and the pooled group (n=9) fed the test diet. Histograms represent means and bars represent standard errors. Results are expressed as μmoles vitamin C/g tissue.

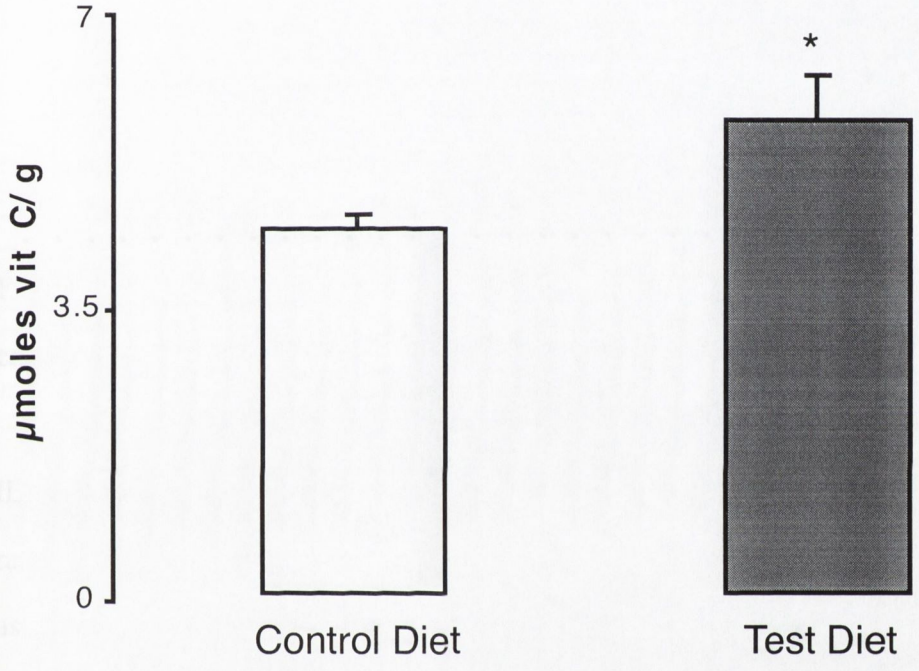

7
3.5
0
µmoles vit C/ g
*
Control Diet
Test Diet

Figure 6.9 Dietary supplementation reverses the stress related increase in the concentration of IL-1ß in cortical tissue.

IL-1ß was measured in hippocampal supernatant prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was an increase, though not statistically significant, in the stressed group (n=5) fed the control diet compared with tissue from the unstressed pooled group fed both diets (n=11). However there was a significant decrease in IL-1ß in stressed animals fed the test diet (n=5) compared with tissue from stressed animals fed the control diet. Histograms represent means and bars represent standard errors. (* $p<0.05$;Student's t-test). Results are expressed as pg IL-1ß/ mg.

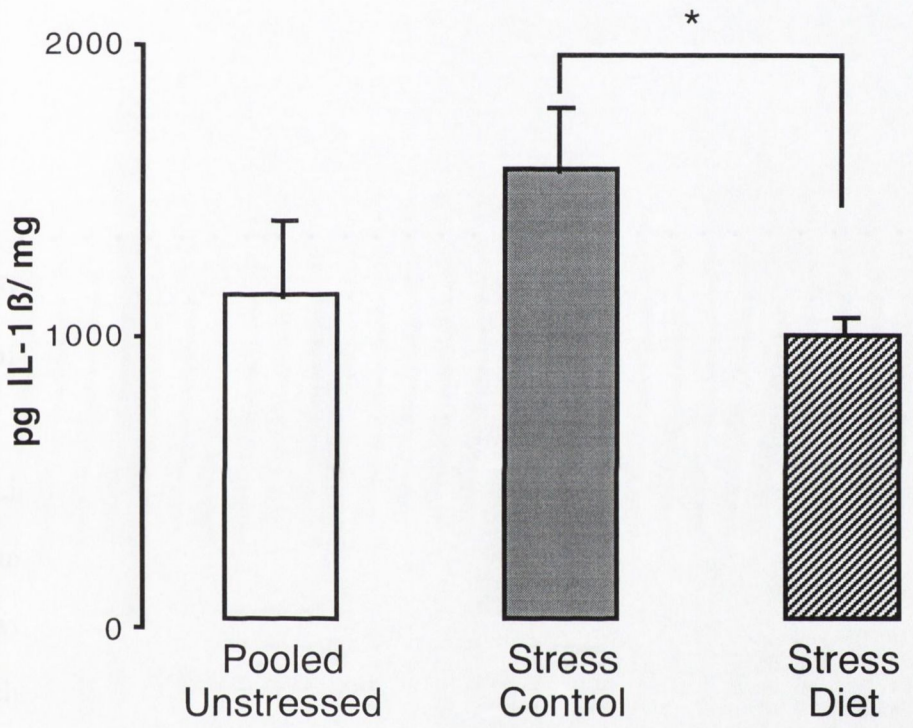

*
2000
1000
0
pg IL-1 ß/ mg
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.10 There is no stress or dietary induced change in lipid peroxidation in the hippocampus.

Lipid peroxidation was measured in hippocampal tissue prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was no change in lipid peroxidation between the unstressed pooled group fed both diets (n=11), the stressed group (n=5) fed the control diet or in stressed animals fed the test diet (n=5). Histograms represent means and bars represent standard errors. Results are expressed as nmoles MDA/g tissue.

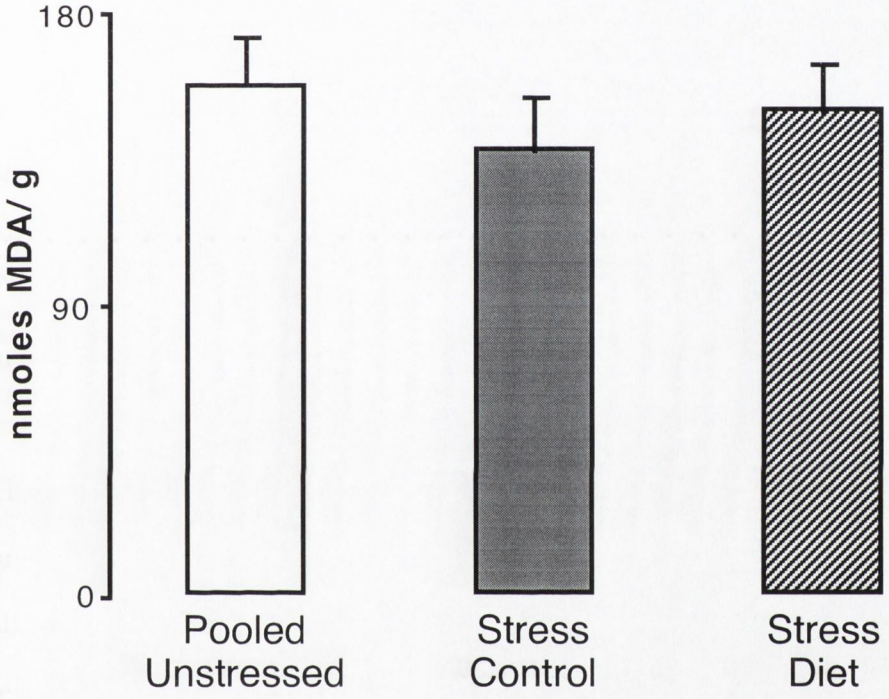

180
90
0
nmoles MDA/ g
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.11 There is no stress or dietary induced change in the concentration of arachidonic acid in the hippocampus.

The concentration of arachidonic acid was measured in hippocampal supernatant prepared from stressed or unstressed rats fed either a control or test diet. The results for all the unstressed animals were pooled as there was no statistical difference between their results. There was no change in aa concentration between the unstressed pooled group fed both diets (n=11), the stressed group (n=5) fed the control diet or in stressed animals fed the test diet (n=5). Histograms represent means and bars represent standard errors. Results are expressed as nmoles AA/mg.

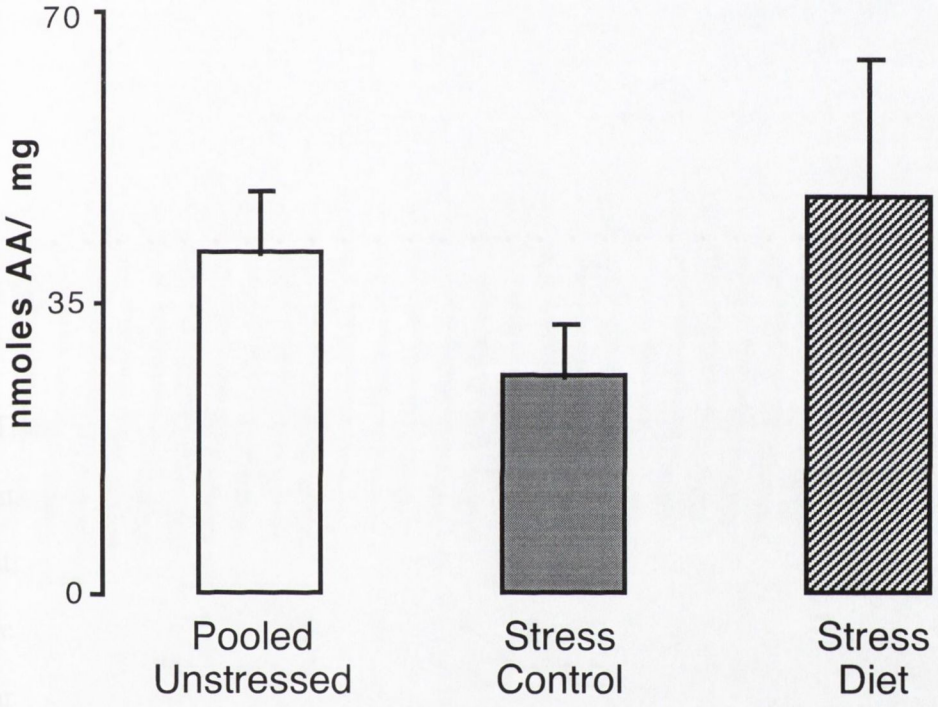
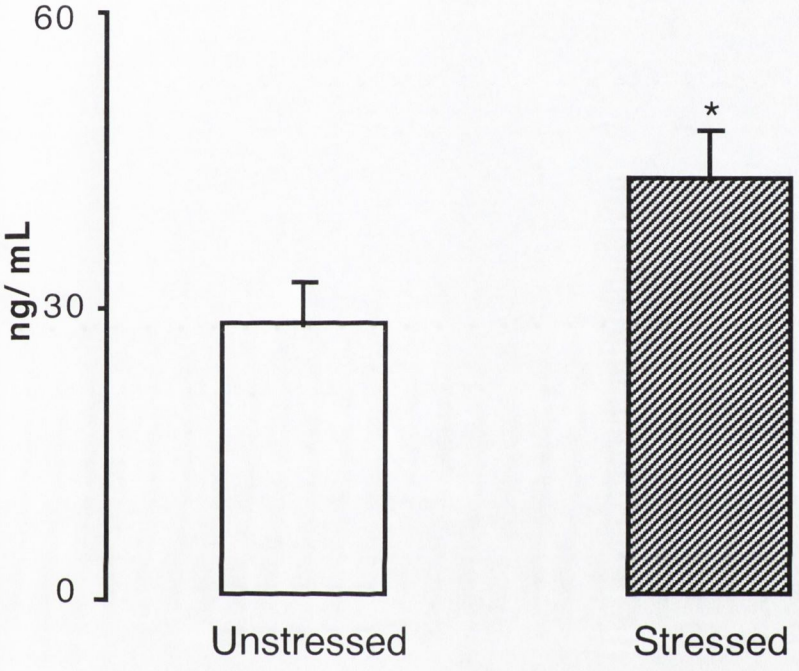

70
35
0
nmoles AA/ mg
Pooled
Unstressed
Stress
Control
Stress
Diet

Figure 6.12 There is a stress -associated increase in plasma corticosterone

The concentration of corticosterone was measured in plasma prepared from stressed or unstressed rats. There was an increase in circulating corticosterone in the plasma of stressed rats (n=7) compared with the plasma of unstressed rats (n=7). Histograms represent means and bars represent standard errors. . (*$p<0.05$;Student's t-test). Results are expressed as ng of corticosterone /ml.


60
ng/mL
30
0
*
Unstressed
Stressed

6.4 **Discussion**

In the previous chapter we observed that injecting IL-1ß *in vivo* induced an oxidative stress on hippocampal tissue and impaired LTP and that this effect could be reversed by dietary supplementation with high doses of the vitamin C and vitamin E. Clearly, in that study, the concentration of IL-1ß used exceeded those found physiologically in neuronal tissue. Isolation stress increases IL-1ß concentrations in the dentate gyrus region of the hippocampus (Murray & Lynch, 1998). Can social isolation cause similar physiological effects in the hippocampus as the i.c.v.injection of IL-1ß? The aim of this study was to examine if short term isolation stress (a) impaired LTP in the dentate gyrus (b) increased IL-1ß concentration (c) affected the oxidative defence system and (d) increased oxidative stress status in the rat hippocampus. A further aim was to investigate if dietary supplementation with antioxidants altered any of the parameters examined.

Isolation stress impaired LTP in the rat dentate gyrus. This observation supports previous findings that demonstrated impairment of LTP in the dentate gyrus after short term social isolation (Murray & Lynch, 1998). Other paradigms that induce psychological stress have also resulted in an impairment in neuronal function. One report observed a marked impairment of LTP in the dentate gyrus of hippocampal slices from rats that were restrained and exposed to tail shock every minute for 30 minutes compared with animals that were just restrained (Foy *et al.*, 1987). Another report observed that after exposure to acute stress there was an impairment in LTP in the CA1 region of mouse hippocampal slices ; this impairment was evident 24 hrs after stress but LTP was restored 48hrs later implying that the impairment to neuronal function induced by stress is reversible and relatively short lasting (Garcia *et al.*, 1997). Other studies have noted that stress can not only impair the increase in the efficiency of synaptic signalling due to tetanic stimulation but also cause a long lasting decrease in synaptic signalling, termed long term depression (LTD). Exposure of rats

to a mild stress, consisting of a tail pinch induced LTD in the CA1 region of the hippocampus. (Xu *et al.*, 1997).

Stress experiments can be conducted by directly injecting corticosterone into animals rather than exposing animals to the less quantifiable paradigms such as psychological stress which can be difficult to replicate and may be associated with unidentifiable variables. Chronic corticosterone treatment over 21 days, to simulate long-term stress, decreased LTP in the dentate gyrus *in vivo*. This effect persisted after 2 days of cessation of the corticosterone treatment (Pavilides *et al.*, 1991). A short term increase in corticosterone, which occurs with sudden brief stress, can also reduce neuronal function. One study demonstrated that a single high dose of corticosterone decreased LTP in the dentate gyrus compared with controls but this effect was not long lasting and was not observed after 48hrs (Pavlides *et al.*, 1993). Glucocorticoid-mediated neuronal impairment was also assessed in behavioural experiments. Long term exposure of rats to high doses of corticosterone has been shown to attenuate spatial memory as assessed in an 8 arm radial maze, whereas removal of the adrenal gland and replacement with basal levels of corticosterone prevents stress-induced cognitive impairment (Luine *et al.*, 1993). The extent of corticosteroid secretion may exert differential effects on neuronal tissue. A bi-phasic influence of corticosterone was observed with primed burst (PB) potentiation, a long term increase in population spike amplitude produced by brief physiologically patterned electrical stimulation. It was shown that low and intermediate concentrations of corticosterone (5-20 μg/dL) correlated positively to the magnitude of the PB potentiation , however higher concentrations of the hormone showed a negative correlation (Diamond *et al.*, 1992). Clearly stress impairs neuronal signalling as assessed by LTP in the rat hippocampus.

The test diet partially restored the ability of these animals to maintain LTP. This implies that the impairment in LTP seen with social isolation may be due to a decrease in the functionality of the tissue possibly due, in part, to an increase in

oxidative stress. There was an increase in ROS production in hippocampal tissue prepared from stressed rats fed the control diet. The underlying cause for this needs to be established. One possible cause is an increase in circulating corticosterone and consistent with this possibility is the finding that glucocorticoids increased ROS production by 10% in cultured rat hippocampal neurons (McIntosh *et al.*, 1996). Another report observed that glucocorticoids enhanced the cell death of cultured hippocampal neurons when co-incubated with two separate oxidate stressors (Behl *et al.*, 1997) implying that increased glucocorticoid secretion may contribute to oxidative stress in neuronal tissue. On the basis of this observation, it might be speculated that increased circulating glucocorticoid concentration may enhance ROS production in this study and it may be predicted that this generates oxidative stress in hippocampal neurons and impairs cell function. It must be also considered that the impairment of LTP may derive from a non-ROS mediated component. It was observed in one report that stress impaired LTP in the CA1 region of the rat hippocampus and that this effect was blocked by competitive NMDA antagonists administered prior to the stress implying that some of the stress effects may be mediated through a subtype of the NMDA receptor (Kim *et al.*, 1996).

Lipid peroxidation in the hippocampal homogenate was unaffected by stress or by diet. This is in apparent conflict with previous findings. For example from one study it was reported that lipid peroxidation significantly increased in the cerebral cortex, cerebellum, hippocampus and midbrain of rats that underwent a severe stress, immobilisation of the animal, compared with non-stress controls. This was accompanied by protein oxidation and DNA damage (Liu *et al.*, 1996). In another study it was shown that chronic severe stress, consisting of 3 weeks of immobilsation of rats for 6 hours a day, caused atrophy of apical dendrites in the CA3 pyramidal neurons of the hippocampus and this was accompanied by specific cognitive deficits in spatial learning and memory effects (Magarinos *et al.*, 1997). The intensity of stress,

and subsequently the duration of increased glucocorticoid secretion, may directly influence the degree of oxidative damage experienced by neuronal tissue and this suggestion is supported by the finding that a single restraint session did not cause any neurodegenerative effects in the CA3 region and was not associated with cognitive deficits (Magarinos *et al.*, 1997). It may be that short term isolation stress does not increase the concentration of circulating glucocorticoids sufficiently to induce lipid peroxidation.

The data from this chapter demonstrated that the concentration of arachidonic acid was unaltered after isolation stress confirming a finding from chapter 5 which noted that polyunsaturated fatty acids do not appear to be a target for lipid peroxidation during short term oxidative stress.

There was an increase in SOD activity in the hippocampus of stressed rats fed the control diet. In another study it was reported CuZnSOD activity was significantly lower in the hippocampus and cortex of rats treated with glucocorticoids for 3 days while MnSOD activity was unaffected (McIntosh *et al.*, 1998). The concentration of glucocorticoid injected in these animals was sufficient to significantly raise plasma concentrations. Such increases in concentration would not be seen in the socially isolated rat as this paradigm is considered a mild stressor only. It is conceivable that, as already mentioned, the intensity and duration of stress dictates the extent and nature of the physiological response.

In this chapter the data showed that GPx activity was similar in all groups of rats while there was a considerable decrease in the activity of catalase in the hippocampus of stressed rats fed the test diet. Catalase is believed to play a minor role in the antioxidative defence system of neuronal tissue (Cohen *et al.*, 1996). While the data show that its activity was down-regulated in hippocampal tissue of stressed rats fed on the test diet, the activity of the enzyme was unaffected in hippocampal tissue of unstressed rats fed the test diet. The increase in SOD activity, in the absence of

concomitant increases in the activities of GPx and catalase observed here present the same pattern observed in hippocampal tissue prepared from aged rats and rats treated i.c.v. with IL-1ß. It is proposed that this may be responsible for the increase in ROS production and the impairment in LTP observed here. In support of this view is the observation that the increase in SOD activity was reversed and LTP partially restored in the stressed rats fed the test diet.

Vitamin C concentration was increased in hippocampal tissue of those animals on the test diet while vitamin E concentrations were unaffected. This finding confirms that of chapter 5 which found short-term dietary supplementation with these vitamins increased the concentration of vitamin C concentration without affecting vitamin E concentration. As mentioned previously, this is consistent with previous findings which showed that long-term ingestion of vitamin E was required before an increase in its concentration was observed in brain tissue (Vatassery *et al.*, 1988).

Isolation stress caused a decrease in the concentration of glutathione in the stressed rats on the control diet. This decrease was not observed in stressed rats on the test diet and may indicate that isolation stress provokes an oxidative stress that depletes GSH in hippocampal neurons without increasing lipid peroxidation. In the isolated rat heart glutathione was selectively depleted, while vitamin E concentrations were unaffected, during brief ischemia-reperfusion (Haramaki *et al.*, 1998). It was suggested that glutathione may be a "front line" antioxidant during cellular oxidative stress and may be preferentially depleted. Another report observed that glutathione depletion decreased the activity of complex I, II and IV of the mitochondrial electron transport chain in whole brain homogenate from mice and increased ROS production in that tissue (Merad-Saidoune *et al.*, 1999). It might be concluded that mild stress selectively depletes glutathione concentration in hippocampal neurons and this may contribute to an increase in ROS production within that tissue.

Isolation stress increased the concentration of IL-1ß in the hippocampus of rats fed the control diet, but this did not reach statistical significance. In a previous study using the same stress paradigm a significant increase IL-1ß concentration was observed (Murray & Lynch, 1998). In this study IL-1ß concentration was assessed in whole hippocampal homogenate. Perhaps the increase in IL-1ß is a localised phenomenon specific to the granular layers of the dentate gyrus and the increase observed in the whole hippocampus failed to reach statistical significance due to a dilution effect? Other studies have reported a stress-associated increase in either IL-1ß gene expression or protein in neuronal tissue. For example immobilisation stress was reported to have induced a rapid rise in IL-1ß mRNA in the rat hypothalamus (Minami *et al.*, 1991). Similarily adrenalectomised rats, supplemented with basal levels of glucocorticoids, exposed to an acute stress exhibited a widespread and robust increase in IL-1ß in the hippocampus and hypothalamus. (Nguyen *et al.*, 1998). IL-1ß also stimulates the activation of the HPA. It has been reported that i.c.v. injection of IL-1ß stimulates CRF secretion and increases plasma corticosterone concentration (Sapolsky *et al.*, 1987). However the precise biochemical relationship between IL-1ß and glucocorticoids is difficult to discern due to negative feedback mechanisms in the CNS. One report observed that glucocorticoids selectively inhibited the transcription of the IL-1ß gene and decreased the stability of IL-1ß mRNA (Lee *et al.*, 1988). While another study showed that 3 day infusion of IL-1ß significantly increased glucocorticoid concentration in the plasma of rats (Van der Meer *et al.*, 1996). However, administration of high doses of dexsamethasone completely abolished this effect of IL-1ß implying that there is an immunoregulatory feedback between the actions of IL-1ß and glucocorticoids (Van der Meer *et al.*, 1996). The exact biochemical relationship between IL-1ß released in the brain and circulating glucocorticoid concentrations is still unclear. Hippocampal tissue from the stressed rats fed the test diet had decreased concentration of IL-1ß in hippocampal tissue. This

implies that the diet reduced oxidative stress in hippocampal tissue and this effect may have reduced IL-1ß release.

In the previous chapter IL-1ß was argued to be a central mediator in initiating oxidative stress within hippocampal tissue possibly through enhancing the activity of SOD and this oxidative stress was responsible for impairing LTP. In the stressed rats we see a similar pattern in hippocampal tissue; an increase in the concentration of IL-1ß, an increase in ROS production, an increase in the activity of SOD and an impairment of LTP in the hippocampus. A diet high in vitamin C and vitamin E increased the concentration of vitamin C in the hippocampus and partially restored LTP. In the previous chapters a link between increased concentrations of IL-1ß in neuronal tissue and increased SOD activity in that tissue was suggested. In this chapter a decrease in IL-1ß brought about by the test diet paralleled a decrease in the activity of this enzyme.

Does IL-1ß initiate an oxidative stress in the hippocampus of stressed rats and does this affect the ability to maintain LTP? The decrease in the concentration of GSH in the hippocampus of the stressed rats fed the control diet may indicate that an oxidative stress is generated and that this is responsible for the decrease in the concentration of this antioxidant. The fact that an increase in the concentration of vitamin C is correlated with a partial restoration of LTP would seem to indicate that increased ROS production may be implicated, in part, in the impairment of LTP. However it must be recognised that antioxidant supplementation only partially restored LTP implying that there are other biochemical components involved in the impairment of neuronal signaling due to stress.

Chapter 7

The Effect of IL-1ß and H_2O_2 Treatment on Markers of Apoptosis in a Neuronal Cell Culture.

7.1 **Introduction.**

In previous chapters it has been demonstrated that IL-1ß concentration is increased in hippocampal tissue of aged and stressed rats. It has been proposed that this imposes an oxidative stress on these tissues and results in impaired cell function. One question arising from this is whether high concentrations of IL-1ß induce apoptosis in neurons? The aim of this experiment is to address this question. Therefore cultured hippocampal neurons prepared from neonatal rats were (a) exposed to long and short term incubation periods with IL-1ß and (b) short term incubation with H_2O_2. These cells were then assessed for markers of apoptosis; caspase-1 and caspase-3 activity and cytochrome c expression in cell supernatant.

7.2 **Methods**

7.2.1 Method of Cell Culture

This method is described in detail in section 2.4.1. Hippocampii from rat pups were dissected free, on ice, and placed in a trypsin solution before trypsin inhibitor was added and the contents allowed to settle. The supernatant was removed and 2 mls of concentrated trypsin inhibitor solution was added. This solution was triturated, filtered through a gauze into a sterile Falcon tube, centrifuged and the supernatant removed. Modified Neurobasal Medium (mNBM) was added and the solution was triturated gently. Drops of the cell suspension were placed onto coverslips in 12 or 48 well plates and incubated for 4 hr at 37°C. mNBM was added to each well after this incubation period.

7.2.2 Preparation of Tissue

This method is described in detail in section 2.4.1. After 3 days the medium was removed from the plates and replaced with mNBM. IL-1ß (3.5ng/ml) or H_2O was added and the plates were incubated for 48 hr. The mNBM solution was removed and

replaced with lysis buffer. The plates were left at 4°C for 20 min before the solution was removed, centrifuged and the supernatant obtained and stored at -20°C. In some experiments plates were incubated with mNBM for 3 days after the preparation of the cells before the addition of either H_2O (control), IL-1ß (3.5ng/ml), H_2O_2 (0.5mM) or a combination of IL-1ß (3.5ng/ml) and H_20_2 (0.5mM). After 30 min the mNBM solution was removed and replaced with lysis buffer. The cell lysate from 6 coverslips which had the same treatment were combined to provide one sample (i.e n=1). The plates were left at 4°C for 20 min before the solution was removed, centrifuged and the supernatant obtained and stored at -20°C.

7.2.3 Analysis of Caspase-1 (ICE) Activity

Cell lysate was added to 100μM ICE substrate (YVAD peptide, Santa Cruz, U.S.A.) and incubated for 1hr at 37°C. Incubation buffer was added to the solution, samples were transferred to cuvettes and emission at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC were prepared and a standard curve of absorbance against concentration was plotted.

7.2.4 Analysis of Caspase-3 Activity

Cell lysate was added to 100μM caspase 3 substrate (DEVD peptide, Alexis Corporation, USA) and incubated for 1hr at 37°C. Incubation buffer was added to the solution, samples were transferred to cuvettes and emission of fluoresence at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC were prepared and a standard curve of absorbance against concentration was plotted.

7.2.5 Analysis of Cytochrome c Expression

A 12% gel was cast , samples were loaded into wells and a current was applied to the gel apparatus. Gels were removed from the gel apparatus, placed on nitrocellulose blotting paper and positioned on a blotter. A constant current was applied before the gel and nitrocellulose paper were removed and the nitrocellulose paper placed in blocking buffer overnight at 4°C. The nitrocellulose paper was washed before the addition of a primary antibody and washed again before a secondary antibody was added. The nitrocellulose paper was washed a final time and ECL solution was added to the paper which was then placed between 2 transparent plastic sheets. A photographic film was placed over the top plastic sheet and left overnight at 4°C. The film was developed and the intensity of the bands that appeared were taken to be proportional to the concentration of cytochrome C present in the sample. The intensity of the bands were measured using a densitometer and the concentration of cytochrome C present was expressed in arbitary units.

7.3 **Results**

7.3.1 **Effect of IL-1ß on Caspase-1 Activity after 48 hr Incubation**

Figure 7.1 indicates that there was a significant decrease in the activity of caspase-1 in hippocampal cell supernatant incubated with IL-1ß compared with the activity of the enzyme in cell supernatant incubated with control (*$p<0.05$; Student's t-test).

7.3.2 **Effect of IL-1ß on Caspase-3 Activity after 48 hr Incubation**

Figure 7.2 indicates that there was a decrease in the activity of caspase-3, which did not reach statistical significance, in hippocampal cell supernatant incubated with IL-1ß compared with the activity of the enzyme in cell supernatant incubated with control.

7.3.3 **Effect of IL-1ß Treatment on Cytochrome c Expression after 48 hr Incubation**

Figure 7.3 indicates that the expression of the protein was similar in hippocampal supernatant incubated with either IL-1ß or control.

7.3.4 **Effect of IL-1ß or H_2O_2 Treatment on Caspase-1 Activity after 30 min Incubation**

Figure 7.4 indicates that there were increases in the activity of caspase-1 in hippocampal cell supernatants incubated with IL-1ß (3.5 ng/ml), H_2O_2 (5mM) and a combination of both IL-1ß and H_2O_2 compared with the activity of the enzyme in cell supernatant incubated with control. These changes did not reach ststistical statistical significance.

7.3.5 **Effect of IL-1ß or H_2O_2 Treatment on Caspase-3 Activity after 30 min Incubation**

Figure 7.5 indicates that there were increases in the activity of caspase-1 in hippocampal cell supernatants incubated with IL-1ß (3.5 ng/ml), H_2O_2 (5mM) and a combination of both IL-1ß and H_2O_2 compared with the activity of the enzyme in cell supernatant incubated with control. These changes did not reach significance.

Figure 7.1 Caspase-1 activity is decreased after a 48 hr incubation with IL-1ß.

Caspase-1 activity was measured in hippocampal cell supernatant prepared from neonatal (0-4 days old) rats. There was a significant decrease in caspase-1 activity in supernatant from cells incubated for 48 hr with IL-1ß (n=8) compared with supernatant from cells incubated with control (n=8). Histograms represent means and bars represent standard errors. (*$p<0.05$; Student t-test). Results are expressed as nmoles AFC/mg.

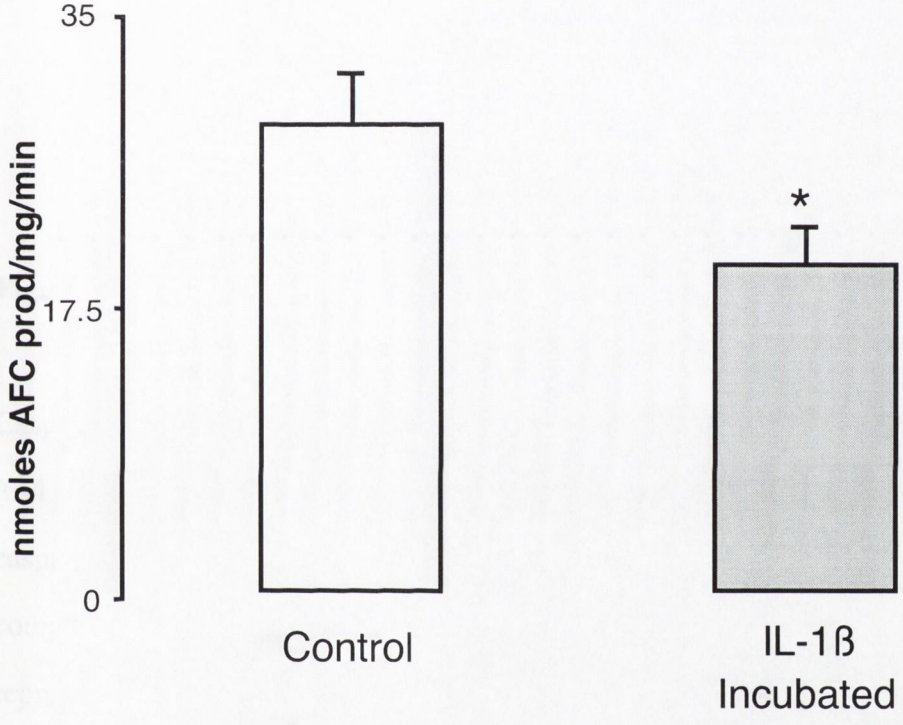

35
17.5
0
nmoles AFC prod/mg/min
*
Control
IL-1ß
Incubated

Figure 7.2 Caspase-3 activity is decreased after a 48 hr incubation with IL-1ß.

Caspase-3 activity was measured in hippocampal cell supernatant prepared from neonatal (0-4 days old) rats. There was a decrease, which did not reach statistical significance, in caspase-3 activity in supernatant from cells incubated for 48 hr with IL-1ß (n=8) compared with supernatant from cells incubated with control (n=8). Histograms represent means and bars represent standard errors. Results are expressed as nmoles AFC/mg.

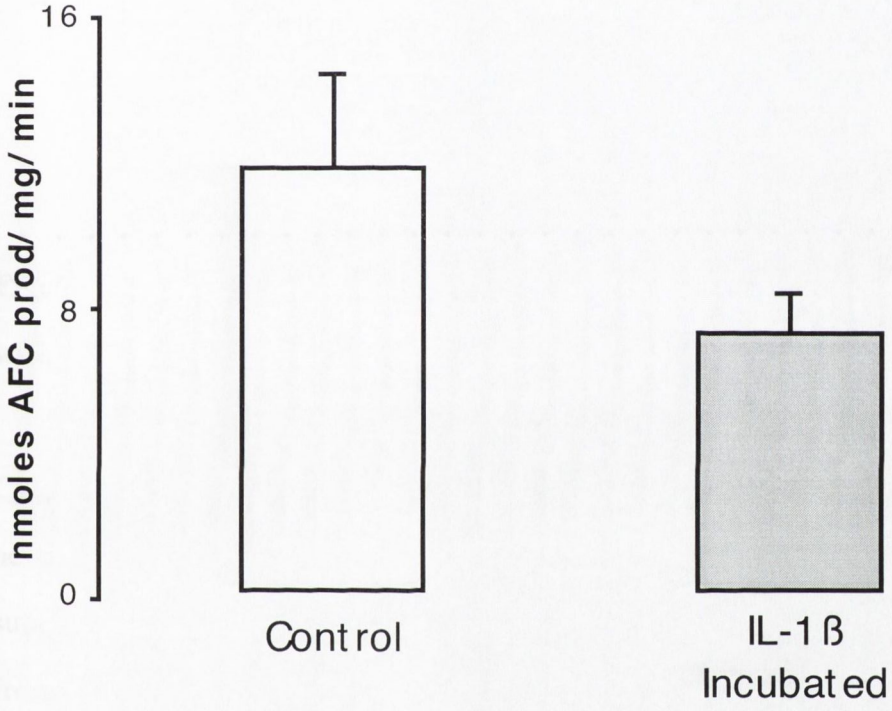

16
8
0
nmoles AFC prod/ mg/ min
Control
IL-1 ß
Incubated

Figure 7.3 There was no change in cytochrome C expression after a 48 hr incubation with IL-1ß.

Cytochrome c expression was measured in hippocampal cell supernatant prepared from neonatal (0-4 days old) rats. There was no change in cytochrome c expression in supernatant from cells incubated for 48 hr with IL-1ß (n=8) compared with supernatant from cells incubated with control (n=8). Histograms represent means and bars represent standard errors. Results are expressed as arbitary units.

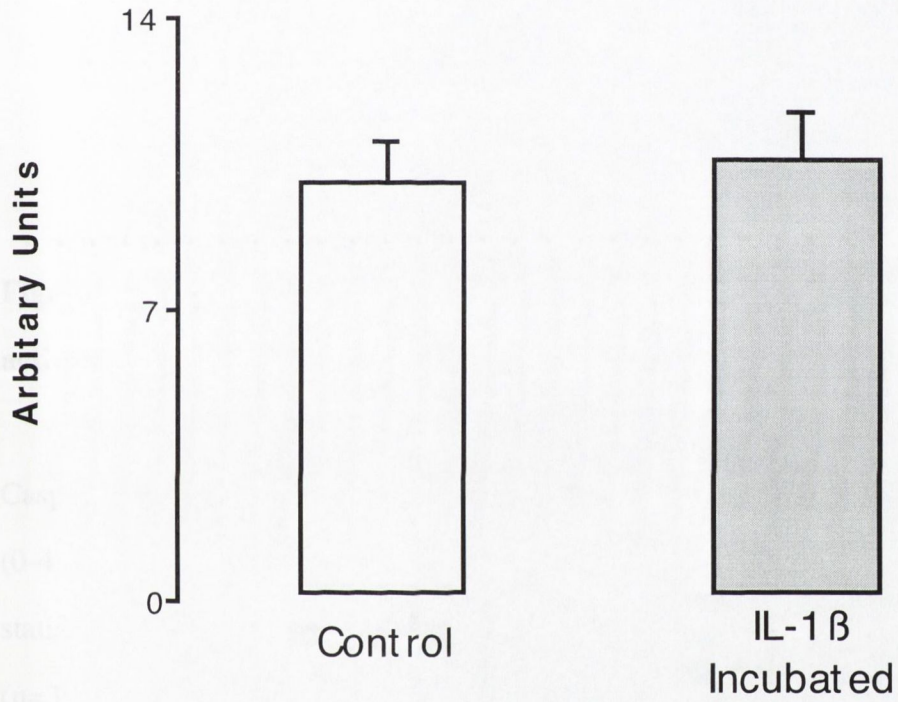

14
7
0
Arbitary Units
Control
IL-1ß
Incubated

Figure 7.4 IL-1ß and H_2O_2 separately and in combination increases caspase-1 activity after a 30 min incubation.

Caspase-1 activity was measured in hippocampal cell supernatant prepared from neonatal (0-4 days old) rats. There was an increase in caspase-1 activity, which did not reach statistical significance, in supernatant from cells incubated for 30 min with IL-1ß or H_2O_2 (n=3) compared with supernatant from cells incubated with control (n=3). There was a further increase in the activity of caspase-1, which did not reach statistical significance, when IL-1ß and H_2O_2 were incubated with cells (n=3) in combination for 30 min. Histograms represent means and bars represent standard errors. Results are expressed as nmoles AFC/mg.

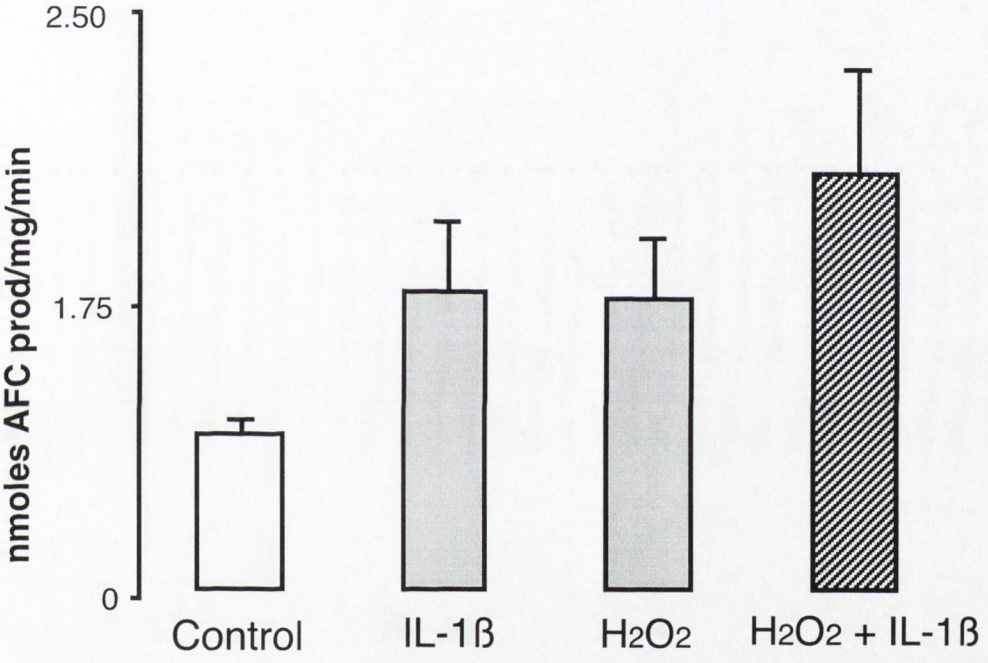
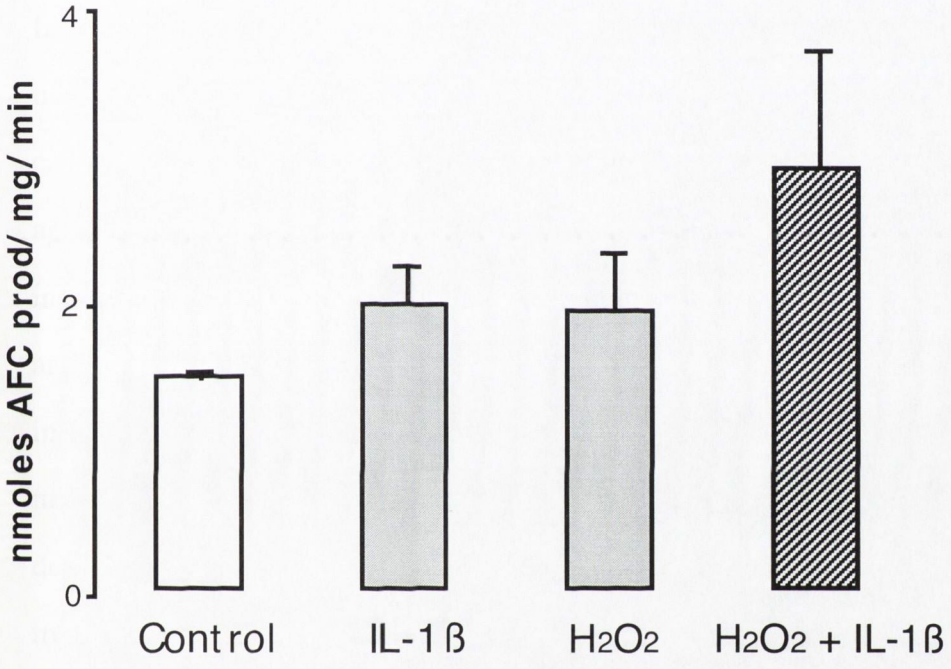

2.50
1.75
0
nmoles AFC prod/mg/min
Control
IL-1ß
H2O2
H2O2 + IL-1ß

Figure 7.5 IL-1ß and H_2O_2 separately and in combination increases caspase-3 activity after a 30 min incubation.

Caspase-3 activity was measured in hippocampal cell supernatant prepared from neonatal (0-4 days old) rats. There was an increase in caspase-3 activity, though not statistically significant, in supernatant from cells incubated for 30 min with IL-1ß or H_2O_2 (n=3; see section 7.2.2.) compared with supernatant from cells incubated with control (n=3). There was a further increase in the activity of caspase-3, though not statistically significant, when IL-1ß and H_2O_2 were incubated with cells (n=3) in combination for 30 min. Histograms represent means and bars represent standard errors. Results are expressed as nmoles AFC/mg.


nmoles AFC prod/ mg/ min
4
2
0
Control
IL-1ß
H2O2
H2O2 + IL-1ß

7.4 **Discussion**

The aim of this study was to assess the effect of short term (30 min) and long term (48 hr) exposure of cultured hippocampal neurons to IL-1ß on markers of apoptosis. The short term (30 min) effect of H_2O_2 incubation was also assessed.

Primary neurons from newborn hippocampus, on culture, acquire many physiological properties of mature neurons when supplied with the appropriate trophic factors (Marks & Berg, 1999). The data from this chapter indicated that incubating neonatal hippocampal neurons with IL-1ß for 30 min increased the activity of both caspase-1 and caspase-3. These results imply that IL-1ß may be triggering an apoptotic response in these cells. In a previous study it was observed that hypoxia-induced apoptosis in cultured neurons could be inhibited by either IL-1ra, neutralising antibodies to IL-1ß or antibodies to IL-1 Type-1 receptor (Freilander *et al.*, 1996) implying that hypoxia induced the cultured cells to release IL-1ß and this had a role in mediating apoptosis. Further evidence for IL-1ß involvement in programmed cell death was provided by the observation that IL-1ra inhibited apoptosis induced by trophic factor withdrawal in cultured primary neurons (Freilander *et al.*, 1996). It was observed in neurons derived from transgenic mice expressing a dominant negative mutation of caspase-1 and unable to synthetise and release IL-1ß, that there was a resistance to trophic factor withdrawal-induced apoptosis (Freilander *et al.*, 1996). This type of transgenic mouse was significantly protected against, the MPTP-induced apoptosis of dopamine neurons in the nigro-striatal pathway (Klevenyi, *et al.*, 1999) implicating a role for IL-1ß in neuronal apoptosis. The evidence from this chapter supports the hypothesis that IL-1ß may directly induce apoptosis in vulnerable cells. It also implies that sudden increases in the concentration of IL-1ß in brain tissue may induce neuronal cell death. Consistent with this hypothesis was a study that noted that there was an increase in the expression of caspase-1 in the mouse hippocampus after experimentally induced ischemia and this was correlated with an increase in IL-1ß in

that tissue (Bhat *et al.*, 1996) and in transgenic mice that were unable to express caspase-1 and did not show evidence for IL-1ß release, there was a significant reduction in brain injury and neurological deficits compared to wild type controls after cerebral ischemia (Bhat *et al.*, 1996 Further evidence demonstrated that a peptide inhibitor of caspase-1 decreased IL-1ß concentration and reduced tissue damage in ischemic mouse and rat brain (Hara *et al.*, 1997).

In previous chapters it was demonstrated that the IL-1ß-induced impairment in neuronal function was related to an increase in SOD activity. It was suggested that IL-1ß mediated an increase in ROS production in neuronal tissue and this generated an oxidative stress. This increase in ROS production may be responsible for the activation of caspase-1 and caspase-3 in hippocampal cell culture. However IL-1ß is also capable of inducing iNOS and subsequently causing the release of $NO^{\bullet}$. Human astrocytes treated with IL-1ß underwent apoptosis and this effect was abrogated by IL-1ra. N-monomethyl-L-arginine, an antagonist of NOS, partially prevented apoptosis and completely inhibited NO production (Chao *et al.*, 1996). This implies that IL-1ß may initiate apoptosis along a variety of biochemical pathways and not only through enhancing the activity of SOD.

The present data show that short-term exposure of neonatal cells to H_2O_2, a potent apoptotic stimulus, also increased the activity of caspase-1 and caspase-3. This is in agreement with other studies in which high doses of H_2O_2 (>100μM) resulted in incomplete apoptosis in isolated rat spinal motorneurons while low doses (50μM) resulted in complete apoptosis (Kaal *et al.*, 1998). It was suggested that the high doses of H_2O_2 used in that study may have caused necrosis, rather than apoptosis, of the neurons and this sugestion must also be considered for the data presented in this chapter. The fact that the increase in caspase-1 and caspase-3 observed did not reach statistical significance may indicate that necrosis was induced to some extent. It was observed that there was a further increase in the activity of caspase-1 and caspase-3 in

hippocampal neurons co-cultured with H_2O_2 and IL-1ß. Both of these agents have been shown to induce oxidative stress (Murray & Lynch, 1999; Halliwell, 1992). In previous chapters it was discussed that an IL-1ß mediated increase in SOD activity without a concomitant increase in catalase or GPx may lead to an accumulation of H_2O_2. Supporting this hypothesis was evidence that demonstrated that in cell lines that had been transfected with the gene for CuZnSOD and had an elevation in the ratio of CuZnSOD activity to GPx activity, higher concentrations of H_2O_2 were observed (de Haan *et al.*, 1996). The data from this chapter would indicate that a combination of increased IL-1ß concentrations and increased production of H_2O_2 in neuronal tissue may enhance caspase-1 and caspase-3 activity and contribute to neuronal cell death *in vivo*.

Incubation of the cells in the presence of IL-1ß for 48 hr led to a decrease in the activity of caspase-1 and caspase-3 compared with control. There was no increase in cytochrome c expression, another marker for apoptosis, in the supernatant of hippocampal cells incubated long-term with IL-1ß and this may imply that there is no change in apoptotic activity within these cultures compared to control. It could be suggested that after the addition of IL-1ß there is an immediate wave of apoptosis within the cultured neurons as evidenced by the increase in activity of caspase-1 and caspase-3 after 30 min but that this effect diminishes after 2 days, possibly through the downregulation of the IL-1 Type I receptor. In a previous study it was reported that there was a 50% decrease in IL-1 Type I receptor mRNA in embryos cultured in the presence of IL-1ß. This downregulation effect was found to be dose dependent with progressive attenuation of receptor mRNA expression with increasing concentration of IL-1ß (Huang *et al.*, 1997). In primary cultured neurons incubated with IL-1ß there was a downregulation of the IL-1 type I receptor and a decrease in hypoxia induced apoptosis (Freidlander *et al.*, 1996). It might be proposed, on the basis of these studies, that sudden increases in IL-1ß in neuronal tissue may increase the activity of

caspase-1 and caspase-3 and initiate apoptosis. This may be relevant in cases of stroke and traumatic brain injury where tissue damage initiates a large and sudden increase in pro-inflammatory cytokines. However in cases of chronic elevation of IL-1ß concentration as observed in aging and stress this increase in the activity of caspase-1 and caspase-3 may not be observed due to downregulation of the type I receptor or other adaptive processes.

VII Final Discussion

Previous studies have shown that there is an age-related increase in ROS production in the cortex and hippocampus of aged rats (Martin, Pers Comm; Murray & Lynch, 1998). The data presented in chapter 3 and chapter 4 was concerned with establishing the underlying cause of this increase in ROS production and for this reason the main components of the antioxidant defence system were assessed; thus activities of SOD, catalase and GPx were investigated as were the concentrations of vitamin E, vitamin C and glutathione. It is important to note that there are other components of the antioxidant defence sysytem which may also play a role in neutralising ROS; these include melatonin, uric acid, selenium and lipoic acid. The most consistent finding from these series of experiments was that there was an increase in SOD activity in tissue prepared from aged rats without a concomitant increase in the activities of GPx and catalase. The significance of this finding is that it may lead to an increase in the production of H_2O_2 and generate oxidative stress within aged tissue. There is evidence from previous studies supporting this hypothesis, for example, in cell lines that had been transfected with the gene for CuZnSOD and had an elevation in the ratio of CuZnSOD activity to GPx activity, higher concentrations of H_2O_2 were produced (de Haan *et al.*, 1996). While another study reported an increase in the ratio of CuZnSOD activity to GPx activity in the aged mouse brain and this was accompanied by an increase in a marker for oxidative stress within that tissue (Criastiano *et al.*,1995).

In the presence of appropriate concentrations of the main antioxidants, vitamin C, vitamin E and GSH, an increase in SOD activity may not result in oxidative damage. However the findings of this thesis indicate that there are age-related decreases in the concentrations of these antioxidants. Some of these decreases do not reach statistical significance however the general trend of a decline in the cellular antioxidant defences coupled with an increase in the activity of SOD suggest this may

be a source of the increase in ROS production observed in the cortex and the hippocampus in the aged rat. The age-related changes in antioxidant concentration which are presented in this thesis confirm many data previously reported which indicate a gradual decline in cellular antioxidant defences. For example electrochemical detection revealed a similar age-related decrease in the basal extracellular vitamin C concentration from the nucleus accumbens (Svensson *et al.*, 1993) while GSH concentration was found to be lower in the cortex, cerebellum, striatum, thalamus and hippocampus of aged Sprague Dawley rats (Bondy *et al.*, 1995).

A number of important questions arise from these findings and these include (1) what triggers the changes observed in the antioxidant defences and (2) what are the consequences of the age-related compromises observed. Previous evidence suggested that one possible trigger might be the cytokine, IL-1ß (Murray & Lynch, 1998; Murray & Lynch, 1999) and this possibility was assessed here. An age-related increase in IL-1ß concentration was observed in both cortex and hippocampus and these results supported a previous finding of an age-related increase in IL-1ß in the dentate gyrus (Murray & Lynch, 1998). Further evidence that IL-1ß may modulate antioxidant defences was provided by the observation that hippocampal tissue incubated in the presence of IL-1ß was found to have an increase in the activity of SOD. This important finding was consistent with findings of others which demonstrated an IL-1ß-mediated effect on SOD expression and activity. For example it was shown that IL-1ß increased Mn-SOD gene expression in a time dependent manner in cultured rat hepatocytes (Antras-Ferry *et al.*, 1997); that IL-1ß increased the activities of both the Mn- and CuZnSOD in rat pancreatic islets (Borg *et al.*, 1992); that IL-1ß induced a significant increase in mRNA concentrations of MnSOD in cells derived from the corpus luteum of rats (Sugino *et al.*, 1998) and that IL-1ß induced the expression of MnSOD in rat neuronal and glial cells (Kifle *et al.*, 1996; Mokuno *et al.*, 1994). In

addition to these findings it was noted in data from this thesis that hippocampal slices incubated in the presence of IL-1ß had an increase in ROS production in parallel with an increase in SOD activity. This suggests that enhanced SOD activity may be responsible for this increase in ROS production. Evidence supporting this hypothesis came from a study which showed that cortical tissue incubated in the presence of IL-1ß and a SOD inhibitor did not show an increase in the activity of SOD or an increase in ROS production (Martin, Pers Comm). In addition to these observations, the findings of a number of studies provide indirect support for the hypothesis that IL-1ß may significantly contribute to increased ROS production. For example, an increase in IL-1ß concentration has been observed in brain tissue of patients suffering from a variety of degenerative diseases in which increased ROS production from affected tissue has been found; these include Alzheimer's Disease, Parkinson's Disease and ALS (Blume,*et al.*, 1989; Mogi *et al.*, 1996; Freidlander *et al.*, 1997), The data presented in this thesis suggests that an age-related increase in the concentration of IL-1ß in neuronal tissue may lead to an increase in the production of ROS in that tissue and this may be the result of an IL-1ß-mediated stimulatory effect on SOD activity.

One consequence of an increase in ROS production might be an increase in lipid peroxidation, which in turn, may affect the concentration of polyunsaturated fatty acids in the membrane. The data presented here addressed this question and the findings showed that lipid peroxidation was increased in the cortex and hippocampus of aged rats, a result that confirms an earlier finding (Murray & Lynch, 1998). The data show that this change was accompanied by a decrease in the polyunsaturated fatty acid (PUFA) arachidonic acid suggesting that PUFAs are target molecules in age-associated lipid peroxidation. A decrease in PUFAs within neuronal membranes increases membrane rigidity (Halliwell, 1992). Evidence supporting this view showed that rat cortical preparations that were artificially peroxidised were found to have a decrease in membrane fluidity and in muscarinic receptor binding (Ghosh *et al.*,

1993). This implies that oxidative stress not only impacts on membrane structures but, by increasing lipid peroxidation, also affects those structures embedded within the neuronal membrane. It may be suggested that this would impair cell function.

The hypothesis that IL-1ß may be a mediator of oxidative stress in neuronal tissue was strengthened by data presented in this thesis from cortex of aged rats that underwent long-term dietary supplementation. These data showed that increased vitamin E concentration in cortical tissue from aged rats paralleled a decrease in; IL-1ß concentration, SOD activity and lipid peroxidation. These findings support the results of a previous study which showed a decrease in lipid peroxidation and a restoration in neuronal function in the hippocampus of aged rats fed a high antioxidant diet for 3 months (Murray & Lynch, 1998). It might be proposed that a destructive feedback mechanism may be initiated in aged tissue whereby a general decline in the antioxidant defence system of aged cortical and hippocampal tissue results in oxidative damage. This damage may, in turn, stimulate the release of IL-1ß and exacerbate the age-related oxidative stress on neuronal tissue by increasing the activity of SOD. It may be suggested that dietary supplementation breaks this destructive cycle by attenuating oxidative damage and thereby removing the stimulus for IL-1ß release.

The increase in SOD activity may appear to be paradoxical as, in some cases, an increase in its activity has a protective effect. For example, in cerebral ischemia an increase in ROS production occurs that leads to neuronal cell death and in focal ischemia studies with transgenic mice expressing either normal, 50% or no CuZnSOD activity it was observed that mice with no CuZnSOD activity showed a high level of blood-brain barrier disruption 1hr after middle cerebral artery occlusion and 100% mortality at 24 hours. Mice with 50% CuZnSOD activity showed 30% mortality and increased neurological compared with wild type controls (Kondo *et al.,* 1997). Clearly enhanced SOD activity in the presence of an external stressor is beneficial but in the absence of an oxidative stress, this increase in SOD activity can, paradoxically,

mediate an increase in ROS production and oxidative damage. IL-1 ß is released in response to injury and inflammation (Rothwell, 1999), conditions that lead to the generation of uncontrolled ROS production. It is not unreasonable to suggest that the IL-1 ß mediated increase in SOD activity is a natural response to counteract a perceived injury and to minimize oxidative damage. However during aging this mechanism appears to be inappropriately activated and contributes to, rather than counteracts, oxidative damage.

The second series of experiments presented in chapter 5 sought (a) to establish the functional consequences of an increase in IL-1ß in the hippocampus and (b) to confirm the link between an increase in the concentration of IL-1ß and ROS production. The data showed that by increasing IL-1ß concentration in the brain through i.c.v. injection, a functional impairment was created in the perforant path/dentate gyrus pathway as assessed by LTP experiments. It was investigated whether this neuronal impairment was due to oxidative stress by examining components of neuronal antioxidant defences. A similar pattern emerged to that in aged tissue; an increase in SOD activity without a concomitant increase in GPx and catalase activities or in the concentrations of vitamin C, vitamin E or GSH. This suggested that an oxidative stress may be generated due to the enhanced activity of SOD and this was confirmed by increases in ROS production and in lipid peroxidation. These data suggests that the impairment in LTP may be due to oxidative stress as previous studies have demonstrated that this can adversely effect neuronal function. For example, one study demonstrated that the addition of H_2O_2 onto guinea pig hippocampal slices impairs LTP while another report showed that in the hippocampal structures of transgenic mice that overexpress the gene for CuZn SOD and , as a result, have an increased production of H_2O_2, there is an inability to initiate and maintain LTP. (Pellmar *et al.*, 1987; Gahtan, *et al.*, 1998). The data presented in this thesis showed that dietary supplementation with antioxidants reversed the IL-1ß-induced

impairment in LTP. This result is consistent with the hypothesis that IL-1ß mediates an increase in oxidative stress in hippocampal tissue. It is of interest that while IL-1ß-treated rats fed the antioxidant-supplemented diet had an increase in SOD activity, there was no increase in lipid peroxidation suggesting the elevation of vitamin C concentration observed in hippocampal tissue after dietary supplementation increased the antioxidant capacity of that tissue and protected it from oxidative stress and the consequences of oxidative stress.

The third series of experiments (chapter 6) attempted (a) to assess the effect of isolation stress on neuronal function and (b) to establish if stress initiates changes in cellular oxidative defences. It is known that isolation stress increases IL-1ß concentration in the dentate gyrus (Murray & Lynch, 1999) and the data from this thesis showed an increase in IL-1ß in the whole hippocampus. LTP was also impaired in the dentate gyrus of stressed rats and, in view of the findings presented in this thesis, it is not unreasonable to suggest that IL-1ß may be mediating the impairment of LTP in this region. The data showed that SOD activity was increased in this region without compensatory increases in the other components of antioxidant defence and that this was accompanied by an increase in ROS production was also observed. There was no increase in lipid peroxidation, suggesting that intensity and duration of stress may influence the extent of oxidative damage. Evidence supporting this hypothesis comes from other studies which showed that chronic severe stress induced widespread atrophy within the hippocampus and was associated with marked neurological deficits while a similar stress for a brief period did not induce any of these effects{Magarinos, 1997}. Dietary supplementation with antioxidant vitamins partially restored LTP in the stressed rat and this suggests that oxidative stress plays a role in mediating stress-induced impairment of LTP. Again the data implicate IL-1ß as mediating this oxidative stress as hippocampal tissue from stressed rats fed the antioxidant diet had; decreased IL-1ß concentration, decreased SOD activity and increased GSH

concentrations compared to tissue from stressed rats on the control diet. Clearly, other factors contribute to the stress-mediated impairment in LTP as dietary supplementation does not completely restore LTP. Other factors were not assessed here but one possibility is that stress may activate a subtype of the NMDA receptor and that this effect may contribute to impaired LTP in the CA1 region of the rat hippocampus (Kim *et al.*, 1996).

The aim of the final results chapter of this thesis was to examine the effect of long- and short- term incubation of IL-1ß on caspase activation in a hippocampal cell culture. The data suggest that short-term incubation of neuronal cells in the presence of IL-1ß may increase the activity of caspase-1 and caspase-3 and initiate apoptosis. It would be interesting to investigate whether this effect is due to an increase in oxidative stress within the cell culture and more specifically to an increase in SOD activity and whether antioxidant supplementation to the cell culture medium would alter this effect. Interestingly, long-term incubation (48 hr) with IL-1ß did not increase caspase activation and this suggests there may be adaptive processes employed by neuronal tissue to combat prolonged IL-1 ß exposure. However further investigation is required to elucidate these putative mechanisms.

On the basis of the data presented in this thesis it seems reasonable to propose that IL-1ß acts as a central mediator of oxidative stress in neuronal tissue. This hypothesis is supported by other studies. For example, intratracheal administration of recombinant IL-1ß into rats caused acute lung injury and treatment with the antioxidant dimethyl sulfoxide (DMSO) prior to administration of the cytokine attenuated this effect (Leff *et al.*,1994). Another study reported that treatment of IL-1ra, to block the actions of IL-1ß, in a rat liver preparation prior to ischemia/reperfusion experiments decreased ROS mediated cellular damage (Shirasugi *et al.*,1997). It is suggested in this thesis that IL-1ß may increase the production of ROS by specifically increasing the activity of SOD. This increase in ROS production

causes oxidative damage to neuronal tissue including increasing lipid peroxidation and these effects impede the functional efficiency of the hippocampus as assessed by LTP experiments. However dietary supplementation removes the oxidative stress by increasing the concentration of antioxidants within tissue and this action restores functional efficiency to the hippocampus and allows LTP to be successfully initiated and maintained.

VIII Future Experiments

This thesis suggests further experiments that may be worth investigating. I would concentrate mainly on cell culture techniques

1. The activity of caspase-1 and caspase-3 and the expression of cytosolic cytochrome C in a cell culture of hippocampal neurons in the presence and absence of different concentrations of IL-1ß for a fixed time period. The aim of this experiment would be to find the concentration of IL-1ß that activates the apoptotic machinery of these cells.
2 A related experiment would analyse the activity of caspase-1 and caspase-3 and the expression of cytosolic cytochrome C in a cell culture of hippocampal neurons in the presence of a fixed concentration of IL-1ß at various timepoints. I would suggest 5min, 10 min, 15 min, 30 min, 1 hr, 6hr, 12 hr, 24hr and 48 hr. This experiment may indicate the time of maximal activation of the apoptotic machinery and may also indicate more clearly if a downregulation in the activation of caspase-1 and caspase-3 occurs.
3 The activity of caspase-1 and caspase-3 and the expression of cytosolic cytochrome C in a cell culture of hippocampal neurons in the presence and absence of a fixed concentration of IL-1ß and antioxidants. These antioxidants may include a combination of the vitamins C and E or each vitamin alone. This experiment would indicate whether these molecules can prevent the apoptotic machinery being activated by IL-1ß and if so this would strongly suggest that ROS were primarily involved in the initiation of apoptosis.
4 The activity of caspase-1 and caspase-3 and the expression of cytosolic cytochrome C in a cell culture of hippocampal neurons in the presence and absence of a fixed concentration of IL-1ß and IL-1ra. This experiment may indicate

whether the activation of the apoptotic machinery within these cells is directly due to IL-1ß acting via its receptor or whether IL-1ß activated these caspases through a non-specific event

5 The activity of caspase-3 and the expression of cytosolic cytochrome C in a cell culture of hippocampal neurons incubated in the presence of IL-1ß and a caspase-1 inhibitor. This experiment may indicate whether the effect of IL-1ß on caspase-3 activation and cytosolic cytochrome C expression is dependent on an increase in the activity of caspase-1.

6 The activity of caspase-1 and caspase-3 and the expression of cytosolic cytochrome C in a cell culture of hippocampal neurons in the presence and absence of variable concentrations of H_2O_2. The concentration of H_2O_2 used throughout the thesis was too high. A concentration of H_2O_2 needs to be found that induces gradual apoptosis in cultured hippocampal cells rather than rapid necrosis.

7 The expression of mRNA using PCR and protein expression of the different isoforms of SOD in hippocampal cell culture incubated with a fixed concentration of IL-1ß for 1 hr. The increase in SOD activity recorded in this thesis appeared to occur rapidly (40 min in chapter 5). This experiment would indicate if this effect was due to *de novo* synthesis of the protein.

8 The activity of SOD in a cell culture of hippocampal neurons in the presence and absence of a fixed concentration of IL-1ß and a kinase inhibitor. This experiment would indicate if any increase in SOD activity is due to a phosphorylation event rather than *de novo* synthesis of the protein.

9 It would be interesting to repeat all the above experiments in a hippocampal cells culture derived from MALE pups to see if there is a differential mechanism in IL-1ß mediated events between the sexes.

Some *in vivo* experiments may include:

1. Altering the stress paradigm in chapter 6. This paradigm is unreliable as the rats can communicate via smell and sound and are not truly isolated. Different pychological stress paradigms may include altering the light/dark cycle or allowing nocturnal starvation. An alternative approach would be to directly inject corticosterone in rats for 5 days during the dietary supplementation phase of the experiment. This would have the advantage of elevating corticosterone levels in all test animals to the same extent for the same period.
2. The experiments in chapter 4,5 and 6 would be interesting to repeat using only vitamin C as the antioxidant in the dietary supplement. This would indicate if this antioxidant alone can induce similar changes to those observed.

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IX Appendix I

Company	Address
Amersham	Amersham International plc., Lincoln Place, Green End, Aylesbury, Buckinghamshire, HP20 2TP, U.K.
Beeline Healthcare	Beeline Healthcare Ltd., Clondalkin Road Clondalkin Dublin 24 Ireland
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Marylands Avenue, Hemel Hempstead, Hertfordshire, HP2 7TD, U.K.
Genzyme Diagnostics	Genzyme Diagnostics,

	1 Kendall Square, Cambridge, MA 2139, U.S.A
IDS, Ltd.	Immunodiagnostic Systems Ltd., Bolden Business Park, Boldon, Tyne and Wear, NE35 9PD, U.K.
Lennox	Lennox Laboratory Supplies, John F. Kennedy Drive, Naas Road, Dublin 12 Ireland.
Molecular Probes	Molecular Probes Europe, Poort Gebouw, Rjinsburgerweg 10, 2333 Leiden, Netherlands
Santa Cruz	Santa Cruz Biotechnology, Inc.,

	2161 Delaware Avenue, Santa Cruz, CA 95060 USA.
Sigma	Sigma Chemical Co. Ltd., Fancy Road, Poole, Dorset, U.K.

XI Appendix II

Solutions used:

Krebs solution

NaCl, 136 mM; KCl, 2.54 mM; KH_2PO_4 , 1.18 mM; $MgSO_4.7H_2O$, 1.18 mM; $NaHCO_3$, 16 mM; $CaCl_2$, 2mM; glucose 10 mM

Trypsin solution (cell culture)

0.25mg trypsin/ml Dulbecco's Modified Phosphate Buffered Saline (DM-PBS)

Trypsin inhibitor (TI) solution (cell culture)

0.025 mg soyabean trypsin inhibitor (SBTI), DNAase (100μl), $MgSO_4$ (100μl)/ ml DM-PBS

Concentrated TI solution (cell culture)

1mg SBTI, DNAase (100μl), $MgSO_4$ (100μl)/ml DM-PBS

Modified Neurobasal Medium (mNBM) (cell culture)

Fetal Calf Serum (10mls), Glutamax (1ml), Penicillin/Streptomycin(1ml))/100mls Neurobasal Medium

Lysis buffer

HEPES (25mM), $MgCl_2$ (5mM), DTT (5mM), EDTA (5mM), PMSF (2mM), leupeptin (10µg/ml) and pepstatin (10µg/ml), pH 7.4

Sample buffer for GSH assay

100mM potassium phosphate buffer, pH 7.5, containing 5mM EDTA

Reaction medium for GPx assay

50mM potassium phosphate buffer (pH 7.0), 1mM EDTA (ethylene diaminetetraacetic acid), 1mM NaN_3 (sodium azide), 0.2mM NADPH, 1 unit of glutathione reductase, 1mM GSH (glutathione) and 1.5mM cumene hydroperoxide

DTC solution for vitamin C assay

0.04g thiourea, 0.005g copper sulphate ($CuSO_4$) and 0.3g dinitrophenylhydrazine brought to a total volume of 10mls with 9N H_2SO_4

Assay mixture for SOD assay

1.8mM xanthine, 2.24mM nitroblue tetrazolium (NBT), 40 units of catalase, xanthine oxidase (7μl/ml) and 1.33mM diethylenetriaminepentaacetic acid (DETAPAC) dissolved in 50mM potassium buffer (pH 7.8)

Assay solution for lipid peroxidation

3.95 ml acetic acid added to 15 ml of distilled H_2O (pH 3.5) was prepared and added to 0.213g sodium dodecylsulfate (SDS) and 0.158g of thiobarbituric acid (TBA). The resultant solution was brought to 50mls by the addition of distilled H_2O.

Sample buffer for gels

Tris-HCL; 0.5M, pH 6.8; glycerol; 10% (v/v); SDS, 10% (w/v); ß-mercaptoethanol 5% (v/v); bromophenol blue, 0.05% (w/v).

Gel Solutions

Separating gel (0.375M Tris, pH 8.8)

Acrylamide/Bisacrylamide (30% v/v, stock), Tris-HCL, 1.5 M, pH 8.8 (stock), SDS, 12% w/v (stock), ammonium persulphate 10% w/v.

Stacking gel

Acrylamide/Bisacrylamide (30% v/v, stock),0.5M,pH 6.8 (stock), ammonium persulphate, 10% w/v

Transfer buffer

Tris base, 25mM, glycine, 192mM, methanol, 20% v/v, SDS, 0.05% w/v

XII Appendix III

Weight changes in rats undergoing isolation stress and 5-day dietary supplementation (see Chapter 6)

Group	**Start (g)**	**End (g)**	**Difference (g)**	**% Change**
Control Stress	333	357	24	6.7
	278	303	25	8.3
	299	316	17	5.4
	274	303	29	9.6
	299	318	19	6.0
	290	315	25	8.0
Diet Stress	256	283	27	9.5
	332	337	5	1.5
	283	280	-3	-1.0
	353	378	25	6.6
	331	356	225	7.0
	334	346	12	3.5
Control Unstressed	284	291	7	2.4
	308	294	-14	-4.8
	252	257	5	2.0
	318	351	33	9.4
	336	350	14	4.0

	404	408	4	1.0
Diet Unstressed	388	414	26	6.28
	400	402	2	.5
	330	336	6	1.8
	386	405	19	4.7
	290	306	16	5.3
	315	329	14	4.3

XIII Publications

1. O'Donnell, E. & Lynch, M.A. "*Dietary Antioxidant Supplementation Reverses Age-related Neuronal Changes*" (1998) Neurobiology of Aging, **19**: 461-467.

2. O'Donnell, E. & Lynch, M.A. '*Analysis of Age-related Changes in Antioxidant Defence Mechanisms in the Rat Hippocampus*', (1998) Society of Neuroscience Abstract, **24**: 1494.

3. O'Donnell, E., Vereker, E., Lynch, M.A. "*Age-related Impairment in LTP is Accompanied by Enhanced Activity of Stress-Activated Protein Kinases: Analysis of Underlying Mechanisms* " (2000) European Journal of Neuroscience, **12**, 345-352.

4. O'Donnell, E. "*Age-related Neuronal Damage and its Reversal by Vitamin Supplementation*" (1999) Irish Scientist Yearbook, p19.

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Eamonn O'Donnell*

Age-related neuronal damage and its reversal by vitamin supplementation

Aging is a universal process and yet it is a remarkably difficult phenomenon to define. The human body theoretically should be able to regenerate itself indefinitely as it has numerous processes that repair and renew time dependent damage. And yet we age. In the Department of Physiology at Trinity College we are interested in the processes that underlie cellular aging and in ways in which these processes can be delayed.

All organisms fundamentally operate on a cellular level. Cells co-operate and co-ordinate to form tissues and organs. To do this they must produce energy, and it is this process, it is hypothetised, that creates the conditions that "cause" aging.

To respire, a mammalian cell must combine oxygen and glucose and, from this reaction, "energy" is released and stored in a chemical form. However life evolved in an oxygen-free environment over a billion years ago, and it is imperfectly suited to handling the very high energy potential present in molecular oxygen. It is this leakage of semi-reacted but highly energetic oxygen or reactive oxygen species (ROS) from respiration sites within the cell that is hypothesised to cause aging. For these moieties rip through the delicate cellular architecture, distorting and inactivating proteins, damaging DNA and hardening membranes. Over a life time the destruction wrought by these agents overwhelms the cells reparitive capacities. Muscle cells whither, skin cells lose flexibility, and brain cells decline. An organism grows "old".

The cell has evolved an elaborate defence mechanism against these toxic by-products of respiration. This system comprises enzymatic and non-enzymatic components. Both arms function to neutralise the ROS before they can interact and damage cellular components.

In the laboratory of Dr Marina Lynch in the Physiology Department of Trinity College, we have been studying a portion of the brain, the hippocampus, believed to be involved in memory formation. The brain is composed of an entangled tapestry of specialised cells called neurons. These cells are unable to regenerate themselves and appear to be especially sensitive to the constant barrage of ROS. Over time, the capabilities of these neurons erode, and we believe this is due to a parallel decline in the antioxidant defence system of these cells.

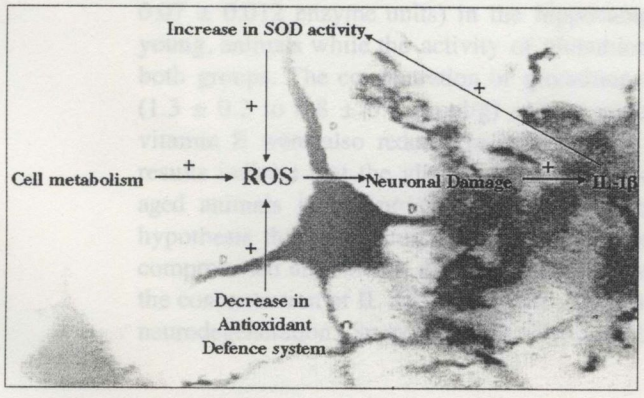
The nonenzymatic portion of the defence system, which is comprised mainly of the vitamins C and E, deteriorates significantly in the aged hippocampus. But also, in aged hippocampal neurons, the activity of a key enzyme involved in trapping ROS is paradoxically increased. This over-activity has deleterious consequences. The enzyme, superoxide dismutase (SOD), converts the toxic ROS into another toxic agent, hydrogen peroxide. This molecule can cause cellular damage if it is not instantly removed, and the enzymes that break down this molecule struggle to cope with its overproduction. The resultant combination of SOD over-activity and the decrease in the concentrations of the vitamins C and E is believed to significantly contribute to the "aging" in these neurons.

It is known that a particular peptide released during infection in the brain, interleukin-1ß (IL-1ß), is also present in high concentration in aged hippocampal neurons. We hypothesised that ROS-mediated neuronal damage causes this peptide to be released and that this in turn stimulates the activity of SOD – thus creating a destructive feedback loop. We postulated that, if this cycle could be stopped by artificially boosting the non-enzymatic portion of the defence system and thus limiting the extent of ROS mediated damage, perhaps the hippocampal neuronal injury witnessed in aging could be deferred?

To test this hypothesis, young and aged rats were fed a diet rich in the vitamins C and E to boost their antioxidant defence system. It was found that there was no increase in concentration in IL-1ß in these animals compared to corresponding controls, SOD activity was not increased, and oxidative damage was also significantly decreased. Furthermore, in a key experiment designed to test hippocampal neuronal function, it was found that aged cells from the diet group recovered their responsiveness to a specific electrical stimulus, and the extent of that response was similar to that of hippocampal neurons from young rats.

We have shown that long-term vitamin supplementation can reverse certain age related changes in hippocampal neurons and that time dependent decline in neuronal function in this portion of the brain can be inhibited.

Mrs Jeanne Calment (122 years old) – can antioxidants delay age-related cellular damage?



Effect of age on hippocampal neurons.

** Eamonn O'Donnell won first prize and the RDS Medal in the Science Communication Forum in November 1998. This is a summary of his winning presentation.*

Contact: Eamonn O'Donnell,
Department of Physiology,
Trinity College, Dublin 2;
E-mail: odonneec@mail.tcd.ie

Message from Mr Chris Shouldice, Chairman, Royal Dublin Society Committee of Science and Technology:

Congratulations to Eamonn O'Donnell on his impressive presentation of an important research project. The subject of the Public Benefits of Contemporary Science Education will be addressed at a conference to be held in the RDS on Wednesday 1 December 1999. Enquiries to Carol Power, Development Executive, Science & Industry, RDS; Tel: 01-668-0866; E-mail: carol.power@rds.ie

ANALYSIS OF AGE-RELATED CHANGES IN ANTIOXIDANT DEFENCE MECHANISMS IN THE RAT HIPPOCAMPUS.

E. O'Donnell* and M. A. Lynch. Department of Physiology, Trinity College, Dublin 2, Ireland.

Previous studies found that interleukin-1β (IL-1β) and lipid peroxidation were increased in hippocampal tissue prepared from the aged rat. IL-1β has been shown to increase the activity of the antioxidant enzyme superoxide dismutase (SOD) while increased SOD activity can cause overproduction of superoxide radicals with resulting damage to neuronal tissue. The activity of SOD was significantly increased (14.9 ± 2.0 to 23.9 ± 3.6 U/mg protein) and the activity of catalase significantly decreased (0.13 ± 0.02 to 0.07 ± 0.012 enzyme units) in the hippocampus of aged, compared with young, animals while the activity of glutathione peroxidase was similar in both groups. The concentration of glutathione was significantly decreased (1.3 ± 0.2 to 0.8 ± 0.1 μmol/g) and the concentrations of vitamin C and vitamin E were also reduced in hippocampal tissue of aged rats. These results indicate that the global antioxidant capacity in the hippocampus of aged animals is compromised. This evidence is consistent with the hypothesis that an accumulation of reactive oxygen species arising from a compromised antioxidant defence system is accompanied by an increase in the concentration of IL-1β and implies a role for this cytokine in age-related neurodegeneration. Supported by BioResearch Ireland.