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An investigation into the roles played by interleukin-1β and reactive oxygen species in the rat brain during ageing.

By

Darren Martin BSc.

Thesis submitted for the degree of Doctor of Philosophy at the University of Dublin, Trinity College.

Submitted September 2001

Department of Physiology,
Trinity College, Dublin 2.

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This thesis is dedicated to Anne, my wife to be.

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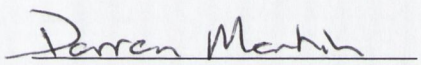
IV Declaration.

I declare that this thesis is entirely my own work with the following exceptions: certain *in vitro* experiments were kindly donated to me by Martina Gooney and Emily Vereker and are acknowledged within this thesis. 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.



Darren Martin

V Abstract.

Ageing is associated with an increase in reactive oxygen species production and an increase in the concentration of the pro-inflammatory cytokine interleukin-1β in the hippocampus. These have both been shown to have deleterious effects on the brain and specifically on a model of learning and memory, long-term potentiation. The ability of aged animals to maintain LTP is impaired and it is postulated that the increases in ROS accumulation and IL-1β concentration may affect one factor which contributes to the expression of LTP in perforant path granule cell synapses, glutamate release. It has been shown that dietary manipulation with antioxidants or essential fatty acids reverses some of the age-related changes observed in the rat brain. In the present study, the ability of the antioxidants, α-lipoic acid and vitamins E and C, and the polyunsaturated fatty acid, eicospentanoic acid, in reversing certain age-related changes in the hippocampus and cortex was assessed.

The evidence suggests that the age-related increase in ROS production in rat cortex occurs as a consequence of an increase in the activity of superoxide dismutase in the absence of concomitant increases in the activities of glutathione peroxidase and catalase. These changes were reversed by dietary supplementation with α-lipoic acid. The increase in ROS was coupled with an increase in lipid peroxidation and a decreased concentration of arachidonic acid; dietary manipulation also reversed these changes. The evidence suggested that the increase in IL-1β concentration in the aged brain may trigger many of the observed changes; dietary manipulation with α-lipoic acid reversed the age-related increase in ICE activity and IL-1β concentration.

Dietary supplementation with EPA reversed the age-related increases in IL-1β concentration, ROS accumulation and p38 activation; these changes were coupled with a restoration of KCl-stimulated glutamate release. Consequently the age-related decrease in LTP was reversed and the pivotal importance of IL-1β in the inhibition of LTP, as well as the efficacy of the diet in reversing the age-related increase in IL-1β, was underscored by the finding that irradiation and LPS increased IL-1β concentration and inhibited the maintenance of LTP. Significantly dietary manipulation reversed this effect.

In parallel experiments in the cortex, supplementation with a diet enriched in EPA led to a reversal of the age-related increase in IL-1β concentration and p38 activation. Evidence from *in vitro* experiments suggested that the increase in p38 activation triggered cytochrome c translocation from the mitochondria and consequently increased the activity of caspase-3. These findings, which are indicative off cell death, were coupled with an increase in PARP cleavage and TUNEL staining while all these measures were absent in the cortex of aged rats fed on the EPA diet. The study indicates that IL-1β plays a significant role in promoting deleterious effects in the brain and the evidence suggests that these effects are mediated by activation of p38.

VI List of Abbreviations.

AA	Arachidonic acid
ACN	Acetonitrile
AD	Alzheimer's Disease
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
APAF-1	Apoptotic protease-activating factor-1
ATP	Adenosine 5'-triphosphate
AFC	7-Amino-4-(trifluoromethyl) coumarin
BHT	Butylated Hydoxytoluene
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin kinase II
CNS	Central nervous system
DAG	Diacylglycerol
DCF	2'7'-dichlorofluorcein
DCFH-DA	2'7'-dichlorofluorcin diacetate
DETAPAC	Diethylenetriaminepentaacetic acid
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithio-bis(2-nitrobenzoic acid)
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbo-diimide hydrochloride
EGTA	Ethylene glycol bis (β-aminoethylether) N,N 'tetraacteic acid
EPA	Eicosapentaenoic acid
EPSP	Excitatory postsynaptic potential

Gpx	Glutathione peroxidase
GSH	Glutathione
HFS	High frequency stimulation
ICE	Interleukin-1β converting enzyme
IL-1β	Interleukin-1β
IL-1ra	Interleukin-1 receptor antagonist
IL-1RI	Interleukin-1 type I receptor
JNK	c-Jun N-terminal kinase
LBP	LPS binding protein
LPS	Lipopolysaccharide
LTP	Long-term potentiation
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
mGluR	metabotropic Glutamate receptor
mRNA	messenger Ribonucleic acid
NADPH	Nictotinamide Adenine Dinucleotide Phosphate
NBT	Nitroblue tetrazolium
NMDA	N-methyl-D-aspartate
NPH-HCL	2-Nitrophenylhydrazine hydrochloric acid
PARP	Poly- (ADP-ribose) polymerase
PKC	Protein kinase C
PLC	Phospholipase C
PMSF	Phenylmethylsulphonyl fluoride
PUFA	Polyunsaturated fatty acid
ROS	Reactive oxygen species
RSK	Ribosomal S6 Kinase
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TBA	Thiobarbituric acid

TCA	Trichloroacetic acid
TETA	Triethylenetetraamine
TMB	3,3',5,5'-Tetramethyl-benzidine
TMP	Tertamethoxypropane
TNF-α	Tumour necrosis factor-α
TUNEL	TdT-mediated dUTP Nick-End-Labelling

Chapter 1:

Introduction

1.1 Ageing.

Ageing can be defined as a "progressive accumulation of changes with time that are associated with or responsible for the ever-increasing susceptibility to disease and death which accompanies advancing age" (Harman, 1981). The changes that occur in a multicellular organism over time are vast, though the reasons for the changes are poorly understood. Many hypotheses of ageing have been presented but few meet the necessary criteria. Reasons why organisms age, why life expectancy or rate of ageing differs between individuals and species are open to debate. How certain experimental procedures appear to prolong life or improve the quality of life also remain unclear. The membrane hypothesis of ageing (Zs-Nagy, 1994) and the free radical theory of ageing (Harman, 1981) fulfil at least some of these criteria. Individually they are adequate hypotheses of ageing, however, combining the basic principles of both theories may be more beneficial. The membrane theory of ageing suggests that there is an age-related decrease in membrane fluidity, which may give rise to many problems such as altered receptor signalling and impaired membrane plasticity. The free radical theory of ageing suggests that the sum of deleterious free radical reactions is a major contributory factor to the ageing process. The free radical theory of ageing offers both a molecular and mechanistic explanation for ageing (Yu, 1994). As will be described later, free radical attack on the cell membrane leads to the reduction of membrane fluidity, therefore, combining both hypotheses.

1.2 Reactive Oxygen Species.

All mammalian cells require molecular oxygen (O_2) for energy production in oxidative phosphorylation. However, reactive oxygen species (ROS) are by-products of chemical reactions that contain oxygen and are damaging to the cell. ROS are formed in all aerobic organisms by both enzymatic and non-enzymatic reactions. They may also be formed by ionising irradiation and during disease states. The main type of ROS are free radicals but other reactive species exist, which are not free radicals, such as hydrogen peroxide (H_2O_2), hypochloric acid (HOCl), singlet oxygen ($^1O_2$) and ozone (O_3). They can inflict damage on many targets molecules *in vivo* including proteins, lipids and DNA (Halliwell, 1994). The body has developed a defence system to combat the accumulation of ROS, which is made up of both enzymatic and non-enzymatic components. The antioxidant enzymes actively convert ROS into safe or less harmful

molecules and the non-enzymatic antioxidants (or antioxidant scavengers) scavenge the radicals and, therefore, terminate the chain reaction that arises from ROS production.

1.2.1 Chemistry of ROS.

A free radical is defined as an atom or molecule that contains one or more unpaired electrons (e^-; Bergendi *et al.*, 1999) and is capable of existing independently (hence the term "free"). Hydrogen is the simplest of free radicals with one unpaired electron. Molecular oxygen itself is biradical, as it possesses two unpaired electrons situated in different orbitals, which gives it its reactivity. The usual route for the metabolism of molecular oxygen is its complete reduction to water (H_2O) by the addition of 4 electrons. However, reduction by the addition of only one electron can form many free radicals and H_2O_2.

The superoxide radical ($O_2^{\bullet-}$) is the best-known free radical as it is the first intermediate in the reduction of molecular oxygen. It is formed as follows:

$$O_2 + e^- \rightarrow O_2^{\bullet-}$$

Mitochondria utilise 90% of the total O_2 consumed and cytochrome oxidase converts it to H_2O by the addition of the 4 e^- from an NADH or $FADH_2$ source. It is thought that between 2 and 5% of the total O_2 consumed may leak out into the cytosol in the form of the superoxide radical. The reactivity of $O_2^{\bullet-}$ itself is relatively low, with a half-life of 10^{-6}s, however, it is unique in that it can lead to the formation of other ROS.

The formation of H_2O_2 can come from the natural dismutation of $O_2^{\bullet-}$.

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

The antioxidant enzyme superoxide dismutase (SOD; described in greater detail later) acts as a catalyst for this reaction and increases the dismutation rate by a factor of about 10^4. H_2O_2 may also be created by other reactions but this is the major source. Though H_2O_2 is not a free radical, it is of a great biological importance because of its ability to diffuse through hydrophobic membranes, such as the mitochondrial and cellular membranes (Yu, 1994). H_2O_2 has some metabolic roles such as in the thyroid gland where it is used by a peroxidase enzyme to iodinate the thyroid hormones (Halliwell,

1994). Due to its ability to diffuse through membranes and relatively stable state (its molecular structure resembles water) it has been extensively researched and is also frequently used as a source of ROS in experiments *in vitro*. H_2O_2 is also biologically important because of its ability to produce further ROS such as the highly reactive hydroxyl radical ($^{\bullet}OH$).

The hydroxyl radical is considered to be the most potent of free radicals but has a very short half-life (10^{-9}s) and, therefore, can only move two molecular diameters before reacting (Yu, 1994). As stated above, it can be formed from H_2O_2 via the Fenton reaction, which involves the reduction of iron as follows:

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

It can also be formed through the Haber-Weiss reaction, which involves the reaction of the superoxide radical and hydrogen peroxide as follows:

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

The hydroxyl radical readily reacts with many molecules especially those found in organic lipids by the removal or addition of hydrogen to unsaturated bonds (Yu, 1994).

Other free radicals may be formed from $^{\bullet}OH$, such as the peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$), with differing degrees of reactivity. Another source of damage from molecules involving oxygen are reactive nitrogen intermediates such as nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^{-}$). These may also be formed from ROS such as the superoxide radical. It is, therefore, clear that a highly damaging chain reaction of free radical production occurs from the initial ROS produced.

1.2.2 Biological sources of ROS.

A major source of ROS is leakage from the mitochondria during oxidative phosphorylation. The normal conversion of O_2 to H_2O by the oxidase process requires the transfer of e^{-}, which, therefore, is associated with free radical production (Yu, 1994). The major source of $O_2^{\bullet-}$ generation within the mitochondria is ubiquinone-cytochrome b, but other sources include the oxidation of ubisemiquinone and NADH dehydrogenase activity in autoxidisable e^{-} carriers (Yu, 1994). Other sources of $O_2^{\bullet-}$

include phagocytic cells, such as macrophages, which actively produce $O_2^{\bullet-}$ by the enzyme NADPH oxidase to combat viruses and bacteria. It may also be produced by other cells such as lymphocytes and fibroblasts and used in cell signalling and growth regulation (Halliwell, 1994). $O_2^{\bullet-}$ may also be produced by autoxidation reactions in which compounds such as catecholamines and reduced flavins can react directly with O_2 to form $O_2^{\bullet-}$. It has been estimated that humans may produce up to 1.72kg of $O_2^{\bullet-}$ per year (Halliwell, 1994).

H_2O_2 is produced by the SOD catalysed dismutation of $O_2^{\bullet-}$, however it may also be generated by the activity of xanthine oxidase. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine, and xanthine to uric acid but during this reaction O_2 is reduced to both $O_2^{\bullet-}$ and H_2O_2. Xanthine oxidase activity increases following stresses such as trauma and oxygen deprivation therefore, increasing the amount of ROS produced (Halliwell, 1994). Phagocytic cells also are believed to produce large amounts of H_2O_2 as well as microsomal and nuclear membranes where cytochrome P-450 and NADPH oxidation increases the generation of this non-radical (Yu, 1994).

The harmful effects of ionising irradiation are thought to be due to the generation of $^{\bullet}OH$. Ionising irradiation exposure is thought to cause fissions of O-H bonds in water to give both $^{\bullet}H$ and $^{\bullet}OH$. Along with generation through the Fenton and Haber-Weiss reactions, $^{\bullet}OH$ can also be generated by phagocytic cells and by catalytic enzymes.

1.2.3 Cellular damage caused by ROS.

ROS and, in particular, $^{\bullet}OH$ can react with biological macromolecules. Proteins may undergo substantial modifications when attacked by ROS. Amino acids are targets as well as the larger macromolecule. H_2O_2 can oxidise essential –SH groups on many enzymes inactivating them (Halliwell, 1994). Three ways in which proteins may be affected are due to fragmentation, aggregation and susceptibility to proteolytic digestion. Oxidation of the polypeptide backbone may result in protein fragmentation. This is initiated when the $^{\bullet}OH$ removes the α-hydrogen atom from an amino acid residue to form a carbon-centred radical (Berlett and Stadtman, 1997). This then reacts rapidly with O_2 to form alkoxyl and alkylperoxide radicals, which begins a chain reaction of destruction. In proteins such as collagen and albumin, $^{\bullet}OH$ attacks proline,

histidine and arginine residues due to their close association with transition metals which may act as catalysts (Yu, 1994). Aggregation occurs by denaturation where $^{\bullet}OH$ has the ability to form cross-bridges with other proteins where, in the absence of O_2, the carbon-centred radical reacts with another radical of the same type to form the cross-bridge (Berlett and Stadtman, 1997). Finally, due to damage and conformational changes, proteins may come under attack from proteolytic enzymes in order to remove dysfunctional proteins from the cell.

ROS attack of lipids, or lipid peroxidation (discussed in greater length below), is widely studied, however, the exact mechanism of lipid peroxidation *in vivo* is not yet fully elucidated. Lipid peroxidation causes damage to the lipid molecular structure and disrupts the cohesive lipid bilayer arrangement. Molecular oxygen does not readily react with unsaturated bonds of fatty acids due to spin forbidden reactions. However, attack by ROS readily occurs. Polyunsaturated fatty acids, such as arachidonic acid (AA) and docosahexaenoic acid (DHA), with more double bonds than monounsaturated fatty acids, are more open to attack from ROS. There are three processes involved in lipid peroxidation, initiation, propagation and termination. Initiation involves the removal of a hydrogen atom from the double bond resulting in a carbon-centred radical as follows where –LH is the side-chain, $R^{\bullet}$ is any free radical and $-L^{\bullet}$ is the carbon centred radical:

$$-LH + R^{\bullet} \rightarrow -L^{\bullet} + RH$$

The propagation stage occurs when the $-L^{\bullet}$ reacts readily with O_2 to form the peroxyl radical ($-LO_2^{\bullet}$) as follows

$$-L^{\bullet} + O_2 \rightarrow -LO_2^{\bullet}$$

The $-LO_2^{\bullet}$ may then be able to react with the double bond adjacent to it. This forms a lipid peroxide ($-LO_2H$) as follows

$$-LO_2^{\bullet} + -LH \rightarrow -LO_2H$$

Lipid peroxides accumulate in membranes destabilising them and making them leaky to ions (Halliwell, 1994). Lipid peroxidation can form aldehydes, which can be cytotoxic. 4-hydroxynonenal is a by-product of 1,6-arachidonic acid and is both cytotoxic and mutagenic (Yu, 1994). Some metals may act as catalysts in the decomposition of the membrane and it has been shown that zinc (Zn^{2+}), which is present in high concentration in the brain following excitation of certain glutamatergic neurons, promotes lipid peroxidation in brain homogenate (Diaz-Arrastia and Hashemi, 2000). Termination only occurs when two peroxyl radicals combine binding both free electrons halting propagation.

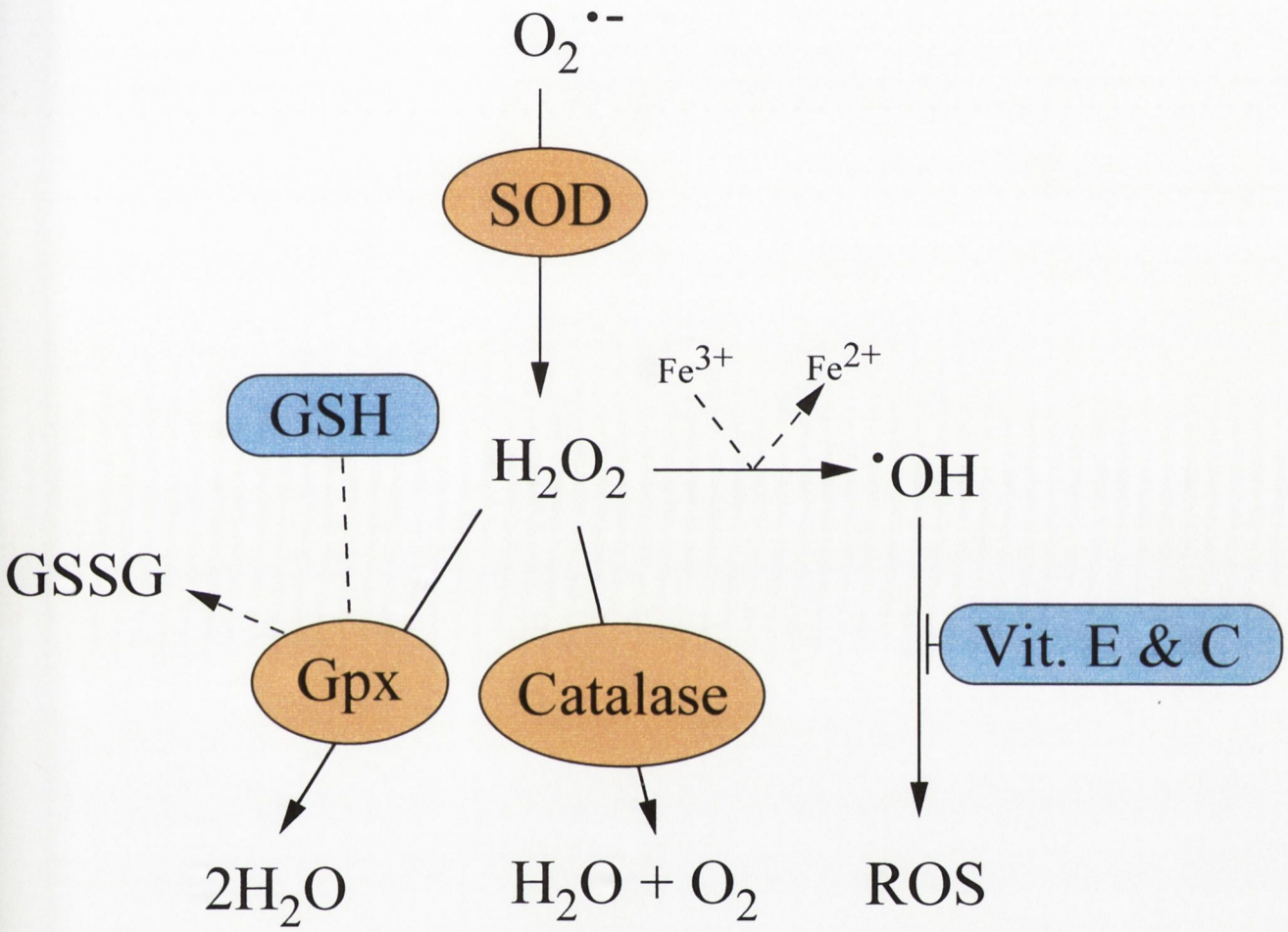
ROS have also been shown to attack deoxyribonucleic acid (DNA). Approximately 20 types of oxidatively altered DNA molecules have been identified (Yu, 1994) and the free radical that inflicts most of the damage is $^{\bullet}OH$. It can attack both deoxyribose sugars and pyridine and pyrimidine bases with thymine and cytosine being the most susceptible. $^{\bullet}OH$ attack on the deoxyribose moiety leads to release of free bases from DNA which generate strand breaks with various sugar modifications, simple abasic sites and DNA-protein cross-links (Brennan *et al.*, 2000). Xanthine oxidase has been shown to produce strand breaks in plasmids and it does this through the actions of $^{\bullet}OH$ as inhibitors of $^{\bullet}OH$ prevent the strand breaks (Yu, 1994). Mitochondrial DNA (mtDNA) is especially susceptible to free radical damage as it is the site of a large production of ROS. It also has fewer repair enzymes and this, together with the increased free radical production, probably explains why the mutation rate for mtDNA is 10-20 times greater than for nuclear DNA (Johnson *et al.*, 1999).

1.3 The antioxidant defence system.

The body has developed a system to counteract the production of ROS. The antioxidant defence system comprises antioxidant enzymes and antioxidant scavengers (see figure 1.1 for antioxidant schematic). The activity or concentration of these is maintained at a level that deals with the normal production of ROS, however, increased damage occurs when the generation of ROS is increased or the defence mechanisms are compromised.

Figure 1.1 The antioxidant defence system.

A simple schematic showing the roles played by the antioxidant enzymes and scavengers in protecting the body from ROS-inflicted damage. SOD converts the superoxide radical into hydrogen peroxide, which is then metabolised by catalase and Gpx into water and molecular oxygen. GSH is utilised during Gpx's metabolism of hydrogen peroxide. Hydrogen peroxide may be converted to the hydroxyl radical through the Fenton reaction, which involves the reduction of iron. The hydroxyl radical, and other ROS, are scavenged by the vitamins E and C.


$O_2^{\bullet-}$
SOD
GSH
H_2O_2
Fe^{3+}
Fe^{2+}
$^{\bullet}OH$
GSSG
Gpx
Catalase
Vit. E & C
$2H_2O$
$H_2O + O_2$
ROS

1.3.1 The antioxidant enzymes.

The main antioxidant enzymes active in the detoxification of ROS within the body are SOD, catalase and glutathione peroxidase. As will be shown, they work in conjunction with each other and their activities relative to each other are very important.

The SOD enzyme is sometimes referred to as the primary defence as it catalyses the dismutation of the superoxide radical to hydrogen peroxide as follows

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

It does this conversion at a rate 4 times greater than natural dismutation (Yu, 1994). Its activity varies between tissues and is found in highest levels in the liver, adrenal glands, kidney and spleen of mammals (Yu, 1994). There are 3 isoenzymes known to exist in mammalian tissue and are distinct from each other depending on which metal is present at the active site or location. All types of SOD bind single charged anions such as azide and fluoride (Matés *et al.*, 1999).

Cu/Zn-SOD (or SOD-1) is made up of two identical subunits of 32kD and has both copper and zinc at its active site bridged by a histamine residue (Matés *et al.*, 1999). It resides in the cytosol of mammalian cells and high immunoreactivity was found in the cerebral cortex and hippocampus of mice, humans and monkeys (Przedborski *et al.*, 1996). Cu/Zn-SOD is the most widely used form of SOD in experiments developed to further understand the damaging effects of ROS on cells. Transgenic mice, with a 2.5 to 3-fold increase in brain Cu/Zn-SOD activity, were shown to be protected in the nigrostriatal dopaminergic pathway against the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (Przedborski *et al.*, 1996). They were also shown to have reduced infarct volume following focal cerebral ischemia (Chan *et al.*, 1996) and were protected from ischemia-reperfusion injury (Wilson 1997). As well as being neuroprotective, a greater than 4-fold increase in Cu/Zn-SOD activity may be neurodegenerative causing cell death and lipid peroxidation (Przedborski *et al.*, 1996).

Mn-SOD (or SOD-2) is a 96kD tetramer and contains one manganese atom per subunit that cycles from Mn(III) to Mn(II) and back to Mn(III) during dismutation (Matés *et al.*, 1999). Mn-SOD resides in the mitochondria and its expression is greatly induced by cytokines, such as tumour necrosis factor-α (TNF-α), but is weakly induced

by oxidants (Matés, *et al.*, 1999). There is half the amount of Mn-SOD as Cu/Zn-SOD present in human tissue and Mn-SOD transgenic mice live for only about 3 weeks whereas Cu/Zn-SOD transgenic mice show no abnormalities until compromised with traumatic injury (Matés *et al.*, 1999). Transfection of cells with Mn-SOD leads to resistance to paraquat (a known $O_2^{\bullet-}$ producer), TNF-α, and radiation induced neoplastic transformations (Matés *et al.*, 1999).

Extracelluar SOD (EC-SOD or SOD-3) has copper and zinc at its active site. It accounts for the majority of SOD activity in plasma, lymph and synovial fluids and is regulated by both cytokines and oxidants (Matés *et al.*, 1999). It is prominently expressed in the hilar region of the hippocampus in mice (Oury and Klann, 1999).

H_2O_2 readily diffuses through membranes easily and because of its ability to form damaging ROS, it represents a threat to cells therefore, its further metabolism is important to cell homeostasis. Glutathione peroxidase and catalase carry out this further metabolism, converting H_2O_2 into H_2O. Catalase (240kD) is a tetrameric enzyme consisting of 4 identical tetrahedrally arranged subunits. Each subunit contains a single ferriprotophorphyin group (Matés *et al.*, 1999) and is found in highest levels in the liver, kidney and red blood cells (Yu, 1994). Within mammalian cells it is present within peroxisomes but it is also found in microsomes and the cytosol of hepatocytes (Yu, 1994). It reacts very efficiently with H_2O_2 and is also associated with peroxidase activity (ROOH; in the presence of H donors such as methanol and ethanol (AH_2)) as follows

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

or

$$ROOH + AH_2 \rightarrow H_2O + ROH + A$$

(Matés *et al.*, 1999). At high H_2O_2 concentrations, organic peroxides are metabolised by catalase (Yu, 1994). Survival of rats exposed to 100% O_2 increased when they were pre-treated with liposomes containing SOD and catalase (Matés *et al.*, 1999). Catalase activity was found to be increased in the rat brain following cortical contusion (Wilson, 1997).

Glutathione peroxidase (80kD) contains a single selenocysteine residue in each of 4 identical subunits (Matés *et al.*, 1999). It is found in the cytosol and mitochondrial

matrix of mammalian cells and is considered to be the most important antioxidant enzymes responsible for the breakdown of H_2O_2 (Yu, 1994). Glutathione peroxidase catalyses the reduction of hydroperoxides using glutathione (GSH; discussed in greater detail later) as follows

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

where GSSG (disulphide GSH) is the oxidised form of GSH. Glutathione peroxidase can also breakdown lipid peroxides (LOOH) as follows

$$2GSH + LOOH \rightarrow GSSG + LOH + H_2O$$

(Halliwell, 1994). There are 5 isoenzymes of glutathione peroxidase found in mammalian tissue with glutathione peroxidase 1 and 4 found in most tissues (Matés *et al.*, 1999). Cancer patients have been shown to have lower glutathione peroxidase activity compared with healthy people and it is thought to be important in the prevention of lipid peroxidation (Yu, 1994). Glutathione peroxidase knockout mice appear to have normal phenotype, but are more susceptible to toxins such as paraquat and their hearts are more sensitive to hypoxia-reperfusion injury (Halliwell, 1999). Melatonin (a powerful scavenger of free radicals) increases glutathione peroxidase activity in maternal and foetal brains of Sprague-Dawley rats (Okatani *et al.*, 2000).

1.3.2 The antioxidant scavengers.

The three main types of antioxidant scavengers are GSH, vitamin E and vitamin C. They act to scavenge free radicals and, therefore, terminate chain reactions. The most abundant scavenger is GSH. Its intracellular concentration is about 0.5mM, but it may reach concentrations as high as 10mM. In its reduced form, γ-glutamyl-cysteine-glycine, it is a nucleophile and effective reductant reacting with H_2O_2, $O_2^{\bullet-}$, $^{\bullet}OH$ and carbon radicals neutralising them by donation of a hydrogen atom (Yu, 1994). GSH is synthesised by γ-glutamylcysteine synthase, which uses glutamate and cysteine as substrates. The product is then combined with glycine (catalysed by GSH synthase) to form GSH (Dringen *et al.*, 2000). It is a reaction partner for the detoxification of xenobiotics and it maintains the thiol redox potential in cells by keeping the –SH

groups of cystolic proteins in a reduced form (Dringen *et al.*, 2000). The actions of glutathione peroxidase oxidises GSH, but this can be reduced to form GSH as follows

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

(Halliwell, 1994). Inhibition of GSH synthesis leads to death of cultured cortical neurons within one day. Death occurs as a result of free radical production as it is prevented by the addition of the antioxidant Trolox (Wilson, 1997). Ionising radiation increases GSH concentration in the hemicerebrum of rabbits (Shimizu *et al.*, 1998), however, stress, as accomplished by immobilisation for 21 days and 6hr/day, causes a depletion in GSH concentration and an increase in lipid peroxidation in the brain of Wistar rats (Madrigal *et al.*, 2001).

Vitamin E is the most widely distributed antioxidant in nature, being found in both the animal and plant kingdom. There are about 8 isomers of vitamin E with α-tocopherol (α-TH) the most potent (Yu, 1994). α-TH is highly lipophilic and is found in high levels in selected tissues such as the adrenal glands, heart, testes and liver. This selectivity is probably due to its high lipid solubility where within the cell α-TH is associated with the mitochondrial membrane and endoplasmic reticulum (Yu, 1994). α-TH is essential in the human diet and severe deprivation may lead to neurological damage (Halliwell, 1994). In the context of this study, an important finding is that the induction of long-term potentiation (LTP; described later) in hippocampal slices is impaired in α-TH deficiency (Vatassery *et al.*, 1999) A decrease in α-TH within the cell ultimately imposes structural and functional damage (Martin *et al.*, 1999). Due to its lipophilic characteristics α-TH is highly effective in protecting membranes against lipid peroxidation by reacting with the lipid peroxyl and alkoxyl ($LOO^\bullet$) radical as follows

$$\alpha\text{-}TH + LOO^\bullet \rightarrow \alpha\text{-}T^\bullet + LOOH$$

where the α-TH radical ($\alpha\text{-}T^\bullet$) is a lot less reactive than the peroxyl radical (Halliwell, 1994). In male Fischer 344 rats, supplementation with α-TH for 2 months increased the concentration of α-TH in the brain with high concentrations found in the hippocampus and cortex. However, a high dose (250 or 500mg/kg) of α-TH reduced the

concentration of vitamin C in these same brain areas (Martin *et al.*, 2000). Extensive research on α-TH supplementation has shown great benefits to humans. A dose of 600IU per day for 6 weeks increased α-TH concentration, reduced superoxide production and reduced lipopolysaccharide- (LPS) induced production of the cytokines interleukin-1β and TNF-α in isolated leukocytes (van Tits, 2000). A dose of 800mg of α-TH per day reduced the H_2O_2 induced DNA damage seen in isolated peripheral blood lymphocytes (Brennan *et al.*, 2000).

Vitamin C or ascorbic acid is hydrophilic. It is an effective antioxidant in aqueous environments especially for protection against lipid peroxidation arising from activated neutrophils (Yu, 1994). Its main function is as a free radical scavenger where it reacts directly with $O_2^{\bullet-}$ and $^{\bullet}OH$ but also scavenges HOCl and O_3 (which is especially important in lung-lining fluids; Halliwell, 1994). Ascorbic acid has an important role in the synthesis of collagen and can scavenge radicals arising from $^{\bullet}OH$ attack on uric acid (Halliwell, 1994). It can restore the antioxidant properties of oxidised α-TH as a consequence of the following reaction

$$\alpha T^{\bullet} + \text{ascorbate} \rightarrow \alpha TH + \text{ascorbate}^{\bullet}$$

(Halliwell, 1994). It is widely distributed in mammals but it is found in high concentrations in adrenal and pituitary glands. It is found to a lesser degree in the liver, spleen, pancreas and brain (Yu, 1994). As well as having antioxidant properties ascorbic acid may also have pro-oxidant properties. Excess concentrations (around 1mM) in the presence of the transition metals Fe^{3+} and Cu^{2+} generates co-factors of activated oxygen radicals during promotion of lipid peroxidation (Yu, 1994). It can easily reduce Fe^{3+} to Fe^{2+}, which is known as a potent free radical inducer and it has been shown to increase oxidative modification of DNA bases (Yu, 1994). Lipid peroxidation of microsomal membrane lipids has been shown to be enhanced by ascorbic acid in α-tocopherol-deficient animals, but in normal animals lipid peroxidation is suppressed by ascorbic acid (Yu, 1994). Ascorbic acid can be oxidised to dehydroasorbic acid (DHAA), which has been found to be both cytotoxic and neurotoxic (Wilson, 1997). Ascorbic acid has been shown to be the first antioxidant oxidised under certain conditions, even in mitochondria where its concentration is low (Vatassery, 1995). As stated above, high doses of α-TH reduce the concentration of

ascorbic acid in the hippocampus and cortex (Martin *et al.*, 2000). Dietary supplementation with ascorbic acid has been shown to act as an antioxidant towards lipids in the plasma of guinea pigs and even protects plasma lipids during iron overload, which has been shown to enhance lipid peroxidation (Chen *et al.*, 2000).

In this laboratory, dietary supplementation with vitamins E and C has been shown to reverse the age-related impairment in LTP and the age-related increases in interleukin-1β (IL-1β; discussed in greater detail later) concentration and ROS production in the hippocampus of rats (Murray and Lynch, 1998b). Vitamin supplementation also reversed the age-related increase in SOD activity, lipid peroxidation and IL-1β in the cortex (O'Donnell and Lynch, 1998).

1.3.3 α-Lipoic acid.

α-Lipoic acid is a potent antioxidant and is found in tissue that has high metabolic activity, such as the heart (Biewenga *et al.*, 1997). It is a lipophilic disulphide-containing co-factor required for reactions catalysed by α-keto acid dehydrogenase enzyme complexes. It has three main antioxidant properties (a) it has metal chelating abilities, which are important for sequestering excess free iron and copper, (b) it can scavenge ROS, and (c) it can regenerate endogenous antioxidants such as vitamin E, vitamin C and GSH (Packer *et al.*, 1997). Dihydrolipoic acid (DHLA), the reduced form of α-lipoic acid, has more potent antioxidant properties than the non-reduced form and in combination the 2 forms are very effective (Biewenga *et al.*, 1997). DHLA and α-lipoic acid have been shown to scavenge $O_2^{\bullet-}$, $HO^{\bullet}$, H_2O_2, the peroxyl radical ($ROO^{\bullet}$), HOCl, $NO^{\cdot}$ and singlet oxygen (Packer *et al.*, 1997).

α-Lipoic acid has been shown to be very potent in combating the increase in lipid peroxidation in patients with diabetic neuropathy (Nickander *et al.*, 1996; Ziegler *et al.*, 1999). In a study by Arivazhagan and Panneerselvam (2000) it was observed that intraperitoneal injection of α-lipoic acid reversed the age-related increase in lipid peroxidation in the cortex and hippocampus. In the same study α-lipoic acid also reversed the age-related decreases observed in the antioxidant scavengers GSH, vitamin E and vitamin C (Arivazhagan and Panneersalvam, 2000). α-Lipoic acid has been shown to reverse the age-related decline in ascorbic acid concentration in rat hepatocytes (Lykkesfeldt *et al.*, 1998). These studies confirm the suggestion that α-lipoic acid can act to regenerate the antioxidant scavengers, however, its ability to

regenerate vitamin E is probably indirect and achieved at the expense of GSH and vitamin C (Biewenga *et al.*, 1997).

α-Lipoic acid has been shown to reverse the age-related changes in long-term potentiation (LTP) and the down-regulated antioxidant status in the rat hippocampus (McGahon *et al.*, 1999b). One factor that may contribute to the reduced ability of aged rats to sustain LTP is a reduction in glutamate release; it was reported that α-lipoic acid reversed this age-related decrease (McGahon *et al.*, 1999b). It has been shown that α-lipoic acid can reverse the age-related decline in mitochondrial function (Hagan *et al.*, 1999). This is not surprising as α-lipoic acid is readily taken up into mitochondria where it acts as an essential co-factor in the oxidative phosphorylation of α-keto acids and confers antioxidant capabilities within the mitochondria (Biewenga *et al.*, 1997).

1.4 ROS in disease and ageing.

Oxidative stress has been suggested as being a cause or a consequence of many diseases. Oxidative stress occurs after all tissue injury and is a secondary phenomenon of most human diseases (Halliwell, 1994). In neuronal tissue oxidative stress is associated with alcoholism, Alzheimer's disease (AD), HIV infection, amyotrophic lateral sclerosis (ALS), dementia, multiple sclerosis, Down's syndrome, epileptic seizures, Parkinson's disease, schizophrenia, shock and tardive dyskinesia (Packer *et al.*, 1997). It has been shown that the familial dominant form of ALS is related to mutations in Cu/Zn-SOD, which not only results in a decrease in the activity of the enzyme but also results in the enzyme becoming toxic (Halliwell, 1994). It is thought that atherosclerosis is caused by an increase in oxidised low-density lipoproteins (LDLs) and cholesterol, which in turn causes an increase in fatty acid, deposits on cells lining the arterial walls. The increase in oxidised LDLs and cholesterol is thought to be related to oxidative stress (Yu, 1994). Oxidative stress is also implicated in cancer. ROS induced damage to DNA and regulators of cell division may cause uncontrolled proliferation of cells. Carcinogen- and radiation-induced tumour production may be caused by an increase in ROS. Tumour cells show reduced activities of Mn and Cu/Zn-SOD and catalase, which results in an increase in $O_2^{\bullet-}$ (Yu, 1994). An increase in lipid peroxidation has been implicated in both hepatitis C in adults and HIV infection in children (Romero *et al.*, 1998). In both cases a decrease in GSH concentration has been detected and this may cause the increase in lipid peroxidation (Romero *et al.*, 1998).

AD has been associated with oxidative stress. ROS are thought to cause the aggregation of β-amyloid which in turn causes a further increase in ROS and this is confirmed in studies which show that β-amyloid toxicity is eliminated by ROS scavengers (Christen, 2000). The lesions found in the brain of AD patients are associated with damage to DNA, protein oxidation, lipid peroxidation and advanced glycosylation end products, all of which may be caused by ROS. AD is also associated with a decrease in mitochondrial cytochrome c oxidase activity, which results in an increase in ROS production. It has been shown that there is a 25-30% decrease in its activity in post-mortem cortex, dentate gyrus and hippocampus taken from AD patients (Christen, 2000). This change may explain the low levels of oxidative phosphorylation associated with AD and why oxidation of mtDNA has been seen in the parietal cortex of AD sufferers. F2-isopropane, a by-product of AA peroxidation, has been shown to be increased in the cerebrospinal fluids of AD patients (Christen, 2000).

An age-related increase in ROS production is known to exist. This may be due to a number of reasons, including a reduction in the efficiency of the mitochondria leading to an increase in the leakage of $O_2^{\bullet-}$ into the cytoplasm from the mitochondria. ROS-inflicted damage builds up within the mitochondria inactivating enzymes and causing mutations in mtDNA. Changes in protein conformation occur and the membrane may lose its fluidity due to polyunsaturated fatty acid peroxidation. The appearance of age pigments serves as a putative link between free radical injury and lipid damage in ageing (Yu, 1994). Lipofuscin is thought to derive from peroxidatively damaged cell organelles that have been phagocytised by liposomes. It is thought to consist of a heterogeneous mixture of cross-linked polymerised lipids and undigested protein products that accumulate with age in various areas of the central nervous system (CNS) (Yu, 1994; Harman, 1981). Free radical production may also be used as a determinant of a rate of ageing theory. Metabolic rate and free radical production are closely linked, however, a relationship also exists between antioxidant activity and maximum life span. For instance, rats have a higher activity of liver glutathione peroxidase and catalase than humans, yet their maximum life span is a lot shorter than humans (Perez-Campo *et al.*, 1998). Their metabolic rate is also higher and this may result in the production of higher concentrations of ROS and, therefore, require a higher activity of antioxidants. There is a negative correlation between H_2O_2 production by

liver mitochondria and maximum life span in mammalian species, which further supports this theory (Perez-Campo *et al.*, 1998).

There is conflicting evidence relating to antioxidant status in age. In this laboratory, previous studies have shown that there is an increase in SOD activity, a decrease in the activity of catalase and no change in the activity of glutathione peroxidase in the hippocampus of aged rats (O'Donnell *et al.*, 2000). This might be interpreted as damaging since there is an increased activity in total (Mn-SOD and Cu/Zn-SOD) SOD increases the conversion of $O_2^{\bullet-}$ to H_2O_2, but without the appropriate concomitant changes in activities of glutathione peroxidase and catalase resulting in an accumulation of H_2O_2. This result is confirmed by some studies (Carrillo *et al.*, 1992), however, other studies have shown that no change in total SOD activity occurs in the hippocampus of aged rats (Danh *et al.*, 1983). Similar discrepancies are seen with age-related changes in the antioxidant scavengers. Previous findings in this laboratory have shown that there is no change in the concentrations of vitamin E and C and a decreased concentration of GSH in the hippocampus of aged rats (O'Donnell *et al.*, 2000). However, Zhang and colleagues (1993) have shown that no changes in GSH concentration occur in the hippocampus of the aged gerbil whereas Arivazhagan and Panneerselvam (2000) reported an age-related decrease in all scavengers in the hippocampus of the rat. In some studies, the age-related decrease in GSH has been suggested to be related to its increased oxidation, together with a decrease in the activities of glutathione peroxidase and GSH transferase. These enzymes are needed in the reduction of oxidised GSH (Yu, 1994). The reason for the differing results quoted by these studies is unclear but may be due to species or strain differences or the condition of the aged rats.

1.5 Polyunsaturated fatty acids and cell membranes.

Fatty acids have 4 major physiological roles. They are the building blocks of phospholipids and glycolipids, they act as fuel molecules, they may act as hormones or intracellular messengers or, attached to proteins, they target proteins to membrane locations. Fatty acids in biological systems usually contain an even number of carbon atoms and the nomenclature assigned to each fatty acid reveals the number of carbons it contains and also the number of carbon-carbon double bonds. For instance, AA has a nomenclature of 20:4, meaning that it has 20 carbon atoms and 4 double bonds. The

position of the first double bond from the carboxy terminal relates to whether a fatty acid is named an omega 3 or omega 6 (ω-3 or ω-6) fatty acid. AA and its precursor, γ-linolenic acid (γLA; 20:3) are ω-6 fatty acids and DHA (22:6) and its precursor, eicosapentaenoic acid (EPA; 20:5) are ω-3 fatty acids. The properties of fatty acids are dependent on their chain length and degree of saturation. The more unsaturated fatty acids, and specifically polyunsaturated fatty acids (PUFA), present in a membrane the more fluid it is. Fluidity refers to the physical state of the fatty acyl chains comprising the membrane bilayer structure, as well as a measure of the different rates of motion of elements within the membrane (Youdim *et al.*, 2000). A more rigid membrane contains more saturated fatty acids in which the carbon-carbon bonds have a trans conformation, whereas a more fluid membrane contains more unsaturated fatty acids and has a gauche conformation.

Another factor in determining the fluidity of a membrane is the concentration of cholesterol present in the membrane. Cholesterol moderates the fluidity of membranes by sterically blocking large motions of fatty acyl chains, which makes membranes less fluid. Roughly 30% of the total lipid content of neuronal membranes is comprised of cholesterol. An appropriate lipid environment of membranes is required for proper functioning of membrane proteins such as receptors, enzymes, ion channels and ion pumps (Lynch, 1998). AA and DHA are both very prominent in neuronal membranes and are important for many signalling pathways and in plasticity. PUFAs, such as AA, EPA and dihomo-γLA are important precursors of eicosanoid biosynthesis (Youdim *et al.*, 2000). The replacement of even a single double bond in these PUFAs is sufficient to have a profound effect on the physical properties of the membrane.

AA concentration in the membrane of the hippocampus is high and it has been implicated as playing a role in synaptic plasticity. It has also been proposed as a retrograde messenger in LTP (Williams *et al.*, 1989). The concentration of AA has been shown to be increased in potentiated tissue and an age-related impairment in LTP has been shown to be correlated with a decreased concentration of AA in the membrane (Lynch and Voss, 1994). The decrease in AA concentration was also correlated with a decrease in the activity of phospholiase A_2 (PLA_2), which, when stimulated by calcium, causes liberation of AA from membrane phospholipids (Lynch and Voss, 1994).

An age-related decrease in DHA in the rat hippocampus has also been shown (McGahon *et al.*, 1999a). The reason for the decrease in PUFAs and, therefore, for the

problems that arise with age, such as an inability to maintain LTP, is probably due to an increase in ROS production (McGahon *et al.*, 1999a). It is proposed that an increase in ROS production leads to increased lipid peroxidation due to the susceptibility of PUFAs to ROS attack. Dietary manipulation with either AA (McGahon *et al.*, 1999a) or DHA (McGahon *et al.*, 1999c) restores the concentration of these fatty acids in the brain; it has been shown that this effect is accompanied by a restoration of the ability of aged rats to maintain LTP (McGahon *et al.*, 1999a and c). A study by Itokazu and colleagues (2000) showed that slices transfused with DHA exhibited a decrease of about 30% in the field excitatory postsynaptic potential (fEPSP) slope in the CA1 region of the hippocampus while LTP was also inhibited. In contrast in the dentate gyrus the fEPSP slope was increased by DHA and LTP was not affected. This shows that DHA exerts regionally different effects on hippocampal transmission (Itokazu *et al.*, 2000).

A study using Greenland Eskimo subjects showed that a correlation exists between low incidence of coronary heart disease and high consumption of fish products (Foulon *et al.*, 1999). Fish products contain high proportions of EPA and DHA and these are the major lipid constituents of cod liver oil. In a study where human subjects were fed a diet enriched in these fatty acids there was an increase in the concentration of these lipids and a decrease in the concentration of ω-6 fatty acids in the plasma of the volunteers (Foulon *et al.*, 1999). A similar result was also found in the brain of rats, where feeding AA decreased DHA and feeding DHA decreased AA concentrations (Wainwright *et al.*, 1999). This suggests that incorporation of both saturated and unsaturated fatty acids into the membrane is rapid (Lynch, 1998). In a report by Liu and colleagues (2001) DHA and EPA were shown to inhibit the phorbol 12-teradecanoate 13-acetate-induced activation of activator protein-1 (AP-1) in JB6 mouse cell line whereas AA had no effect.

1.6 Long-term potentiation.

1.6.1 The hippocampal formation and LTP

The hippocampal formation consists of the dentate gyrus, hippocampus and subiculum and is part of the limbic system. It is located on the medial aspect of each hemisphere and lies at the floor of the lateral ventrical and beneath the cortical structures. There are two main pathways linking the hippocampus and the rest of the

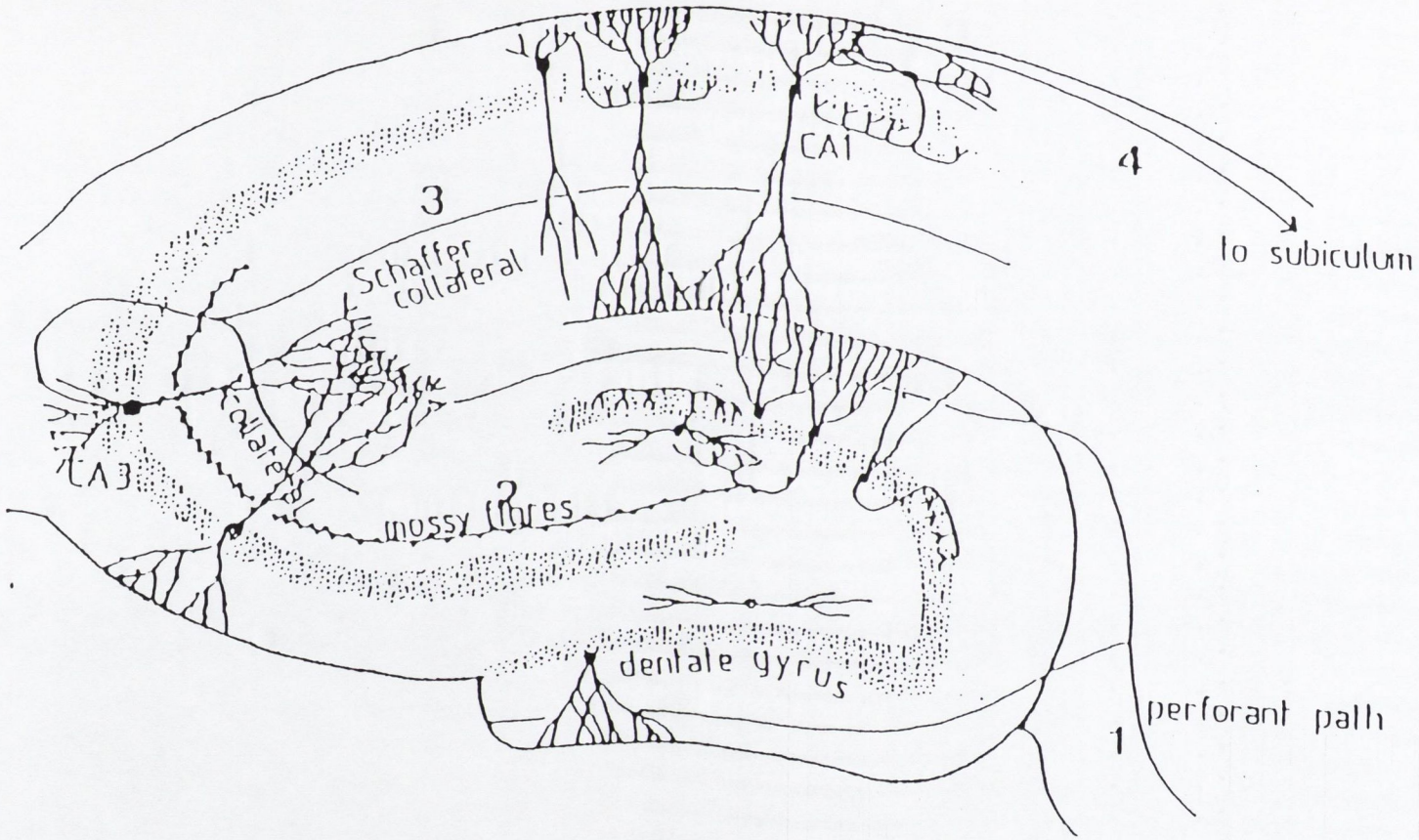
brain. The fornix links the hippocampus with the septal area, the thalamus and the hypothalamus while the perforant pathway links the hippocampus with the temporal lobe, specifically with the entorhinal cortex. The perforant pathway runs from the subiculum to the granule cells in the hilus of the dentate gyrus (see figure 1.2). The axons of the granule cells form the mossy fibre pathway, form the afferent input to the pyramidal cells in the CA3 region of the hippocampus. The pyramidal cell axons form the Schaffer collateral pathway and inputs to the pyramidal cells in the CA1 region. The hippocampus is known to be important for storage of memory (Kandel *et al.*, 1992) and neurons within the hippocampus show plasticity of a sort required for associative learning (Bliss and Lømo, 1973). Around the turn of the 20th century the Spanish neurologist Santiago Ramon y Cajal stated that neurons communicate with each other at specialised junctions which Sherrington later called synapses.

Bliss and Collingridge (1993) stated that LTP is expressed as "a persistent increase in the size of the synaptic component of evoked response, recorded from individual cells or from populations of neurons". LTP was first described by Bliss and Lømo (1973) in the dentate area of the hippocampus in anaesthetised rabbits. The authors showed that the population response recorded from granule cells in the dentate gyrus to single perforant path stimulations, was potentiated for periods ranging from 30min to 10hr following one or more conditioning trains of stimuli at 10-20/s for 10-15s or 100/s for 3-4s. They suggested that 2 independent mechanisms were responsible for LTP: an increase in the efficiency of synaptic transmission at the perforant path synapses and an increase in the excitability of the granule cell population (Bliss and Lømo, 1973). LTP can be expressed in the dentate gyrus, CA3 and CA1 regions of the hippocampus and in other areas of the brain (Andersen *et al.*, 1977). However, LTP is not similar in each area, for example LTP in the CA1 and dentate gyrus is associative LTP and in the CA3 is non-associative (Kandel *et al.*, 1992). LTP in the CA1 and dentate gyrus has three fundamentally important properties: co-operativity, associativity and input-specificity (McNaughton *et al.*, 1978; Andersen *et al.*, 1977; Lynch *et al.*, 1977; Bliss and Collingridge, 1993).

Co-operativity describes the existence of an intensity threshold for induction of LTP, which implies that more than one fibre must be activated to obtain LTP (McNaughton *et al.*, 1978). The threshold needed to induce LTP depends on intensity and pattern of tetanic stimulation. Associativity means that a weak tetanus may be able

Figure 1.2 The hippocampus.

The diagram depicts a transverse section of the hippocampus, illustrating the various regions and inputs. The perforant path (1) inputs to the granule cells of the dentate. The granule cells project via the mossy fibres (2) to the CA3 pyramidal cells. These cells input via the Schaffer collaterals (3) to the CA1 pyramidal cells. These cells project to the subiculum (4).


CA1
4
3
to subiculum
Schaffer collateral
CA3
2
mossy fibres
dentate gyrus
1
perforant path

to induce LTP if, at a separate but convergent input, a strong tetanus is active at the same time as the weak tetanus (McNaughton *et al.*, 1977). Finally, input-specificity of LTP means that inputs that are not active at the time of tetanus are not potentiated, implying that LTP is specific to the active pathway (Andersen *et al.*, 1977; Lynch *et al.*, 1977).

1.6.2 Induction of LTP.

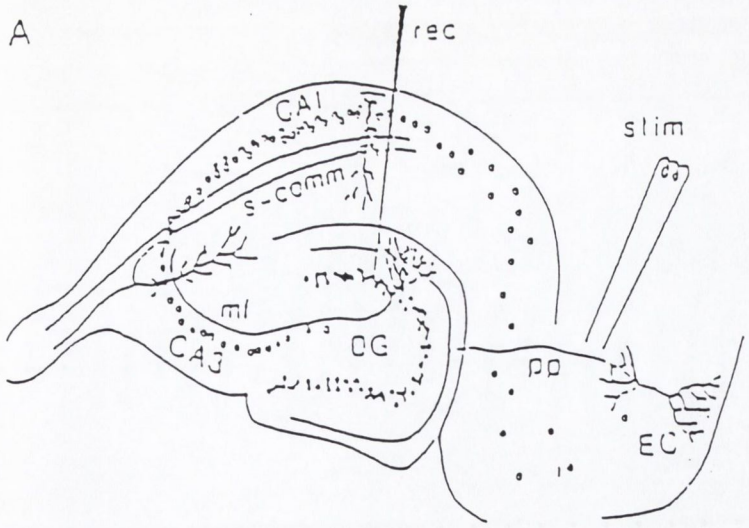
Induction of LTP depends on the depolarisation of the postsynaptic cell. In hippocampal synapses this relies on the presynaptic release of the excitatory amino acid neurotransmitter L-glutamate, upon potentiation and the opening of amino acid receptors on the postsynaptic membrane. In the present study, presynaptic release of glutamate is achieved by a tetanic stimulation protocol of 3 trains of stimuli at 250Hz for 200msec with an inter-burst interval of 30sec (see figure 1.3). There are 4 subtypes of glutamate receptor: N-methyl-D-aspartate (NMDA), kainate, kainate-quisqualate A (also known as a α-amino-3 hydroxy-5 methyl-4 isoxazole propionic acid (AMPA) receptor) and quisqualate B (a metabotropic, second-messenger linked receptor; Kandel *et al.*, 1992). NMDA receptors play a key role in the induction of LTP and are clustered on the heads of the spines and not the shafts of the dendrites (Kandel *et al.*, 1992). The receptors consist of binding sites for glutamate and an associated cation channel (MacDermott *et al.*, 1986). Under resting conditions ion movement, especially through the channel, is prevented by the presence of a magnesium ion (Mg^{2+}). Under conditions of strong depolarisation, Mg^{2+} is displaced from the channel allowing the movement of Na^{+} and Ca^{2+} into the cell and K^{+} out of the cell (McDermott *et al.*, 1986). It is, therefore, called a double-gated channel. Mg^{2+} acts as a block on the channel and is only removed when the cell is depolarised and glutamate is bound to the receptor (Collingridge *et al.*, 1983). Co-operativity is, therefore, the need for depolarisation to reduce the level of the Mg^{2+} block of the NMDA channel. A stimulus from a weak tetanus cannot depolarise the cell sufficiently to remove the Mg^{2+} block (McNaughton *et al.*, 1978). Associativity is, therefore, the ability of the depolarisation to come from another input. Experimentally this can come from electrical stimulation of the pathway (McNaughton *et al.*, 1978). Input-specificity is the need for a sufficient concentration of glutamate to activate adequate numbers of NMDA receptors to induce LTP (Andersen *et al.*, 1977; Lynch *et al.*, 1977). Blocking the NMDA receptor with 2-

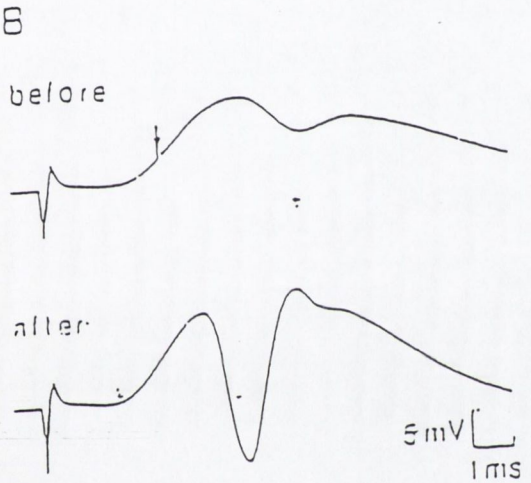
Figure 1.3 Long-term potentiation.

A) Depicts a drawing of LTP in the dentate gyrus in vivo. The stimulating (stim) electrode stimulates the perforant path axons and the recording (rec) electrode receives the electrical activity produced by the granule cells.

B) The graph depicts the evoked response the population EPSP before and after high frequency stimulation recorded from the dentate gyrus granule cells. A base line is normalised at 0% change in the EPSP taken from recordings one hour before high frequency stimulation and the increase in the % change of the EPSP slope is shown.

Taken from "A synaptic model of memory: long-term potentiation in the hippocampus." (1993) Bliss, T.V.P. and Collingridge, G.L. *Nature*, **361**, 31-39.


A
rec
CA1
stim
s-comm
ml
CA3
DG
pp
EC


B
before
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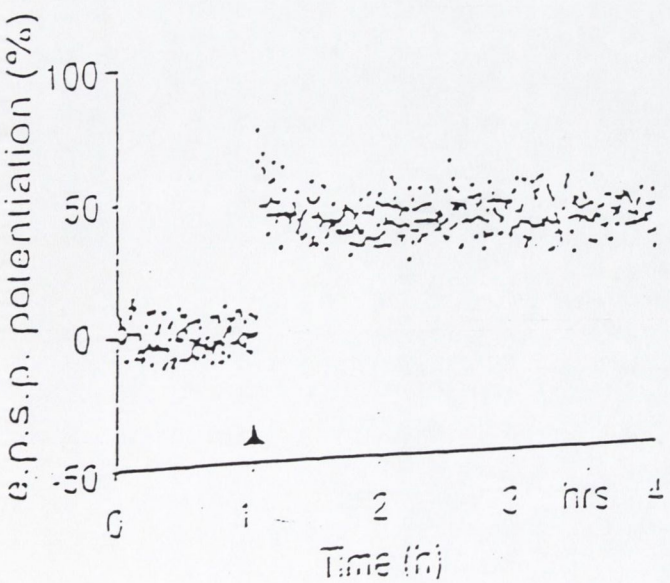
5mV
1ms


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

amino-5-phosphonopentanoate (AP5) prevents LTP induction, but, application of NMDA alone does not induce LTP (Collingridge *et al.*, 1983).

It has been demonstrated that the key event is Ca influx (Lynch *et al.*, 1983). It is suggested that LTP is caused by a modification of the postsynaptic neuron and that its induction depends on the level of free Ca (Lynch *et al.*, 1983). It has been shown that intracellular injection of the Ca^{2+} chelator, EGTA, prevents the induction of LTP (Lynch, *et al.*, 1983). Ca^{2+} entry into the cell through the NMDA receptor is augmented by release of Ca^{2+} from intracellular stores of Ca^{2+}. This is probably aided by the formation of the second-messenger inositol 1,4,5-triphosphate ($InsP_3$), which occurs through glutamate-induced activation of the metabotropic glutamate receptor (mGluR; Sugiyama *et al.*, 1987). Activation of the mGluR activates a G-protein, which then binds to phospholipase C (PLC) that leads to the formation of $InsP_3$ (Curtis, *et al.*, 1960).

1.6.3 Maintenance of LTP.

Once Ca^{2+} has entered the postsynaptic cell it initiates the persistent enhancement of synaptic transmission by activating 2 Ca^{2+}-dependent protein kinases: Ca^{2+}/calmodulin kinase II (CaMKII) and Ca^{2+}/phospholipid-dependent protein kinase C (PKC; Kandel *et al.*, 1992). Inhibitors of PKC are known to inhibit LTP and α-CaMKII knockout mice show severely impaired LTP (Silva *et al.*, 1992). It has been reported that PKC activation, following the blockade of NMDA receptors, rescued LTP in anaesthetised mice (Kleschevnikov and Routtenberg, 2001) indicating a pivotal role for PKC activation. However, although activation of PKC is necessary for LTP it may not be sufficient to induce it (Bliss and Collingridge, 1993). Activation of PKC and CaMKII enhances the phosphorylation of other receptors such as AMPA and ion channels (Malinow *et al.*, 1989). To ensure the long lasting maintenance of LTP, protein synthesis must occur (Krug *et al.*, 1984). It has been shown that protein synthesis is increased in the rat brain following high frequency stimulation (Fazeli *et al.*, 1993; Mullany and Lynch, 1997). Pre-treatment with protein synthesis inhibitors, such as cycloheximide and anisomycin (which prevents the transcription of mRNA from DNA), not only block protein synthesis but also block maintenance of LTP (Otani *et al.*, 1992; Mullany and Lynch, 1997). Pre-existing mRNA is required for the maintenance of LTP during the first phase of LTP whereas newly synthesised mRNA

appears to be required for the more persistent phase (Otani *et al.*, 1989). The identity of the proteins which are newly synthesised and which are essential for LTP, is not known. However, certain IEGs like c-*fos* upregulation, occurs following induction of LTP in unanaesthetised rats and may be important for the most persistent form of LTP (Dragunow *et al.*, 1989).

An increase in neurotransmitter release from the presynaptic terminal occurs during LTP at least in perforant-path granule cell synapses (Lynch and Voss, 1994). If an increase in release is required for maintenance of LTP it must be hypothesised that a signal from the postsynaptic cell will be passed to the pre-synaptic cell. Therefore, the existence of a retrograde messenger has been proposed (Williams *et al.*, 1989). In order for a substance to be considered as a potential retrograde messenger, the following criteria must be fulfilled: it must be released from the postsynaptic cell in response to tetanic stimulation or following application of glutamate; it must appear in the perfusate following induction of LTP and it must act on the presynaptic terminal to evoke an increase in neurotransmitter release. One such candidate is AA (Williams *et al.*, 1989). Weak activation of the perforant path leads to a persistent increase in synaptic efficiency when administered in the presence of AA (Williams *et al.*, 1989). This potentiation was accompanied by an increase in glutamate release (Williams *et al.*, 1989). Inhibitors of PLA_2, which removes AA from phospholipids, were shown to block the induction of LTP (Williams and Bliss, 1989) consistent with a role for AA. In the postsynaptic terminal, along with the activation of PLC through mGluR activation, PLA_2 is also activated. This may result in the liberation of AA from phospholipids, which leads to the release of AA into the synaptic cleft which may then stimulate the presynaptic cell and eventually lead to the release of glutamate (Williams *et al.*, 1989).

1.6.4 Age and LTP.

It is generally accepted that age is associated with memory failure and a deficit in spatial memory (Barnes, 1987). It has also been shown that there is an impairment in the maintenance of LTP in the aged brain (Barnes, 1979; Landfield *et al.*, 1978; Lynch and Voss, 1994; McGahon *et al.*, 1997; McGahon *et al.*, 1999a, b and c). However, the magnitude of LTP has been found to be similar in both young and aged rats where LTP studies were carried out in freely moving rats (Barnes, 1979). While it is generally accepted that there is an impairment in the ability of aged animals to sustain LTP there

are conflicting views over whether the induction of LTP is impaired in the aged animal. Some research groups suggest that induction of LTP is unaffected by age but that the rate of decay of LTP is accelerated in aged animals (Barnes, 1979 and Deupree *et al.*, 1993). Lynch and Voss (1994) showed that the mean percentage increase in the EPSP slope following tetanic stimulation of the perforant path in aged rats was reduced and this was supported by more recent studies (McGahon *et al.*, 1997 and 1999c). This may be due to the impairment in temporal summation of multiple EPSPs during high frequency stimulation (Rosenzweig *et al.*, 1997).

The underlying cause of the impairment in the maintenance of LTP is not known, however, several important findings have been reported. Firstly, there is an age-related decrease in the concentration of AA in the hippocampus of rats (Lynch and Voss, 1994) which impacts on both membrane fluidity and the ability of AA to act as a retrograde messenger. It has been proposed that this may be a consequence of the increase in lipid peroxidation in the hippocampus of the aged rat (McGahon *et al.*, 1999a; Arivazhagan and Panneerselvam, 2000). IL-1β has been shown to inhibit LTP in the CA1 of the rat (Bellinger *et al.*, 1993), the CA3 region of the mouse (Katsuki *et al.*, 1990) and in the rat dentate gyrus *in vitro* (Cunningham *et al.*, 1996) and *in vivo* (Murray and Lynch, 1998a). It has been shown to be increased in the hippocampus of aged rats (Murray and Lynch, 1998b; O'Donnell *et al.*, 2000; McGahon, *et al.*, 1999a and b). Decreases in glutamate release (Lynch and Voss, 1994 and McGahon *et al.*, 1997) and calcium channel numbers and activity have been described in the hippocampus of aged rats (Verkhratsky *et al.*, 1994). In addition protein synthesis in the entorhinal cortex is reduced in the aged rat (Mullany and Lynch, 1997). If it is argued that LTP relies on an appropriate activity of each of these then the finding that LTP is inhibited with age is not surprising.

1.7 Interleukin-1.

One of the most abundant cytokines in mammalian systems are the interleukins. There are, to date, 18 characterised interleukins, which variously possesses both anti-inflammatory and pro-inflammatory cytokines. The most studied of these is interleukin-1 (IL-1). IL-1 affects nearly every cell type and is induced by nearly all microbial products (Dinarello, 1996). There are three members of the IL-1 family. IL-1α and IL-1β are agonists of the specific IL-1 receptor types I and II (IL-1RI and IL-1RII) and IL-

1 receptor antagonist (IL-1ra) is, as its name suggests, an antagonist of IL-1RI (Dinarello, 1996). Both IL-1α and IL-1β are synthesised as precursors with a molecular weight of 31kD and IL-1β is cleaved by a specific cellular protease called interleukin-1β converting enzyme (ICE), to form the mature 17kDa protein. IL-1ra is produced as a single protein and is transported into and out of cells. IL-1RI has a larger intracellular domain than the IL-1RII and induces a cascade of signalling events once stimulated. IL-1RII is thought to be a decoy receptor as binding of IL-1 does not induce any intracellular signalling and this is probably due to its short intracellular domain (Colotta *et al.*, 1994). Both receptors can exist as soluble forms in health and disease. Binding of IL-1 to IL-1RI induces the intracellular binding of IL-1R accessory protein (IL-1R-AcP), which forms a tightly bound complex with IL-1 and IL-1RI (Greenfeder *et al.*, 1995).

1.7.1 IL-1α.

Pro-IL-1α is fully active when translated and remains intracellular. It shares 19-26% homology with IL-1β, which is the predominant form of IL-1 in the brain and other tissues (Ayala *et al.*, 1994), but they are derived from different gene products (Feghali and Wright, 1997). In humans, blood monocytes do not process or secrete mature IL-1α, however, murine IL-1α is more widespread (Dinarello, 1996). Pro-IL-1α is cleaved by extracellular proteases and can also be cleaved by calpain. It is believed that pro-IL-1α may be involved in cell differentiation and is found in high concentrations in keratinocytes of the skin (Hauser *et al.*, 1986). IL-1α binds to IL-1RI, which is then internalised and the complex may act as transcription factor by binding to DNA (Mizel *et al.*, 1987).

1.7.2 IL-1β.

Constitutive expression of IL-1β in brain occurs in neurons, astrocytes, oligodendrocytes and endothelial cells. However, another source of IL-1β, following brain insult maybe invading macrophages (Touzani *et al.*, 1999). IL-1β contains a clear TATA box sequence, unlike the IL-1α gene, and other promoter regions are found over several thousand base pairs (Touzani *et al.*, 1999). Pro-IL-1β remains in the cytosol and is only marginally active. Release of mature IL-1β is linked to processing by ICE (Black *et al.*, 1988) but pro-IL-1β can also be cleaved by trypsin, elastase and

chymotrypsin at different amino acid sites (Dinarello, 1996). There are many non-microbial inducers of IL-1β, which include hypoxia, ultraviolet B, γ-irradiation, kainic acid, melatonin, collagen and phorbol esters (Dinarello, 1996). Depending on the stimulus, IL-1β mRNA levels can increase within 15 minutes but in general start to decline after 4 hours (Jarrous and Kaempfer, 1994). Once released from cells, mature IL-1β can trigger the IL-1RI on neighbouring and distant cells from the site of release, and it is this characteristic that implicates IL-1β in systemic disease (Dinarello, 1996). The cells in the brain, which have been shown to produce IL-1,β are neurons and glial cells (Eriksson *et al.*, 1999).

1.7.3 ICE.

ICE (also known as caspase-1) cleaves IL-1β at the aspartic acid-alanine position, which corresponds to amino acids 116-117 (Black *et al.*, 1988). It is part of a growing group of cysteine proteases called the caspases. It is highly conserved among rodents and humans, suggesting that rodent models of neuroinflammation may be predictive of efficacy of ICE inhibitors in humans (Vasilakos and Shivers, 1996). ICE exists as a 45kD precursor and requires two internal cleavages to form the active heterotetramer consisting of two 10kD and 20kD homodimers (Dinarello, 1996; Hengartner, 2000). This cleavage is achieved by another caspase, caspase-11 (Friedlander and Yuan, 1998). It is a mammalian homologue of CED-3, which is a protein involved in cell suicide in the nematode *Caenorhabditis elegans* (Friedlander and Yuan, 1998). ICE also regulates the secretion of interferon-γ indirectly through the regulation of IL-18 (Friedlander and Yuan, 1998). There are 5 human isoforms of ICE (ICEα, β, γ, δ, and ε). ICEε is a truncated form of ICE and is thought to inhibit ICE activity by binding to the p20 chain (Dinarello, 1996). ICE is also known to cleave poly- (ADP- ribose) polymerase (PARP), which is a DNA repair enzyme, however, cleavage of PARP requires 50 to 100 times the activity of that needed for IL-1β (Gu *et al.*, 1995). Agents inhibiting ICE activity have been shown to reduce IL-1β-induced damage (Dinarello, 1996).

1.7.4 IL-1ra.

As described above, IL-1ra is the endogenous antagonist of IL-1. Three different isoforms exist. The secreted form (IL-1ra1) is a glycosylated extracellular

protein with a molecular weight of 22kD (Palin *et al.*, 2001). IL-1ra2 (18kD) and IL-1ra3 (16kD) are intracellular unglycosylated proteins (Palin *et al.*, 2001). IL-1ra inhibits cellular responses to IL-1 by binding to IL-1RI without inducing cellular transduction. IL-1ra has two binding sites, which are similar to IL-1β. However, it is thought that one site is more homologous to IL-1β than the other and it binds to the receptor by this part of the protein (Grutter *et al.*, 1994). Lacking the second binding site, to which the IL-1RI-AcP is thought to bind on IL-1β, prevents signal transduction (Dinarello, 1996).

1.7.5 Biological actions of IL-1β in the brain.

Expression of IL-1β has been shown to be induced by both systemic and local insult (Rothwell and Luheshi, 1994). An increased expression of IL-1β has been observed in many neurodegenerative conditions, including AD (Holden and Mooney, 1995; Griffin *et al.*, 1989; Blum-Degen *et al.*, 1995), Parkinson's disease (Blum-Degen *et al.*, 1995) and Down's syndrome (Griffin *et al.*, 1989). Kainic acid administration has been shown to lead to the downstream production of IL-1β and it has been used to localise the expression of IL-1β in the brain. Increased expression of IL-1β was observed in the CA1 field and dentate gyrus of the hippocampus, the entorhinal and perirhinal regions of the cortex and in the hypothalamus, 12 hours after intraperitoneal injection of kainic acid (Eriksson *et al.*, 1999). IL-1β has also been shown to be produced by excitotoxic stimulation, for example by microglial cells 4 hours following infusion of the NMDA agonist 2,4-methanoglutamate into the cortex while expression was increased in astrocytes 2 days after stimulation (Pearson *et al.*, 1999). Similar increases in ICE mRNA and expression have been observed in the hippocampus of guinea pigs following global forebrain ischemia (Bhat *et al.*, 1996).

IL-1β has been found to be increased in the hippocampus (McGahon *et al.*, 1999a and b) and in the cortex (O'Donnell and Lynch, 1998) of aged rats and was found to be elevated in the urine of older humans (Dinarello, 1996). It has been shown to increase ROS production and lipid peroxidation in the hippocampus (Murray and Lynch, 1998a) and lipid peroxidation in the cortex (O'Donnell and Lynch, 1998) *in vitro*. IL-1β has been shown to inhibit LTP in the dentate gyrus of rats both *in vivo* (Murray and Lynch, 1998a) and *in vitro* (Cunningham *et al.*, 1996) and has been shown to inhibit glutamate release in synaptosomes prepared from the dentate gyrus *in vivo*

(Vereker *et al.*, 2000a). Low concentrations of IL-1β (1-10ng/ml) have been shown to inhibit the K^+-induced rise in intracellular Ca^{2+} in cortical synaptosomes prepared from rats (Campbell *et al.*, 1998). However, higher concentrations of IL-1β (100-500ng/ml) were shown to markedly increase intracellular Ca^{2+} concentrations in young and middle aged rats but not in aged rats (Campbell *et al.*, 1998).

1.8 p38 mitogen activated protein kinase.

Once a stimulus, such as the binding of IL-1β to IL-1RI, has been received by a cell, a cascade of signalling events occurs which co-ordinate appropriate responses. A group of proteins called the mitogen activated protein kinases (MAPK) have been shown to been involved in this cascade (Robinson and Cobb, 1997). The MAPKs can be divided into 2 categories; extracellular signal regulated protein kinases (ERKs), which are activated by growth factors promoting survival, and the stress activated protein kinases (SAPKs), which include p38 and c-Jun N-terminal kinase (JNK) and are activated by stresses such as cytokines and irradiation and initiate apoptosis (Legos *et al.*, 2001). These MAPKs are activated by MAPK kinase (MEK), which is activated by MEK kinase (MEKK). They afford this activation by dual tyrosine/threonine phosphorylation in subdomain VIII of the catalytic domain (Robinson and Cobb, 1996; Kyriakis and Avruch, 1996).

p38 has been extensively studied in relation to its ability to promote cell death following exposure to stressors such as cytokines, irradiation, osmotic shock, UV protein synthesis inhibitors and DNA-damaging agents (Robinson and Cobb, 1996). Stimulation of the cell by these stressors leads to an activation of GTP binding proteins, such as Cdc42, activate Ste20p-related kinases. These activate MEKK1-3, which in turn activates MEK3 and 6. These are the kinases that phosphorylate p38 (Robinson and Cobb, 1996). Like the yeast HOG1P, p38 contains the motif TGY whereas JNK and ERK have the motif TEY (Kyriakis and Avruch, 1996). p38 has four isoforms with p38α and β being relatively ubiquitous, p38γ is expressed in muscle and p38δ is expressed in the lung and kidney (Maruyama *et al.*, 2000).

Inhibition studies have revealed the involvement of p38 in many models of cell death. SB203580 has been shown to block apoptosis induced by nerve growth factor (NGF) withdrawal from CESS B cells (Rosini *et al.*, 2000) and was also shown to block apoptosis induced by sodium arsenite in cultured cortical neurons (Namgung and

Xia, 2000). p38 has been implicated as playing a pivotal role in the changes associated with neuronal injury caused by focal ischemia (Legos *et al.*, 2001; Barone *et al.*, 2001) and has also been implicated in the IL-1β induced impairment of LTP. p38 phosphorylation has been shown to be increased in the dentate gyrus of rats in circumstances in which LTP was inhibited by intracerebroventricular (icv) injection of IL-1β (Vereker *et al.*, 2000a). However, SB203580 has been shown to block the IL-1β-induced inhibition of LTP in dentate gyrus of the rat *in vitro* (Coogan *et al.*, 1999). ROS has also been implicated in p38 activation. H_2O_2 has been shown to increase p38 activation in hippocampal synaptosomes *in vitro* (O'Donnell *et al.*, 2000).

1.9 Apoptosis.

Cells undergoing apoptosis exhibit distinct morphological changes. These include membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation. The remains of the cell are then engulfed by neighbouring cells, activated macrophages and microglia. Apoptosis is a necessary phenomenon during development and optimal body maintenance requires that about 10 million cells die per day to balance the numbers of new cells produced by mitosis (Heemels, 2000). However, in the CNS, neuronal cell death is a feature of acute and chronic neurodegenerative diseases, including AD and Parkinson's disease (Yuan and Yankner, 2000). The trigger which initiates apoptosis is unknown but requires the activation of a family of cysteine proteases called caspases. All caspases contain a cysteine active site and cleave other proteins at aspartic acid residues. There are at present 14 known caspases with roughly two thirds proposed to play a role in cell death (Thornberry and Lazebnik, 1998). They are synthesised as inert zymogens and pro-caspases contain 3 domains; an N-terminal domain, a p20 subunit and a p10 subunit (Hengartner, 2000). They can cleave structural proteins such as lamins and fodrins, as well as DNA repair enzymes such as PARP (Thornberry and Lazebnik, 1998).

There are at least three possible ways in which caspases can become activated (Hengartner, 2000). First they can be processed by upstream caspases - this infers that at least one type of caspase is already activated. It is possible that autocatalytic activation occurs; cleavage between the p20 and p10 domains and between the pro-domain and the p20 domain is required, and this occurs between aspartic acid and its neighbouring residues on the pro-caspase (Thornberry *et al.*, 1997). Caspase-3, -6 and

–7 are effector caspases and are probably activated in this way (Hengartner, 2000). Secondly, activation can occur by the process known as induced proximity. This occurs when a ligand binds to the CD95 receptor, which aggregate and form a membrane bound signalling complex. Pro-caspase-8 and pro-caspase-10 contain a death effector domain that binds to the aggregated receptors. Due to the close proximity they can then mutually cleave and activate each other (Hengartner, 2000). Thirdly, association with regulatory subunit can occur. The most complex activation of a caspase is that of caspase-9 which requires the persistent binding of apoptotic protease-activating factor-1 (APAF-1; Li *et al.*, 1997). Depletion of caspase-9 from cell extracts prevents the cytochrome c-inducible activation of other caspases such as caspase-3, -6, -7 and –10 (Slee *et al.*, 1999). Caspase-9 contains a caspase activation and recruitment domain (CARD), which allows caspase-9 to activate the effector caspases especially caspase-3 (Hengartner, 2000).

Neuronal apoptosis, like other cell types, requires the activation of APAF-1. In the nematode *Caenorhabditis elegans* the Bcl-2 homologue, CED-9, binds to the APAF-1 homologue, CED-4, although this has not been observed in mammals (Yuan and Yankner, 2000). In mammals, the Bcl-2 family of mitochondrial proteins regulates the translocation of mitochondrial proteins such as cytochrome c. They may effect release by forming channels, interacting with other proteins to form channels, or may rupture the outer mitochondrial membrane (Yuan and Yankner, 2000). Cytochrome c forms a complex with APAF-1, which then activates caspase-9.

The Bcl-2 family all contain one or more Bcl-2 homology domains and are either pro-apoptotic or anti-apoptotic. Bcl-2 and Bcl-x_L are anti-apoptotic and are localised on the mitochondrial outer membrane whereas Bax and Bcl-Xs are pro-apoptotic and reside in the cytosol (Yuan and Yankner, 2000). Bax is widely expressed in the nervous system while Bcl-2 expression is high in the CNS during development but declines after birth; this contrasts with the periphery where the expression remains high during life (Yuan and Yankner, 2000). Bcl-2 and Bax can homo- or heterodimerise and each can neutralise the other's effect suggesting that their relative concentration in mitochondrial membranes can determine whether or not cell death will occur (Kannan and Jain, 2000). It has also been shown that the both p38 and JNK are involved in regulating apoptosis (Legos *et al.*, 2001). One possible way in which they might achieve this is through the activation of Ribosomal S6 Kinase (RSK). RSK can

phosphorylate Bad, another pro-apoptotic Bcl-2 family member (Yuan and Yankner, 2000). However, RSK can also phosphorylate CREB, which promotes cell survival through the transcription of Bcl-2 (Yuan and Yankner, 2000). It, therefore, appears that the SAPKs may have a dual role in apoptosis.

H_2O_2, and trimethyltin, a organometal shown to induce neuronal apoptosis, have been shown to increase cytochrome c translocation and increase caspase-3 activation in Jurkat T cells within 2hr of treatment (Stridh *et al.*, 1998). Cleavage of caspases-1 and –3 and an increase in DNA fragmentation have been shown in the human brain following traumatic brain injury (Clark *et al.*, 1999). An inhibitor of caspase-3, N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone, has been shown to block neuronal death following traumatic brain injury in rats (Clark *et al.*, 2000). This adds to the evidence that caspase-3 mediates neuronal apoptosis.

1.10 γ-Irradiation.

Whole-body γ-irradiation can result in one of the most devastating forms of damage that can be inflicted in the CNS. A large range of apoptosis-inducing agents are stimulated and even low doses of irradiation can affect cellular activity. γ-, x-ray-, UV- and ionising-irradiation all cause damage to cells and tissues. Irradiation can cause an increase in ROS production, especially increasing the production of $^{\bullet}OH$ (Packer *et al.*, 1997). Ionising irradiation has been linked to an increase in lipid peroxidation (Tolliver and Pellmar, 1987; Varshney and Kale, 1995) perhaps as a consequence of an increase in ROS accumulation. Irradiation can also stimulate IL-1β production (Dinarello, 1996) and p38 activation (Robinson and Cobb, 1996). γ-Irradiation was shown to increase JNK activation and DNA fragmentation in Jurkat T cells (Chen *et al.*, 1996a and b). IL-1β concentration was shown to be increased in the presence of macrophages isolated from irradiated animals (Ibuki and Goto, 1999) and also shown to increase cyclooxygenase-2 and prostaglandin E_2 in PC-3 cells (Steinauer *et al.*, 2000).

An increase in certain markers of apoptosis, such as caspases, cytochrome c, Bcl-2 family and PARP have also been observed following irradiation. Whole-body ionising irradiation has been shown to increase the expression of the precursors of caspase-1, -2, -3 and –8 in the cerebellum of neonatal rats (Ferrer, 1999). In the same study the author reported that an increase in the activity of caspase-3 was observed but that injection of the caspase-3 inhibitor Y-DEV-cmk prevented this increase. However,

no change was observed in the biochemical characteristics of apoptosis in the rats that were irradiated and treated with the inhibitor (Ferrer, 1999). This would imply that apoptosis was not reliant on caspase-3 activation. An increase in the 89kD (cleaved form) PARP protein was seen following irradiation in this same study (Ferrer, 1999).

An increase in cytochrome c release was reported in RPMI-8226 (a myeloma cell line) following γ-irradiation (Filippovich *et al.*, 2001) and in the same study an increase in Bcl-2/Bax heterodimerisation in irradiated compared with non-irradiated cells was observed (Filippovich *et al.*, 2001). mRNA expression of Bcl-2 was shown to be increased in spleenocytes isolated from irradiated mice fed an antioxidant diet containing vitamins E and C compared with mice that did not receive the antioxidant supplement (Ushakova *et al.*, 1999). In the same study, expression of SOD and catalase mRNA was shown to be decreased in spleenocytes isolated from irradiated mice fed on the control diet. The antioxidant diet was shown to be effective in preventing the decrease in catalase mRNA expression (Ushakova *et al.*, 1999).

It has been reported that the hippocampus is particularly sensitive to irradiation (Tolliver and Pellmar, 1987). Apoptosis was shown to be increased in dentate gyrus granule cells of x-ray-irradiated mice (Nagai *et al.*, 2000) while whole-body γ-irradiation was reported to disturb hippocampal cellular activity in the rabbit (Bassant and Court, 1978). This was corroborated by data presented by Pellmar and Lepinski (1993) who reported that irradiation exerted damaging effects in CA1 and dentate gyrus. The authors reported that 5Gy of ionising irradiation impaired the acquisition of new learning tasks while 10Gy of radiotherapy induced altered firing patterns and modified synaptic potentials in human patients (Pellmar and Lepinski, 1993).

1.11 Lipopolysaccharide.

LPS is an integral part of the cell wall of gram-negative bacteria. In healthy subjects, gram-negative bacteria reside mostly in the gut, and only after antibiotic agents or host immune modulators lyse the bacteria, is the host exposed to LPS. LPS contains a lipid A moiety and two 3-deoxy-D-manno-octulosonic acid residues (Marsh and Wewers, 1996). LPS activates cells by binding to a 55kD protein called CD14 through its lipid A moiety (Marsh and Wewer, 1996). CD14 is a glycosylphosphatidylinositol (GPI)-anchored membrane bound protein, however, it is not the signalling receptor (O'Neill and Dinarello, 2000). LPS is known to bind to a

LPS binding protein (LBP) which accelerates its binding to CD14 (Hailman *et al.*, 1994). This complex, consisting of LPS, LBP and CD14, then binds to the signalling receptor, toll-like receptor-4 (TLR-4; O'Neill and Dinarello, 2000). This recruits MyD88 within the cell and an intracellular signalling cascade commences (O'Neill and Dinarello, 2000).

Intracerebroventricular injection of LPS has been shown to increase p38, JNK and caspase-1 activity, ROS production and IL-1β concentration in the hippocampus of rats (Vereker *et al.*, 2000b). The increase in IL-1β concentration and JNK activity in the hippocampus of the same study was reversed with pre-treatment with icv injection of the caspase-1 inhibitor Ac-YVAD-cmk (Vereker *et al.*, 2000b). Intraperitoneal injection of rats was also reported to increase the concentration of IL-1ra in neurons and microglia in the hippocampus (Palin *et al.*, 2001). LPS-induced changes have also been shown in the periphery. An increase in the activation of p38 was seen in human neutrophils following LPS challenge *in vitro* (Nick *et al.*, 1996). It has also been shown to induce IL-1β, IL-6 and corticosterone production in the serum of Sprague-Dawley rats that received intraperitoneal injection of LPS (Hansen *et al.*, 2000).

LPS has been shown to impair LTP in perforant-path granule cells in rats following icv injection (Vereker *et al.*, 2000b). This LPS-induced impairment was associated with a decrease in glutamate release, however, the LPS-induced impairment in LTP was blocked by pre-treatment with the caspase-1 inhibitor Ac-YVAD-cmk (Vereker *et al.*, 2000b). In the same study, the caspase-1 inhibitor also prevented the LPS-induced increases in IL-1β concentration, caspase-1 activity, PARP cleavage and cytochrome c translocation in the entorhinal cortex (Vereker *et al.*, 2000b).

1.12 Objectives.

The main objectives of this study are:

- To assess the age-related changes that occur in the cortex of the rat and to determine whether dietary manipulation with the antioxidant α-lipoic acid has the ability to reverse any of these age-related changes.

- To assess the ability of the ω-3 fatty acid eicosapentaenoic acid to reverse age-related changes in the hippocampus and cortex, specifically the age-related increases in ROS production and IL-1β concentration and the age-related impairment in LTP.

- To assess the effect of whole-body γ-irradiation on LTP and on hippocampal IL-1β concentration and ROS accumulation

- To assess the ability of EPA and vitamins E and C to protect the hippocampus against the deleterious effects of γ-irradiation.

- To assess the ability of EPA and vitamins E and C to protect the hippocampus against the deleterious effects of LPS challenge.

Chapter 2:

Materials and Methods

2.1 Materials

Materials	*Source*
Acetic acid	Sigma
Acetonitrile	Lennox
Acrylamide	Sigma
7-Amino-4-(trifluoromethyl) coumarin	Sigma
Anti-rabbit IgG	Amersham
Anti-rat Bax antibody	Santa Cruz
Anti-rat Bcl-2 antibody	Santa Cruz
Anti-rat cytochrome c antibody	Santa Cruz
Anti-rat glutamate antibody	Sigma
Anti-rat p38 antibody	Santa Cruz
Anti-rat PARP antibody	Santa Cruz
Aprotinin	Sigma
Ascorbic acid (Diet)	Beeline Healthcare
Bio-Rad	Bio-Rad
Laboratories	
Bovine serum albumin	Sigma
Butylated hydroxytoluene	Sigma
Calcium chloride	Lennox
Catalase	Sigma
Cobalt Chloride	Sigma
Copper sulphate	Lennox
Cumene hydroperoxide	Sigma
DeadEnd™ Colorimetric Apoptosis Detection System kit	Promega
DEVD peptide	Alexis
2'7' Dichlorofluorescein	Sigma
2'7' Dichlorofluorescin diacetate	Molecular Probes
Diethylenetriaminepentaacetic acid	Sigma
Dimethyl sulphoxide	Sigma
Dinitrophenylhydrazine	Sigma
5,5'-Dithio-bis(2-nitrobenzoic acid)	Sigma
dL-α-tocopheryl acetate (Diet)	Beeline Healthcare

DNAse	Sigma
Docosahexaenoic acid (Diet)	Laxdale Research
DuoSet ELISA IL-1β kit	Genzyme
Eicosapentaenoic acid (Diet)	Laxdale Research
Enhanced Chemiluminescence Detection kit	Amersham
Ethanol	Lennox
Ethylenediaminetetraacetic acid	Sigma
1-Ethyl-3-(3-dimethylaminopropyl)carbo-diimide hydrochloride	Sigma
Ethylene glycol bis (β-aminoethylether) N,N 'tetraacteic acid	Sigma
Ferrous Sulphate	Sigma
Glucose	Lennox
Glutathione	Sigma
Glutathione reductase	Sigma
Glycine	Sigma
HEPES	Sigma
Hexane	Lennox
Hydrochloric acid	Lennox
Hydrogen peroxide	Sigma
Hyperfilm	Amersham
IL-1β antibodies	R&D Systems
Leupeptin	Sigma
α-Lipoic acid (Diet)	Laxdale Research
Lipopolysaccharide	Sigma
Magnesium sulphate	Lennox
Malondialdehyde	Sigma
β-Marcaptoethanol	Sigma
Methanol	Lennox
Nicotinamide Adenine Dinucleotide Phosphate	Sigma
Nitroblue tetrazolium	Sigma
Nitrocellulose extra blotting membrane	Sartorius
2-Nitrophenylhydrazine hydrochloric acid	Sigma
Paraformaldehyde	Agar Scientific Ltd.
Pepstatin	Sigma

Phenylmethylsulphonyl Fluoride	Sigma
Potassium chloride	Lennox
Potassium hydroxide	Sigma
Potassium phosphate	Sigma
Potassium thiocyanate	Sigma
Protease X	Sigma
Protease XIV	Sigma
SB203580	Calbiochem
Sodium azide	Sigma
Sodium carbonate	Sigma
Sodium chloride	Sigma
Sodium dodecylsulphate	Sigma
Sodium hydrocarbonate	Sigma
Sodium hydroxide	Lennox
Sodium phosphate	Sigma
Sucrose	Lennox
Sulphuric acid	Lennox
SuperSignal	Pierce
Tetramethoxypropane	Sigma
Thiobarbituric acid	Sigma
TNF-α Immunoassay kit	Biosource
Trichloroacetic acid	Sigma
Triethylenetetraamine	Sigma
Tris acid	Sigma
Tris base	Sigma
Triton X-100	Sigma
Tween-20	Lennox
Urethane	Sigma
Whatman filter paper	Lennox
Xanthine oxidase	Sigma
Xanthine	Sigma
YVAD peptide	Alexis

2.2 Animals.

2.2.1 Housing of animals.

All animals used were male Wistar rats. "Young" rats were aged between 2 and 4 months and were an inbred strain supplied by the BioResources Unit (BRU) of Trinity College Dublin or Bantham and Kingman, UK and weighed between 250 and 350g. Animals were housed in groups of 6 unless otherwise stated. In some experiments the effects of age were assessed and male Wistar rats were used. "Aged" rats were between 20 and 24 months old and were an inbred strain supplied by Bantham and Kingman, UK. These weighed between 500 and 600g and were generally housed in pairs unless otherwise stated. All rats were maintained under a 12-hour light-dark cycle in the BRU and food (normal laboratory chow, unless otherwise stated) and water was available *ad libitum*. Ambient temperature was controlled between 22 and 23°C.

2.2.2 Dietary manipulation

In all but one series of experiments rats were fed on specific diets (see below). Animals were randomly divided into subgroups and were housed in pairs, in groups of 3 or in groups of 6.

In one series of experiments young and aged rats were fed for 8 weeks a diet enriched in α-lipoic acid. One subgroup of rats received the experimental diet which consisted of the following-in terms of lipid content: 20% γ-linolenic-lipoic acid diol (racemic mixture), 40% tuna oil containing 24% docosahexaenoic acid and 1.7% arachidonic acid and 40% microbial oil containing 40% arachidonic acid, 3% γ-linolenic acid and 4% dihomo-γ-linolenic acid (Laxdale Research, Stirling, Scotland). The overall composition of the diet, in terms of polyunsaturated fatty acids, was, therefore, 20mg γ-linolenic-lipoic acid diol, 9.6mg docosahexaenoic acid, 16.4mg arachidonic acid, 1.2mg % γ-linolenic acid and 1.6mg dihomo-γ-linolenic acid. One group of both aged and young rats received normal laboratory chow supplemented with 100mg experimental lipid/rat/day (100% α-LPA diet). A further group of aged rats received laboratory chow supplemented with 50mg experimental lipid/rat/day (50% α-LPA diet). A third group of rats were fed a control diet consisting of normal laboratory chow to which corn oil was added to ensure isocaloric content to all diets. Food intake was measured for 2 weeks before the start of the dietary period to establish daily food

and water intake. Sufficient diet was then prepared for 2-3 days and daily allowances were stored in airtight containers at -80°C and rats were offered their full daily requirement each day.

In a second series of experiments young and aged rats were fed for 8 weeks a diet enriched in docosahexaenoic acid (DHA; 10mg/rat/day). Rats were randomly divided into groups and received either the DHA diet (26% w/v in tuna oil; Laxdale Research, Stirling, Scotland) or the control diet to which corn oil was added to ensure isocaloric intake. Food intake was measured for 2 weeks before the start of the dietary period to establish daily food and water intake. Sufficient diet was then prepared for 2-3 days and daily allowances were stored in airtight containers at -80°C and rats were offered their full daily requirement each day. Animal husbandry in these two dietary manipulation experiments was supervised by Dr. Bernadette McGahon who kindly provided me with samples for the experiments described in this thesis.

In a further series of experiments, young and aged animals were randomly divided into groups which were fed for 8 weeks an experimental diet of normal laboratory chow enriched with ethyl-eicosapentaenoic acid (EPA diet; 10mg/rat/day for 5 weeks and 20mg/rat/day for 3 weeks; EPA) or a control diet of normal laboratory chow. Both diets had an isocaloric value. The composition of the EPA diet was 20:5(n-3) eicosapentaenoate (ethyl eicosapentaenoate), which was greater than 95% pure with 0.2% dl-tocopherol as an antioxidant. Food and water intake was measured for 2 weeks before the start of the dietary period to establish daily food and water intakes. Sufficient diet was then prepared for 2-3 days and daily allowances were stored in airtight containers at -80°C and rats were offered their full daily requirement each day.

In a further series of experiments, young rats were divided randomly into groups and fed for 4 weeks a diet enriched in EPA at a concentration of either 1% or 2% of daily intake of food. The EPA and control diet was the same as described previously (see above).

In the final series of dietary manipulation experiments, young rats were fed a diet enriched in vitamins E and C. The rats were divided into groups and were fed an experimental diet of laboratory chow to which dL-α-tocopheryl acetate (vitamin E; 250mg/rat/day; Beeline Healthcare, Dublin, Ireland) dissolved in vegetable oil enriched in monounsaturated fatty acids. Rats received drinking water *ad libitum* in which L-ascorbic acid (vitamin C; 205mg/rat/day; Beeline Healthcare, Dublin, Ireland) was

dissolved. Control groups were given normal laboratory chow to which the monounsaturated oil was added to ensure both groups received an isocaloric intake. Food intake was measured for 2 weeks before the start of the dietary period to establish daily food and water intakes. Sufficient diet was then prepared for 2-3 days and daily allowances were stored in airtight containers at -80°C and rats were offered their full daily requirement each day.

2.2.3 Irradiation experiments.

In some experiments rats were subjected to whole body γ-irradiation. This took place in the Department of Biology, National University of Ireland, Maynooth. In the first series of experiments young rats were irradiated at a dose of 10Gy @ 20Gy/min using a Nordion Gammacell Caesium Irradiator. The rats were left to recover for 4 days before long-term potentiation (LTP) was assessed. In the second series of experiments involving γ-irradiation a dose of 10Gy @ 10Gy/min was used. Again rats were allowed to recover for 4 days before LTP studies were carried out.

2.3 Preparation of tissue.

Rats were killed by cervical dislocation and decapitation and the brains were rapidly removed and dissected on ice. The hippocampus, dentate gyrus, entorhinal cortex and cortex were dissected free. This procedure took approximately 2 minutes.

2.3.1 Preparation of slices for freezing.

Freshly dissected tissue was sliced bidirectionally to a thickness of 350μm using a McIlwain tissue chopper and placed in microfuge tubes containing 1ml Krebs solution (composition in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4$.7H2O 1.18, $NaHCO_3$ 16, glucose 10) with added $CaCl_2$ (2mM final concentration). Slices were allowed to settle and were rinsed once more with Krebs solution containing $CaCl_2$. The slices were rinsed a further two times with Krebs solution containing $CaCl_2$ containing 10% DMSO and stored at -80°C until required for later analyses.

2.3.2 Preparation of cytospun cells.

Freshly dissected and, in some cases, frozen entorhinal cortical and cortical slices were dissociated and spun onto glass microscope slides. Slices were allowed to

equilibrate in oxygenated Krebs solution containing $CaCl_2$ for 30min at 30°C and then incubated in Krebs solution containing $CaCl_2$ containing protease X (1mg/ml), protease XIV (1mg/ml) and DNAse (1600 Kunitz) for 30min at 30°C. Washed slices were resuspended in 1ml Dulbecco's modified essential medium containing DNAse (1600 Kunitz), triturated with a glass pasteur pipette and passed through a nylon mesh filter to remove tissue clumps. Suspensions of dissociated cells were cytospun (Shandon cytospin II) at 600rpm for 2min to facilitate adherence onto glass microscope slides. The slide-mounted cells were immersed gently into methanol in order to fix the cells and were stored at room temperature, in the dark until required for analysis.

2.4 Protein quantification using the Bradford protein assay.

Protein quantification was assessed according to Bradford (1976). Samples (10μl) were added to 150μl dH_2O in a 96-well plate (microtest plate; Sarstedt, Ireland) in duplicate. Standards were prepared from a stock solution of 200μg/ml Bovine Serum Albumin (BSA). This was diluted in dH_2O to prepare a range of standards (including a blank of dH_2O) ranging from 200μg/ml to 3.125μg/ml. Duplicate aliquots (160μl) of standard were added to the 96-well plate and Bio-Rad (Bio-Rad, Hertfordshire, UK) reagent (40μl) was added to both samples and standards. Absorbance was assessed at 630nm using a 96-well plate reader (EIA Multiwell reader, Sigma, Dorset, UK). A regression line was plotted and the concentration of protein was calculated and converted to mg protein/ml.

2.5 Induction of LTP *in vivo*.

2.5.1 Preparation of rats.

Rats were anaesthetised by an intraperitoneal injection of urethane (1.5g/kg). The absence of a pedal reflex was used to confirm deep anaesthesia and if needed a further top-up dose was administered (to a maximum of 2g/kg). Fur on the scalp was clipped and the head was positioned in a head holder in a stereotaxic frame. A midline incision was made by a scalpel and the skin pulled back to reveal the skull. The periosteum was scraped clear and a dental drill was used to remove a window of skull that would allow correct placement of the electrodes. The dura mater was carefully peeled away so as to expose the brain. 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 50Hz cycle noise.

2.5.2 Electrode implantation.

Bipolar stimulating electrodes and unipolar recording electrodes (Clark Electromedical, UK) were used in this study. The stimulating electrode was placed on the surface of the brain, 4.4mm lateral to lambda. The recording electrode was placed on the surface of the brain 2.5mm lateral and 3.9mm posterior to bregma. The positions of the electrodes were carefully monitored as they were lowered through the cortical and hippocampal layers to the perforant path granule cell layer of the dentate gyrus. This was carried out by generating 0.1msec duration, 2msec delay, 4V pulse through the stimulating electrode at a frequency of 0.1Hz. Evoked responses were picked up by the recording electrode and displayed on an Apple Macintosh computer (Performa 200). The stimulating electrode was lowered in increments into the perforant path. The recording electrode was lowered into the dentate gyrus until the characteristic perforant path granule cell synapse response was observed. The electrodes were finely adjusted so as to maximise the response. The final depth of the recording electrode was between 2.5 and 3.5mm and for the stimulating electrode was between 2.5 and 3mm. Stimuli were then delivered at 30sec intervals.

2.5.3 EPSP recordings.

The population field post-synaptic potential (field EPSP) was used as a measure of excitatory synaptic transmission in the dentate gyrus. EPSPs were achieved by passing a single square wave of current at low frequency (0.033Hz, 0.1sec, 2msec delay) generated by a constant isolation unit (IsoFlex, UK), to the bipolar stimulating electrode. The evoked response was transmitted via a pre-amplifier (DAM 50; Differential Amplifier; gain 75, World Precision Instruments, USA) 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). This was customised to control both the generation of the square wave pulses and recording of the evoked potentials. The field EPSPs were displayed on-line and could be analysed at the time of recording or in the case of these studies at a later date.

The slope of the EPSP was taken as the main indicator of excitatory synaptic transmission. After a period of stabilisation, test shocks at 1/30sec were recorded for a 10min control period to establish stable baseline recordings. This was followed by delivery of 3 trains of stimuli (250Hz for 200msec) at 30sec intervals. Recording at test shock frequency then resumed for 40min.

In one series of experiments young rats were treated intraperitoneally with either lipopolysaccharide (LPS; 1ml; 100μg/kg) or saline (1ml; 0.9%) 3hr before the induction of LTP.

2.6 Analysis of samples.

2.6.1 Analysis of fatty acid concentration

Fatty acid concentrations were measured as previously described (Miwa *et al.*, 1986). Slices prepared from hippocampal tissue were homogenised in Krebs solution containing $CaCl_2$ and aliquots (150μl) were added to chloroform:methanol (2:1 v/v; 1ml) and the fatty acids and lipids were extracted by vigorous shaking for 10min followed by centrifugation at 2000rpm for 2min to separate the phases. The aqueous layer was discarded and the top (chloroform) layer was removed and evaporated under nitrogen, samples were frozen at -80°C until required. When required for analysis, samples were resuspended in ethanol and the fatty acids were analysed as their 2-nitrophenolhydrazine derivatives. The samples (10μl) were derivatised by adding 20μl 2-nitrophenolhydrazine-HCl (0.02M; 2-NPH-HCl in 40mM HCl-ethanol (3:1, v/v)) to 40μl EDC solution (1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide hydrochloride; 0.25M EDC in ethanol mixed in equal volumes with 3% ethanolic pyridine) and incubated at 60°C for 20min. KOH (10μl; 15% w/v in MeOH:H_2O, 80:20) was added and samples were incubated for 15min at 60°C. After cooling, *n*-hexane (300μl) and phosphate buffer (400μl; 0.033M, pH6.4 in 0.5M HCl) were added and samples were vortex mixed for 30sec. They were centrifuged at 2000rpm for 2min and the hexane layer was removed and evaporated to dryness under nitrogen. For HPLC analysis, the samples were resuspended in methanol and were placed in an autosampler (SpectraSYSTEM AS3000), from which aliquots (30μl) were automatically injected. The HPLC machine consisted of a SP8800 Ternary HPLC pump linked to a Hypersil BDS C18 column. This, in turn, was connected to a UV detector (Spectra 100 Variable Wavelength Detector) attached to a computer (Dell Optiplex PC 5133L), which

allowed recording and analysis of data. Fatty acid derivatives were separated in isocratic mode with a mobile phase of 85% ACN: 15% water (maintained at pH4.5 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 μmol/mg of tissue corrected for protein (see section 2.4 for protein analysis and appendix II and III for list of solutions used and addresses of companies).

2.6.2 Analysis of lipid peroxidation.

Lipid peroxidation was measured by analysis of thiobarbiturate reactive substances (TBARS) as previously describes by Dexter *et al.* (1989). Slices prepared from hippocampal and cortical tissue were homogenised in Krebs solution containing $CaCl_2$ and aliquots (30μl) were incubated for 1hr at 90°C in an assay buffer (570μl; 3.95ml acetic acid added to 15ml water pH3.5). Sodium dodecylsulphate (0.213g) and thiobarbituric acid (0.158g)were added and water was subsequently added to bring the assay buffer volume to 50ml. Standards of tetramethoxypropane (30μl; 6.25-100μM) were also incubated in assay buffer for 1hr at 90°C. Samples were allowed to cool and duplicate aliquots (200μl) were removed and placed in a 96-well plate and read at 545nm on a Sigma Diagnostics EIA Multiwell Reader (Dorset, UK). TBARS were expressed as nmol malondialdehyde (MDA)/mg tissue corrected for protein (see section 2.4 for protein analysis).

2.6.3 Analysis of vitamin E concentration.

Vitamin E was assessed by HPLC according to a modification of the method of Vatassery *et al.*, 1994. Slices prepared from hippocampal and cortical tissue were thawed and homogenised in Krebs solution containing $CaCl_2$. Samples of homogenate (150μl) were incubated in the presence of ethanol containing 0.025% butyl-hydroxytoluene (BHT, 150μl), 25% ascorbic acid (70μl) and 10% potassium hydroxide (135μl) for 30min at 60°C. Hexane (540μl) containing 0.025% BHT was added and vortex-mixed for 1min before centrifuged at 1500 rpm for 6min. The upper hexane phase was removed and evaporated to dryness under nitrogen; the recovery of vitamin E under this procedure was between 70% and 80% (correction for recovery was not made). For HPLC analysis, dried samples were resuspended in methanol (150μl)

containing 0.025% BHT and were placed in an autosampler (SpectraSYSTEM AS3000). Aliquots (30μl) volumes of standard and samples were automatically injected into the column. The HPLC machine consisted of a SP8800 Ternary HPLC pump linked to an Inertsil 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. Separation of α-TH was achieved using a mobile phase of 75% acetonitrile (ACN):25% methanol at a flow rate of 1.2ml/min and samples were detected by UV spectroscopy at 292nm. Vitamin E concentration was estimated by the external standard method (6.25-100μg/ml) and expressed as μg/g tissue corrected for protein (see section 2.4 for protein analysis).

2.6.4 Analysis of vitamin C concentration.

Vitamin C concentrations were analysed by a modification of the method previously described by Omaye *et al.*, 1997. Slices prepared from cortical and hippocampal tissue were homogenised in Krebs solution containing $CaCl_2$ containing 5% TCA and centrifuged at 15,000 rpm for 10min at 4°C and the supernatant (100μl) added to a 2,4-dinitrophenylhydrazine/thiourea/copper (DTC) solution (50mM thiourea, 2mM copper sulphate and 150mM dinitrophenylhydrazine in 9N H_2SO_4; 20μl) and incubated for 3h at 37°C. Ice-cold H_2SO_4 (65%; 150μl) was added to stop the reaction. Samples were vortex mixed and incubated at room temperature for 30min and aliquots (100μl) were transferred to 96-well plates for assessment by UV spectroscopy at 545nm (Sigma Diagnostics EIA Multiwell Reader, Dorset, UK). The 96-well plates were loaded and read within 5min. Results were expressed as μmol/g tissue corrected for protein (see section 2.4 for protein analysis) with reference to a standard curve of ascorbic acid (6.25-100μg/ml) prepared in 5% TCA.

2.6.5 Analysis of glutathione concentration.

Total glutathione concentration was measured as previously described (Tietze, 1969) with some modifications. Slices prepared from cortical tissue were homogenised in Krebs solution containing $CaCl_2$ containing 5% TCA and centrifuged at 15,000rpm for 10min at 4°C. Samples of supernatant (30μl) were incubated for 1min on ice with sample buffer (100mM potassium phosphate containing 5mM EDTA, pH 7.5; 450μl), in the presence of glutathione reductase (100μl; 5U/ml) and 10mM 5,5'-dithiobis-2-

nitrobenzoic acid (50μl). NADPH (2.4mM; 100μl) was added and the absorbance monitored for 2min at 412nm using a colorimeter linked to a MacLab 2E Data Acquisition System connected to a Macintosh Performa 5200. A standard curve of change of absorbance with time was constructed using glutathione (6.25-100μM) and the slope of trace was calculated. Results were expressed as μmol GSH/g tissue corrected for protein (see section 2.4 for protein analysis).

2.6.6 Analysis of superoxide dismutase activity.

Total superoxide dismutase (SOD) activity was measured by a modification of the method described by Spitz and Oberley, 1989. Slices prepared from hippocampal and cortical tissue were homogenised in Krebs solution containing $CaCl_2$ and centrifuged at 15,000rpm for 10min at 4°C. In some experiments cortical tissue was incubated with IL-1β (1ng/ml) for 15min at 37°C. The samples were centrifuged at 2000rpm for 2min and the supernatant discarded and the slices were resuspended in Krebs solution containing $CaCl_2$ and treated as above. In all cases the resulting supernatant was diluted in a reaction mixture containing 1.8mM xanthine, 2.24mM nitroblue tetrazolium (NBT) 40 units of catalase, 7μl/ml xanthine oxidase and 1.33mM diethylenetriaminepentaacetic acid (DETAPAC) in 50mM potassium buffer to achieve the dilutions; 1:2, 1:5, 1:10, 1:20 and 1:30. Aliquots (100μl) of each sample were added to the reaction mixture (900μl)in a 1.5ml cuvette. Potassium phosphate buffer (100μl; 100mM KH_2PO_4 and 100mM K_2HPO_4 pH 7.0) was used as a blank. The absorbance was measured at 560nm for 5min using a UV spectrometer (Phillips Pye Unicam 8625 UV/VIS Spectrometer, Phillips, UK) linked to a MacLab 2E Data Acquisition System connected to a Macintosh Performa 5200. Results were calculated as the rate of reduction of NBT, which was inhibited with increasing protein concentrations. The data was plotted as percentage inhibition of NBT reduction (100-((sample slope/blank slope)*100)) versus protein concentration. One unit of activity was defined as the amount of protein necessary to decrease the rate of the reduction of NBT by 50%. Results were expressed as units of SOD activity per mg protein (U/mg; see section 2.4 for protein analysis).

2.6.7 Analysis of catalase activity

Catalase activity was determined as previously described (Cohen *et al.*, 1996) with modifications. Slices prepared from cortical tissue were homogenised in Krebs solution containing $CaCl_2$ and centrifuged at 15,000rpm for 10min at 4°C. The resulting supernatant (100μl) was added to microfuge tubes containing phosphate buffer (800μl). Water was used as controls. The reaction was initiated by the addition of 60mM H_2O_2 (diluted from stock 30% H_2O_2, 100μl) followed by gentle mixing. At 2 and 10min intervals, duplicate aliquots (100μl) were removed and quenched by addition of samples to 0.6N H_2SO_4 (4ml) and 10mM ferrous sulphate ($FeSO_4.7H_2O$; 1ml) at room temperature. Colour was developed by addition of 2.5mM potassium thiocyanate (400μl). Two aliquots of 200μl from each tube were transferred to a 96-well plates (Sarstedt) and absorbance read at 492nm (Sigma Diagnostics EIA Multiwell Reader, Dorset, UK). 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 $A1$ and $A2$ represent the absorbance at the two time points and t is the difference between these points. Results are expressed as enzyme units per gram of protein (see section 2.4 for protein analysis).

2.6.8 Analysis of glutathione peroxidase activity.

Glutathione peroxidase activity was measured according to the method of Lawrence and Burk, 1976. Slices prepared from cortical tissue were homogenised in Krebs solution containing $CaCl_2$ and centrifuged at 15,000rpm for 10min. Samples of supernatant (100μl) were added to an incubation medium (900μl) consisting of 50mM potassium phosphate buffer (pH 7.0), 1mM EDTA, 1mM sodium azide, 0.2mM NADPH, 1 unit glutathione reductase, 1mM glutathione and 1.5mM cumene hydroperoxide (15mM; 100μl). Absorbance was recorded at 340nm for 5min using a UV spectrometer (Phillips Pye Unicam 8625 UV/VIS Spectrometer, Phillips, UK) linked to a MacLab 2E Data Acquisition System connected to a Macintosh Performa 5200. The activity was calculated from the slope of the trace obtained. A standard curve of NADPH (6.25-100μM) was used to translate the results to μmol NADPH oxidised/mg protein/min (see section 2.4 for protein analysis).

2.6.9 Analysis of interleukin-1β concentration.

The Enzyme Linked ImmunoSorbent Assay (ELISA) method was used to determine the concentration of interleukin-1β (IL-1β) as described before (Murray and Lynch, 1998a; IL-1β; Genzyme Diagnostics, MA, USA). 96-well plates (Nalge Nunc, International) were coated with 100μl capture antibody (2.0μg/ml, diluted in 0.1M sodium carbonate buffer, pH 9.5; monoclonal hamster anti-mouse IL-1β antibody) and incubated overnight at 4°C. Wells were washed three times with phosphate buffered saline-Tween 20 (PBS-T; 80mM Na_2HPO_4, 20mM NaH_2PO_4, 100mM NaCl; containing 0.05% Tween 20) and blocked for 2h at 37°C with 250μl blocking buffer, (PBS, pH7.3; 0.01M with 4% bovine serum albumin (BSA)). Slices prepared from hippocampal and cortical tissue were homogenised in Krebs solution containing $CaCl_2$. In a separate set of experiments hippocampal tissue prepared from young, untreated rats was incubated in the presence of H_2O_2 (200μM) or a control buffer (Krebs solution containing $CaCl_2$) for 15min at 37°C. The tissue was homogenised in Krebs solution containing $CaCl_2$ and the samples treated as follows. The plates were again washed in PBS and triplicate aliquots of homogenate or IL-1β standard (100μl; 0-1000pg/ml) were added to each well and incubated for 1h at 37°C. After the plates had been washed again, secondary antibody (100μl; final concentration 0.8μg/ml in PBS containing 0.05% Tween 20 and 1% BSA; biotinylated polyclonal rabbit anti-mouse IL-1β antibody) was added to each well and the plates were incubated at 37°C for 1h. The plates were washed and the detection agent (100μl; horseradish peroxidase conjugated streptavidin; 1:10 dilution in PBS-T and 1% BSA) was added to all wells and incubated for 15min at 37°C. Plates were washed and substrate (100μl; tetramethylbenzidine (TMB) liquid substrate; Sigma, Dorset, UK) was added to each well and were incubated at room temperature for 10min. Absorbance was read at 450nm within 30min (Sigma Diagnostics EIA Multiwell Reader, Dorset, UK).

Due to a changeover in manufacturers a second IL-1β ELISA assay was used (IL-1β antibodies, R&D systems, Oxford, UK) in some experiments. Briefly, 96-well plates were coated in the capture antibody (anti-rat IL-1β; 100μl; 0.1mg/ml in PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, and 1.5mM KH_2PO_4) pH7.4) and incubated overnight at room temperature. The plates were washed in PBS-T 3 times, a blocking buffer was added to the wells (300μl; 1% BSA, 5% sucrose and 0.05% NaN_3 in PBS) and the plates were incubated for 1hr at room temperature. Standards (0-

1000pg/ml) were made from recombinant rat IL-1β diluted in 1% BSA in PBS (R&D systems, Oxford, UK). The plates were washed 3 times in PBS-T and 100μl standard or samples (as prepared above) were incubated for 2hr at room temperature. After this incubation period the plates were washed 3 times in PBS-T and the detection antibody was added (100μl; biotinylated anti-rat IL-1β; 350ng/ml in diluent) and incubated for 2hr at room temperature. The plates were washed 3 times in PBS-T and streptavidin-HRP conjugate (100μl) was added and incubated for 20min at room temperature. The plates were washed 3 times in PBS-T and Substrate Solution (100μl; 1:1 reagent A (H_2O_2):reagent B (TMB); R&D Systems, Oxford, UK) was added to the wells and incubated in the dark for 20min, which created a colour change to blue. Stop solution of 1M H_2SO_4 (50μl) was added and the plates were read at 450nm within 30min (Sigma Diagnostics EIA Multiwell Reader, Dorset, UK). A standard curve was constructed by plotting the standards against their absorption. Results are expressed as pg IL-1β/mg tissue corrected for protein (see section 2.4 for protein analysis).

3.6.10 Analysis of tumour necrosis factor-α. concentration.

The commercially available rat TNF-α Immunoassay Kit (Biosource International, Fleurus, Belgium) ELISA system was used. Slices prepared from cortical tissue were homogenised in Krebs solution containing $CaCl_2$ and were added (50μl) to the pre-coated (TNF-α capture antibody) 96-well plate. Standards (0-1000pg/ml recombinant rat TNF-α; 50μl) were added and to this biotinylated anti-TNF-α (50μl) was added. Plates were incubated for 1hr 30min at room temperature and then washed in the wash buffer provided. Strepavidin-HRP Working Solution (100μl) was added to the wells and incubated for 45min at room temperature. The plates were washed and the Stabilised Chromogen (100μl) was added to the wells and incubated for 30min at room temperature. The Stop Solution (100μl) was added and the plates were read at 450nm (Sigma Diagnostics EIA Multiwell Reader, Dorset, UK). A standard curve was constructed by plotting the standards against their absorption. Results are expressed as pg TNF-α/mg tissue corrected for protein (see section 2.4 for protein analysis).

2.6.11 Analysis of reactive oxygen species production.

The production of reactive oxygen species was assessed in the impure synaptosomal preparation (P_2) as previously described (Lebel and Bondy, 1990).

Briefly, synaptosomes were prepared by homogenising slices (15 up and down strokes) prepared from hippocampal and cortical tissue in 500μl ice-cold sucrose (0.32M). Homogenate was centrifuged at 5,000rpm for 5min at 4°C and the supernatant was placed in another microfuge tube and centrifuged at 15,000rpm for 15min at 4°C. The resulting pellet, P_2, was an impure synaptosome-enriched preparation. The supernatant was removed and the resultant pellet was resuspended in 1ml ice-cold 40mM Tris buffer (pH 7.4). In a separate experiment P_2 prepared from the cortex of young, untreated rats was resuspended in either Krebs solution containing $CaCl_2$ (vehicle), interleukin-1β (IL-1β; 1ng/ml), the superoxide dismutase inhibitor, triethylenetetraamine (TETA; 100μM; De Man *et al.*, 1996) or both IL-1β and TETA, and incubated for 15min at 37°C. After incubation samples were centrifuged at 2000 rpm for 2min at 4°C and the supernatant was discarded. The resultant pellet was resuspended in 1ml Tris. In all experiments, the samples were incubated with the non-fluorescent probe, 2',7-dichlorofluorescin diacetate (DCFH-DA, 10μl; final concentration 5μM from a stock of 500μM in methanol), at 37°C for 15min in the dark. The reaction was stopped by centrifuging the samples at 13,000 rpm for 8min at 4°C. The pellet was resuspended in 40mM Tris buffer (1.5ml; pH 7.4). The samples were stored at 37°C in the dark until the fluorescence was monitored at a constant 37°C at 488nm excitation (bandwidth 5nm) and 525nm emission (bandwidth 20nm) in a fluorescent spectrometer linked to a MacLab 2E Data Acquisition System connected to a Macintosh Performa 5200. Reactive oxygen species production was quantified from a standard curve of dichlorofuorescein (DCF) in methanol (range 0.05-1μM). Results were expressed as nmol DCF/mg tissue corrected for protein (see section 2.4 for protein analysis).

3.6.12 Analysis of interleukin-1β converting enzyme activity.

Interleukin-1β converting enzyme (ICE) activity was measured by the cleavage of the ICE substrate, YVED (Alexis Corporation, Nottingham, UK), to its fluorescent product. Slices prepared from hippocampal and cortical tissue were homogenised in lysis buffer (400μl; 25nM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mM PMSF, 10μg/ml leupeptin and 10μg/ml pepstatin, pH 7.4) and subjected to 4 freeze-thaw actions. The samples were centrifuged at 15,000 rpm for 10min at 4°C and the resulting supernatant (90μl) was added to ICE substrate (10μl; 5μM; DEVD peptide).

These were incubated at 37°C for 60min. The resulting 100μl was added to 900μl of incubation buffer (100mM HEPES containing 10mM DTT; pH 7.4) and transferred to cuvettes to measure the fluorescence at excitation and emission wavelengths of 400nm and 505nm, respectively on a fluorescent spectrometer (Labsystems Fluorskan Ascent FL, Medical Supply Company Ltd. Dublin, Ireland). Standards of AFC (6.25-100μM) were measured at the same wavelengths and a standard curve was constructed. Results are expressed as nmol AFC/mg tissue corrected for protein (see section 2.4 for protein analysis).

2.6.13 Analysis of caspase-3 activation.

Slices prepared from cortical tissue were incubated in lysis buffer (as above) for 20min and homogenised in the lysis buffer. They were centrifuged at 15,000rpm for 10min at 4°C. In one experiment cortical tissue prepared from young rats was incubated in the control buffer (HEPES; 145mM NaCl, 5mM KCl, 1mM $MgCl_2$, 2mM $CaCl_2$, 1mM Mg_2SO_4, 1mM KH_2PO_4, 10mM glucose and 30mM HEPES; pH 7.4), in the presence of IL-1β (3.5ng/ml), interleukin-1β receptor antagonist (IL-1ra; 350ng/ml), the p38 inhibitor SB203580 (SB; 50μM) or a combination of IL-1β + IL-1ra or IL-1β + SB for 2hr at 37°C. In another experiment cortical tissue prepared from young rats was incubated in the presence of H_2O_2 (200μM), SB203580 (50μM), H_2O_2 + SB203580 or the control buffer for 1hr at 37°C. After incubation the slices were allowed to settle and the supernatant was removed and the samples were treated as described first. For all samples the supernatant (90μl) was removed following centrifugation and added to caspase-3 substrate (10μl; 100μM; DEVD peptide; Alexis Corporation, Nottingham, UK) 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. Emission at 505nm was measured upon excitation at 400nm in a fluorescent spectrometer. Standards of AFC (6.25μM to 100μM) were prepared and a standard curve of absorbance against concentration was plotted. Results were expressed as nmol AFC/mg protein/min (see section 2.4 for protein analysis).

2.6.14 Analysis of glutamate release.

The synaptosomal preparation P_2 was prepared as described in section 2.6.11. The pellet was resuspended in oxygenated Krebs solution containing $CaCl_2$, which had been brought to the assay temperature of 37°C. In one experiment dentate gyri from young rats were preincubated in the control buffer (Krebs solution containing $CaCl_2$), in the presence of IL-1β (10pg/ml), SB203580 (50μM) for 20min at 37°C. After incubation these samples were treated as before. For all experiments, aliquots (25μl) of synaptosomes were transferred onto a filtration manifold in which cellulose acetate strips (pore size 0.45μm) were placed. Samples were washed under vacuum at least 10 times with oxygenated Krebs solution containing $CaCl_2$ (250μl) maintained at 37°C. Synaptosomes were incubated for 3min at 37°C with either Krebs solution containing $CaCl_2$ or Krebs solution containing $CaCl_2$ and KCl (50mM), to depolarise the synaptic plasma membrane. Filtrates were collected under vacuum and stored at -20°C for later analysis of glutamate concentration by immunoassay. This work was carried out by Martina Gooney and the *in vitro* work by Emily Vereker.

2.6.15 Analysis of glutamate concentration by immunoassay.

Endogenous glutamate was measured in stored samples (see 2.6.14) according to the method of Ordronneau and colleagues (1991). 96-well plates were coated with coating buffer (250μl; 100mM NaH_2PO_4, 0.5% glutaraldehyde v/v pH 4.5) and incubated for 1hr at 37°C, after which time plates were washed twice with NaH_2PO_4 buffer (250μl; 100mM, pH 8.0). Samples of filtrate (50μl) and glutamate standards (12.5nM to 500nM) were added to the coated wells. Plates were covered and incubated for 2hr at 37°C and washed 4 times with the NaH_2PO_4 buffer. Non-specific binding was blocked in 2 steps; firstly for 1hr at 37°C with ethanolamine (250μl; 0.1M in Na_2HPO_4 buffer) and then for 1hr at 37°C with normal donkey serum (250μl; 3% v/v in PBS-T). Plates were washed in PBS-T thereafter.

Anti-glutamate antibody (Sigma, Dorset, UK) was diluted 1:5,000 with normal donkey serum (3% v/v in PB-T) and 100μl was added to each well. To assess background absorbance, normal donkey serum (3% v/v in PBS-T) was added in the absence of anti-glutamate antibody. Plates were covered and incubated overnight at 4°C. The plates were washed and the secondary antibody (100μl; HRP-linked anti-rabbit IgG, Amersham, Buckinghamshire, UK) was diluted 1:10,000 with normal

donkey serum (3% v/v in PBS-T) and added to the plates. This was incubated for 1hr at room temperature. The plates were washed and TMB liquid substrate (100μl; Sigma, Dorset, UK) solution was added to each well and incubated until a colour change to blue occurred. The reaction was stopped by the addition of H_2SO_4 (50μl; 4M). The plates were agitated for 15sec and the absorbencies were read at 450nm using Sigma Diagnostics EIA Multiwell Reader (Dorset, UK). The concentration of glutamate in the samples was calculated with reference to the standard curve and the concentration of glutamate was expressed as μmol glutamate/mg protein (see section 2.4 for protein analysis).

2.6.16 Analysis of TUNEL positive cells.

The DeadEnd™ Colorimetric Apoptosis Detection System was used and this system end-labels the fragmented DNA of apoptotic cells using a modified TUNEL (TdT-mediated dUTP Nick-End Labelling) assay. Cytospun cells were prepared as described in section 2.3.2, circled with a pap pen and fixed with 4% paraformaldehyde for 30min at room temperature by placing a drop of the solution on top of the cells. The cells were washed by incubating in PBS for 5min. This procedure was repeated twice. The cells were permeablised with PBS-Triton (1μl Triton:1ml PBS; 1:500 dilution), and washed three times with PBS. Excess liquid was removed gently from the slide, cells were covered with equilibration buffer (50μl; 200mM potassium cacodylate (pH 6.6 at 25°C), 25mM Tris-HCl (pH 6.6 at 25°C), 0.2mM DTT, 0.25mg/ml BSA 2.5mM cobalt chloride) and equilibrated for 5min at room temperature. The cells were incubated with 30μl of Terminal deoxynucleotidyl transferase reaction mixture (TdT; 98μl equilibration buffer, 1μl biotinylated nucleotide mix, 1μl TdT enzyme) at 37°C for 1hr and the slides were covered to prevent desiccation. The reaction was terminated by covering cells with 100μl 2XSCC (1:10; 2XSCC: deionised water) for 15min at room temperature. Cells were washed 3 times in PBS, endogenous peroxides were blocked with 100μl 0.3% H_2O_2 for 5min at room temperature and cells were washed 3 times in PBS. Streptavidin-HRP solution, diluted in PBS (100μl; 1:500), was added to the cells and incubation continued at room temperature for 30min to allow binding to the biotinylated nucleotides. The slides were washed 3 times in PBS. A diaminobenzidine (DAB) solution was used to stain apoptotic nuclei brown by binding to the streptavidin-HRP. DAB solution (1ml; 50μl DAB substrate 20X buffer, 50μl

DAB 20X chromogen, 50μl H_2O_2 20X, 950μl deionised water) was added to the cells for a 10min incubation period. The cells were washed several times with deionised water. The slides were mounted with glycerine jelly (10g gelatine, 60ml distilled water; 70ml glycerine; 0.25g phenol). The cells were counted using a light microscope and the proportion of positively stained cells was calculated and expressed as the percentage of the total cells counted.

2.6.17 Analysis of protein synthesis.

Protein synthesis was estimated by assessing [^{35}S]-methionine incorporation into proteins. Slices prepared from entorhinal cortical tissue were pre-incubated in oxygenating Krebs solution containing $CaCl_2$ (500μl) for 10min at 37°C. The supernatant was removed and the reaction buffer was added (500μl; Krebs solution containing $CaCl_2$, ATP (3.5mM) and [^{35}S]-methionine (specific activity 37 TBq/mmol; 0.2μl/ml)). Samples were incubated for 1hr at 37°C under continuos oxygenation. Ice-cold trichloroacetic acid (TCA; 10%; 50μl) was added and the samples placed on ice. Slices were homogenised and aliquots (50μl) were placed on filters (cellulose acetate strips, pore size 0.45μm) in a manifold. Samples were washed under vacuum 10 times with excess TCA (5% w/v). Filter papers were removed and placed in scintillant (4ml) and [^{35}S]-methionine incorporation into TCA-precipitated proteins was analysed by liquid scintillation counting.

2.7 SDS-PAGE.

2.7.1 Gel electrophoresis.

12.5% and 14% gels (see Appendix III) were cast by setting them between 2 gel plates, which were mounted on an electrophoresis unit (Sigma Techware, Dorset, UK) using spring clamps. The upper and lower reservoirs of the unit were filled with electrode running buffer (25mM Tris Base; 200mM glycine; 17mM SDS). Slices were homogenised in lysis buffer (400μl; 25nM HEPES, 5mM $MgCl_2$, 5mM DTT, 5mM EDTA, 2mMPMSF, 10μg/ml leupeptin and 10μg/ml pepstatin, pH 7.4) or Krebs solution containing $CaCl_2$. In some experiments, cortical tissue prepared from young, untreated rats was incubated in the control buffer (HEPES), in the presence of IL-1β (3.5ng/ml), interleukin-1β receptor antagonist (IL-1ra; 350ng/ml), the p38 inhibitor SB203580 (SB; 50μM) or a combination of IL-1β + IL-1ra or IL-1β + SB203580 for

2hr at 37°C. In another experiment hippocampal or cortical tissue prepared from young, untreated rats was incubated in the presence of H_2O_2 (200μM), SB203580 (50μM), H_2O_2 + SB203580 for 1hr at 37°C. In a further experiment, hippocampal tissue was incubated in the presence of IL-1β (100ng/ml) or in the control buffer (Krebs solution containing $CaCl_2$) for 15min at 37°C. This final *in vitro* experiment was carried out by Emily Vereker. Following these experiments the samples were homogenised in Krebs solution containing $CaCl_2$ or lysis buffer and treated as follows. The protein concentrations were assessed (see section 2.4) and were equalised. An equal volume of sample buffer (0.5M Tris-HCl pH 6.8; 10% glycerol (v/v); 10% SDS (w/v); 5% β-marcaptoethanol (v/v); 0.05% bromophenol blue (w/v)) to sample was added and samples boiled for 5min. The samples (10μl) were loaded into the wells, a 32mA current was applied to the gel apparatus and migration of the bromophenol blue was monitored. The current was switched off when the blue dye band reached the bottom of the gel (approximately 30min).

2.7.2 Western immunoblotting.

The gel was removed from the gel apparatus and washed gently in transfer buffer (25mM Tris-Base; 192mM glycine; 20% methanol (v/v); 0.05% SDS (w/v)). It was placed on top of a sheet nitrocellulose blotting paper (Sartorius, Dublin, Ireland) wetted in the transfer buffer, cut to the size of the gel. One piece of filter paper (Whatman No.3) was placed on top of the gel and one piece was placed beneath the nitrocellulose paper forming a sandwich. The sandwich was soaked in transfer buffer and placed on the graphite electrode (anode) of a semi-dry blotter (Biometra, UK). Air bubbles were removed from the sandwich by gently rolling a pasteur pipette over it. The lid of the blotter (cathode) was placed down firmly on top of the sandwich. A constant current of 250mA for 1hr 15min was applied. The nitrocellulose membrane was blocked (either non-fat dry milk or BSA was used; see below) for non-specific binding and probed with an antibody raised against the protein sought. This was washed off and a secondary antibody was added which was HRP-linked. A chemi-luminescence detection chemical (either SuperSignal Ultra (Pierce, Leiden, The Netherlands) or Enhanced Chemiluminescence (ECL) detection reagent (Amersham, Buckinghamshire, UK) was used; see below) was added and the membrane was

exposed to photographic (Hyperfilm, Amersham, Buckinghamshire, UK) film and developed using a Fuji Processor.

In the case of p38 phosphorylation, non-specific binding was blocked by incubating the nitrocellulose membrane in Tris buffered saline-Tween 20 (TBS-T; 20mM Tris-HCl; 150mM NaCl; containing 0.05% Tween 20; pH 7.6; 15ml) containing 5% BSA overnight at 4°C. The membrane was washed for 15min 3 times in TBS-T (10ml) and the primary antibody added (anti-phospho-p38; Santa Cruz, CA, USA; 1:500 dilution in TBS-T containing 0.1% BSA; 10ml). This was incubated for 2hr at room temperature and it was washed for 15min 3 times in TBS-T. The secondary antibody was added (anti-rabbit IgM-HRP linked; Sigma, Dorset, UK; 1:5000 dilution in TBS-T containing 0.1% BSA; 10ml) and incubated for 2hr at room temperature. The membrane was washed for 15min 6 times in TBS-T. SuperSignal Ultra (Pierce, Leiden, The Netherlands) was added for 5min and membranes were exposed to the photographic film for 2min in the dark after which time the film was developed.

In the case of cytochrome c expression the non-specific binding was blocked by incubating the membrane in PBS-T containing 6% non-fat dry milk (Marvel) for 2hr at room temperature. The membrane was washed for 15min 3 times in PBS-T and the primary antibody was added (rabbit anti-rat polyclonal cytochrome c; Santa Cruz, CA, USA; 1:250 dilution in PBS-T containing 2% non-fat dry milk) and incubated overnight at 4°C. The membrane was washed for 15min 3 times and the secondary antibody added (sheep anti-rabbit polyclonal IgG-HRP linked; Amersham, Buckinghamshire, UK; 1:1500 dilution in PBS-T containing 2% non-fat dry milk). Membranes were incubated for 1hr at room temperature and washed for 15min 8 times in PBS-T. ECL detection reagent (Amersham, Buckinghamshire, UK) was added to the membrane for 1min and exposed to the photographic film for 1hr at 4°C in the dark before being developed.

In the case of Poly-(ADP-ribose)-polymerase (PARP) expression, non-specific binding was blocked by incubating the membrane in PBS-T containing 6% non-fat dry milk overnight at 4°C. Membranes were washed for 15min 3 times in PBS-T and the primary antibody added (CSSA PARP; Santa Cruz, CA, USA; 1:250 dilution in PBS-T containing 2% non-fat dry milk) and incubated for 2hr at room temperature. Membranes were washed for 15min 3 times and the secondary antibody added (sheep anti-rabbit polyclonal IgG-HRP linked; Amersham, Buckinghamshire, UK; 1:2000

dilution in PBS-T containing 2% non-fat dry milk). Membranes were incubated for 1hr at room temperature and washed for 15min 4 times in PBS-T. ECL detection reagent was added to membranes, incubated for 1min and exposed to photographic film overnight at 4°C in the dark before being developed.

In the case of Bax and Bcl-2 expression, membranes were probed using antibodies against both proteins. The non-specific binding was blocked by incubating membranes in TBS-T containing 5% non-fat dry milk for 2hr at room temperature. Membranes were washed for 15min 3 times in TBS-T, the primary antibodies added (anti-Bax, 1:150 dilution and anti-Bcl-2, 1:400 dilution in TBS-T containing 2% non-fat dry milk; Santa Cruz, CA, USA) and incubated overnight at 4°C. Membranes were washed for 15min 3 times and the secondary antibody added (sheep anti-rabbit polyclonal IgG-HRP linked; Amersham, Buckinghamshire, UK; 1:1000 dilution in TBS-T containing 2% non-fat dry milk). Membranes were incubated for 1hr at room temperature and washed for 15min 6 times in TBS-T. SuperSignal Ultra reagent was added for 5min and the membrane was exposed to the photographic film for 2min in the dark after which time the film was then developed.

In all cases the intensity of the bands exposed onto the photographic film was measured by densitometry using a ZeroDscan package. The values are expressed as arbitrary units.

Chapter 3:

Results

ANALYSIS OF CHANGES IN CORTICAL TISSUE PREPARED FROM YOUNG AND AGED RATS FED ON A DIET SUPPLEMENTED WITH α-LIPOIC ACID.

The putative antioxidant α-lipoic acid was assessed for its ability to reverse the age-related changes in the cortex. Previous studies in this laboratory have assessed the antioxidant capabilities of polyunsaturated fatty acids such as AA (McGahon *et al.*, 1999a) and DHA (McGahon *et al.*, 1999c) in the aged rat. For the present study α-lipoic acid was chosen as it has been reported to have important antioxidant properties. The three main antioxidant properties are: (a) it has metal chelating abilities, which are important for sequestering excess free iron and copper, (b) it can scavenge ROS, and (c) it can regenerate endogenous antioxidants such as vitamin E, vitamin C and GSH (Packer *et al.*, 1997). Dihydrolipoic acid (DHLA), the reduced form of α-lipoic acid, has more potent antioxidant properties than the non-reduced form and in combination the 2 forms are very effective (Biewenga *et al.*, 1997). α-Lipoic acid has been shown to be very potent in combating the increase in lipid peroxidation in patients with diabetic neuropathy (Nickander *et al.*, 1996; Ziegler *et al.*, 1999) and in reversing the age-related increase in lipid peroxidation in the cortex and hippocampus of rats (Arivazhagan and Panneerselvam, 2000). α-Lipoic acid has also been shown to reverse the age-related changes in long-term potentiation (LTP) and the down-regulated antioxidant status in the rat hippocampus (McGahon *et al.*, 1999b).

The following data was obtained from the cortical tissue prepared from the rats of the same study. Young (4 months old) and aged (22 months old) were fed on a control diet (corn oil) or an experimental diet enriched in α-lipoic acid (100mg/rat/day; 100% α-LPA diet or 50mg/rat/day; 50% α-LPA diet) for 8 weeks. At the end of the dietary period the following biochemical analyses were assessed in cortical tissue (see Appendix I for mean data).

3.1 Age is associated with an increase in cortical Reactive Oxygen Species production.

Reactive Oxygen Species (ROS) production in cortical synaptosomes prepared from young and aged rats fed on the control diet was found to be markedly increased, however, this increase did not reach statistical significance. There was no significant

increase seen in tissue prepared from aged rats fed the 50% or 100% α-LPA diet compared with tissue prepared from young rats fed on the 100% α-LPA diet, although in the case of the 100% α-LPA diet this may be due to the large standard error. A significant increase in ROS production was seen in tissue prepared from aged rats fed on the control diet compared with young rats fed on the 100% α-LPA diet (*$p<0.01$ *b* v *c*, Student's t-test for independent means). The age-related increase in ROS production was reduced by the α-LPA diet.

3.2 Age is associated with an increase in the activity of superoxide dismutase, but not in the activities of catalase or glutathione peroxidase in the cortex.

In an effort to explain the age-related increase in ROS production, the antioxidant capabilities of the rats within the dietary groups were assessed. In Figure 3.2 the activities of the antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (Gpx), are presented. Figure 3.2A shows that SOD activity is significantly increased in cortical tissue prepared from aged rats fed on the control diet compared with tissue prepared from young rats fed on the control diet (*$p<0.01$ *a* v *c*, Student's t-test for independent means). The activity of SOD was similar in tissue prepared from young rats fed the α-LPA diet and aged rats fed on both α-LPA diets. The age-related increase in SOD activity was not seen in tissue prepared from those rats fed the 50% and 100% α-LPA diets with a significant difference between tissue prepared from aged rats fed the control and those fed the 50% α-LPA diet (*$p<0.05$ *c* v *d*, Student's t-test for independent means). The age-related increase in SOD activity was reduced by the 50% α-LPA diet and possibly by the 100% α-LPA diet.

Catalase activity in tissue prepared from young and aged rats fed on control and 100% α-LPA diet is shown in Figure 3.2B; there was insufficient tissue obtained from the aged rats fed on the 50% α-LPA diet to analyse enzyme activity. Catalase activity was similar in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed the control diet. The activity of catalase was also similar in tissue prepared from young and aged rats fed the 100% α-LPA diet. A significant increase was seen in aged rats fed the 100% α-LPA diet compared with those fed on control diet (*$p<0.05$ *c* v *d*, Student's t-test for independent means).

A significant decrease in Gpx activity in cortical tissue prepared from the aged rats fed the control diet compared with the young rats fed the 100% α-LPA diet is

shown in Figure 3.2C (*p<0.05 *b* v *c*, Student's t-test for independent means). This significant difference was not seen in tissue prepared from young rats fed control diet. Although a decrease in Gpx activity was evident between the tissue prepared from the young rats and the tissue prepared from the aged rats fed the 50% or 100% α-LPA diet there was no significant difference between them, indicating a possible restoration of enzyme antioxidant capability in aged rats following dietary supplementation with α-lipoic acid.

3.3 The activity of superoxide dismutase in the hippocampus.

Figure 3.3 shows that in addition to the observed changes in cortical tissue (above) there was a marked age-related increase in SOD activity in the hippocampus of the same rats. This increase was also seen in the tissue prepared from aged rats fed on the 50% α-LPA diet but was absent in the tissue prepared from the aged rats fed the 100% α-LPA diet.

3.4 The antioxidant scavengers in the cortex of young and aged rats.

The concentrations of vitamin C, vitamin E and glutathione (GSH) in cortical tissue prepared from aged and young rats fed on control and α-LPA diets are shown in Figure 3.4. The concentration of vitamin C (Figure 3.4A) was similar in the cortical tissue prepared from all dietary groups. There was no age-related or diet-related change observed. The concentration of vitamin E was similar in the tissue prepared from all dietary groups (Figure 3.4B) although a slight increase was seen in the tissue prepared from the aged rats fed on the 100% α-LPA diet compared with all other groups.

The concentration of GSH was significantly increased in cortical tissue prepared from the aged rats fed on the 100% α-LPA diet compared with the tissue prepared from the young rats fed the control diet (*p<0.05 *a* v *e*, Student's t-test for independent means; Figure 3.4C). As there was no significant difference between the tissue prepared from young rats fed either the control or α-LPA diet the data were pooled which resulted in achieving a significant difference between this value and the value obtained in tissue prepared from the aged rats fed the 100% α-LPA diet (p<0.05, Student's t-test for independent means, data not shown). No other changes were observed.

3.5 Age is associated with an increase in cortical lipid peroxidation.

An increase in ROS may cause an increase in lipid peroxidation and so this measure was assessed and the results are shown in Figure 3.5. Lipid peroxidation was increased in cortical tissue prepared from aged rats fed on the control diet compared with the tissue prepared from the young rats fed either the control or 100% α-LPA diet. This difference was not significant but as there was no significant difference between the tissue prepared from young rats fed either the control or α-LPA diet the data were pooled which resulted in achieving a significant age-related increase ($p<0.05$, Student's t-test for independent means, data not shown). The age-related increase was not seen in those aged rats fed the 50% and 100% α-LPA diets.

3.6 Age is associated with a decrease in cortical arachidonic acid concentration.

Polyunsaturated fatty acids, like AA, are substrates for lipid peroxidation. The concentration was assessed in the cortical tissue and the data are shown in Figure 3.6. There was a decrease in AA concentration in cortical tissue prepared from aged rats fed the control diet compared with young rats fed the control diet although this did not reach statistical significance. There was a significant decrease in the tissue prepared from aged rats fed the control diet compared with the young rats fed the 100% α-LPA diet (*$p<0.05$ *b* v *c*, ANOVA). This age-related decrease was not seen in the tissue prepared from the rats fed the 50% and 100% α-LPA diets with a significant difference existing between the tissue prepared from the aged rats fed the control and 100% α-LPA diet (*$p<0.05$ *c* v *e*, ANOVA).

3.7 IL-1β increases SOD activity and ROS production in cortical tissue *in vitro*.

The ability of the pro-inflammatory cytokine interleukin-1β (IL-1β) to increase ROS production and SOD activity *in vitro* was assessed in tissue prepared from young untreated rats and the results are shown in Figure 3.7A and B respectively. Addition of IL-1β (1ng/ml) to the incubation medium significantly increased ROS production (*$p<0.05$ *a* v *b*, ANOVA). The SOD inhibitor triethylenetetraamine (TETA; 100μM) was assessed for its ability to prevent the IL-1β induced increase in ROS and the data indicate that there was no increase in ROS production in the tissue incubated in the presence of TETA or IL-1β + TETA. However there was a significant difference in SOD activity between the tissue incubated in IL-1β and that incubated in IL-1β +

TETA ($p<0.05$ *b* v *d*, ANOVA), suggesting that the IL-1β induced increase in ROS may be mediated through an increase in SOD.

This observation was consolidated by the finding that IL-1β (1ng/ml) significantly increased SOD activity in cortical tissue (*$p<0.05$ *a* v *b*, Student's t-test for independent means).

3.8 The activity of Interleukin-1β Converting Enzyme in cortical tissue.

As IL-1β increased ROS production, IL-1β concentration and the activity of Interleukin-1β Converting Enzyme (ICE) were assessed in cortical tissue. The results are shown in Figures 3.8 and 3.9. ICE activity was increased in cortical tissue prepared from aged rats fed the control diet compared with young rats fed the control or 100% α-LPA diet. This did not reach statistical significance but as there was no significant difference between the tissue prepared from young rats fed either the control or α-LPA diet the data were pooled which resulted in achieving a significant difference ($p<0.05$, Student's t-test for independent means, data not shown). This increase was not seen in the tissue prepared from aged rats fed the 50% or 100% α-LPA diets. In the case of the values from the 50% diet, large standard errors were observed and probably contributed to the lack of effect.

3.9 Age is associated with an increase in cortical interleukin-1β concentration.

IL-1β concentration was significantly increased in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed either the control or 100% α-LPA diet (*$p<0.05$ *a* v *c*, Student's t-test for independent means). This increase was not seen in the tissue prepared from aged rats fed either the 50% or 100% α-LPA diet with a significant difference existing between the tissue prepared from the aged rats fed the control and the 50% α-LPA diets ($p<0.01$ *c* v *d*, Student's t-test for independent means).

3.10 Age is associated with an increase in interleukin-1β concentration in the hippocampus.

IL-1β concentration was assessed in the hippocampus of the same rats and the results are shown in Figure 3.10. IL-1β was significantly increased in hippocampal tissue prepared from aged rats fed the α-LPA diet compared with tissue prepared from

young animals fed the control and 100% α-LPA diets (*p<0.01 *a* v *c* & *b* v *c*, Student's t-test for independent means). This significant increase was not seen in the tissue prepared from aged rats fed either the 50% or 100% α-LPA diets with a significant difference existing between the tissue prepared from the aged rats fed the control and the 100% α-LPA diets (*p<0.05 *c* v *e*, Student's t-test for independent means).

Figure 3.1: Age is associated with an increase in cortical reactive oxygen species production.

Reactive oxygen species (ROS) production in cortical tissue prepared from aged rats fed the control diet (n=7) was significantly increased compared with tissue prepared from young rats fed the 100% experimental (n=6), but not the control (n=4), diet (*$p<0.01$ *b* v *c*, Student's t-test for independent means). In contrast, there was no significant difference in tissue prepared from the 50% (n=5) or the 100% (n=5) α-LPA diet. The values are expressed as μmol DCF/mg tissue corrected for protein and are the means ± the standard error of the means.

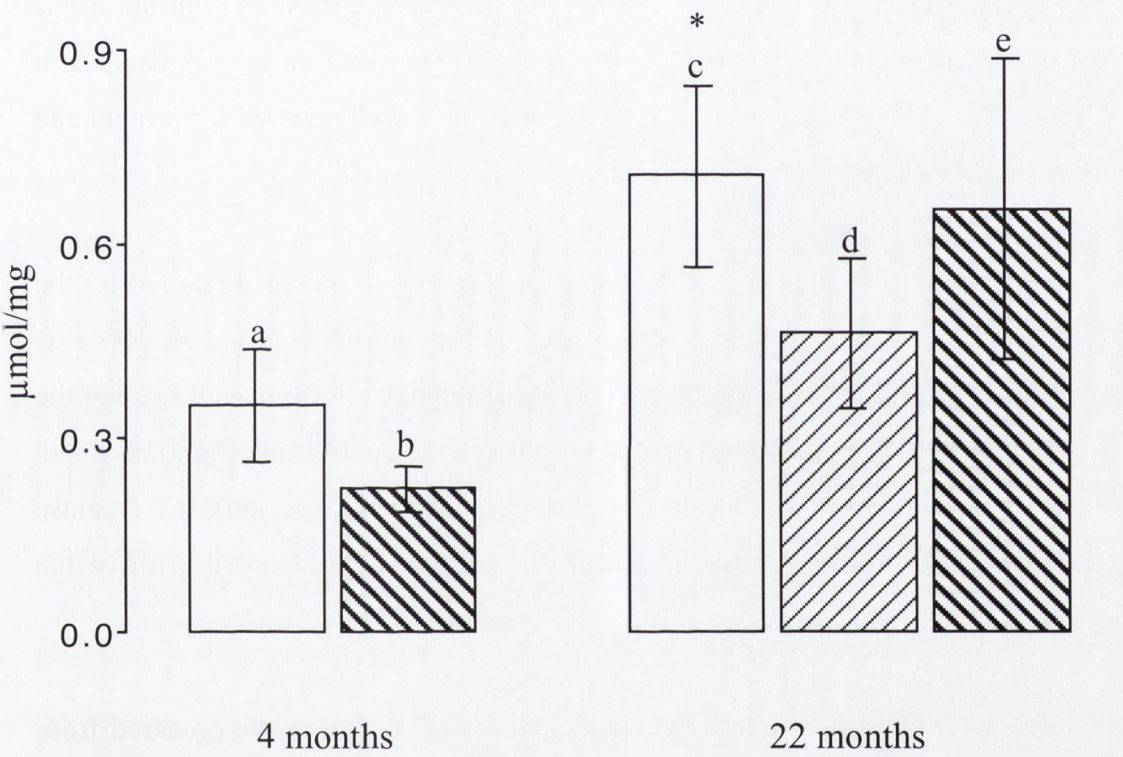

Control Diet
50% α-LPA Diet
100% α-LPA Diet
µmol/mg
0.9
0.6
0.3
0.0
a
b
*
c
d
e
4 months
22 months

Figure 3.2: Age is associated with an increase in the activity of superoxide dismutase, but not in the activities of catalase or glutathione peroxidase in the cortex.

A) Superoxide dismutase (SOD) activity was significantly increased in tissue prepared from the young (n=4) compared with aged (n=7) rats fed on the control diet (*$p<0.01$, Student's t-test for independent means). SOD activity in the cortex prepared aged rats fed on the 50% (n=8) and 100% (n=8) α-LPA diet was decreased compared with the tissue prepared from aged rats fed the control (*$p<0.05$ *c* v *d*, Student's t-test for independent means). Values are expressed as units of SOD activity/mg tissue corrected for protein and are the means ± the standard error of the means.

B) Catalase activity was similar in cortical tissue prepared from the aged rats fed on the control diet (n=10) compared with the young rats fed on the control (n=3) and the 100% α-LPA diet (n=5). A significant increase was seen in tissue prepared from aged rats fed the 100% α-LPA diet (n=6) compared with the aged rats fed the control diet (*$p<0.05$ *c* v *d*, Student's t-test for independent means). Values are expressed as units of catalase activity/mg tissue corrected for protein and are the means ± the standard error of the means.

C) Glutathione peroxidase (Gpx) activity was decreased in cortical tissue prepared from aged rats fed on the control diet (n=11) compared with tissue prepared from young rats fed on the control (n=6) or 100% α-LPA diet (n=7; *$p<0.05$ *b* v *c*, Student's t-test for independent means). Dietary manipulation with the 50% diet (n=11) only partially reversed this effect whereas the 100% diet (n=12) had no effect. Values are expressed as μmol Gpx activity/mg tissue corrected for protein/min and are the means ± the standard error of the means.

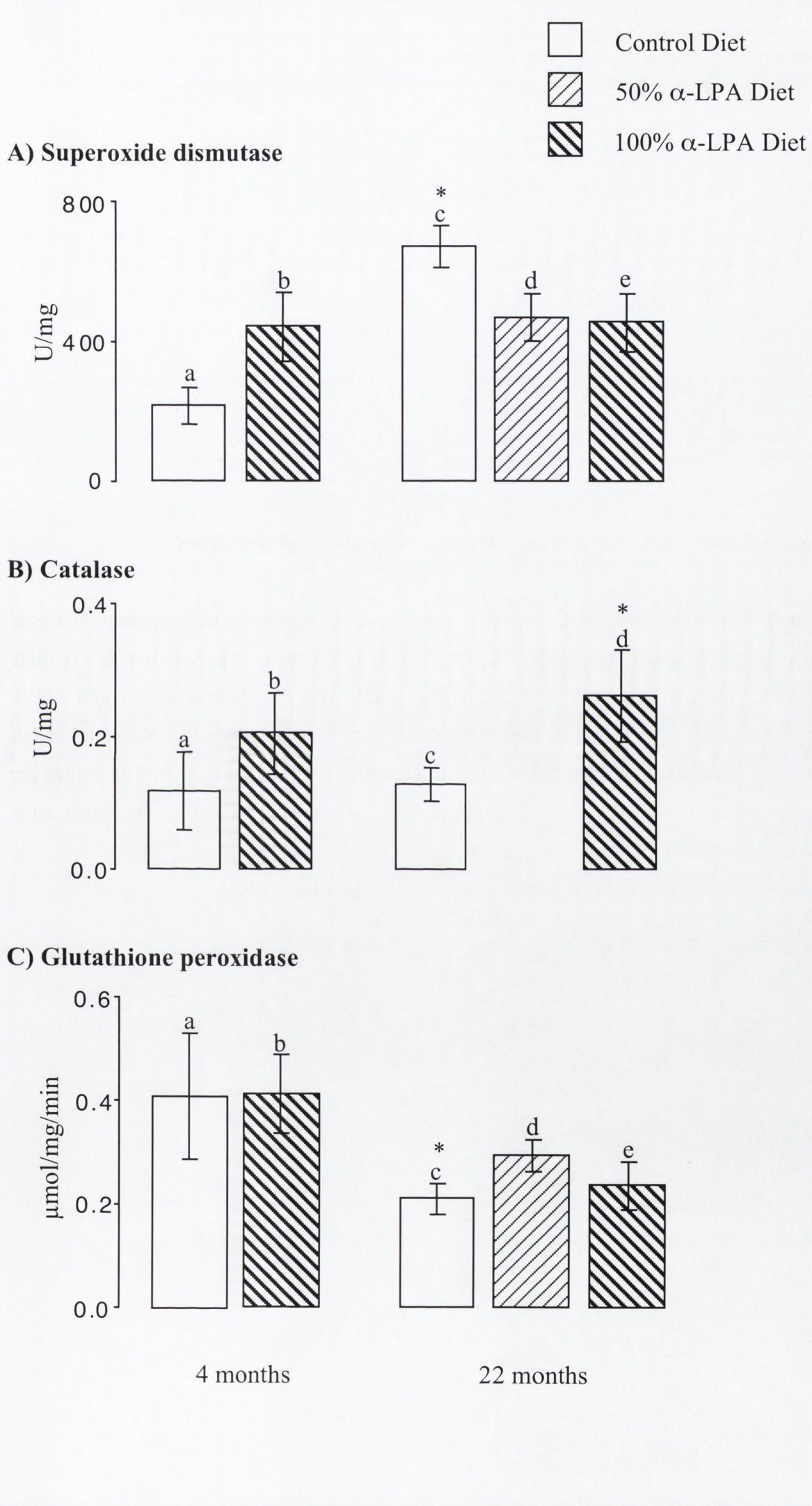

Control Diet
50% α-LPA Diet
100% α-LPA Diet
A) Superoxide dismutase
800
400
0
U/mg
a
b
*
c
d
e
B) Catalase
0.4
0.2
0.0
U/mg
a
b
c
*
d
C) Glutathione peroxidase
0.6
0.4
0.2
0.0
µmol/mg/min
a
b
*
c
d
e
4 months
22 months

Figure 3.3: The activity of superoxide dismutase in the hippocampus.

Superoxide dismutase (SOD) activity was markedly increased in hippocampal tissue prepared from aged rats fed the control (n=10) and 50% experimental (n=9) diets compared with tissue prepared from young rats fed the control (n=4) and 100% experimental (n=4) diets. This increase did not reach statistical significance and was not seen in tissue prepared from aged rats fed the 100% α-LPA diet (n=13). The values are expressed as units of SOD activity/mg tissue corrected for protein and are the means ± the standard error of the means.

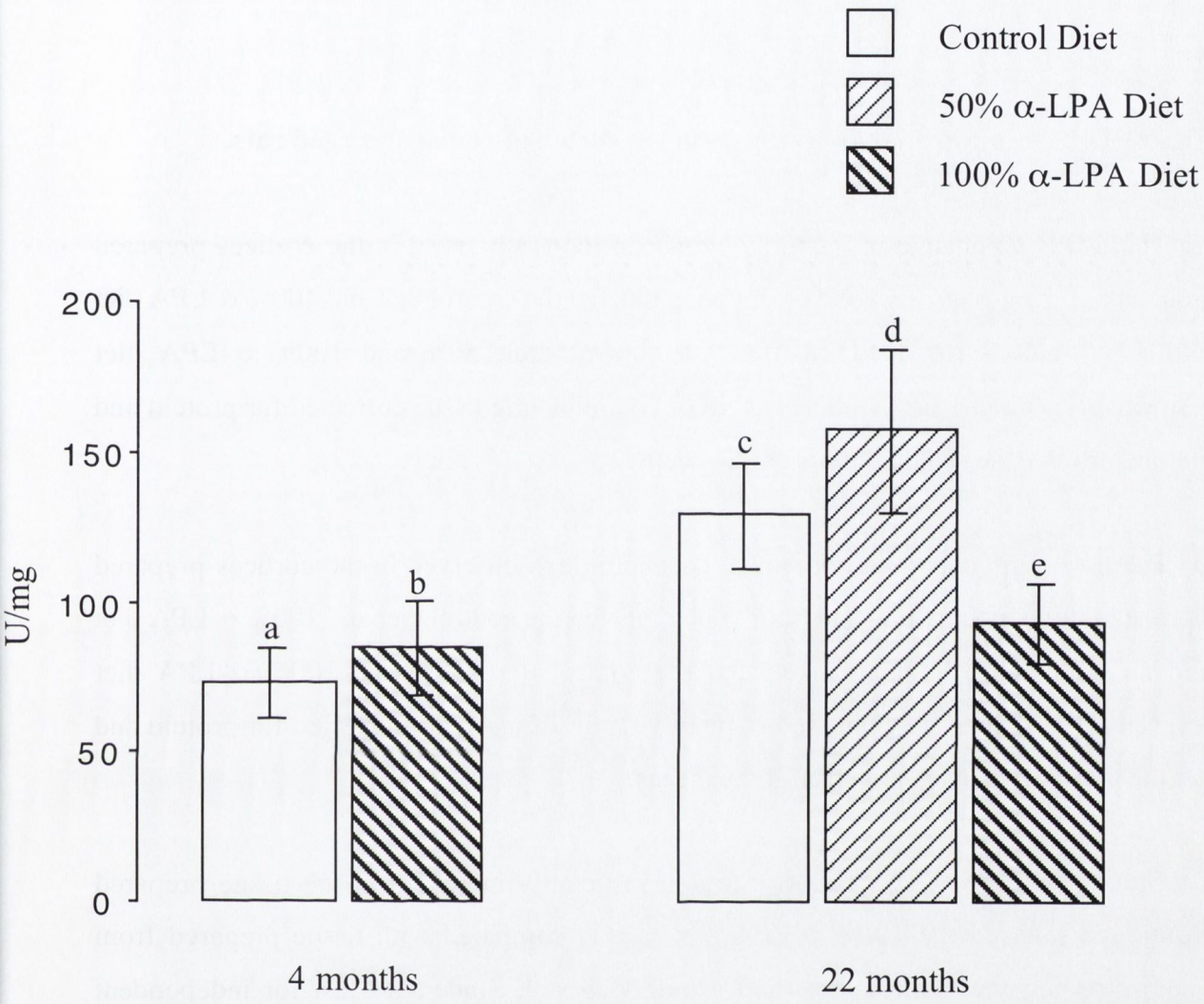

Control Diet
50% α-LPA Diet
100% α-LPA Diet
200
150
100
50
0
U/mg
a
b
c
d
e
4 months
22 months

Figure 3.4: The antioxidant scavengers in the cortex of young and aged rats.

A) There was no change in vitamin C concentrations observed in the cortices prepared from any of the groups (n=4 and 6 for young rats fed the control diet and 100% α-LPA diet and n=7,7 and 8 for aged rats fed the control diet, 50% and 100% α-LPA diet respectively). Values are expressed as μmol vitamin C/mg tissue corrected for protein and are the means ± the standard error of the means.

B) There were no change in vitamin E concentrations observed in the cortices prepared from any of the groups (n=4 and 6 for young rats fed the control diet and 100% α-LPA diet and n=12,11 and 13 for aged rats fed the control diet, 50% and 100% α-LPA diet respectively). Values are expressed as pmol vitamin E/mg tissue corrected for protein and are the means ± the standard error of the means.

C) Glutathione (GSH) concentration was significantly increased in the tissue prepared from aged rats fed the 100% α-LPA diet (n=11) compared with tissue prepared from young rats fed the control diet (n=7; *$p<0.05$ *a* v *e*, Student's t-test for independent means). There was no significant difference in tissue prepared from either young rats fed the 100% α-LPA diet (n=4) or aged rats fed the control (n=10) or 50% α-LPA diet (n=8). Values are expressed as μmol GSH/mg tissue corrected for protein and are the means ± the standard error of the means.

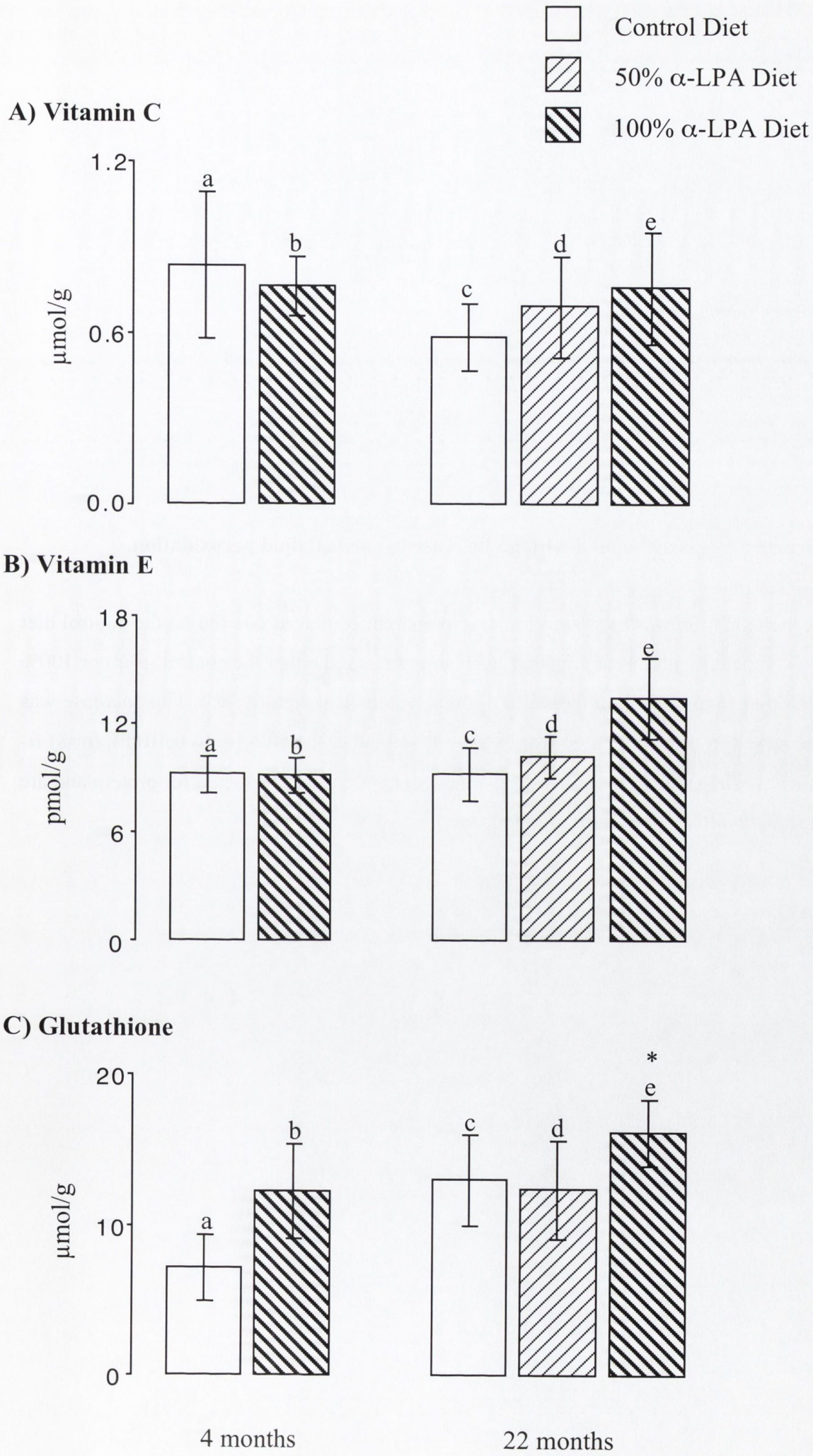

Control Diet
50% α-LPA Diet
100% α-LPA Diet
A) Vitamin C
1.2
0.6
0.0
µmol/g
a
b
c
d
e
B) Vitamin E
18
12
6
0
pmol/g
a
b
c
d
e
C) Glutathione
20
10
0
µmol/g
a
b
c
d
*
e
4 months
22 months

Figure 3.5: Age is associated with an increase in cortical lipid peroxidation.

Lipid peroxidation was increased in tissue prepared from aged rats fed on the control diet (n=9) compared with tissue prepared from young rats fed either the control (n=8) or 100% α-LPA diet (n=5) although these did not reach statistical significance. This increase was not observed in tissue prepared from aged rats fed either the 50% (n=9) or 100% (n=8) α-LPA diets. The values are expressed as nmol MDA/mg tissue corrected for protein and are the means ± the standard error of the means.

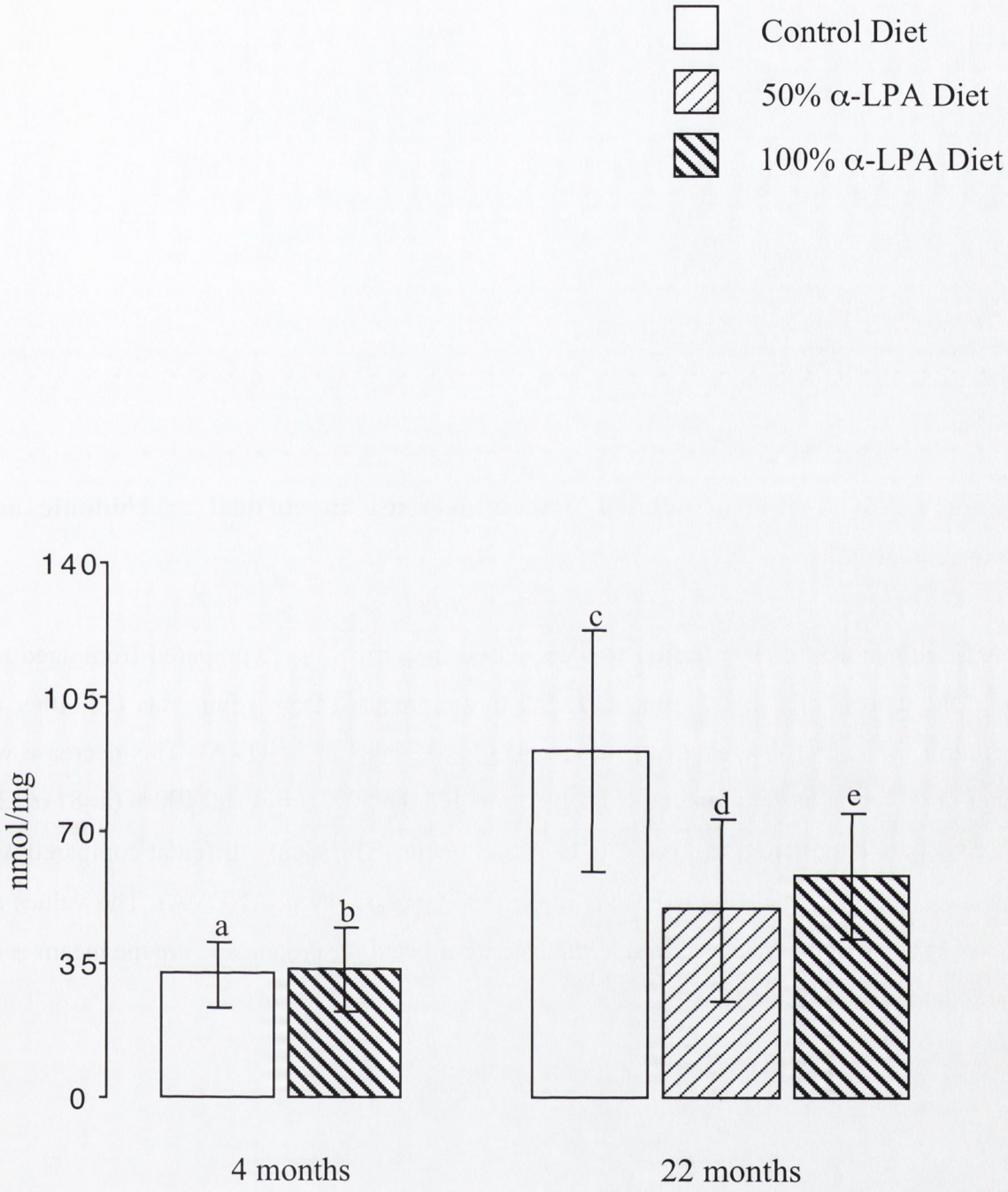

Control Diet
50% α-LPA Diet
100% α-LPA Diet
140
105
70
35
0
nmol/mg
a
b
c
d
e
4 months
22 months

Figure 3.6: Age is associated with a decrease in cortical arachidonic acid concentration.

Arachidonic acid concentration was decreased in cortical tissue prepared from aged rats fed the control diet (n=8) compared with tissue prepared from young rats fed either the control (n=5) or 100% experimental (n=4) (*$p<0.05$ *b* v *c*, ANOVA). This decrease was not observed in tissue prepared from aged rats fed the 50% (n=6) and 100% (n=8) α-LPA diets, with values from the 100% α-LPA diet being significantly different compared with values from the aged rats fed the control diet (*$p<0.05$ *c* v *e*, ANOVA). The values are expressed as μmol arachidonic acid/mg tissue corrected for protein and are the means ± the standard error of the means.

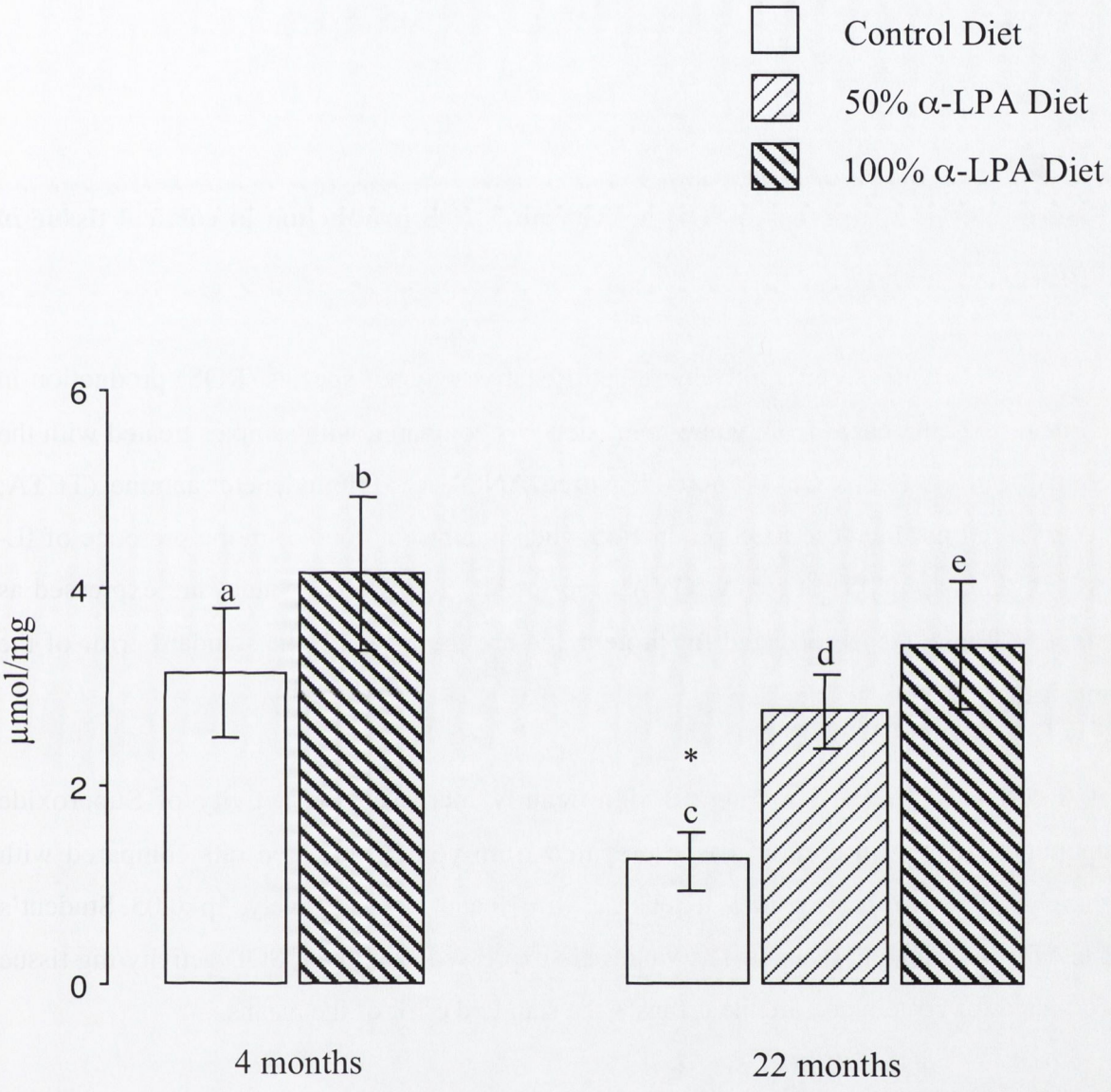

Control Diet
50% α-LPA Diet
100% α-LPA Diet
µmol/mg
6
4
2
0
a
b
c
*
d
e
4 months
22 months

Figure 3.7: IL-1β increases SOD activity and ROS production in cortical tissue *in vitro*.

A) IL-1β (1ng/ml) significantly increased Reactive oxygen species (ROS) production in cortical tissue prepared from young untreated rats compared with samples treated with the control buffer, Krebs Ca^{2+} (*$p<0.05$, paired ANOVA). Triethylenetetraamine (TETA; 100μM) did not increase ROS production when incubated alone or in the presence of IL-1β (1ng/ml; +$p<0.05$, paired ANOVA; n=6 for all groups). The values are expressed as nmol DCF/mg tissue corrected for protein and are the means ± the standard error of the means.

B) Interleukin-1β (IL-1β; 1ng/ml) significantly increased the activity of Superoxide dismutase (SOD) in cortical tissue prepared from young untreated rats compared with samples treated with the control, Krebs Ca^{2+} (n=10 and 11 respectively; *$p<0.05$, Student's t-test for independent means). The values are expressed as units of SOD activity/mg tissue corrected for protein and are the means ± the standard error of the means.

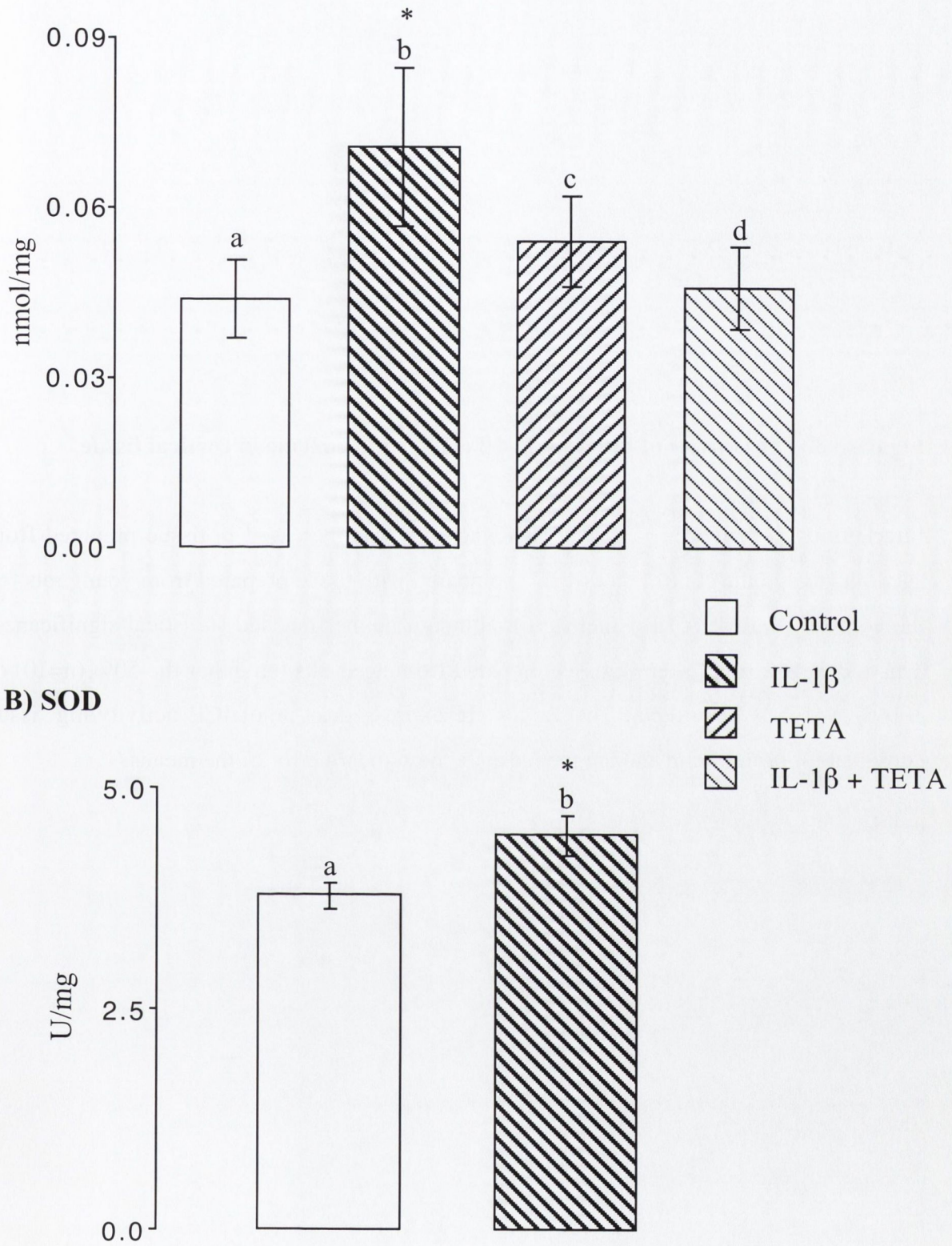

A) ROS
0.09
0.06
0.03
0.00
nmol/mg
a
*
b
c
d
Control
IL-1β
TETA
IL-1β + TETA
B) SOD
5.0
2.5
0.0
U/mg
a
*
b

Figure 3.8: The activity of interleukin-1β converting enzyme in cortical tissue.

Interleukin-1β converting enzyme (ICE) activity was increased in tissue prepared from aged rats fed on the control diet (n=11) compared with tissue prepared from young rats fed the control (n=5) or α-LPA diet (n=6), although neither reached statistical significance. This increase was not seen in tissue prepared from aged rats fed either the 50% (n=10) or 100% (n=12) α-LPA diets. The values are expressed as pmol ICE activity/mg tissue corrected for protein/min and are the means ± the standard error of the means.

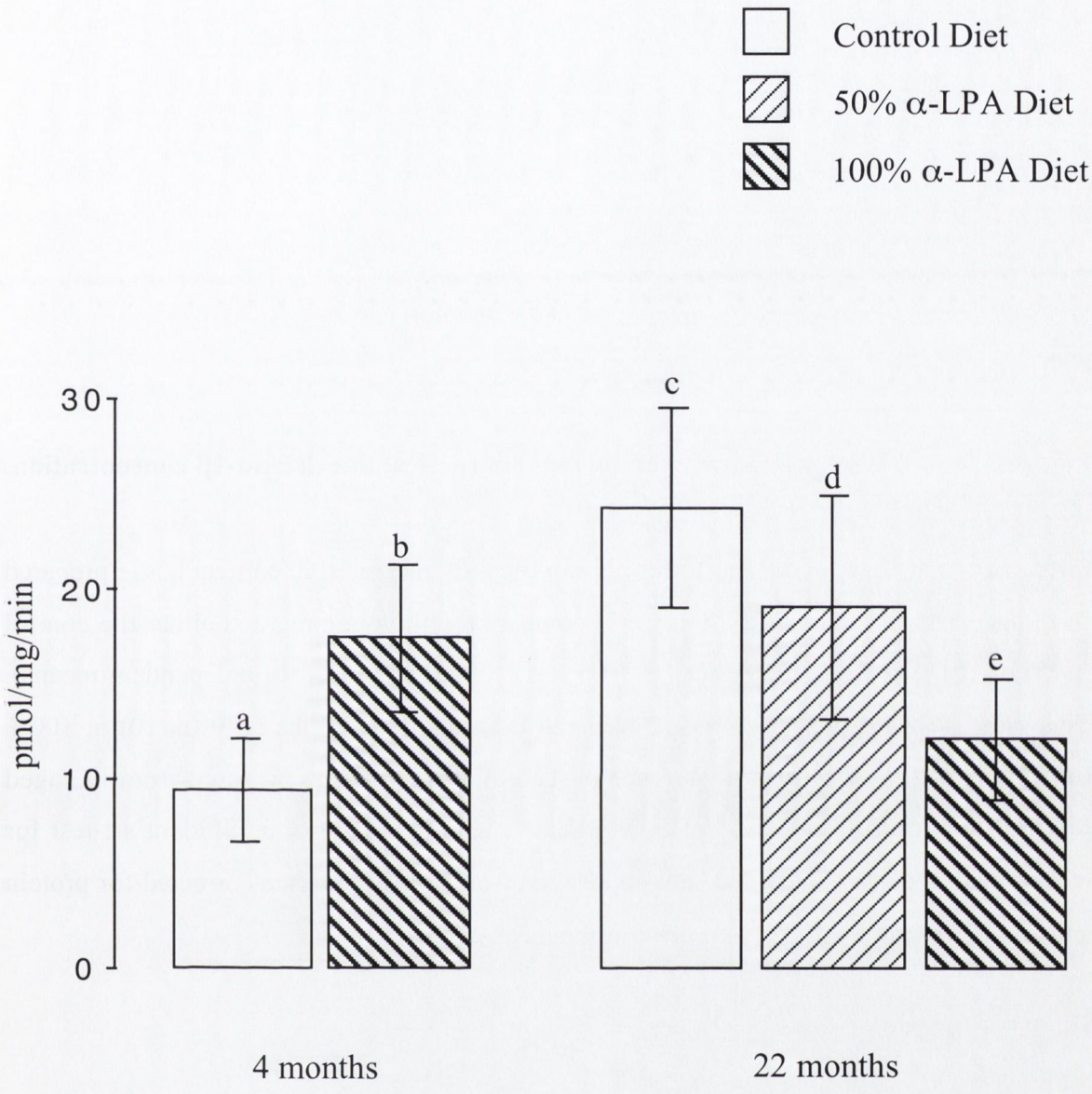

Control Diet
50% α-LPA Diet
100% α-LPA Diet
pmol/mg/min
30
20
10
0
a
b
c
d
e
4 months
22 months

Figure 3.9: Age is associated with an increase in cortical interleukin-1β concentration.

Interleukin-1β (IL-1β) concentration was significantly increased in cortical tissue prepared from aged rats fed the control diet (n=7) compared with young rats fed either the control (n=5) or 100% α-LPA diet (n=6; *$p<0.05$ *a* v *c*, Student's t-test for independent means). This increase was not seen in tissue prepared from age rats fed the 50% (n=10) or 100% (n=9) diet with a significant difference observed between tissue prepared from the aged rats fed the control diet and the 50% α-LPA diet (*$p<0.01$ *c* v *d*, Student's t-test for independent means). The values are expressed as ng IL-1β/mg tissue corrected for protein and are the means ± the standard error of the means.

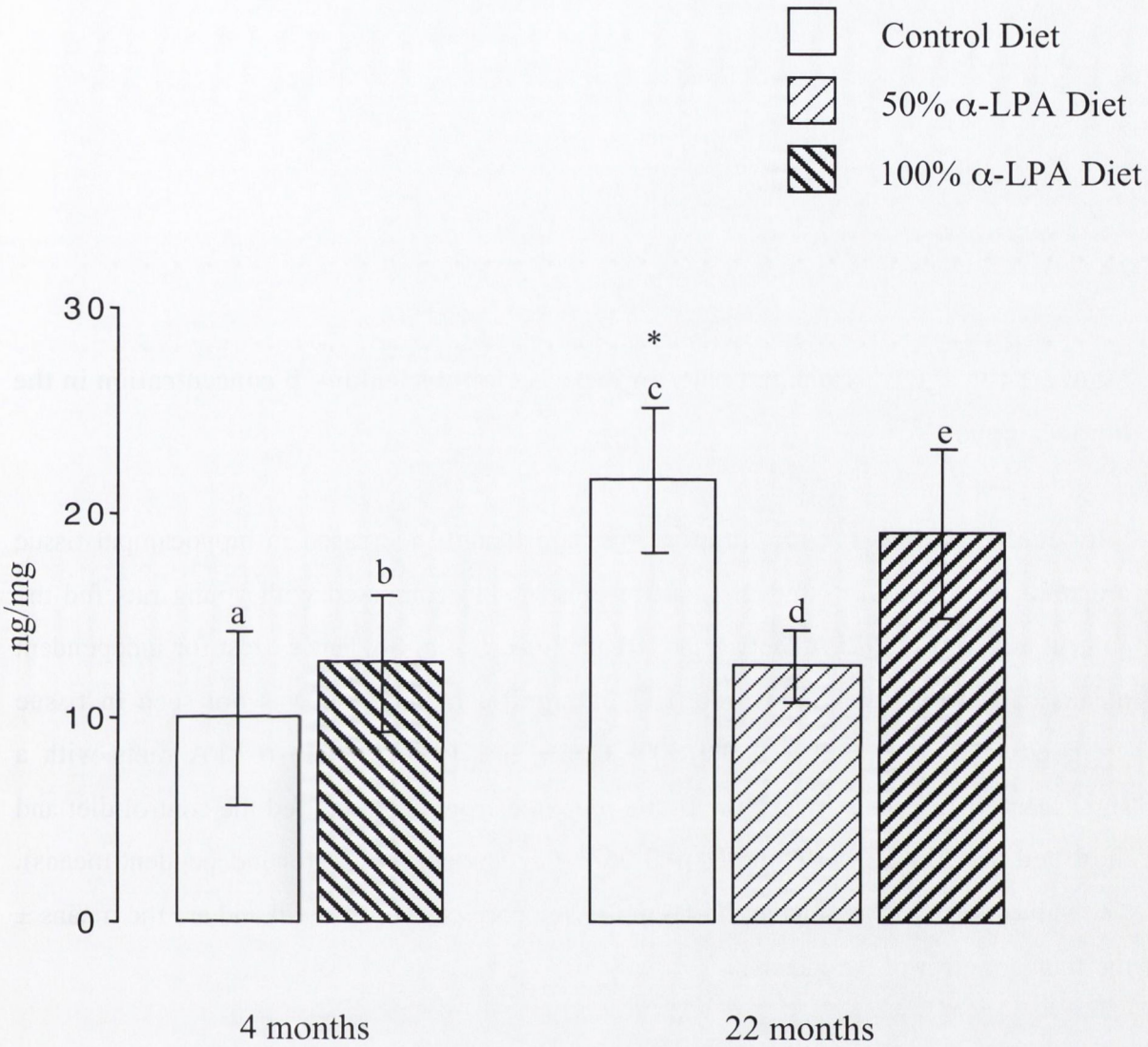
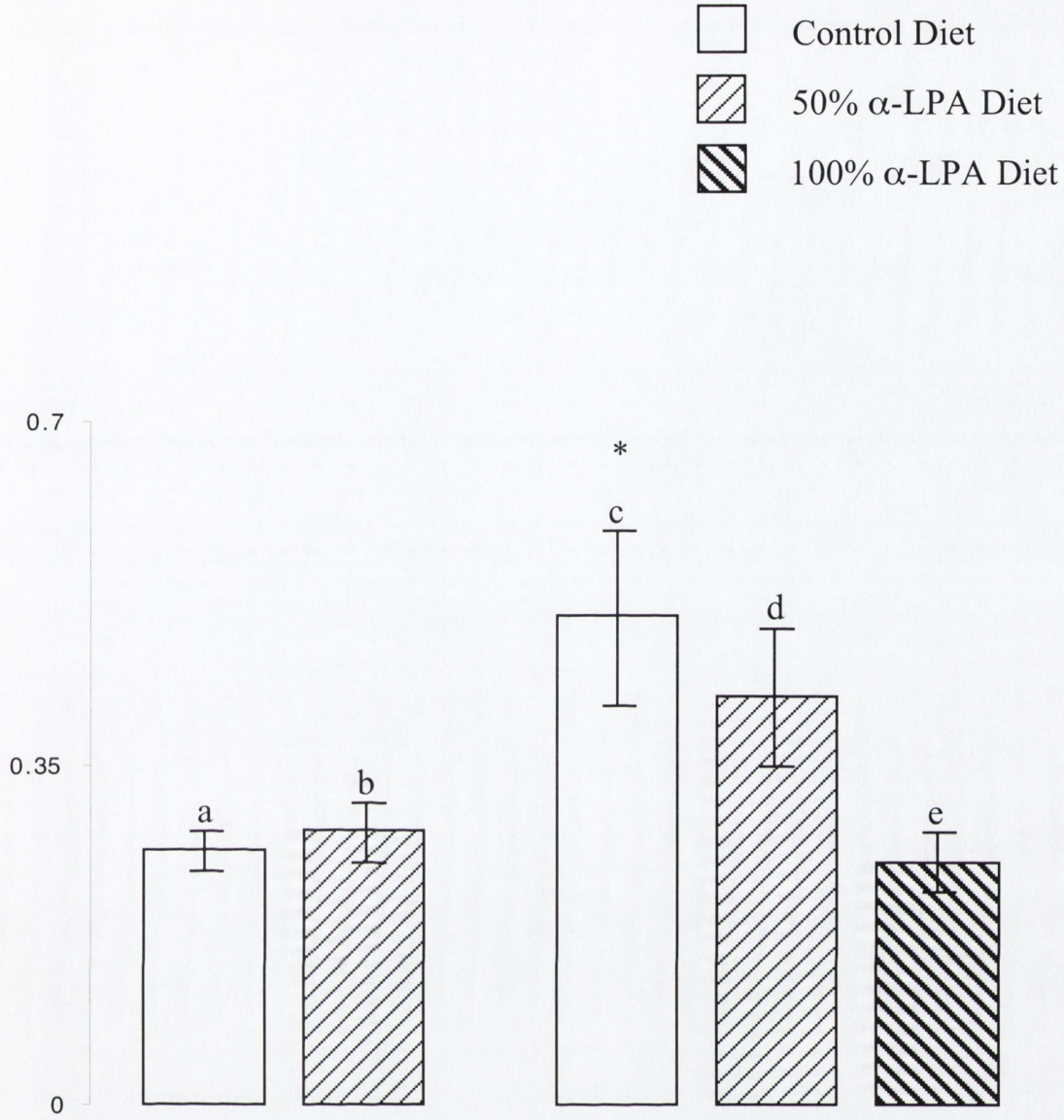

Control Diet
50% α-LPA Diet
100% α-LPA Diet
30
20
10
0
ng/mg
a
b
*
c
d
e
4 months
22 months

Figure 3.10: Age is associated with an increase in interleukin-1β concentration in the hippocampus.

Interleukin-1β (IL-1β) concentration was significantly increased in hippocampal tissue prepared from aged rats fed the control diet (n=11) compared with young rats fed the control and 100% α-LPA diets (*$p<0.01$ *a* v *c* & *b* v *c*, Student's t-test for independent means, n=8 for each young group). This significant increase was not seen in tissue prepared from aged rats fed the 50% (n=9) and 100% (n=11) α-LPA diets with a significant difference between the tissue prepared from aged rats fed the control diet and those fed the 100% α-LPA diet (*$p<0.05$ *c* v *e*, Student's t-test for independent means). The values are expressed as ng IL-1β/mg tissue corrected for protein and are the means ± the standard error of the means.


Control Diet
50% α-LPA Diet
100% α-LPA Diet
0.7
0.35
0
a
b
*
c
d
e

ANALYSIS OF CHANGES IN HIPPOCAMPAL TISSUE PREPARED FROM AGED AND YOUNG RATS FED ON A DIET SUPPLEMENTED WITH EICOSAPENTAENOIC ACID.

The polyunsaturated fatty acid eicosapentaenoic acid (EPA) was assessed for its ability to reverse the age-associated changes in the hippocampus. It has been previously shown that aged rats have an impaired ability to maintain LTP (Barnes, 1979; Landfield *et al.*, 1978; Lynch and Voss, 1994; McGahon *et al.*, 1997; McGahon *et al.*, 1999a, b and c). These second series of experiments were designed to further elucidate the factors that lead to an impaired ability to maintain LTP in the aged brain. A possible link between the previously observed increases in both ROS production and IL-1β concentration in the aged rat was also sought. Previous studies in this laboratory have successfully shown the ability of PUFAs such as AA (McGahon *et al.*, 1999a) and DHA (McGahon *et al.*, 1999c) to reverse the age-related impairment in LTP. In this experiment EPA was also assessed for its ability to not only reverse the age-related impairment in LTP but also whatever factors may lead to this impairment.

Young (4 months old) and aged (22months old) rats were fed a control diet (oil enriched in monounsaturated fatty acids) or an experimental diet enriched in EPA (10mg/rat/day for 3 weeks and then 20mg/rat/day for 5weeks). Following dietary supplementation the rats underwent LTP studies (see Appendix I for mean data).

3.11 Body weights.

The mean body weights of the young rats significantly increased during the course of the dietary period (*$p<0.001$ *a* v *b* & *c* v *d*, ANOVA; Figure 3.11) to a similar extent in both dietary groups, which was as expected with young rats still growing. The body weights of the aged rats were significantly greater compared with the young rats but their mean body weight remained unchanged during the course of the experiment so that at the end of the 8 weeks of dietary supplementation, the body weights were similar (in both groups of aged rats) to the mean body weights at the start of the experiment.

3.12 Age is associated with an impairment in long-term potentiation in the dentate gyrus.

After the 8 week dietary period, rats were anaesthetised by intraperitoneal injection of urethane. There was no significant difference in the amount of urethane needed to induce deep anaesthesia in the different age and dietary groups (1.89 ± 0.08 and 2.13 ± 0.1 g/kg for young rats fed the control and EPA diet respectively and 1.7 ± 0.1 and 1.71 ± 0.1 g/kg for aged rats fed on control and EPA diet respectively). The mean stimulus strength was similar in all groups of rats (0.461 ± 0.065 and 0.539 ± 0.071 V for young rats fed the control and EPA diet respectively and 0.506 ± 0.084 and 0.505 ± 0.084 V for aged rats fed on control and EPA diet respectively). Figure 3.12 shows that delivery of a high frequency train of stimuli to perforant path granule cell synapses (at time point 0) resulted in an immediate increase in the mean population EPSP slope in all groups of rats.

3.13 Age is associated with a decrease in the percentage change in the EPSP slope in the dentate gyrus.

The mean percentage change in the EPSP slope in the first 2min following tetanic stimulation (compared with the mean value in the 5min prior to stimulation) in the young rats fed on control and EPA diets were 190.62 ± 11.01 and 184.41 ± 7.95 respectively. The corresponding values in aged rats were 134.76 ± 4.59 and 176.60 ± 6.42 for those fed on control and EPA diet respectively. The mean percentage change in the EPSP slope in the last 5min of recording following tetanic stimulation (compared with the mean value in the 5min prior to stimulation) in the young rats fed on control and EPA diets were 143.83 ± 2.63 and 152.09 ± 5.43 respectively. The corresponding values in aged rats were 109.80 ± 1.84 and 148.73 ± 2.91 for those fed on control and EPA diet respectively (Figure 3.13).

3.14 Reactive oxygen species production in the hippocampus.

In hippocampal tissue prepared from aged rats fed on the control diet there was a trend towards an increase in ROS production compared with tissue prepared from young rats fed either the control or EPA diet (Figure 3.14). This increase did not reach statistical significance (Figure 3.14). The EPA diet was not successful in reversing this age-related increase in ROS production.

3.15: The effects of a diet enriched in docosahexaenoic acid on hippocampal ROS production.

ROS was also assessed in rats fed a diet enriched in docosahexaenoic acid, of which EPA is a precursor (Figure 3.15). ROS production was marginally increased in hippocampal tissue prepared from aged rats fed on the control diet (corn oil) compared with tissue prepared from young rats fed either the control or DHA diet. The absence of significance may be due to the fact that the standard errors of both young groups were quite large. This is probably as a result of the small n number in each group (n=4 control and =5 EPA diet). This increase was not seen in tissue prepared from aged rats fed on the DHA diet.

3.16 Activity of the antioxidant enzymes is unchanged with age in the hippocampus.

In an attempt to explain the age-related increase in ROS, the activities of the antioxidant enzymes were assessed in the hippocampus. Figure 3.16A indicates that SOD activity was similar in both age and dietary groups. Similarly the activity of catalase was unchanged by age or diet (Figure 3.16B). The activity of Gpx was significantly increased in the tissue prepared from aged rats fed the control diet compared with the tissue prepared from young rats fed the EPA diet (*$p<0.05$ *b* v *c*, Student's t-test for independent means; Figure 3.16C). Gpx activity was similar in all other groups.

3.17 Correlation between reactive oxygen species and superoxide dismutase in the hippocampus.

A significant positive correlation exists with a r^2 value of 0.25 between the activity of SOD and ROS production (Figure 3.17). This implies that (in the absence of other changes) with increasing SOD activity there is a theoretical concomitant increase in ROS production.

3.18 Age is associated with an increase in hippocampal lipid peroxidation.

One cause of increased ROS production is an increase in lipid peroxidation, which was analysed in parallel with the change in ROS production. Figure 3.18 shows that lipid peroxidation was significantly increased in hippocampal tissue prepared from

aged rats fed on the control and EPA diet compared with tissue prepared from young rats fed both diets (*p<0.05 *a/b* v *c/d*, ANOVA). Therefore the diet was unsuccessful in reversing the age-related increase in lipid peroxidation in the hippocampus

3.19 Age is associated with an increase in hippocampal interleukin-1β concentration.

IL-1β concentration was assessed, as it has been shown to increase ROS production. Figure 3.19 shows that IL-1β concentration was increased in hippocampal tissue prepared from aged rats fed the EPA diet compared with tissue prepared from young rats fed the control diet, although this did not reach statistical significance. However, a significant increase in IL-1β concentration was observed between aged rats fed on the control diet and young rats fed on the EPA diet (*p<0.01 *b* v *c*, Student's t-test for independent means). This increase was not seen in tissue prepared from aged rats fed the EPA diet.

3.20 Effect of H_2O_2 on IL-1β concentration in hippocampal tissue *in vitro*.

To further assess the relationship between IL-1β and ROS, the effect of H_2O_2 (which increases ROS) was assessed on IL-1β concentration *in vitro*. Hydrogen peroxide (H_2O_2; 200μM) slightly increased IL-1β concentration in hippocampal tissue prepared from young untreated rats compared with tissue incubated in the control buffer although this did not reach statistical significance (Figure 3.20).

3.21 H_2O_2 increases p38 phosphorylation in hippocampal tissue *in vitro*.

H_2O_2 was assessed for its ability to increase the activity of mitogen-activated protein kinase p38 *in vitro*. Figure 3.21A one sample immunoblot indicates that in lane 2 (H_2O_2; 200μM) p38 expression was increased in hippocampal tissue compared with lane 1 (untreated tissue). Densitometric analysis reveals that H_2O_2 significantly increases p38 phosphorylation. (*p<0.05 *a* v *b*, Student's t-test for independent means, Figure 3.21B).

3.22 Interleukin-1β increases p38 phosphorylation in hippocampal tissue.

IL-1β (100ng/ml) was assessed for its ability to increase p38 phosphorylation in hippocampal tissue. One sample immunoblot indicates that in lane 2 (H_2O_2 treated;

200μM) p38 phosphorylation was increased compared with lane 1 (control; Figure 3.22). Figure 3.22B shows the mean data obtained following densitometric analysis of the immunoblots and shows that IL-1β significantly increased p38 (*$p<0.05$ *a* v *b*, Student's t-test for independent means).

3.23 Age is associated with an increase in p38 mitogen-activated protein kinase phosphorylation in the hippocampus.

Since age is associated with an increase in IL-1β concentration and ROS production and since both were shown to stimulate p38 activity *in vitro* the age-related and dietary-related effects on p38 phosphorylation were assessed. In Figure 3.23A a sample immunoblot shows that p38 phosphorylation was increased in hippocampal tissue prepared from aged rats fed the control diet (lane 3) compared with tissue prepared from young rats fed the control (lane 1) and experimental (lane 2) diets and the aged rats fed the experimental (lane 4) diet. Densitometric analysis revealed that mean p38 phosphorylation was significantly increased in aged rats fed the control diet compared with tissue prepared from the young rats fed the control diet (*$p<0.05$ *a* v *c*, Student's t-test for independent means; Figure 3.23B). This age-related increase is not seen in the tissue prepared from the aged rats fed the EPA diet.

3.24 Interleukin-1β inhibits glutamate release in dentate gyrus tissue *in vitro*.

Previous data showed that age was associated with a decrease in glutamate release and similarly IL-1β has been shown to decrease glutamate release. It was considered that the effect of IL-1β may be related to its ability to increase p38 phosphorylation. Therefore, IL-1β (10pg/ml) in the presence and absence of the p38 inhibitor SB203580 (SB) was assessed for its ability to inhibit glutamate release in Figure 3.24. Addition of KCl (40mM) significantly increased glutamate release in synaptosomes prepared from the dentate (*$p<0.05$ *a* v *b*, Student's t-test for independent means). Pre-treatment with IL-1β attenuated this KCl-stimulated glutamate release. This effect was probably due to the increased basal concentration of glutamate released. This may occur due to excess leakage of glutamate from IL-1β affected cells. Synaptosomes pre-treated with both IL-1β and SB (50μM) restored the response to KCl such that KCl-stimulated glutamate release was significantly increased

compared with the unstimulated release (*p<0.05 *e* v *f*, Student's t-test for independent means).

3.25 The effect of age and dietary manipulation on glutamate release in the dentate gyrus.

The data shown in Figure 3.24 suggests that IL-1β may inhibit glutamate release by stimulating p38 activity. It was predicted that glutamate release would be decreased in aged rats in parallel with the increase in IL-1β concentration and p38 activity. KCl-stimulated glutamate release was increased in tetanized tissue compared with untetanized tissue in tissue prepared from dentate gyri of young rats fed on the control and EPA diet and aged rats fed on the EPA diet, although this does not reach statistical significance (Figure 3.25). No increase was observed in tissue prepared from aged rats fed on the control diet. Data presented are means of KCl (40mM)-stimulated release minus unstimulated release in each case.

3.26 The effect of age and dietary manipulation on p38 phosphorylation in the entorhinal cortex.

The cell bodies of the perforant path synapses of the dentate gyrus are found in the entorhinal cortex and, therefore, age-related changes were assessed in this brain area. One sample immunoblot shows that p38 phosphorylation appears to be increased in tissue prepared from aged rats fed on the control (lane 3) and experimental (lane 4) diet compared with tissue prepared from young rats fed the control (lane 1) and experimental (lane 2) diet (Figure 3.26A). Figure 3.26B shows the mean data derived from densitometric analysis and indicates that p38 phosphorylation was slightly increased in tissue prepared from aged rats fed either the control and EPA diet compared with tissue prepared from young rats fed both the control and EPA diet, however, this increase did not reach statistical significance.

3.27 Age is associated with an increase in the proportion of TUNEL positive cells in the entorhinal cortex.

TUNEL staining which allows identification of cells containing nicked DNA is considered to be one indicator of cell death. Figure 3.27 shows that the percentage of TUNEL positive cells was significantly increased in cells prepared from the entorhinal

cortex of aged rats fed the control diet compared with cells prepared from young rats fed the control diet and young and aged rats fed the EPA diet (*$p<0.001$ *a*/*b*/*d* v *c*, ANOVA). This suggests dietary manipulation with EPA has the ability to spare cells of the entorhinal cortex from cell death, which is associated with age.

Figure 3.11: Body weights.

Mean body weight of young rats was significantly greater at the end compared with the start of the experimental period (*p<0.001 *a* v *b* & *c* v *d*, ANOVA). Body weights of aged rats remained unchanged.

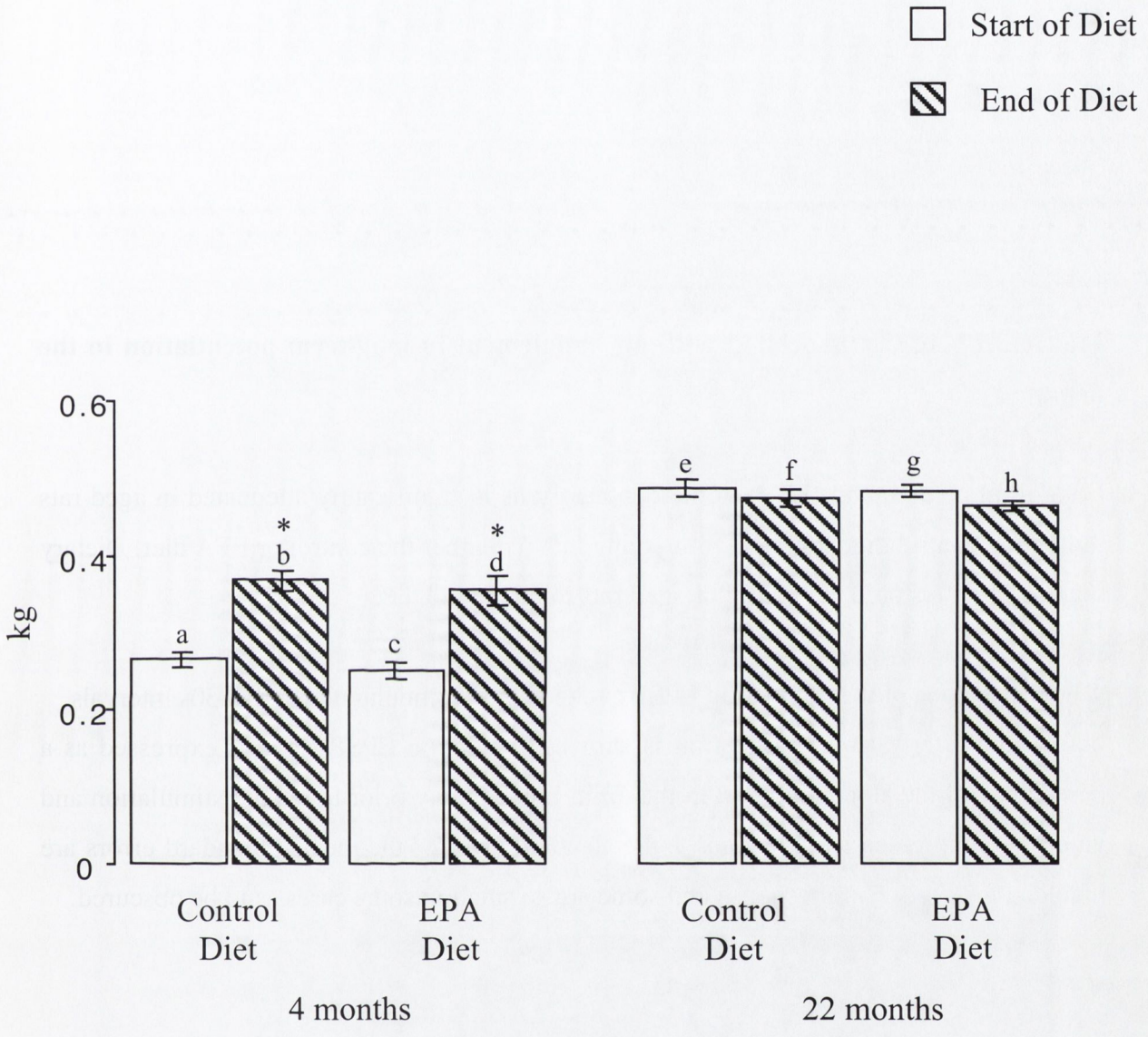

Start of Diet
End of Diet
0.6
0.4
0.2
0
kg
a
b
c
d
e
f
g
h
*
*
Control Diet
EPA Diet
Control Diet
EPA Diet
4 months
22 months

Figure 3.12 Age is associated with an impairment in long-term potentiation in the dentate gyrus.

Long-term potentiation (LTP) in the dentate gyrus is significantly attenuated in aged rats fed on the control diet compare with young rats fed either the control or EPA diet. Dietary manipulation restored the ability of aged rats to maintain LTP.

The mean slope of the population EPSP evoked by test stimuli delivered at 30s intervals before and after tetanic stimulation is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5min immediately prior to tetanic stimulation and values are expressed as the means ± the standard error of the means. Standard errors are included for every tenth response and some are so small in some cases as to be obscured.

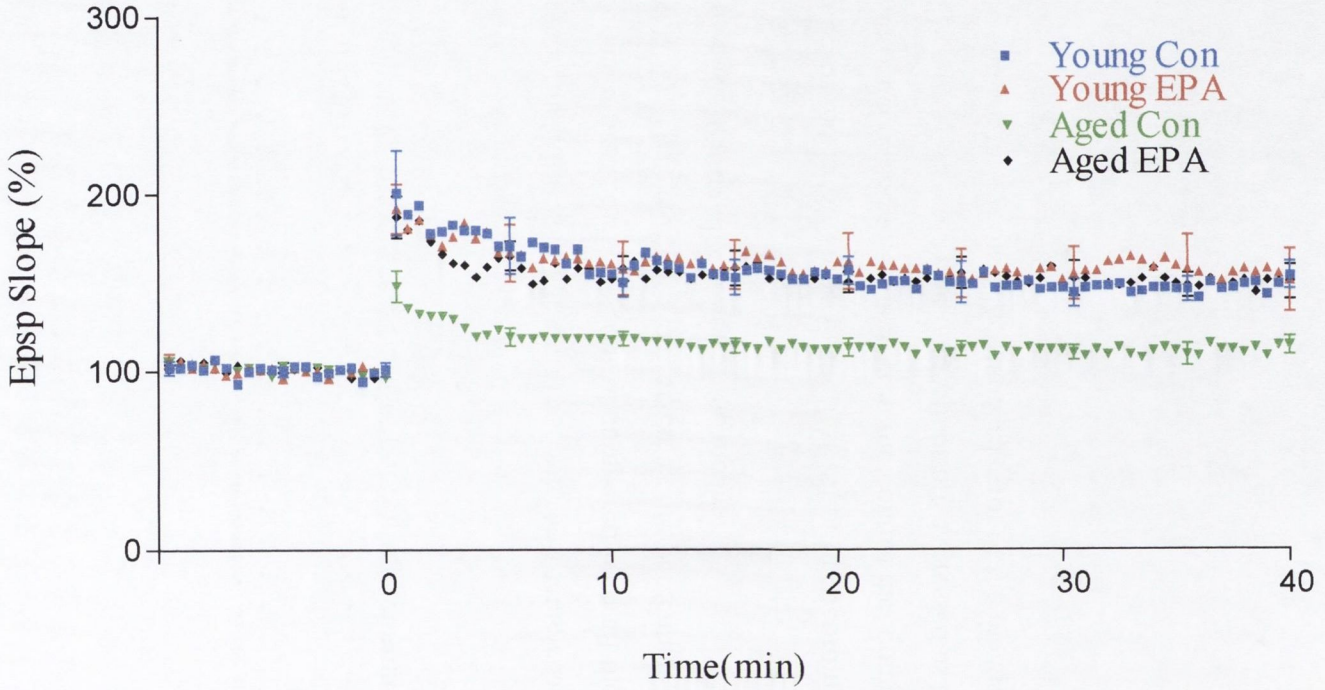

300
200
100
0
Epsp Slope (%)
0
10
20
30
40
Time(min)
Young Con
Young EPA
Aged Con
Aged EPA

Figure 3.13: Age is associated with a decrease in the percentage change in the EPSP slope in the dentate gyrus.

A) The percentage change in the EPSP slope in the first 2min post tetanic stimulation was significantly decreased in aged rats fed on the control compared with all the other groups (*p<0.01 *a/b/d* v *d*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

B) The percentage change in the EPSP slope in the last 5min of recording post tetanic stimulation was significantly decreased in aged rats fed the control diet compared with the all other groups (*p<0.001 *a/b/d* v *d*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

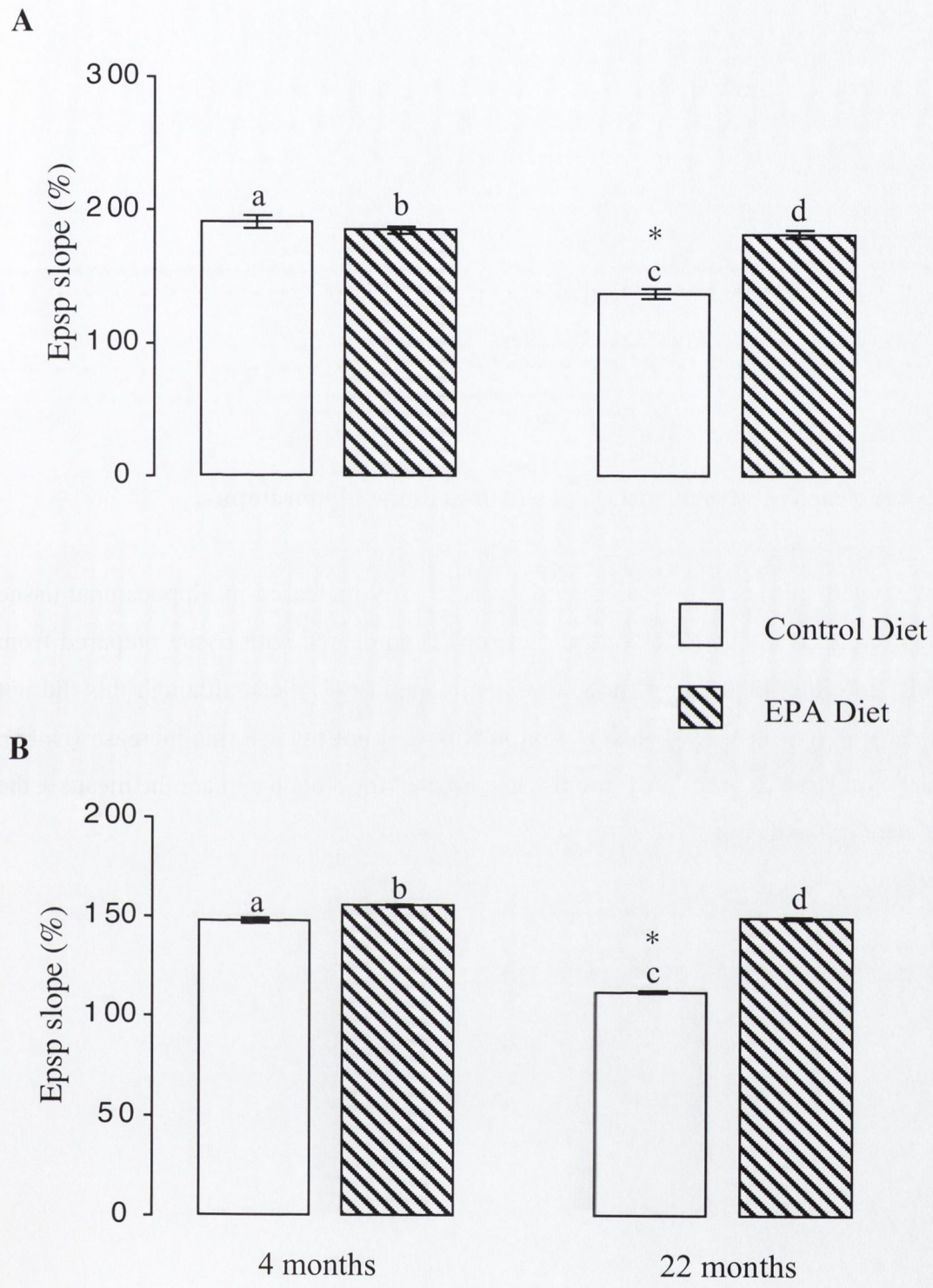

A
300
200
100
0
Epsp slope (%)
a
b
*
c
d
Control Diet
EPA Diet
B
200
150
100
50
0
Epsp slope (%)
a
b
*
c
d
4 months
22 months

Figure 3.14: Reactive oxygen species production in the hippocampus.

Reactive oxygen species (ROS) production was slightly increased in hippocampal tissue prepared from aged rats fed the control diet (n=12) compared with tissue prepared from young rats fed either the control (n=8) or experimental (n=7) diets, although this did not reach statistical significance. Dietary manipulation did not reverse this increase (n=13). Values are expressed as μmol DCF/mg tissue corrected for protein and are the means ± the standard error of the means.

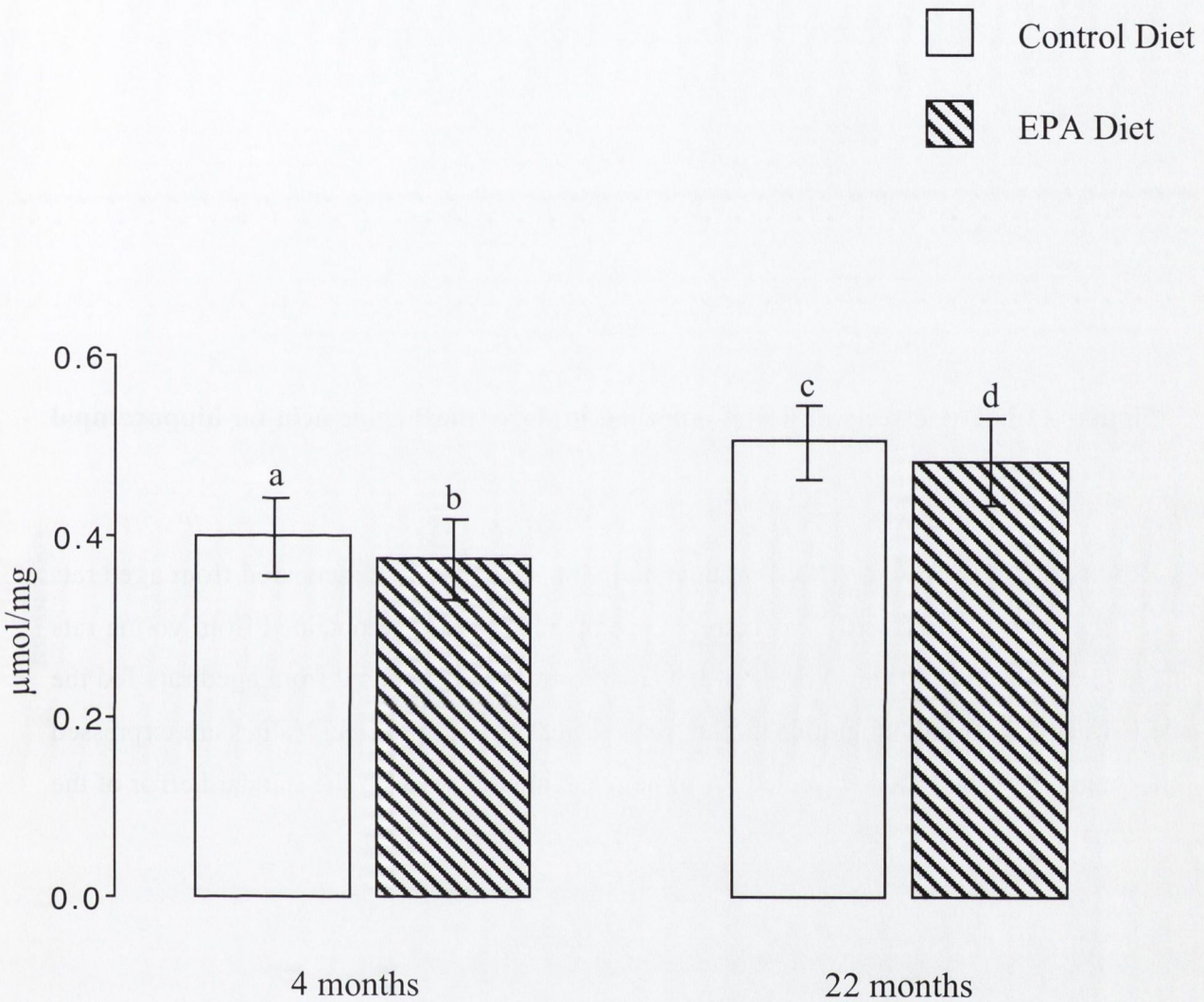

Control Diet
EPA Diet
0.6
0.4
0.2
0.0
µmol/mg
a
b
c
d
4 months
22 months

Figure 3.15: The effects of a diet enriched in docosahexaenoic acid on hippocampal ROS production.

Reactive oxygen species (ROS) production in hippocampal tissue prepared from aged rats fed the control diet (n=10) was increased compared with tissue obtained from young rats fed either the control (n=4) or EPA diet (n=5) or in tissue prepared from aged rats fed the EPA diet (n=10), although this did not reach statistical significance. Values are expressed as nmol DCF/mg tissue corrected for protein and are the means ± the standard error of the means.

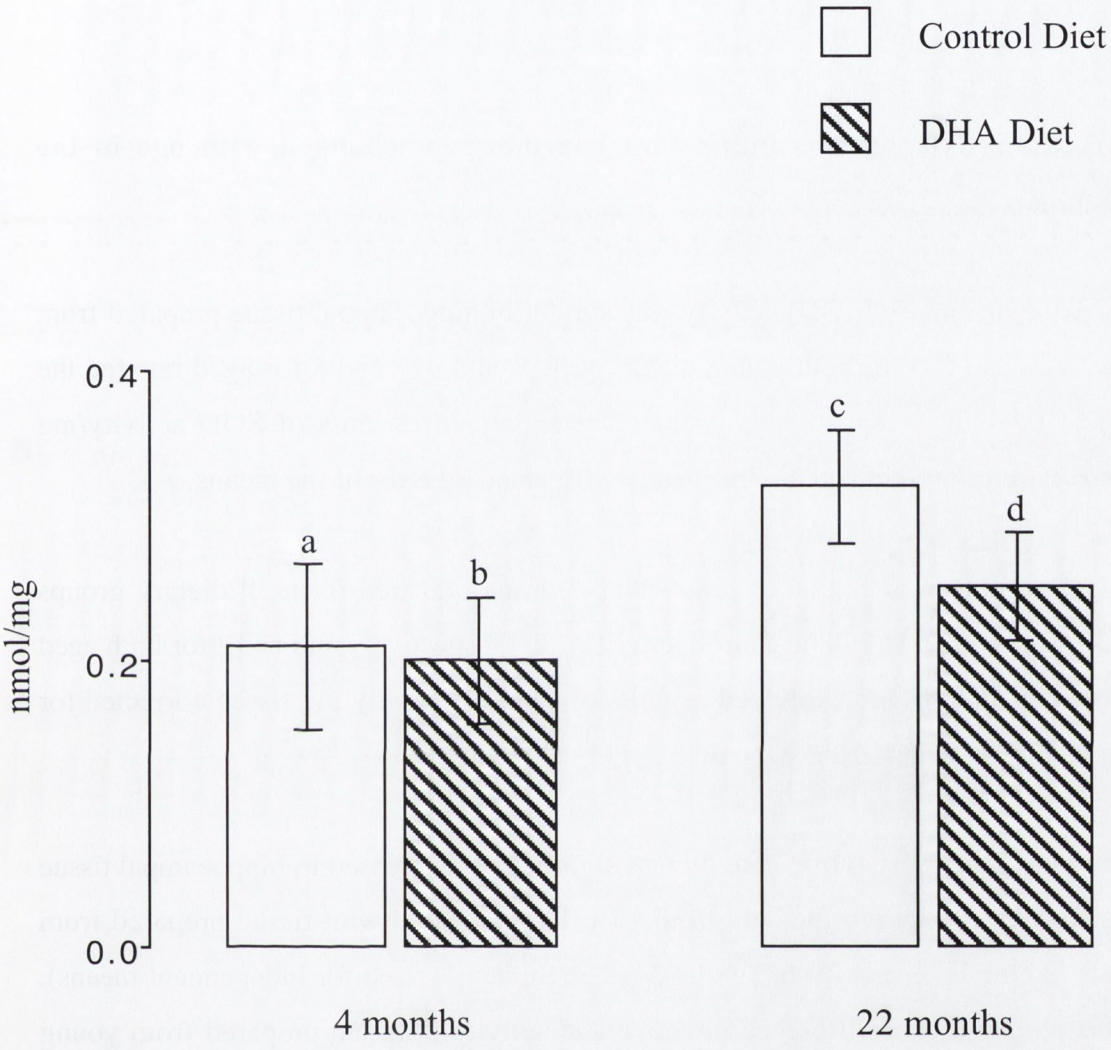

Control Diet
DHA Diet
nmol/mg
0.4
0.2
0.0
a
b
c
d
4 months
22 months

Figure 3.16: Activity of the antioxidant enzymes is unchanged with age in the hippocampus.

A) Superoxide dismutase (SOD) activity was similar in hippocampal tissue prepared from all dietary groups (n=6 for both young dietary groups and n=7 and 8 for aged rats fed the control and EPA diet respectively). Values are expressed as units of SOD activity/mg tissue corrected for protein and are the means ± the standard error of the means.

B) Catalase activity was similar in hippocampal tissue prepared from all dietary groups (n=9 and 7 for young rats fed the control and EPA diet respectively and n=12 for both aged dietary groups). Values are expressed as units of catalase activity/mg tissue corrected for protein and are the means ± the standard error of the means.

C) Glutathione peroxidase (Gpx) activity was significantly increased in hippocampal tissue prepared from aged rats fed the control diet (n=11) compared with tissue prepared from young rats fed the EPA diet (n=6; *$p < 0.05$ *b* v *c*, Student's t-test for independent means). There was no significant difference in enzymatic activity in tissue prepared from young rats fed the control diet (n=8) or aged rats fed the EPA diet (n=13). Values are expressed as μmol Gpx activity/mg tissue corrected for protein/min and are the means ± the standard error of the means.

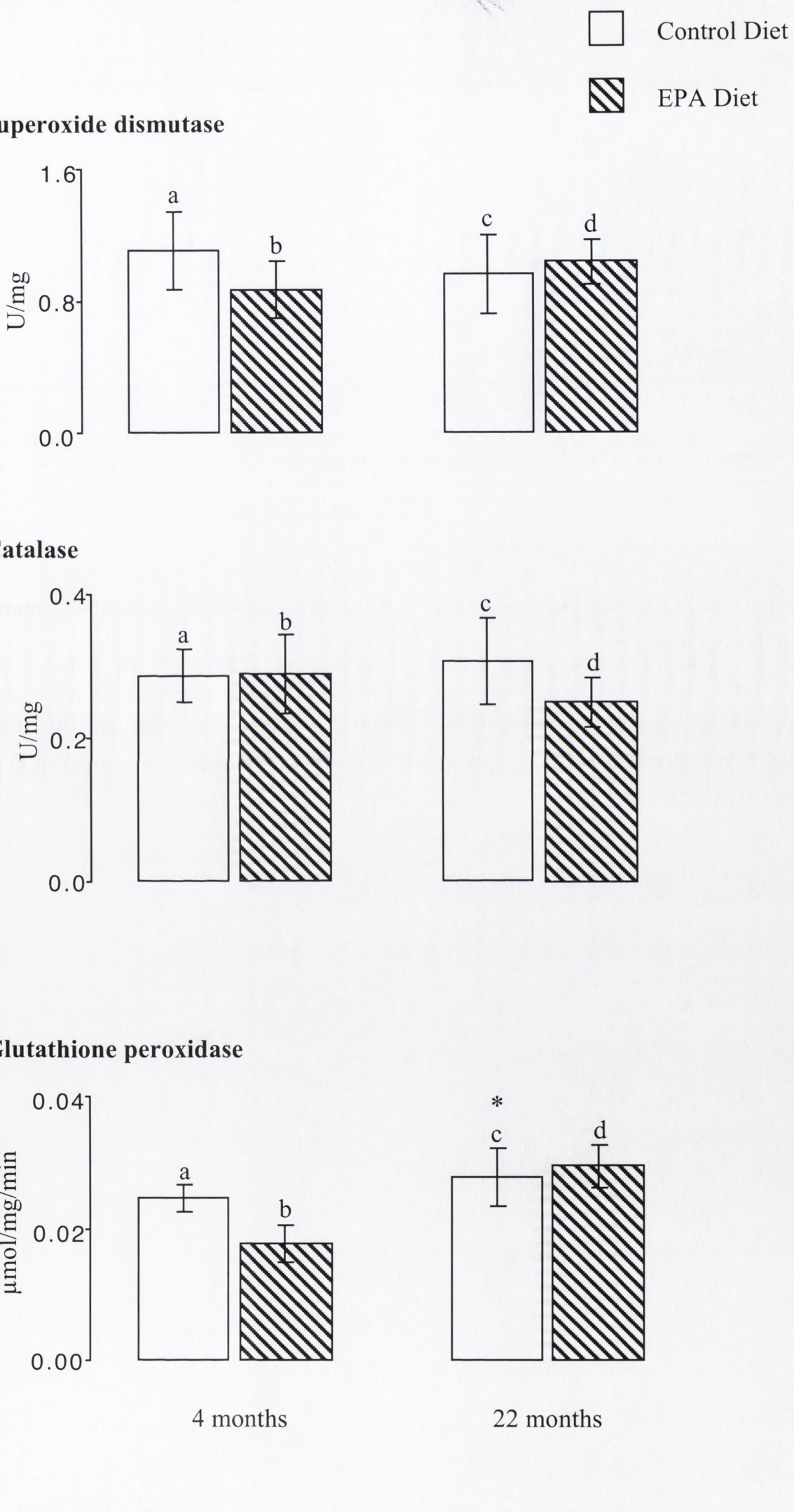

Control Diet
EPA Diet
A) Superoxide dismutase
1.6
0.8
0.0
U/mg
a
b
c
d
B) Catalase
0.4
0.2
0.0
U/mg
a
b
c
d
C) Glutathione peroxidase
0.04
0.02
0.00
µmol/mg/min
a
b
*
c
d
4 months
22 months

Figure 3.17: Correlation between reactive oxygen species and superoxide dismutase in the hippocampus.

A significant positive correlation exists between reactive oxygen species (ROS) and superoxide dismutase (SOD; $p<0.01$; $r^2=0.25$). The values are expressed as nmol DCF/ mg and units of SOD activity/mg of protein.

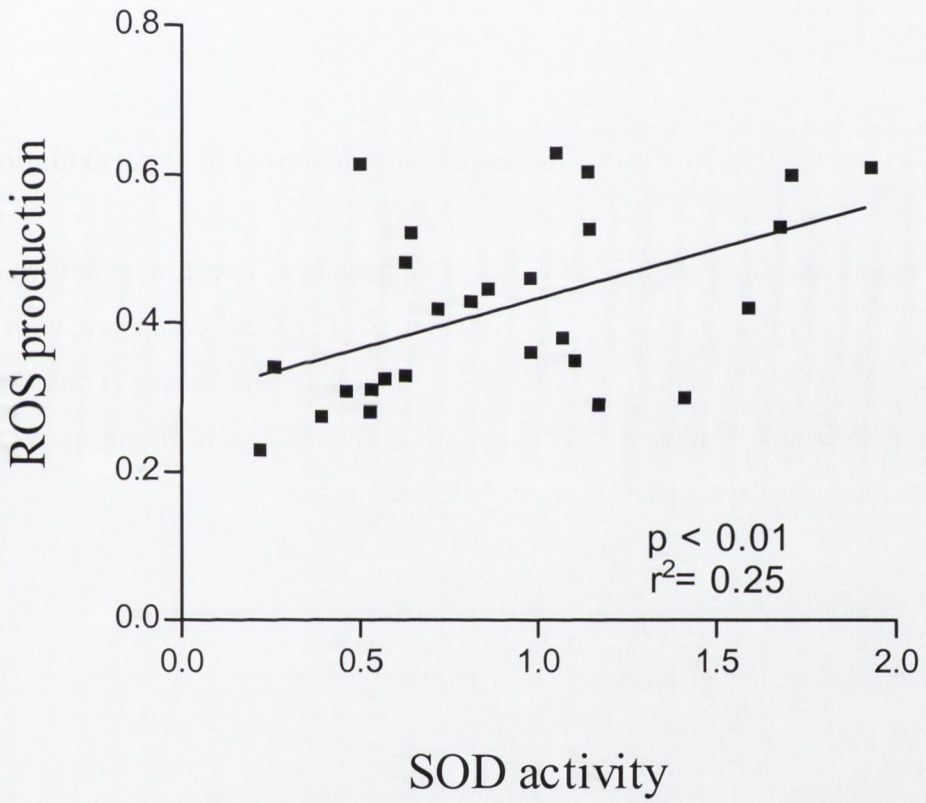

ROS production
0.8
0.6
0.4
0.2
0.0
0.0
0.5
1.0
1.5
2.0
p < 0.01
r2= 0.25
SOD activity

Figure 3.18: Age is associated with an increase in hippocampal lipid peroxidation.

Lipid peroxidation was significantly increased in hippocampal tissue prepared from aged rats fed either the control (n=11) or experimental (n=12) diets compared with tissue prepared from young rats fed either the control (n=9) or experimental (n=7) diet (*$p<0.05$ in both cases, ANOVA). Values are expressed as μmol MDA/mg tissue corrected for protein and are the means ± the standard error of the means.

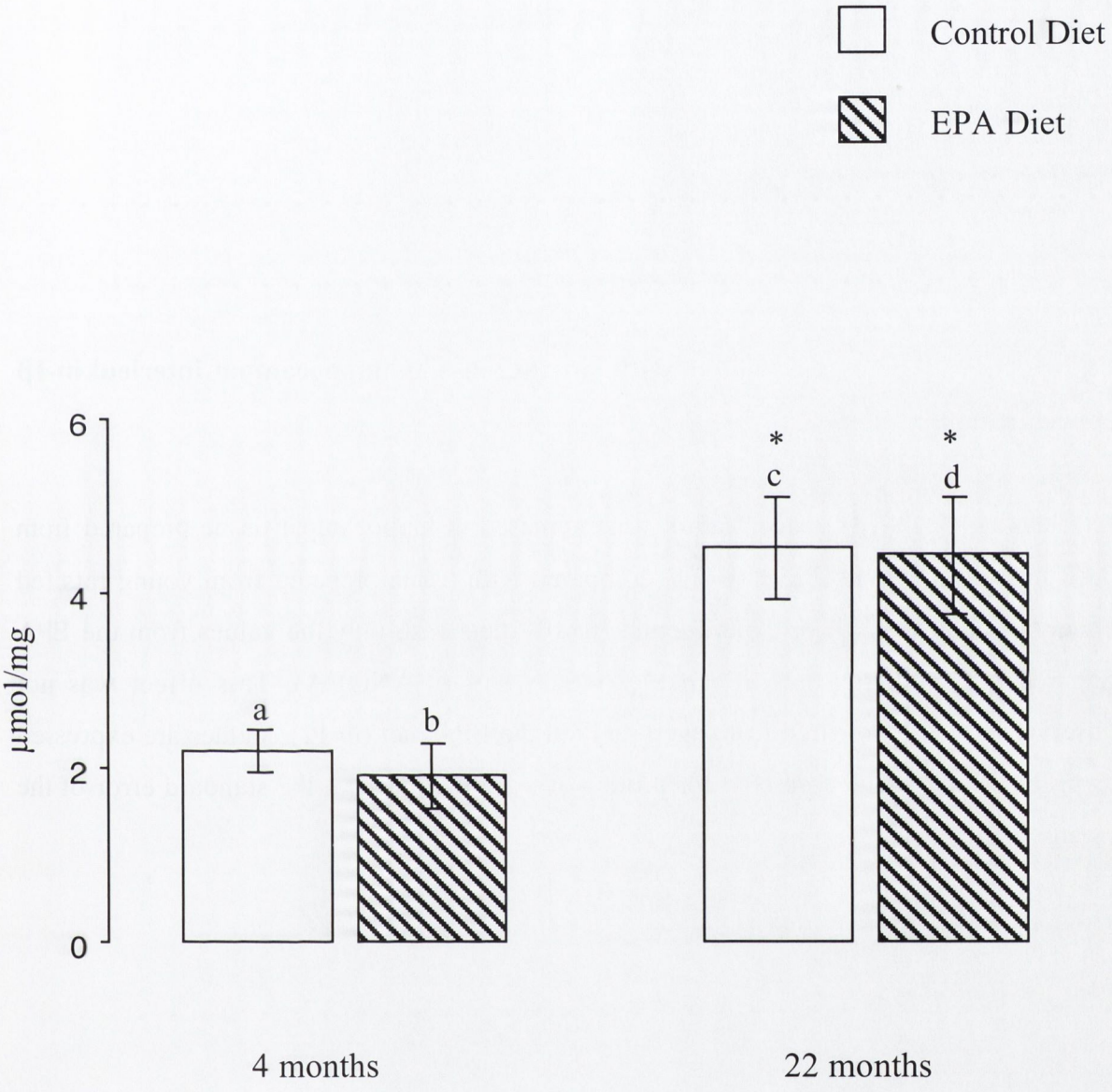

Control Diet
EPA Diet
µmol/mg
6
4
2
0
a
b
*
c
*
d
4 months
22 months

Figure 3.19: Age is associated with an increase in hippocampal interleukin-1β concentration.

Interleukin-1β (IL-1β) concentration was increased in hippocampal tissue prepared from aged rats fed the control diet (n=11) compared with tissue prepared from young rats fed either the control (n=9) or experimental (n=10) diets with only the values from the EPA diet reaching statistical significance (*$p<0.05$ *b* v *c*, ANOVA). This effect was not observed in tissue prepared from aged rats fed the EPA diet (n=12). Values are expressed as pg IL-1β/mg tissue corrected for protein and are the means ± the standard error of the means.

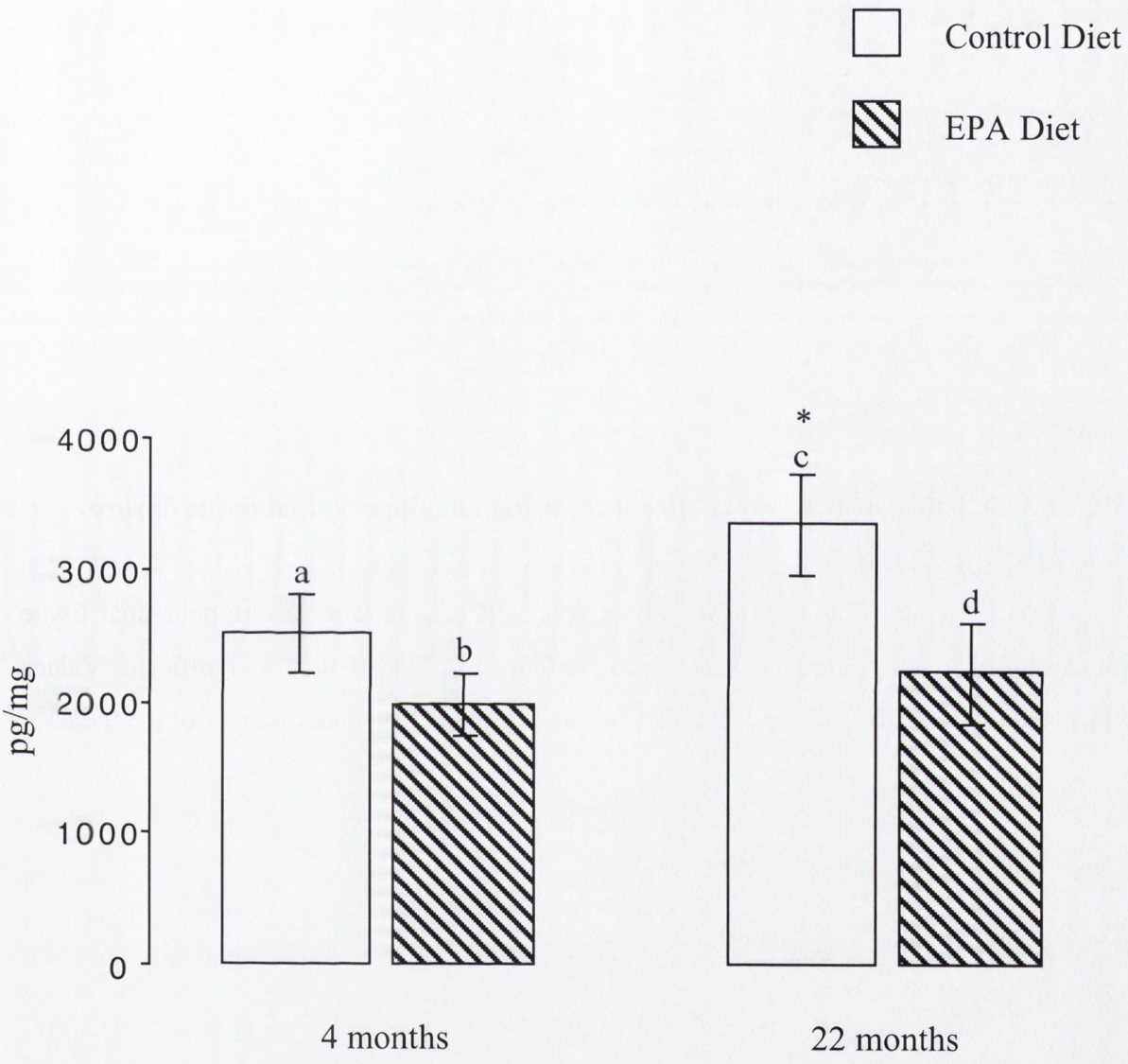

Control Diet
EPA Diet
4000
3000
2000
1000
0
pg/mg
a
b
*
c
d
4 months
22 months

Figure 3.20: Effect of H_2O_2 on IL-1β concentration in hippocampal tissue *in vitro*.

Hydrogen peroxide (H_2O_2; 200μM) increased IL-1β concentration in hippocampal tissue prepared from young untreated rats but this effect was not statistically significant. Values are expressed as ng IL-1β/mg protein and are the means ± the standard error of the means.

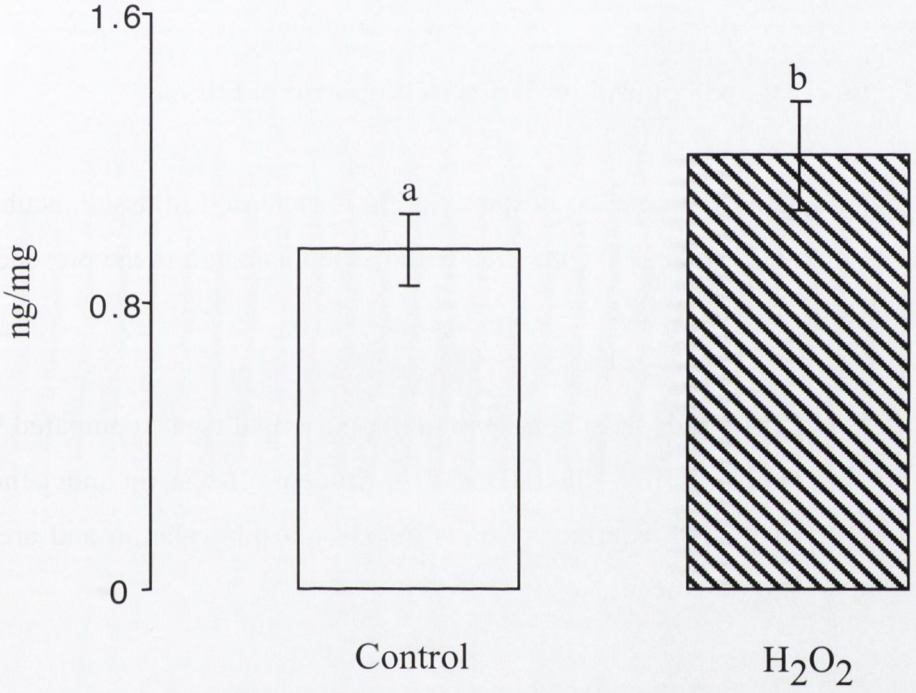
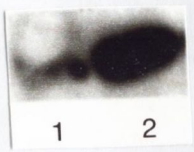

1.6
0.8
0
ng/mg
a
b
Control
H_2O_2

Figure 3.21: H_2O_2 increases p38 phosphorylation in hippocampal tissue.

A) A sample immunoblot shows that p38 phosphorylation is increased in tissue incubated in the presence of H_2O_2 (200μM; lane 2) compared with tissue incubated in the presence of the control buffer (Krebs Ca^{2+}; lane 1).

B) H_2O_2 significantly increases p38 phosphorylation in hippocampal tissue compared with tissue incubated in the control buffer (*$p<0.05$ *a* v *b*, Student's t-test for independent means). The values are expressed as arbitrary units of p38 phosphorylation and are the means ± the standard error of the means.

A



B

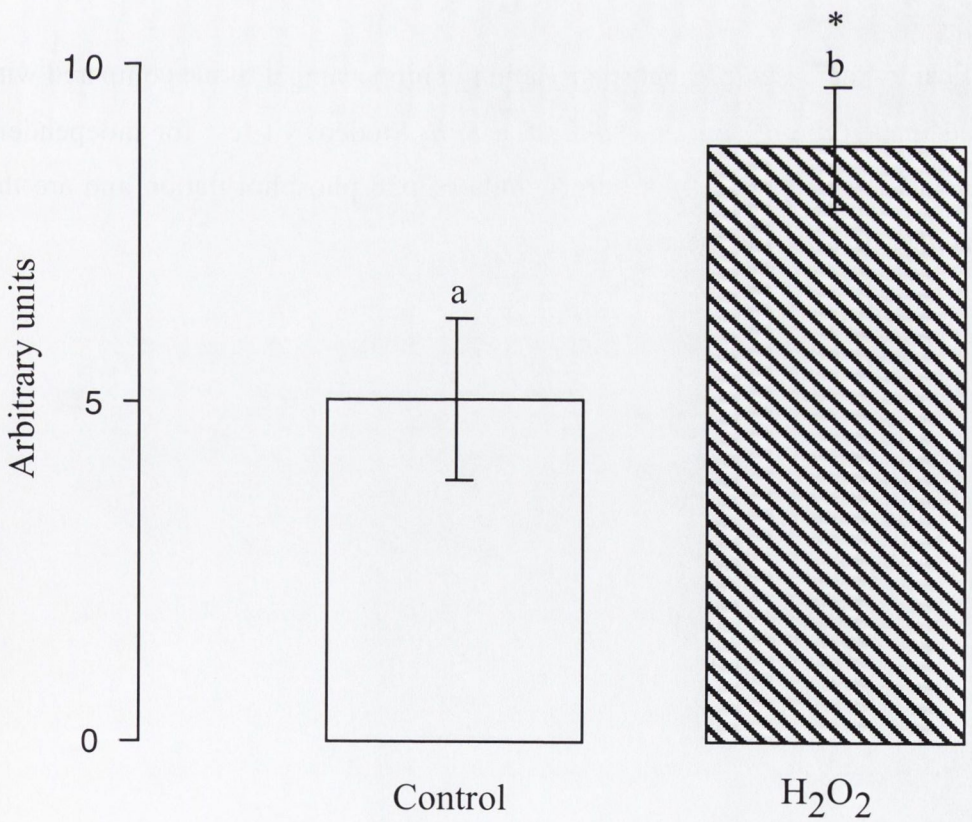
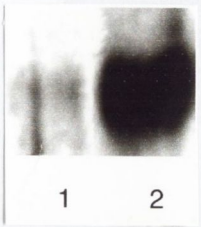


Figure 3.22: Interleukin-1β increases p38 phosphorylation in hippocampal tissue.

A) A sample immunoblot shows that p38 phosphorylation is increased in tissue incubated in the presence of interleukin-1β (IL-1β; 100ng/ml; lane 2) compared with tissue incubated in the presence of the control buffer (Saline; lane 1).

B) IL-1β significantly increased p38 phosphorylation in hippocampal tissue compared with tissue incubated in the control buffer (*$p<0.05$ *a* v *b*, Student's t-test for independent means). The values are expressed as arbitrary units of p38 phosphorylation and are the means ± the standard error of the means.

A



B

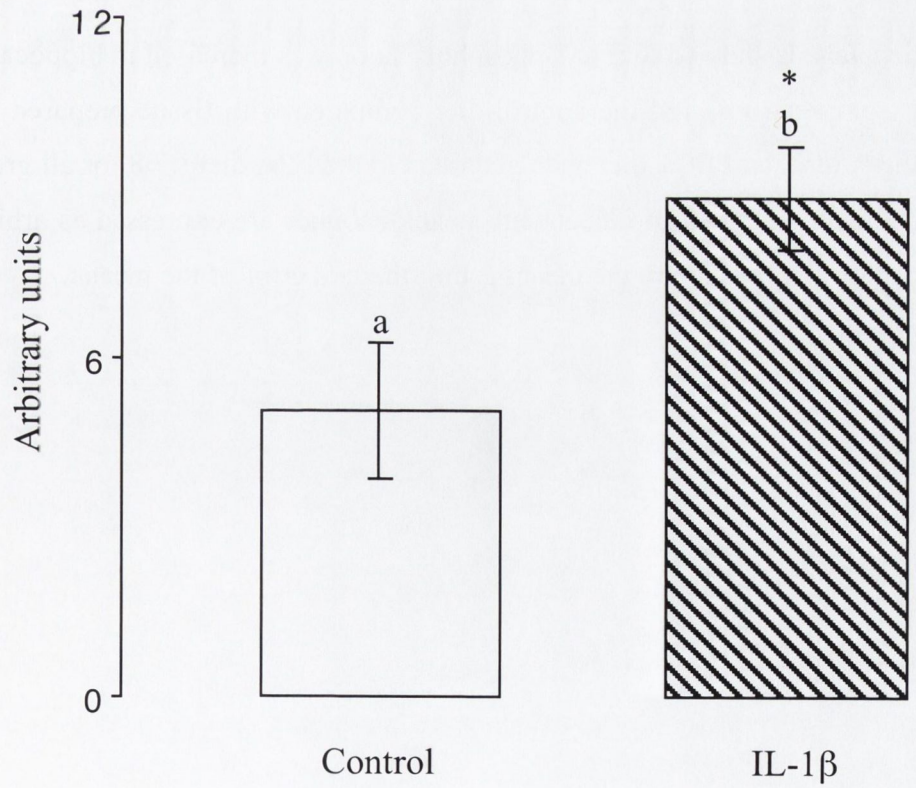


Figure 3.23: Age is associated with an increase in p38 mitogen-activated protein kinase phosphorylation in the hippocampus.

A) One sample immunoblot shows that p38 phosphorylation was increased in hippocampal tissue prepared from aged rats fed on the control diet (lane 3) compared with tissue prepared from young rats fed on the control (lane 1) or experimental (lane 2) diet or tissue prepared from aged rats fed the EPA diet (lane 4).

B) Densitometric analysis showed that p38 phosphorylation was increased in hippocampal tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed the control and EPA diet and aged rats fed the EPA diet (n=8 for all groups; *$p<0.05$ *a* v *c*, Student's t-test for independent means). Values are expressed as arbitrary units of p38 phosphorylation and are the means ± the standard error of the means.

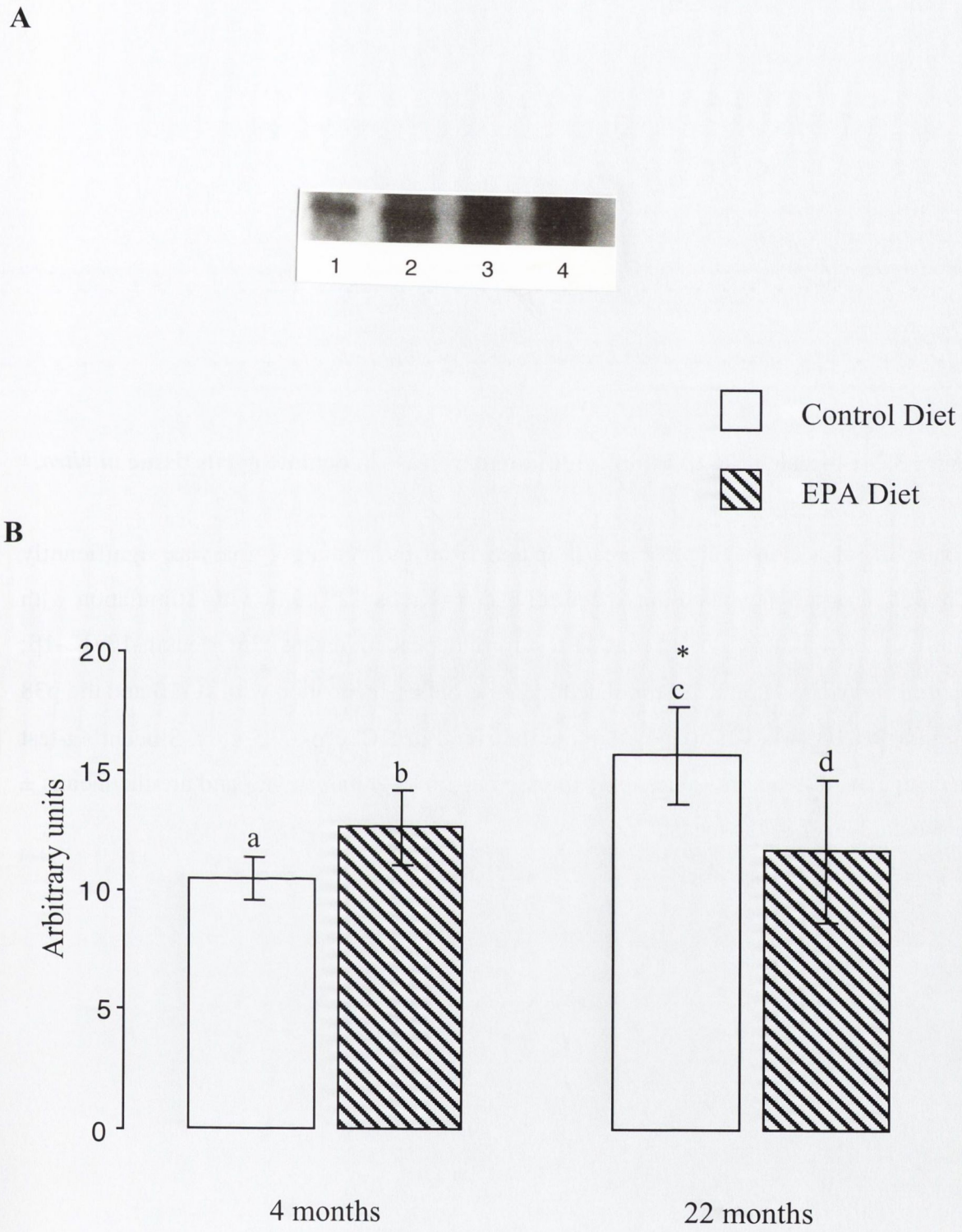

A
1
2
3
4
Control Diet
EPA Diet
B
20
15
10
5
0
Arbitrary units
a
b
*
c
d
4 months
22 months

Figure 3.24: Interleukin-1β inhibits glutamate release in dentate gyrus tissue *in vitro*.

Glutamate release in synaptosomes prepared from the dentate gyrus was significantly increased in tissue incubated in the control buffer (Krebs Ca^{2+}) following stimulation with 40mM KCl (*$p<0.05$ *a* v *b*, Student's t-test for independent means). Interleukin-1β (IL-1β; 10pg/ml) prevented this KCl stimulated increase but co-incubation with IL-1β and the p38 inhibitor SB203580 (SB; 50μM) restored the effect of KCl (*$p<0.05$ *e* v *f*, Student's t-test for independent means). Values are expressed as μmol glutamate/mg and are the means ± the standard error of the means.

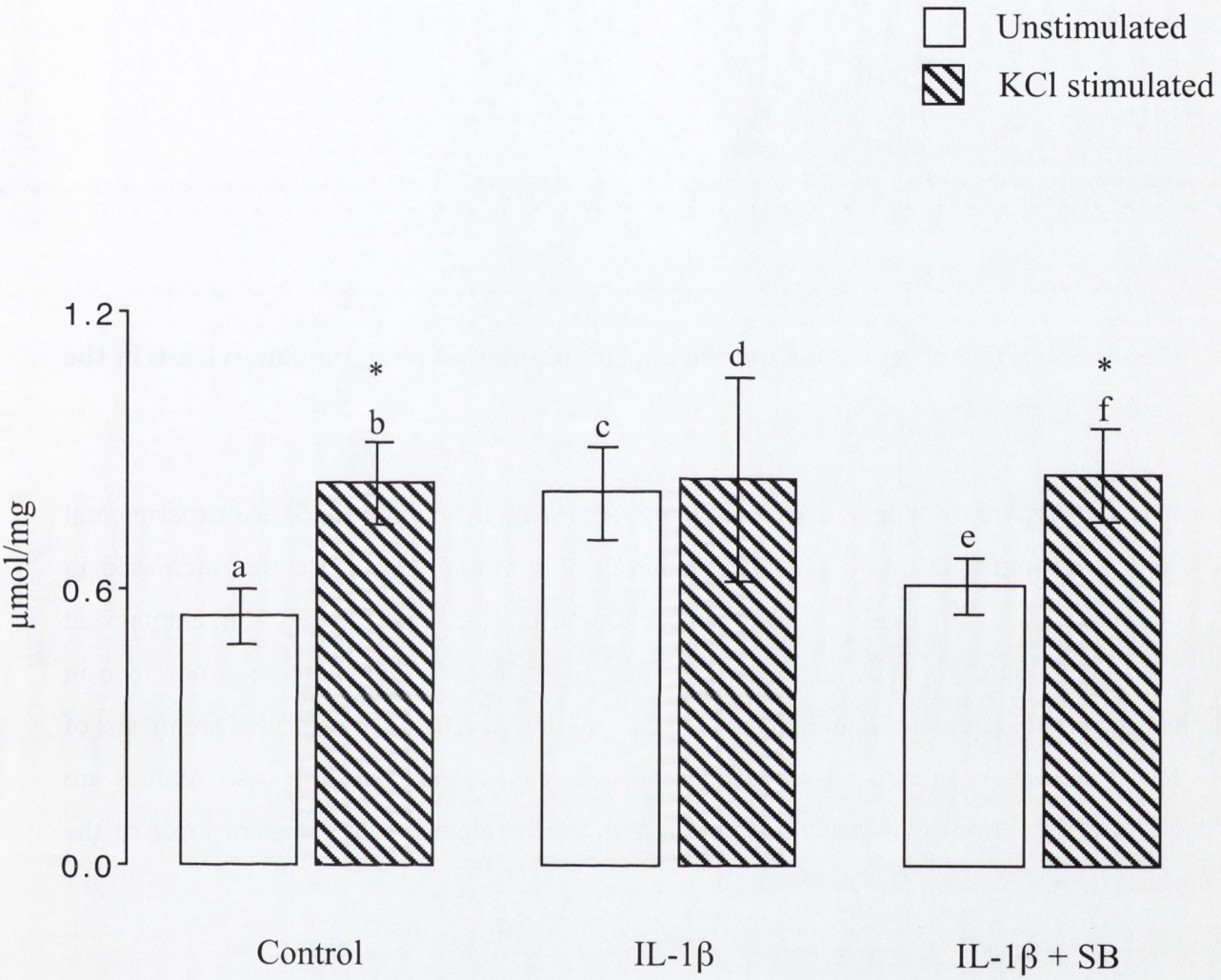

Unstimulated
KCl stimulated
1.2
0.6
0.0
µmol/mg
a
*
b
c
d
e
*
f
Control
IL-1β
IL-1β + SB

Figure 3.25: The effect of age and dietary manipulation on glutamate release in the dentate gyrus.

In tissue prepare from dentate gyri of young rats fed on the control (n=5) and experimental (n=6) diet and aged rats fed the EPA diet (n=11) glutamate release was increased in tetanized tissue compared with basal concentrations of glutamate released from untetanized tissue, although this does not reach statistical significance. This increase is not seen in tissue prepared from aged animals fed the control diet (n=10). Data presented are means of KCl (40mM) stimulated release (minus unstimulated release) in each case. Values are expressed as μmol glutamate/mg protein and are the means ± the standard error of the means.

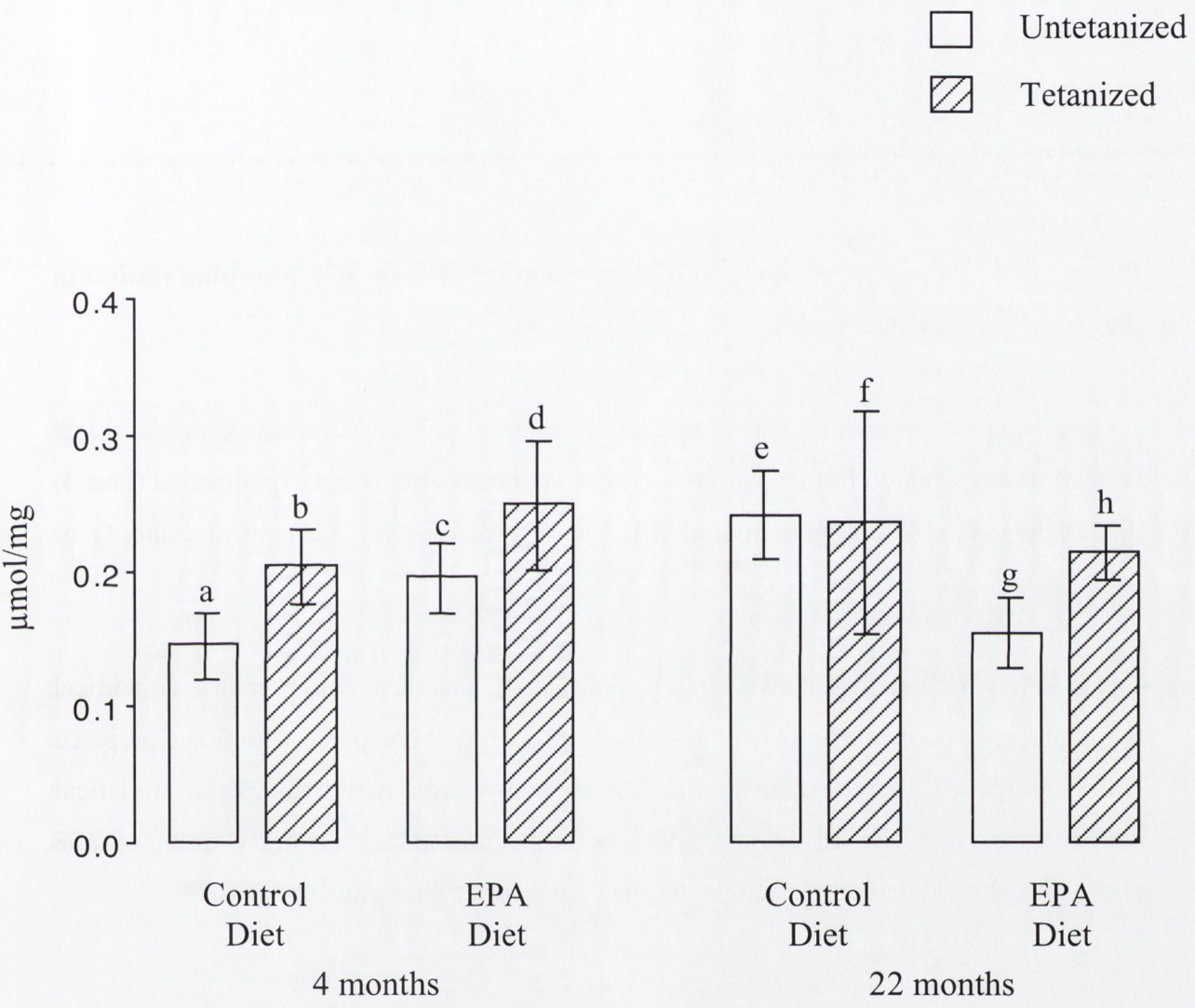
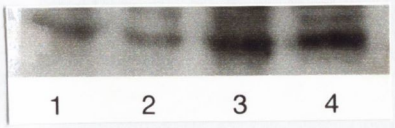

Untetanized
Tetanized
0.4
0.3
0.2
0.1
0.0
µmol/mg
a
b
c
d
e
f
g
h
Control Diet
EPA Diet
Control Diet
EPA Diet
4 months
22 months

Figure 3.26: The effect of age and dietary manipulation on p38 phosphorylation in the entorhinal cortex.

A) One sample immunoblot shows that p38 phosphorylation was increased in entorhinal cortical tissue prepared from aged rats fed on the control (lane 3) and experimental (lane 4) diet compared with tissue prepared from young rats fed on the control (lane 1) or experimental (lane 2) diet.

B) Densitometric analysis shows p38 phosphorylation was increased in entorhinal cortical tissue prepared from aged rats fed the control and EPA diet compared with tissue prepared from young rats fed the control or EPA diet, although it did not reach statistical significance (n=5 for all groups). Values are expressed as arbitrary units of p38 phosphorylation and are the means ± the standard error of the means.

A


1
2
3
4

B

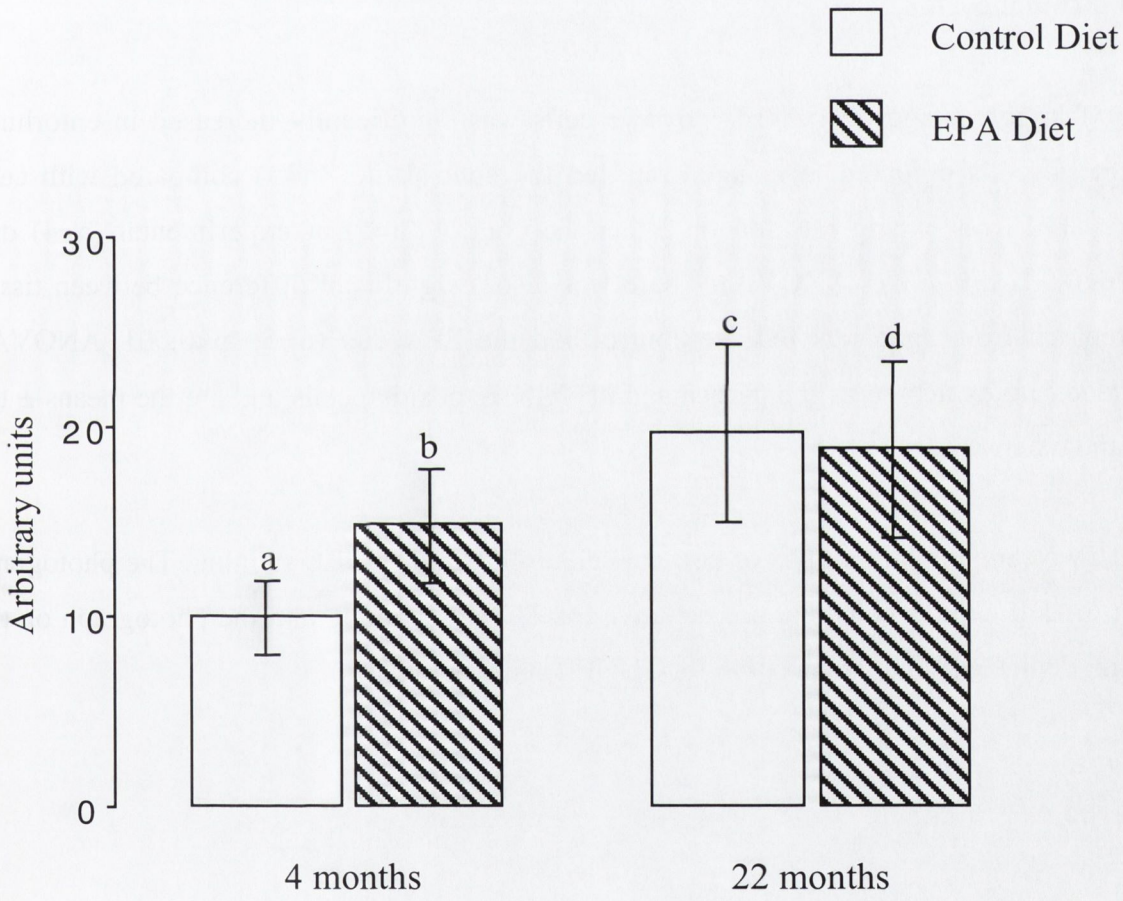
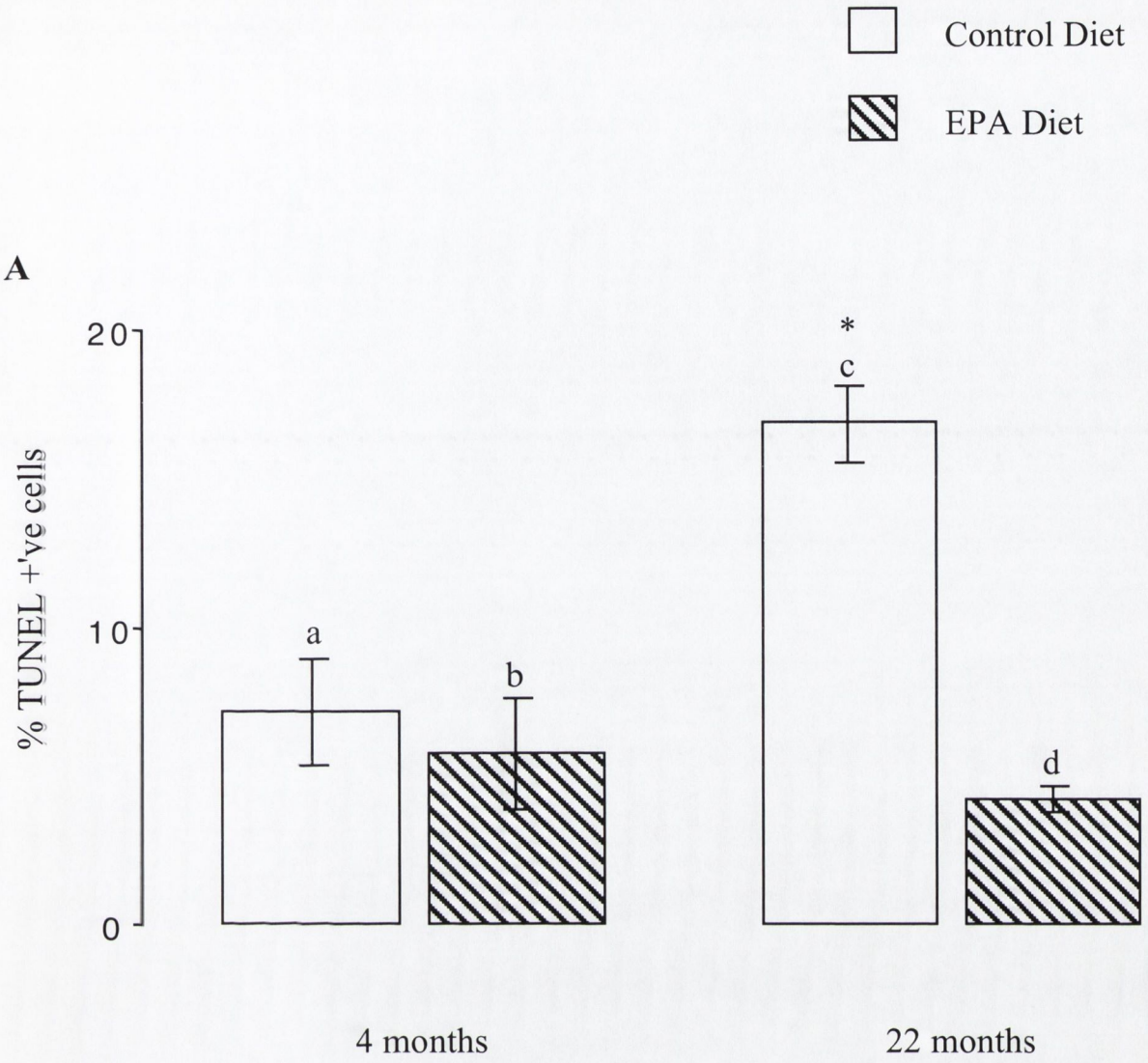

Control Diet
EPA Diet
Arbitrary units
30
20
10
0
a
b
c
d
4 months
22 months

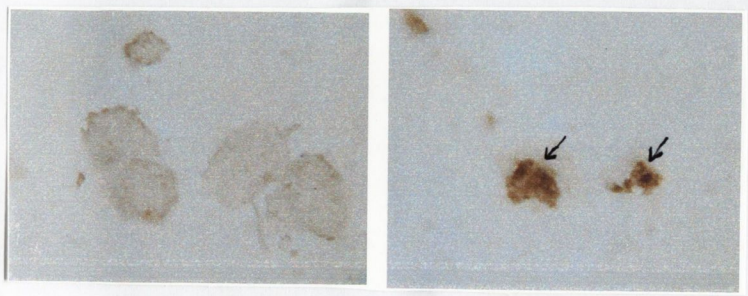
Figure 3.27: Age is associated with an increase in TUNEL positive cells in the entorhinal cortex.

A) The percentage of TUNEL positive cells was significantly increased in entorhinal cortical cells prepared from aged rats fed the control diet (n=5) compared with cells prepared from young rats fed on either the control (n=5) or experimental (n=4) diet (*$p<0.001$ *a/b/d* v *c*, ANOVA). There was also a significant difference between tissue prepared from aged rats fed the control and the EPA diet (n=6; $^{+}p<0.001$, ANOVA). Values are expressed as the percentage of TUNEL positive cells and are the means ± the standard error of the means.

B) Two sample photographs of cortical cells following TUNEL staining. The photograph on the left depicts cells that are negative for TUNEL staining and the photograph on the right depict cells that are positive for TUNEL staining (arrows).


Control Diet
EPA Diet
A
20
10
0
% TUNEL +'ve cells
a
b
*
c
d
4 months
22 months

B



ANALYSIS OF CHANGES IN CORTICAL TISSUE PREPARED FROM AGED AND YOUNG RATS FED ON A DIET SUPPLEMENTED WITH EICOSAPENTAENOIC ACID.

As an increase in apoptosis was observed in the entorhinal cortex of aged rats, the pathway that may lead from an increase in ROS production or IL-1β concentration to cell death was assessed in the cortex of young and aged rats. Apoptotic signals been suggested to lead to an increase in the release of cytochrome c from the mitochondria (Wang and Cortopassi, 1998) which, in turn, may lead to an increase in the activation of caspase-3. The ability of the EPA diet to reverse any observed changes was assessed. This may be due to a possible anti-inflammatory role, thereby reducing the age-related increase in IL-1β.

Analysis of the cortical tissue obtained from same aged and young rats fed the EPA diet (10mg/rat/day for 3 weeks and then 20mg/rat/day for 5weeks) is shown in the next set of results (see Appendix I for mean data).

3.28 Reactive oxygen species production in the cortex.

ROS production was marginally increased in cortical tissue prepared from aged rats fed the control (oil enriched in monounsaturated fatty acids) compared with tissue prepared from young rats fed on either diet, although this did not reach statistical significance (Figure 3.28). The EPA diet did not affect this marginal increase, and this was also seen in the hippocampus of the same study.

3.29 Age is associated with an increase in cortical interleukin-1β concentration.

IL-1β concentration was assessed in cortical tissue and was shown to be significantly increased in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed the control diet (*$p<0.05$ *a* v *c*, Student's t-test for independent means; Figure 3.29). There was no increase seen in tissue prepared from the aged rats fed on the EPA diet. Although the diet did not seem to be effective in reversing the marginal age-related increase in ROS production in the cortex it was effective in reversing the age-related increase in IL-1β concentration.

3.30 Tumour Necrosis Factor-α concentration in the cortex.

The concentration of a second proinflammatory cytokine Tumour Necrosis Factor-α (TNF-α) was assessed in tissue prepared from young and aged rats fed either diet as a concomitant increase in TNF-α and IL-1β has been reported in several conditions. However, as Figure 3.30 shows, the concentration of TNF-α was similar in all dietary and age groups.

3.31 Age is associated with an increase in cortical p38 phosphorylation.

IL-1β concentration and p38 phosphorylation were found to be closely linked in other studies (see section 3.23 and 3.24) and, therefore, p38 activity was assessed in this study. Figure 3.31A shows one sample immunoblot indicating an increase in p38 phosphorylation in tissue prepared from aged rats fed the control diet (lane 3) compared with tissue prepared from young rats fed the control (lane 1) or young (lane 2) or aged (lane 4) rats fed the EPA diet. Figure 3.31B shows the data obtained following densitometric analysis; this reveals a significant increase in p38 phosphorylation in cortical tissue prepared from aged rats fed the control diet compared with young rats fed on the EPA diet (*$p<0.05$ *b* v *c*, Student's t-test for independent means). The EPA diet appeared to be successful in reversing this age-related increase, however, due to a large standard error the statistical difference between the cortical tissue prepared from aged rats fed on the control diet and those fed on the EPA diet did not reach significance.

3.32 Interleukin-1β increases p38 phosphorylation in cortical tissue *in vitro*.

Since IL-1β has already been shown to increase p38 phosphorylation in hippocampal tissue its ability of IL-1β to increase p38 phosphorylation was assessed in cortical tissue *in vitro*. One sample immunoblot indicates that IL-1β (3.5ng/ml; lane 2) increased p38 phosphorylation in cortical tissue prepared from young untreated rats compared with tissue incubated in the control buffer (Krebs Ca^{2+}; lane 1; Figure 3.32A). Mean data obtained from densitometric analysis revealed that IL-1β significantly increased p38 phosphorylation in cortical tissue (*$p<0.05$ *a* v *b*, Student's t-test for independent means; Figure 3.32B).

3.33 Age is associated with an increase in cortical cytochrome c translocation.

Cytochrome c, a protein of the inner membrane of the mitochondria, is released into the cytosol during times of cellular stress. To assess stress in tissue prepared from aged rats cytochrome c translocation to cytosol (hereafter called "cytochrome c expression") was assessed in cytosolic fractions prepared from cortical tissue. Figure 3.33A shows one sample immunoblot indicating that cytochrome c was increased in cortical tissue prepared from aged rats fed the control diet (lane 3) compared with tissue prepared from young rats fed the control diet (lane 1) and young (lane 2) and aged (lane 4) rats fed the EPA diet. Mean data obtained from densitometric analysis shows that cytochrome c was significantly increased in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed the control diet (*$p<0.05$ *a* v *c*, Student's t-test for independent means; Figure 3.33B). This significant increase was not seen in tissue prepared from aged rats fed the EPA diet.

3.34 Interleukin-1β increases cytochrome c translocation in cortical tissue *in vitro*; inhibition by the interleukin-1β receptor antagonist.

IL-1β in the presence and absence of the IL-1β receptor antagonist (IL-1ra) was assessed for its ability to increase cytochrome c expression in cytosolic fractions of cortical tissue. In Figure 3.34 (A) one sample immunoblot indicates that IL-1β (3.5ng/ml; lane 2) increased cytochrome c expression in cortical tissue prepared from young untreated rats compared with tissue incubated in the control buffer (lane 1), IL-1ra (350ng/ml; lane 3) or IL-1β + IL-1ra (lane 4; Figure 3.34A). Mean data obtained from densitometric analysis shows that IL-1β significantly increased cytochrome c expression in cytosolic fractions of cortical tissue compared with tissue incubated in the control buffer, IL-1ra or IL-1β + IL-1ra (*$p<0.01$ *a/c/d* v *b*, ANOVA; Figure 3.50B).

3.35 Interleukin-1β increases cytochrome c translocation in cortical tissue *in vitro*; inhibition by a specific p38 inhibitor.

The effect of the p38 inhibitor SB203580 (SB) was assessed on IL-1β-induced change in cytochrome c expression in cytosolic fractions of cortical tissue. One sample immunoblot indicates that IL-1β (3.5ng/ml; lane 2) increased cytochrome c expression in cortical tissue prepared from young untreated rats compared with tissue incubated in the control buffer (lane 1), SB (50μM; lane 3) or IL-1β + SB (lane 4; Figure 3.35A).

Mean data obtained from densitometric analysis shows that IL-1β significantly increased cytochrome c expression in cortical tissue compared with tissue incubated in the control buffer, SB or IL-1β + SB (*p<0.05 *a/c/d* v *b*, ANOVA; Figure 3.35B). These findings suggest that one way in which IL-1β increases cytochrome c expression in the cytosol of cortical tissue is via the actions of p38.

3.36 H_2O_2 increases cytochrome c translocation in cortical tissue *in vitro*.

The effect of H_2O_2 on cytochrome c expression in cytosolic fractions of cortical tissue was assessed. One sample immunoblot indicates that H_2O_2 (200μM; lane 2) increased cytochrome c expression in cortical tissue prepared from young untreated rats compared with tissue incubated in the control buffer (lane 1; Figure 3.52A). Mean data obtained from densitometric analysis shows that H_2O_2 significantly increased cytochrome c expression compared with tissue incubated in the control buffer (*p<0.05 *a* v *b*, Student's t-test for independent means).

3.37 Expressions of Bcl-2 or Bax are unchanged with age or dietary manipulation in cortical mitochondrial preparations.

Two proteins which may contribute to the release of cytochrome c from the mitochondria into the cytosol are Bcl-2 and Bax. The expression of these proteins was assessed in mitochondrial fractions prepared from cortical tissue of the young and aged rats. One sample immunoblot indicates that the expression of Bcl-2 was similar in tissue prepared from young rats fed the control (lane 1) and EPA diet (lane 2) and aged rats fed the control (lane 3) and EPA (lane 4; Figure 3.37A) diet. Figure 3.37B shows the mean data obtained from densitometric analysis and these data confirm that the expression of Bcl-2 was similar in all groups. One sample immunoblot indicates that the expression of Bax was similar in tissue prepared from young rats fed the control (lane 1) and experimental (lane 2) and aged rat fed the control (lane 3) and experimental (lane 4; Figure 3.37C) diet. Figure 3.37D shows the mean data obtained from densitometric analysis and these data confirm that the expression of Bax was similar in all groups.

3.38 The ratio of Bcl-2 to Bax is also unchanged in cortical mitochondrial preparations.

The ratio of Bcl-2, which is anti-apoptotic, and Bax, which is pro-apoptotic, is considered to be important in determining cell survival. Densitometric analysis of the individual bands in the separate experiments allowed ratios of Bcl-2 to Bax to be calculated. Figure 3.38B shows that the ratio of Bcl-2:Bax was similar in all groups.

3.39 Age is associated with an increase in cortical caspase-3 activation.

Figure 3.39 shows that caspase-3 activation was significantly increased in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed either the control or EPA diet and tissue prepared from aged rats fed the EPA diet (*$p<0.05$ *a/b/d* v *d*, ANOVA). The diet was effective in reversing the age-related increase in cortical caspase-3 activation.

3.40 Interleukin-1β increases caspase-3 activation in cortical tissue *in vitro*; inhibition by the receptor antagonist.

The ability of IL-1β to increase caspase-3 activation was assessed in cortical tissue prepared from young untreated rats. Figure 3.40 shows that IL-1β (3.5ng/ml) significantly increased caspase-3 activation in cortical tissue compared with tissue incubated in the control buffer (*$p<0.05$ *a* v *b*, Student's t-test for independent means). The ability of the receptor antagonist to block this increase was also assessed and the data show that the IL-1β-induced increase in caspase-3 activation was attenuated in tissue incubated with both IL-1β and IL-1ra (350ng/ml).

3.41 Interleukin-1β increases caspase-3 activation in cortical tissue *in vitro*; inhibition by the p38 inhibitor.

The effect of the p38 inhibitor SB203580 (SB) was assessed on the IL-1β-induced increase in caspase-3 activation was assessed. Figure 3.41 shows that IL-1β (3.5ng/ml) significantly increased caspase-3 activation in cortical tissue prepared from young untreated rats compared with tissue incubated in the control buffer (Krebs Ca^{2+}) and with the p38 inhibitor SB (*$p<0.05$ *a/c/d* v *b*, Student's t-test for independent means). This increase was not seen in tissue incubated in the presence of both IL-1β +

SB. Therefore, one way in which IL-1β increases caspase-3 activation is likely to be via the actions of p38.

3.42 H_2O_2 increases caspase-3 activity in cortical tissue *in vitro*; inhibition by a p38 inhibitor.

The effect of H_2O_2 (200μM) on caspase-3 activity in the presence and absence of the p38 inhibitor was assessed. Figure 3.42 shows that H_2O_2 significantly increases caspase-3 activation in cortical tissue prepared from young, untreated rats compared with tissue incubated in either the control buffer (Krebs Ca^{2+}), the p38 inhibitor SB203580 (SB) or H_2O_2 + SB (*p<0.05 *a/c/d* v *b*, ANOVA).

3.43 Age is associated with a decrease in poly-(ADP-ribose) polymerase expression in cortical tissue.

Poly- (ADP-ribose) polymerase (PARP) is a 116kD protein that can be cleaved by caspase-3 resulting in its inactivation which in turn prevents its ability to act as a DNA repair enzyme. With an age-related increase in caspase-3 already shown, PARP expression was assessed. One sample immunoblot indicates that the 116kD PARP expression was decreased in cortical tissue prepared from aged rats fed the control diet (lane 3) compared with tissue prepared from young rats fed the control (lane 1) and young (lane 2) and aged (lane 4) rats fed the EPA diet (Figure 3.43A). Mean data obtained from densitometric analysis shows that there was a significant decrease in PARP expression in cortical tissue prepared from aged rats fed the control diet compared with young rats fed the control diet (*p<0.05 *a* v *c*, Student's t-test for independent means; Figure 3.43B). This age-related decrease was not seen in the tissue prepared from the aged rats fed the EPA diet suggesting that the EPA-diet attenuates the aging effect on PARP.

3.44 Age is associated with an increase in TUNEL positive cortical cells.

TUNEL staining is considered to be an indicator of cell death. With a compromise in the ability of PARP to carry out its role as a DNA repair enzyme, cells may undergo cell death; this was assessed in cells prepared from young and aged rats fed either the control or EPA diet. Figure 3.44 shows that the mean percentage of TUNEL positive cells was significantly increased in the cortex prepared from aged rats

fed the control diet compared with cells prepared from young rats fed either the control or EPA diet (*$p<0.05$ *a/b* v *c*, ANOVA). This increase was not seen in the cells prepared from aged rats fed the EPA diet and suggests that dietary manipulation may spare the cells from DNA nicking.

Figure 3.28: Reactive oxygen species production in the cortex.

Reactive oxygen species (ROS) production was marginally increased in cortical tissue prepared from aged rats fed the control (n=12) and experimental (n=13) diets compared with tissue prepared from young rats fed either the control (n=10) or experimental (n=10) diets. These values did not reach statistical significance. Values are expressed as nmol DCF/mg tissue corrected for protein and are the means ± the standard error of the means.

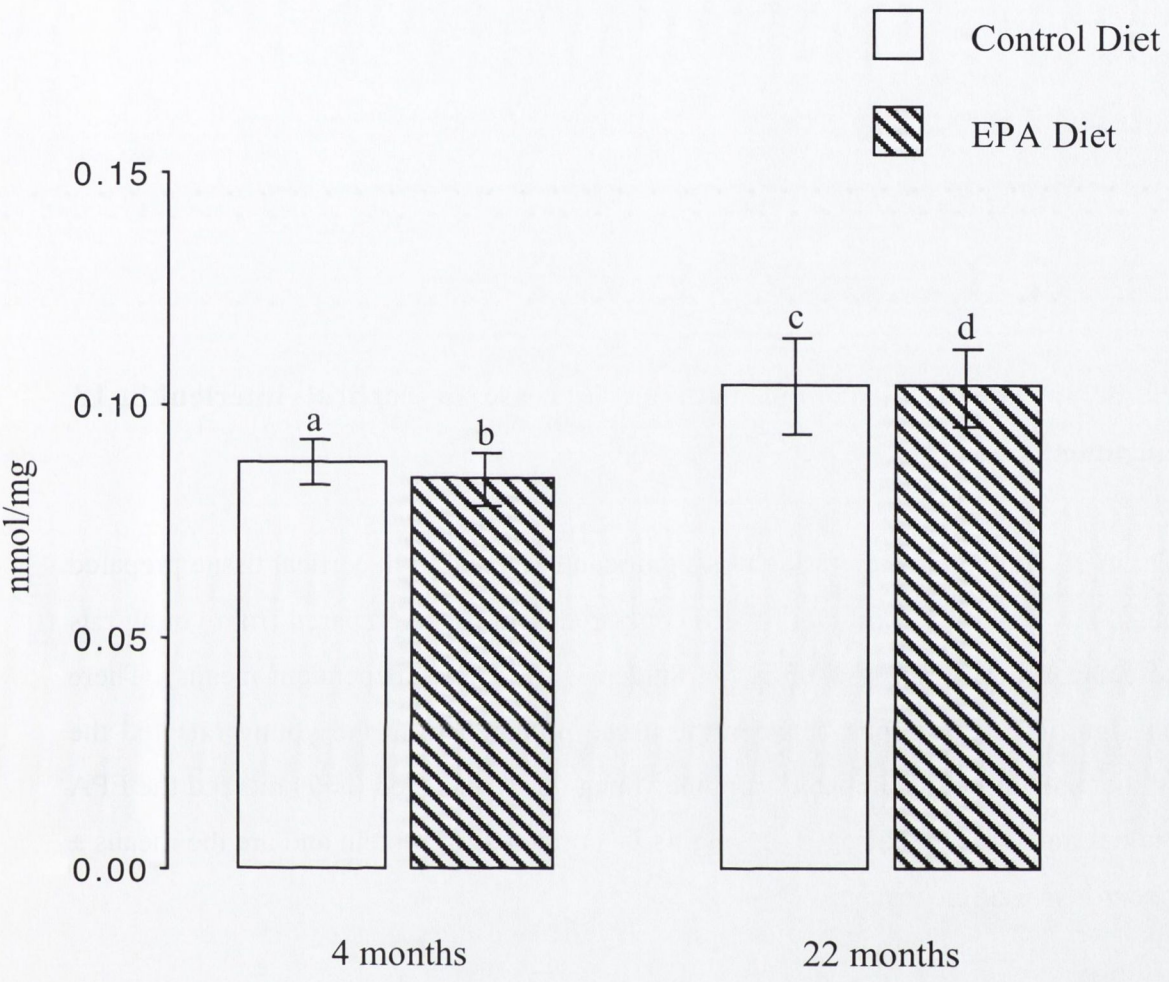

Control Diet
EPA Diet
0.15
0.10
0.05
0.00
nmol/mg
a
b
c
d
4 months
22 months

Figure 3.29: Age is associated with an increase in cortical interleukin-1β concentration.

Interleukin-1β (IL-1β) concentration was significantly increased in cortical tissue prepared from aged rats fed the control diet (n=10) compared with tissue prepared from young rats fed the control diet (n=8; *$p<0.05$ *a* v *c*, Student's t-test for independent means). There was no significant difference between the tissue prepared from the young rats fed the control diet and the tissue prepared from the young (n=7) and aged (n=7) rats fed the EPA diet. Values are expressed as pg IL-1β/mg tissue corrected for protein and are the means ± the standard error of the means.

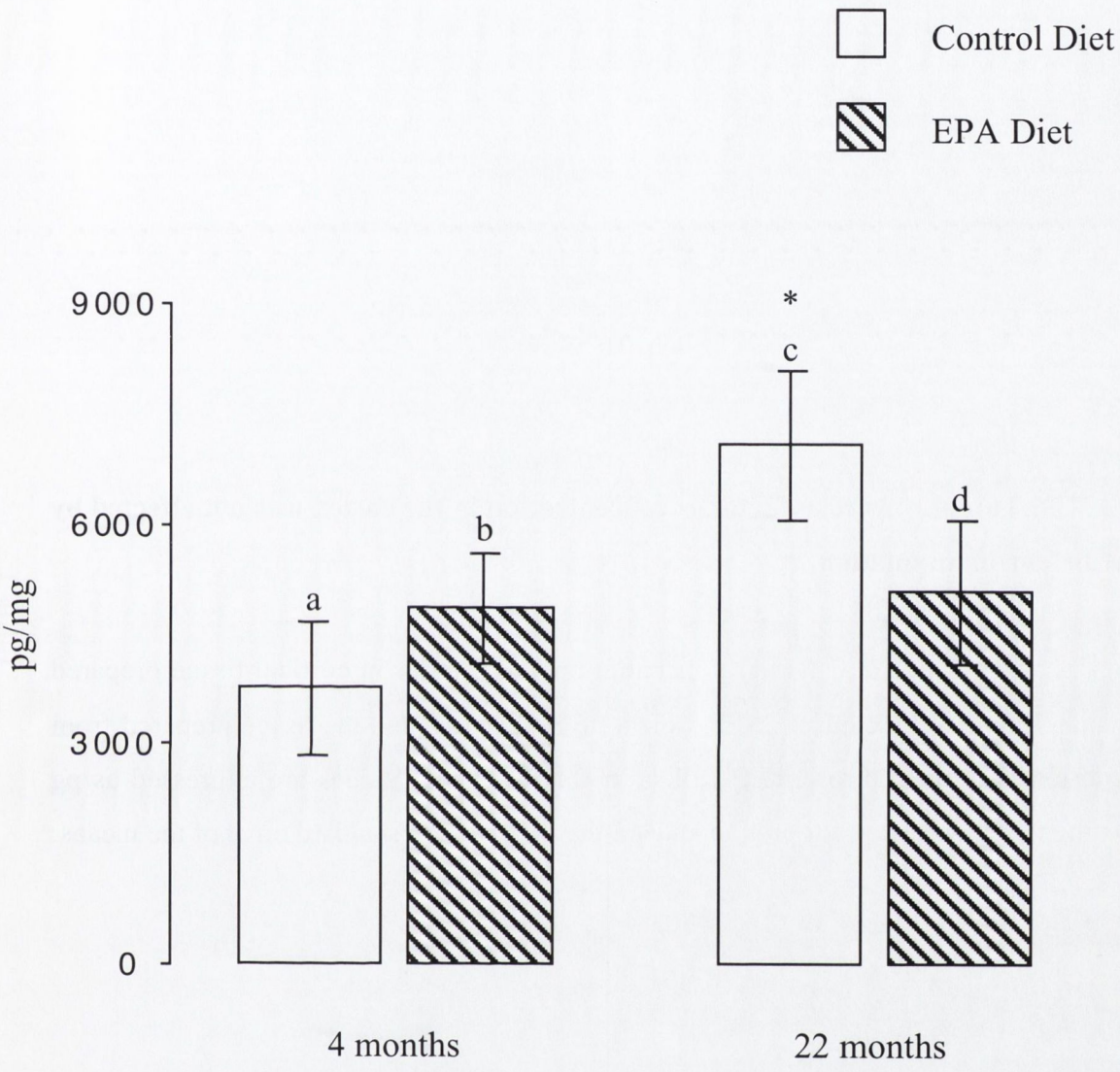

Control Diet
EPA Diet
9000
6000
3000
0
pg/mg
a
b
*
c
d
4 months
22 months

Figure 3.30: Tumour necrosis factor-α concentration in the cortex was not effected by age or dietary manipulation.

Tumour necrosis factor-α (TNF-α) concentration was similar in cortical tissue prepared from young rats fed the control or EPA diets (n=8 for each) and the tissue prepared from the aged rats fed the control or EPA diets (n=11 for each). Values are expressed as pg TNF-α/mg tissue corrected for protein and are the means ± the standard error of the means.

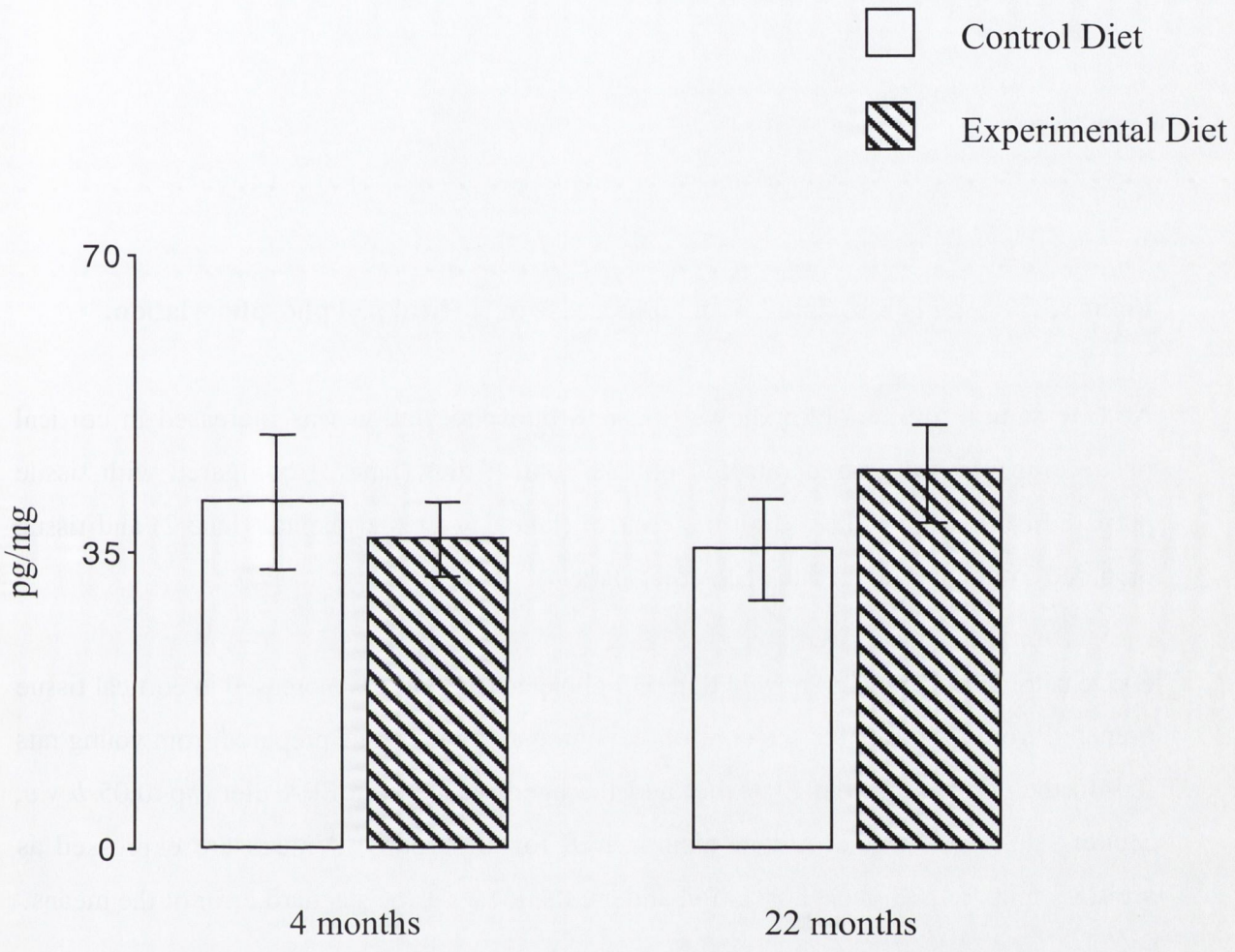

Control Diet
Experimental Diet
70
35
0
pg/mg
4 months
22 months

Figure 3.31: Age is associated with an increase in cortical p38 phosphorylation.

A) One sample immunoblot shows that p38 phosphorylation was increased in cortical tissue prepared from aged rats fed on the control diet (lane 3) compared with tissue prepared from young rats fed on the control (lane 1) or experimental (lane 2) and tissue prepared from aged rats fed the EPA diet (lane 4).

B) Densitometric analysis showed that p38 phosphorylation was increased in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed on the control and the EPA diet and the aged rats fed the EPA diet (*$p<0.05$ *b* v *c*, Student's t-test for independent means; n=8 for all groups). Values are expressed as arbitrary units of p38 phosphorylation and are the means ± the standard error of the means.

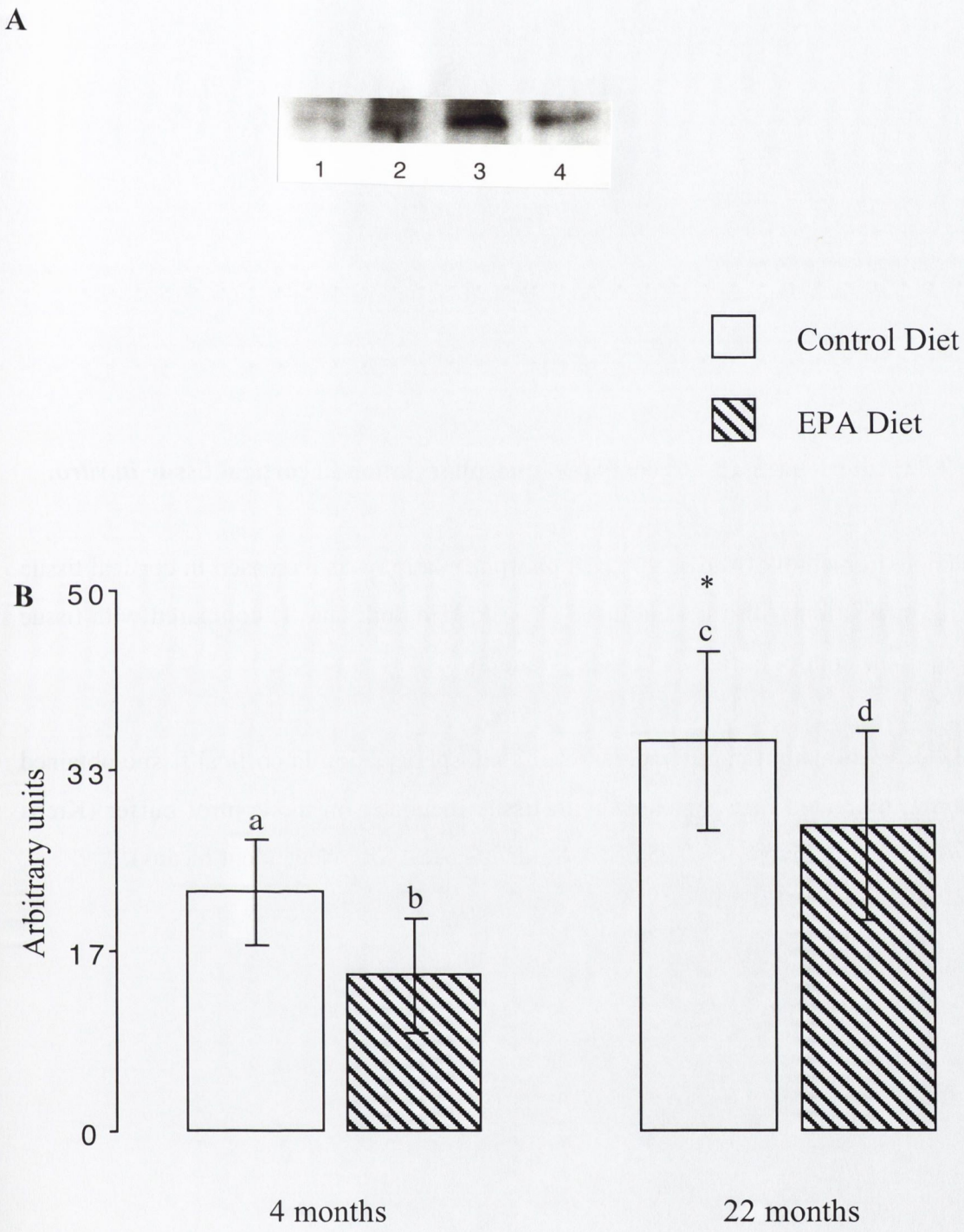

A
1
2
3
4
Control Diet
EPA Diet
B
50
33
17
0
Arbitrary units
a
b
*
c
d
4 months
22 months

Figure 3.32: Interleukin-1β increases p38 phosphorylation in cortical tissue *in vitro*.

A) A sample immunoblot shows that p38 phosphorylation was increased in cortical tissue following incubation with interleukin-1β (IL-1β; 3.5ng/ml; lane 1) compared with tissue incubated in the control buffer (Krebs Ca^{2+}; lane 2).

B) IL-1β (1ng/ml) significantly increased p38 phosphorylation in cortical tissue obtained from young untreated rats compared with tissue incubated in the control buffer (Krebs Ca^{2+}; n=6 for both groups; *$p<0.05$ *a* v *b*, Student's t-test for independent means).

A



B

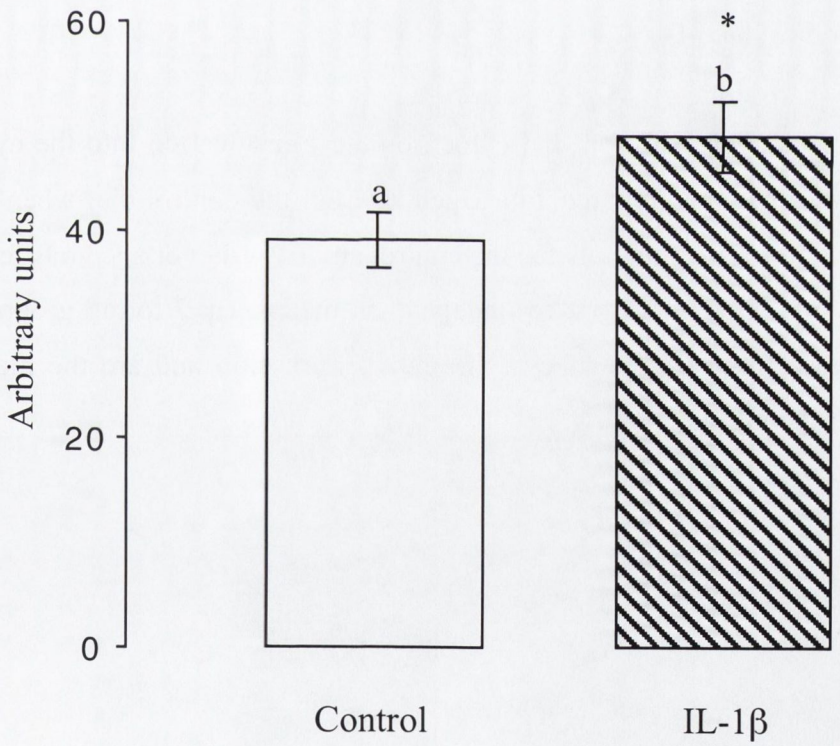


Figure 3.33: Age is associated with an increase in cortical cytochrome c translocation.

A) One sample immunoblot shows that cytochrome c translocation was increased in cortical tissue prepared from aged rats fed on the control diet (lane 3) compared with tissue prepared from young rats fed on the control (lane 1) or experimental (lane 2) diet or aged rats fed the EPA diet (lane 4).

B) Densitometric analysis showed that cytochrome c translocation into the cytosol was increased in cortical tissue prepared from aged rats fed the control diet when compared with tissue prepared from young rats fed the control and EPA diet or aged rats fed the EPA diet (*$p<0.05$ *a* v *c*, Student's t-test for independent means; (n=7 for all groups). Values are expressed as arbitrary units of cytochrome c expression and are the means ± the standard error of the means.

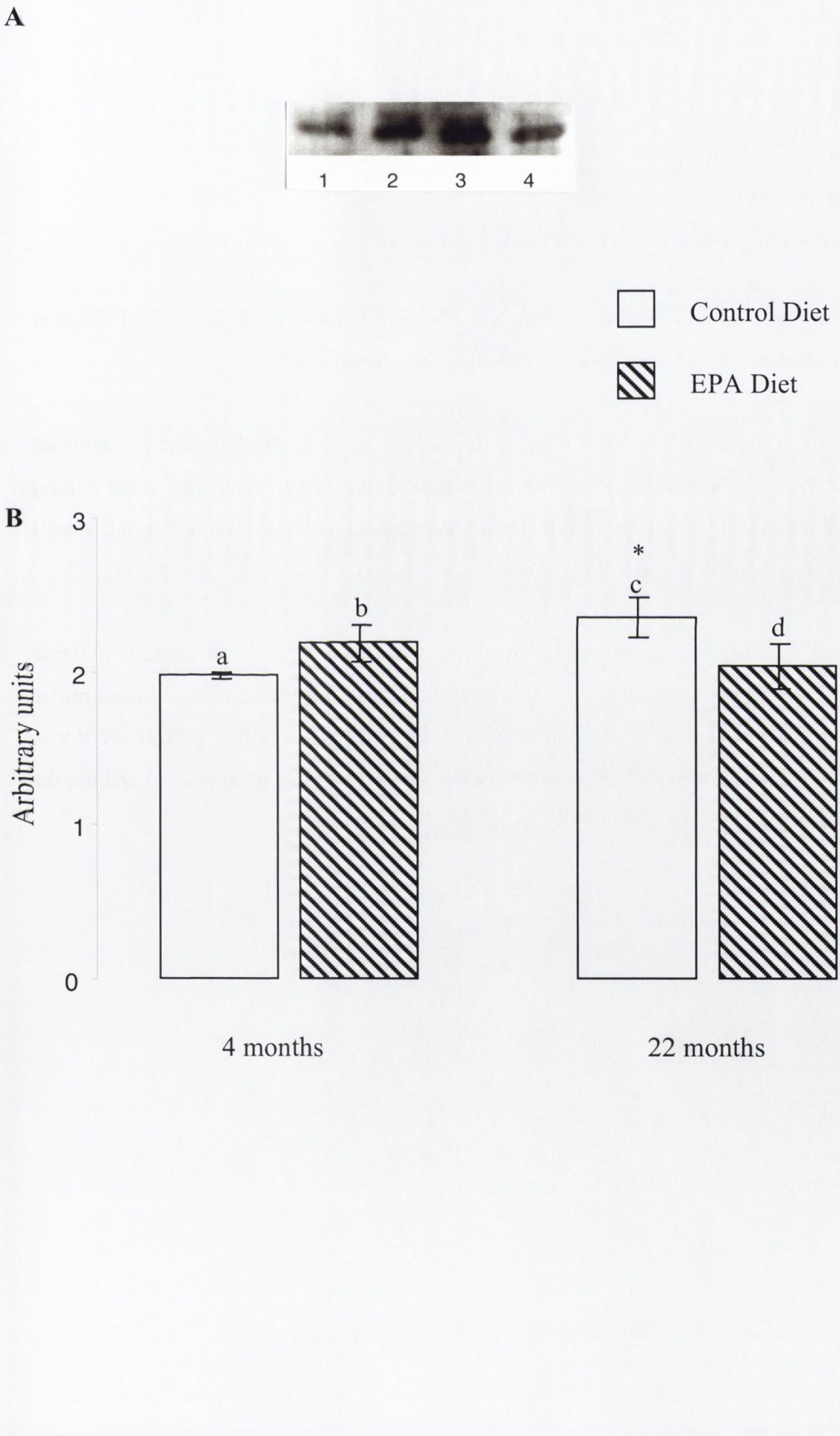
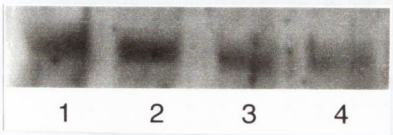

A
1
2
3
4
Control Diet
EPA Diet
B
Arbitrary units
3
2
1
0
a
b
*
c
d
4 months
22 months

Figure 3.34: Interleukin-1β increases cytochrome c translocation in cortical tissue *in vitro*; inhibition by the interleukin-1 receptor antagonist.

A) A sample immunoblot shows that interleukin-1β (IL-1β; 3.5ng/ml; lane 2) increased cytochrome c expression in cortical tissue compared with tissue incubated in the control buffer (Krebs Ca^{2+}; lane 1), the interleukin-1 receptor antagonist (IL-1ra; 350ng/ml; lane 3) or co-incubation with IL-1β + IL-1ra (lane 4)

B) IL-1β significantly increased cytochrome c expression when incubated in tissue prepared from young untreated rats compared with tissue incubated in the control buffer (n=7 for both) or IL-1ra or co-incubation with IL-1β (n=4 for both; *$p<0.01$ *a/c/d* v *b*, ANOVA). Values are expressed as arbitrary units of cytochrome c expression and are the means ± the standard error of the means.

A


1
2
3
4

B

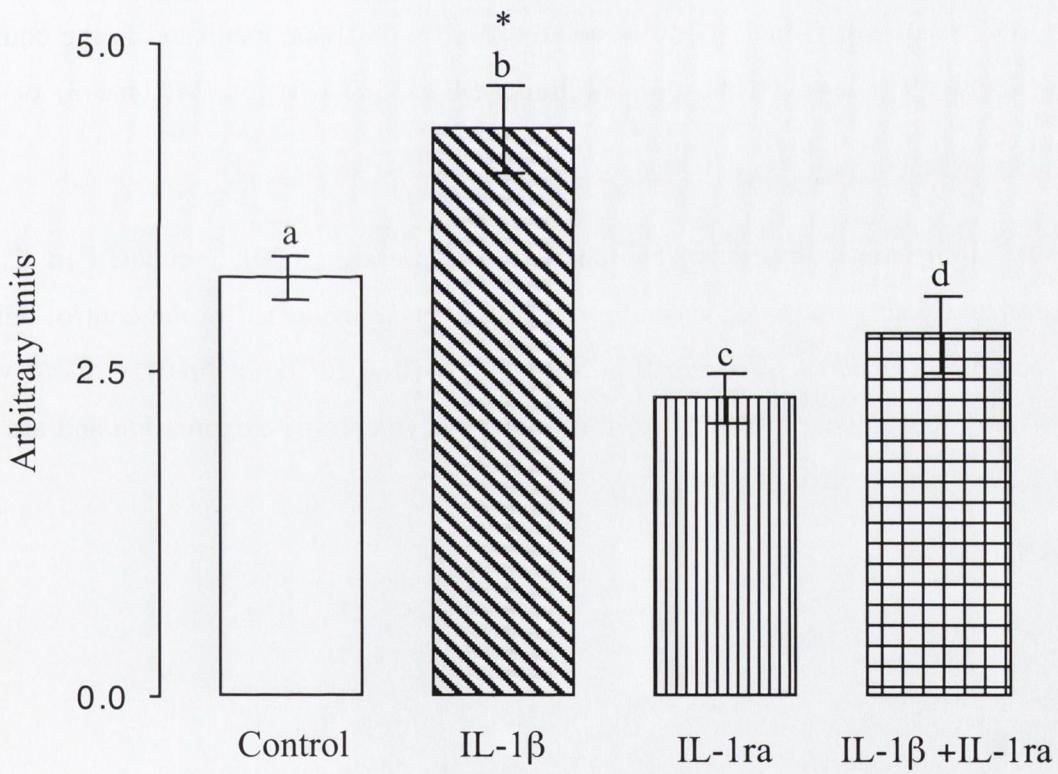
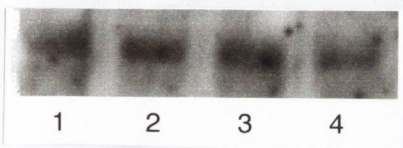

5.0
2.5
0.0
Arbitrary units
*
a
b
c
d
Control
IL-1β
IL-1ra
IL-1β +IL-1ra

Figure 3.35: Interleukin-1β increases cytochrome c translocation in cortical tissue *in vitro*; inhibition by a specific p38 inhibitor.

A) A sample immunoblot shows that interleukin-1β (IL-1β; 3.5ng/ml; lane 2) increased cytochrome c expression in cortical tissue compared with tissue incubated in the control buffer (Krebs Ca^{2+}; lane 1), the p38 inhibitor, SB203580 (SB; 50μM; lane 3) or co-incubation with IL-1β (lane 4)

B) IL-1β significantly increased cytochrome c expression when incubated in tissue prepared from young untreated rats compared with tissue incubated in the control buffer (n=7 for both) or SB or co-incubation with IL-1β (n=4 for both; *$p<0.05$ *a/c/d* v *b*, ANOVA). Values are expressed as arbitrary units of cytochrome c expression and are the means ± the standard error of the means.

A



B

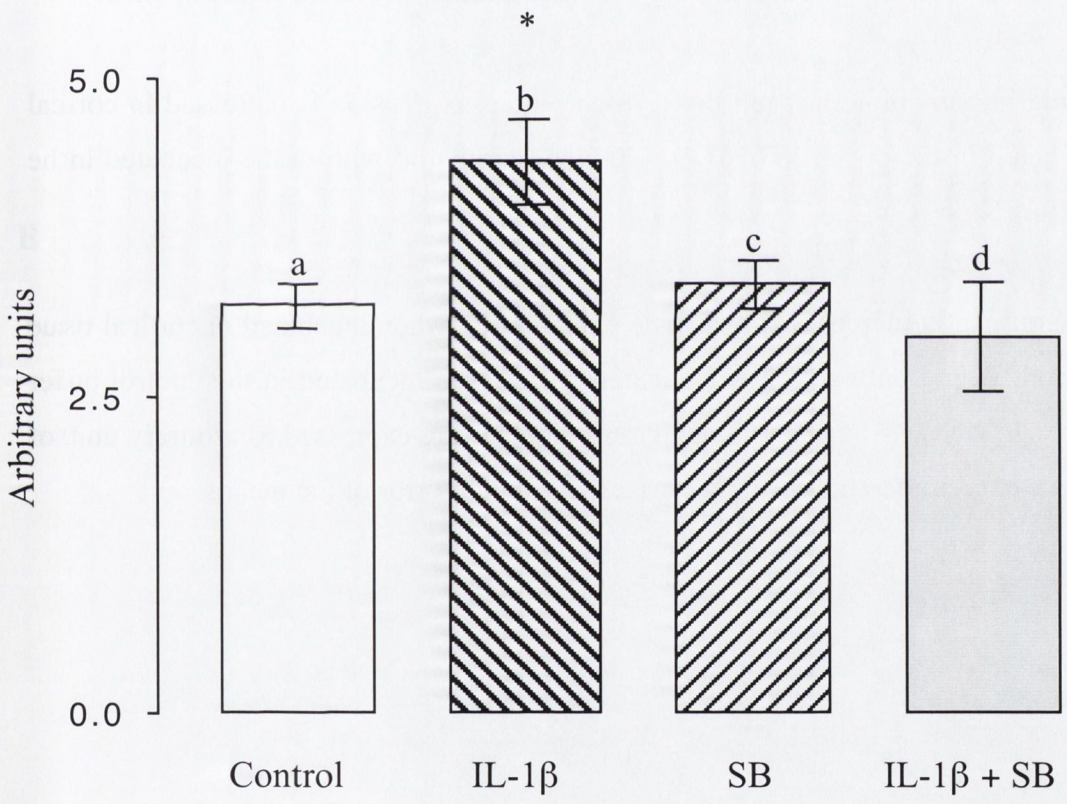
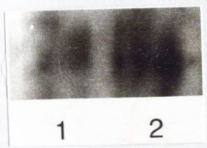


Figure 3.36: H_2O_2 increases cytochrome c translocation in cortical tissue *in vitro*.

A) A sample immunoblot showing that cytochrome c expression is increased in cortical tissue incubated in the presence of H_2O_2 (200μM) compared with tissue incubated in the control buffer (Krebs Ca^{2+}).

B) H_2O_2 significantly increases cytochrome c expression when incubated in cortical tissue prepared from young untreated rats compared with tissue incubated in the control buffer (*$p<0.05$ *a* v *b*, ANOVA; n=12 for each group). Values are expressed as arbitrary units of cytochrome c expression and are the means ± the standard error of the means.

A



B

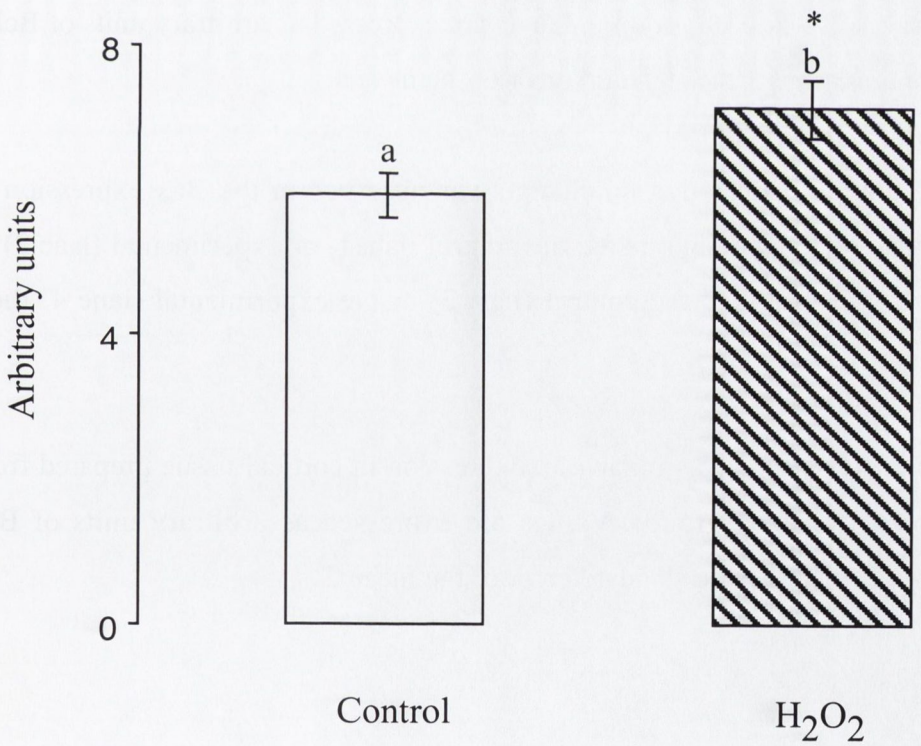


Figure 3.37: Expressions of Bcl-2 or Bax are unchanged with age or dietary manipulation in cortical mitochondrial preparations.

A) A sample immunoblot shows that no change was observed in the Bcl-2 expression in cortical tissue prepared from young rats fed the control (lane1) or experimental (lane 2) or tissue prepare from aged rats fed the control (lane 3) or the experimental (lane 4) diets (Bcl-2=25kD).

B) Densitometric analysis showed similar Bcl-2 expressions in cortical tissue prepared from all the groups (n=12 for each group). Values are expressed as arbitrary units of Bcl-2 expression and are the means ± the standard error of the means.

C) A sample immunoblot shows that no change was observed in the Bax expression in cortical tissue prepared from young rats fed the control (lane1) or experimental (lane 2) or tissue prepare from aged rats fed the control (lane 3) or the experimental (lane 4) diets (Bax=23kD).

D) Densitometric analysis showed similar Bax expression in cortical tissue prepared from all the groups (n=12 for each group). Values are expressed as arbitrary units of Bax expression and are the means ± the standard error of the means.

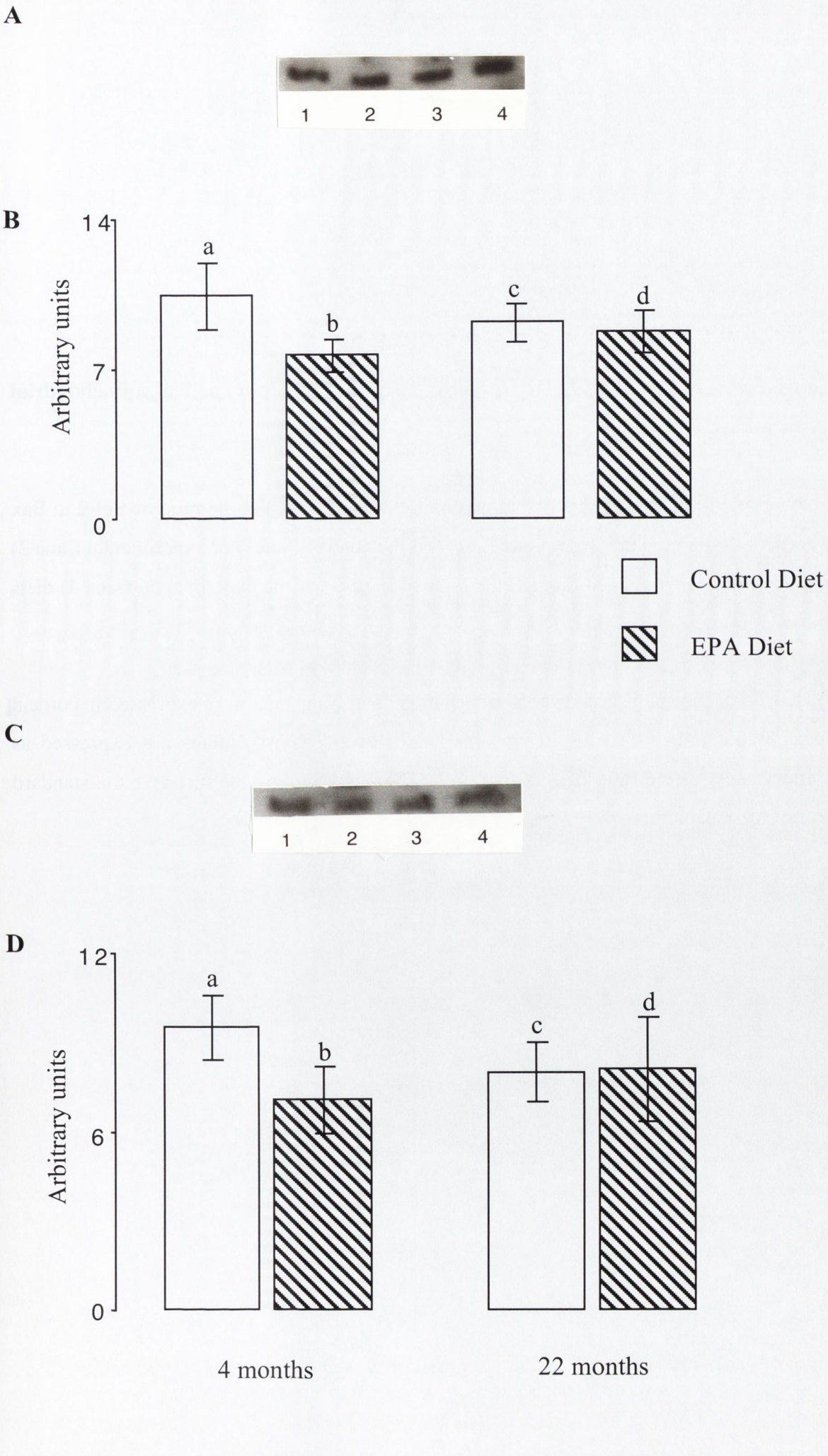
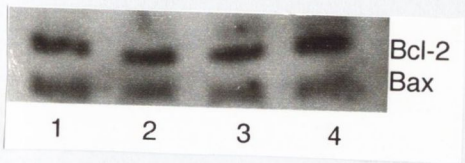

A
1
2
3
4
B
14
7
0
Arbitrary units
a
b
c
d
Control Diet
EPA Diet
C
1
2
3
4
D
12
6
0
Arbitrary units
a
b
c
d
4 months
22 months

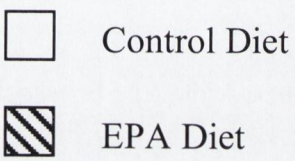
Figure 3.38: The ratio of Bcl-2 to Bax is also unchanged in cortical mitochondrial preparations.

A) A sample immunoblot shows that no change was observed in the ratio of Bcl-2 to Bax in cortical tissue prepared from young rats fed the control (lane1) or experimental (lane 2) or tissue prepare from aged rats fed the control (lane 3) or the experimental (lane 4) diets (Bax=23kD and Bcl-2=25kD).

B) Densitometric analysis showed similar ratios of Bcl-2 to Bax were expressed in cortical tissue prepared from all the groups (n=12 for each group). Values are expressed as arbitrary units of the ratio of Bax to Bcl-2 expression and are the means ± the standard error of the means.

A


Bcl-2
Bax
1
2
3
4


Control Diet
EPA Diet

B

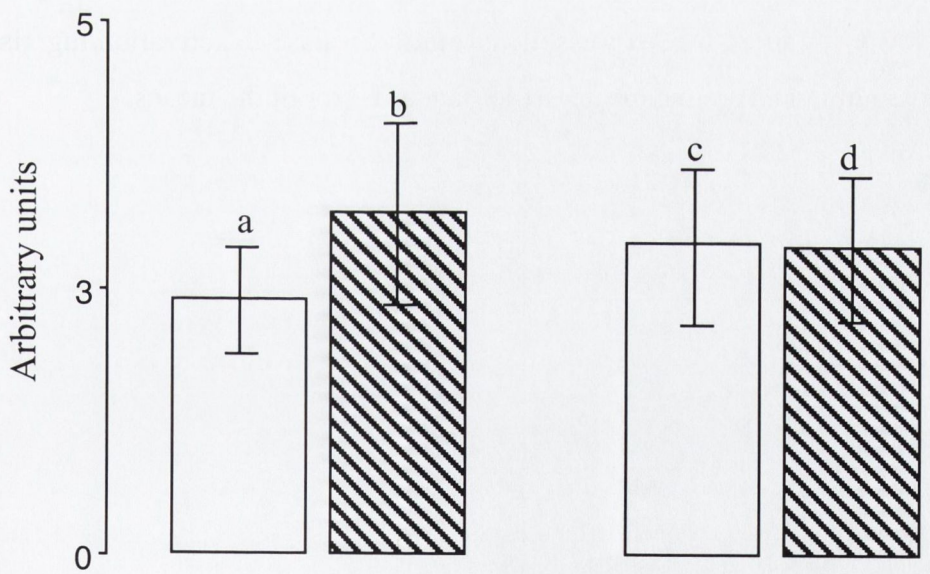

Arbitrary units
5
3
0
a
b
c
d

Figure 3.39: Age is associated with an increase in cortical caspase-3 activation.

Caspase-3 activation was significantly increased in cortical tissue prepared from aged rats fed the control diet (n=12) compared with tissue prepared from young rats fed either the control (n=9) or experimental (n=10) diet and aged rats fed the EPA diet (n=10; *$p<0.05$ *a*/*b*/*d* v *d*, ANOVA). Values are expressed as nmol Caspase-3 activation/mg tissue corrected for protein/min and are the means ± the standard error of the means.

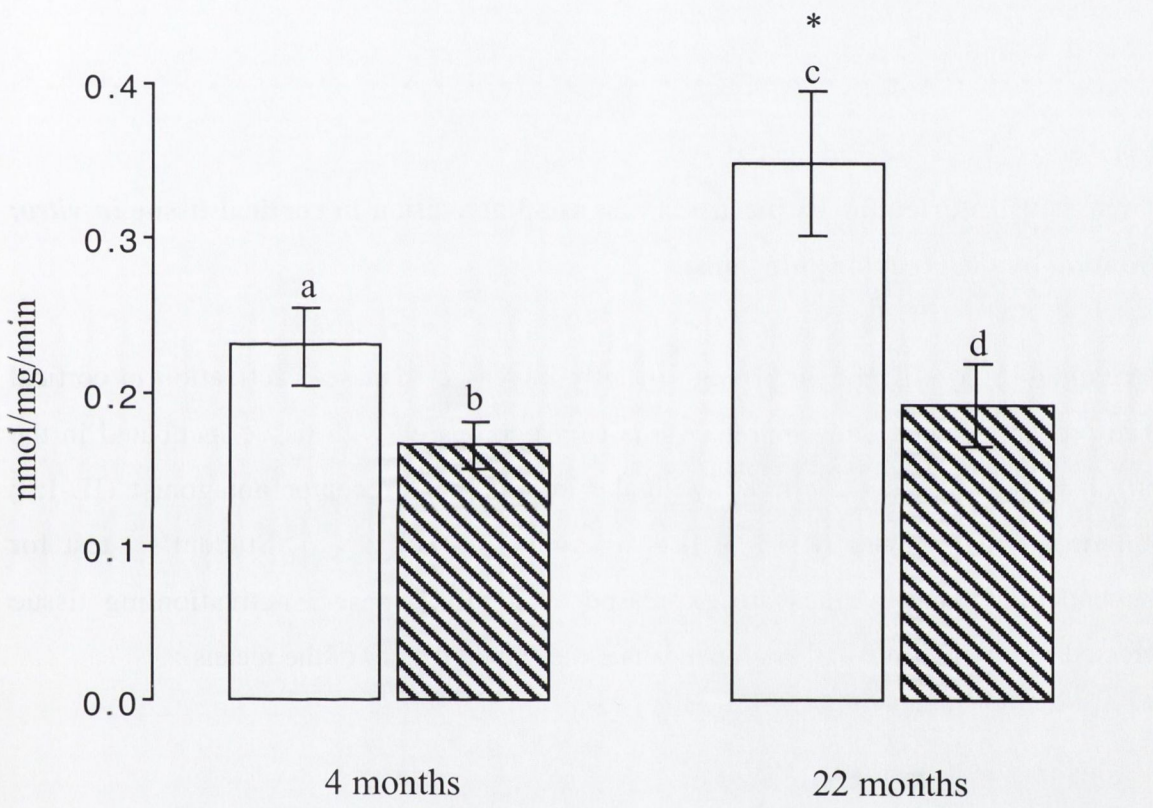

Control Diet
EPA Diet
nmol/mg/min
0.4
0.3
0.2
0.1
0.0
a
b
*
c
d
4 months
22 months

Figure 3.40: Interleukin-1β increases caspase-3 activation in cortical tissue *in vitro;* inhibition by the receptor antagonist.

Interleukin-1β (IL-1β; 3.5ng/ml) significantly increased caspase-3 activation in cortical tissue prepared from young untreated rats (n=5) compared with tissue incubated in the control buffer (Krebs Ca^{2+}; n=8), with the interleukin-1 receptor antagonist (IL-1ra; 350ng/ml; n=6) or with IL-1β + IL-1ra (n=5; *$p<0.05$ *a* v *b*, Student's t-test for independent means). Values are expressed as nmol Caspase-3 activation/mg tissue corrected for protein/min and are the means ± the standard error of the means.

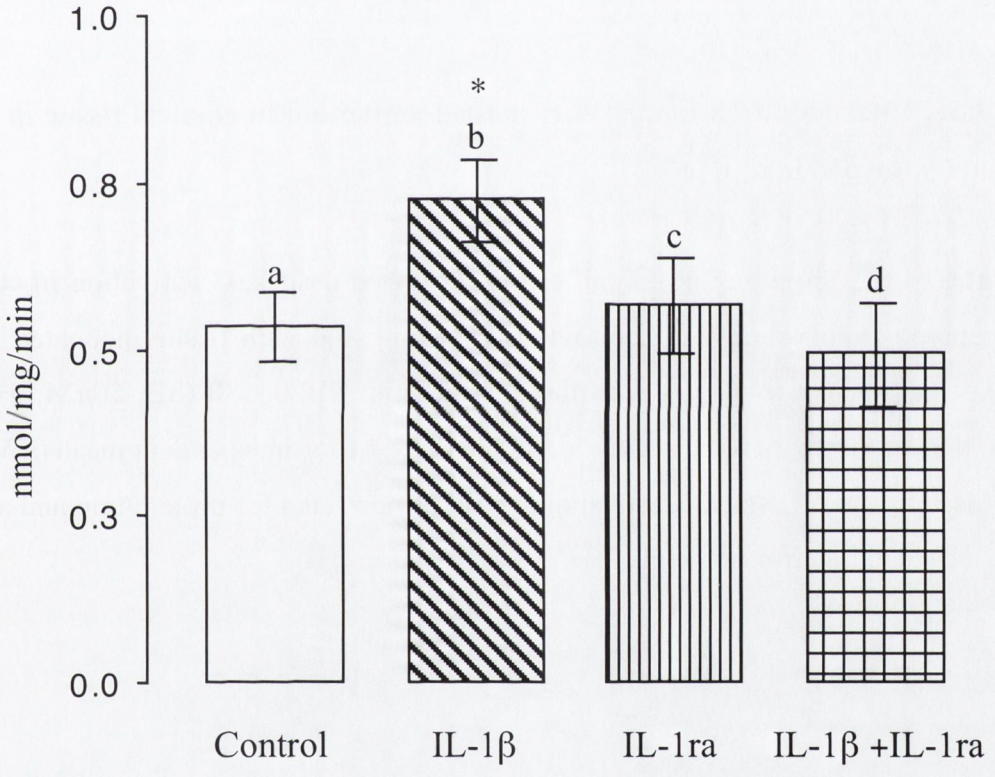

1.0
0.8
0.5
0.3
0.0
nmol/mg/min
*
a
b
c
d
Control
IL-1β
IL-1ra
IL-1β +IL-1ra

Figure 3.41: Interleukin-1β increases caspase-3 activation in cortical tissue *in vitro*; inhibition by the p38 inhibitor.

Interleukin-1β (IL-1β; 3.5ng/ml) significantly increased caspase-3 activation in cortical tissue prepared from young untreated rats (n=5) compared with tissue incubated in the control buffer (Krebs Ca^{2+}; n=8), with the p38 inhibitor SB203580 (SB; 50μM; n=4) or with IL-1β + SB (n=5; *p<0.05 *a/c/d* v *b*, Student's t-test for independent means). Values are expressed as nmol Caspase-3 activation/mg tissue corrected for protein/min and are the means ± the standard error of the means.

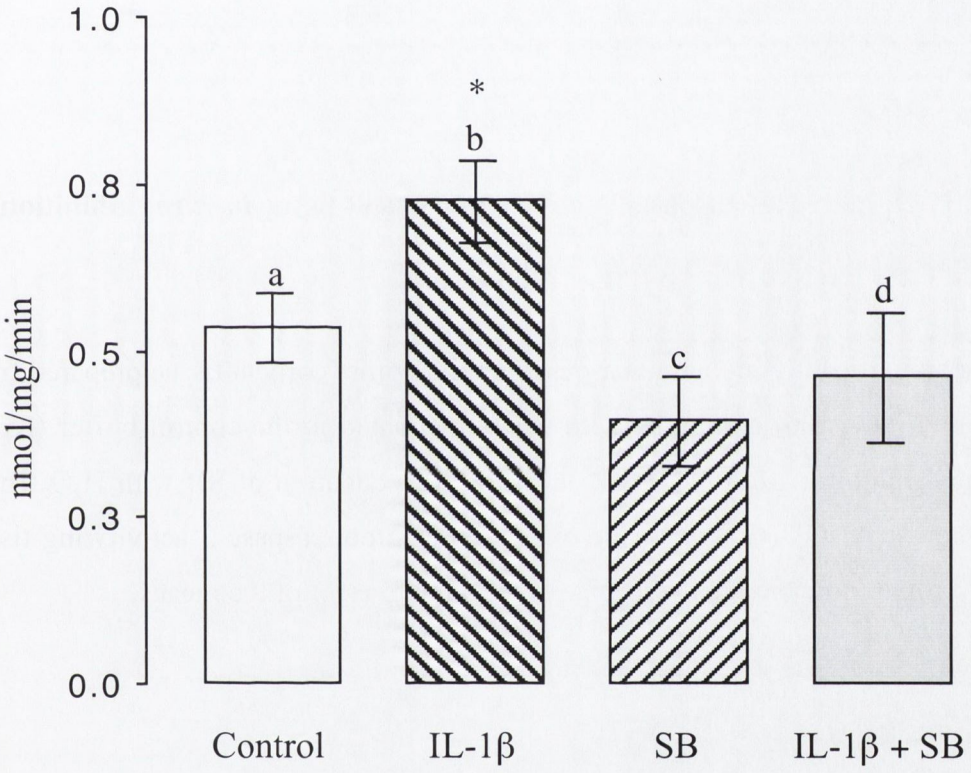

1.0
0.8
0.5
0.3
0.0
nmol/mg/min
*
a
b
c
d
Control
IL-1β
SB
IL-1β + SB

Figure 3.42: H_2O_2 increases caspase-3 activity in cortical tissue *in vitro*; inhibition by a p38 inhibitor.

H_2O_2 (200μM) significantly increased caspase-3 activation in cortical tissue prepared from young untreated rats (n=6) compared with tissue incubated in the control buffer (Krebs Ca^{2+}; n=4), p38 inhibitor SB203580 (SB; n=6) or co-incubation of SB with H_2O_2 (n=6; *p<0.05 *a/c/d* v *b*, ANOVA). Values are expressed as nmol caspase-3 activity/mg tissue corrected for protein/min and are the means ± the standard error of the means.

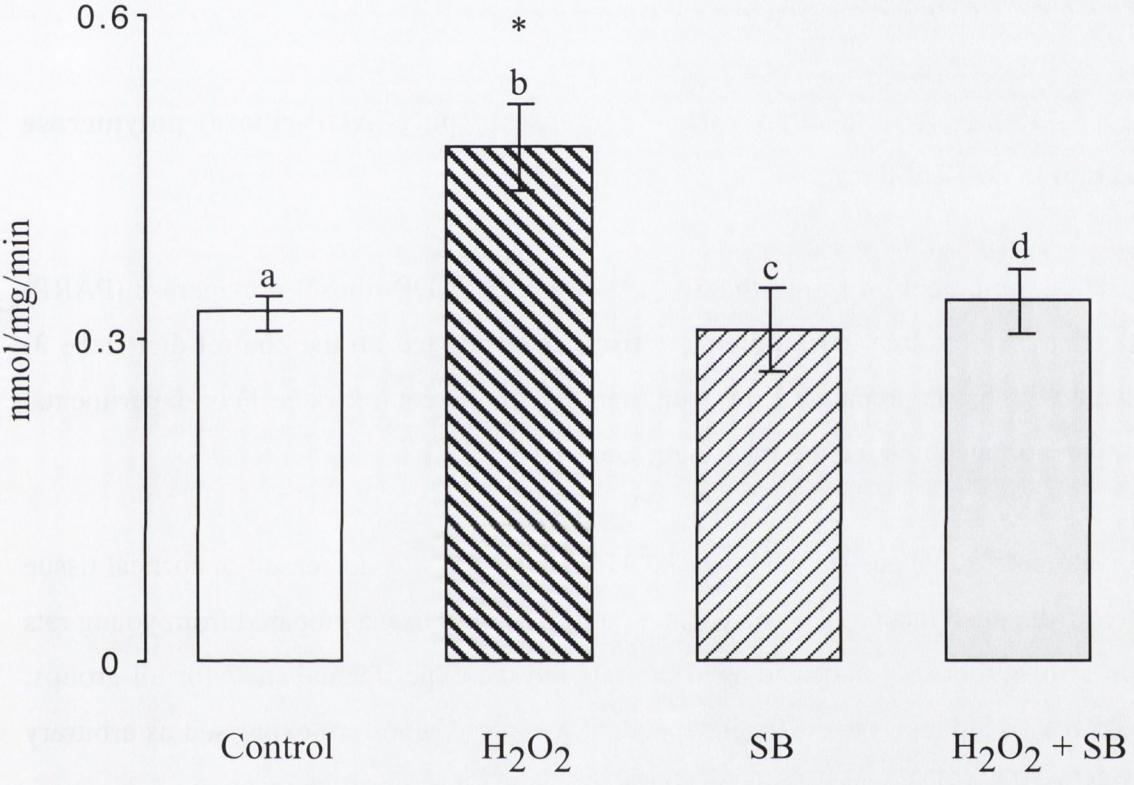
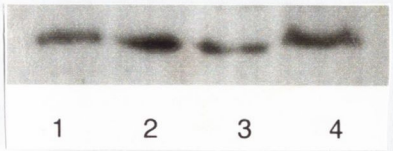

0.6
0.3
0
nmol/mg/min
*
a
b
c
d
Control
H_2O_2
SB
H_2O_2 + SB

Figure 3.43: Age is associated with a decrease in poly-(ADP-ribose) polymerase expression in cortical tissue.

A) A sample immunoblot shows that the 116kDa poly-(ADP-ribose) polymerase (PARP) was decreased in cortical tissue prepared from aged rats fed on the control diet (lane 3) compared with tissue prepared from young rats fed on the control (lane 1) or experimental (lane 2) diet or aged rats fed the EPA diet (lane 4).

B) Densitometric analysis showed that the 116kDa PARP was decreased in cortical tissue prepared from aged rats fed the control diet compared with tissue prepared from young rats fed the control diet or young and aged the rats fed the experimental (n=7 for all groups; *$p<0.05$ *a* v *c*, Student's t-test for independent means). Values are expressed as arbitrary units of PARP (116kDa) and are the means ± the standard error of the means.

A


1
2
3
4

B

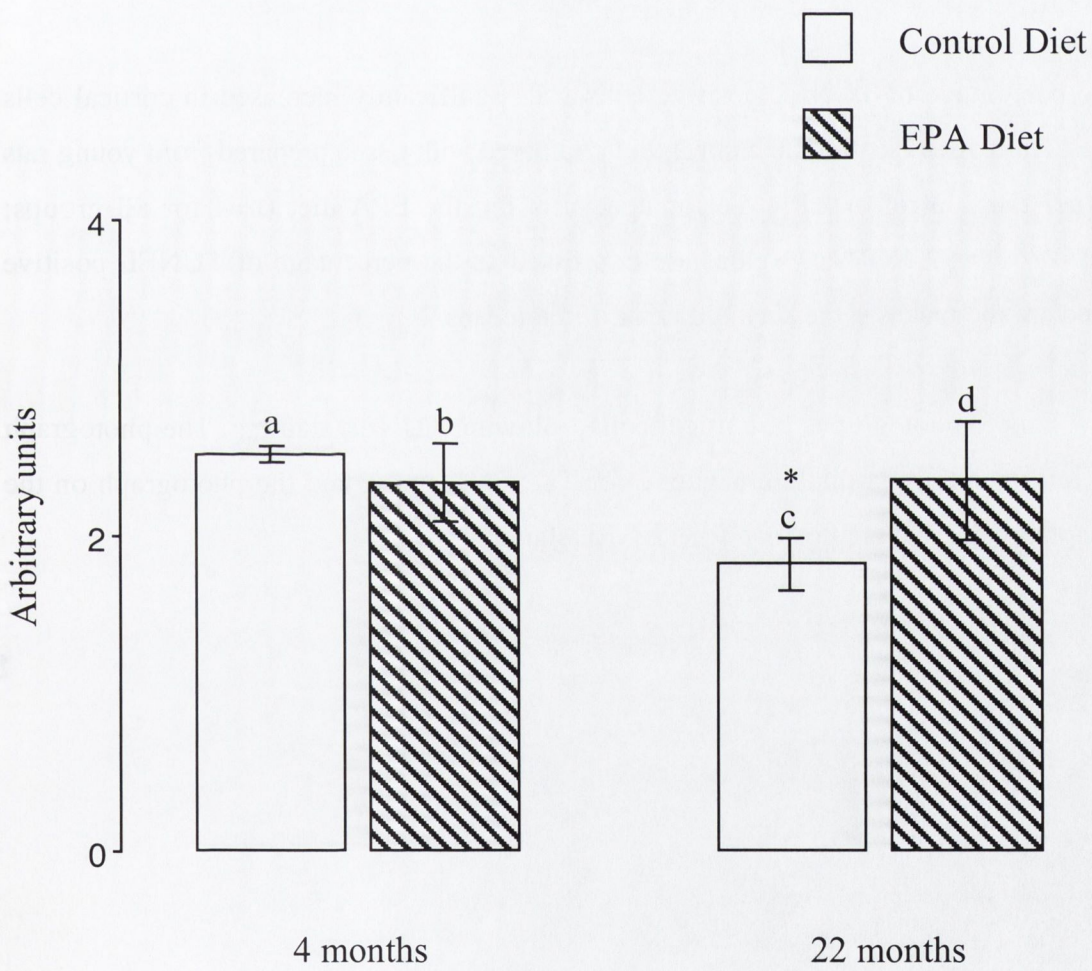
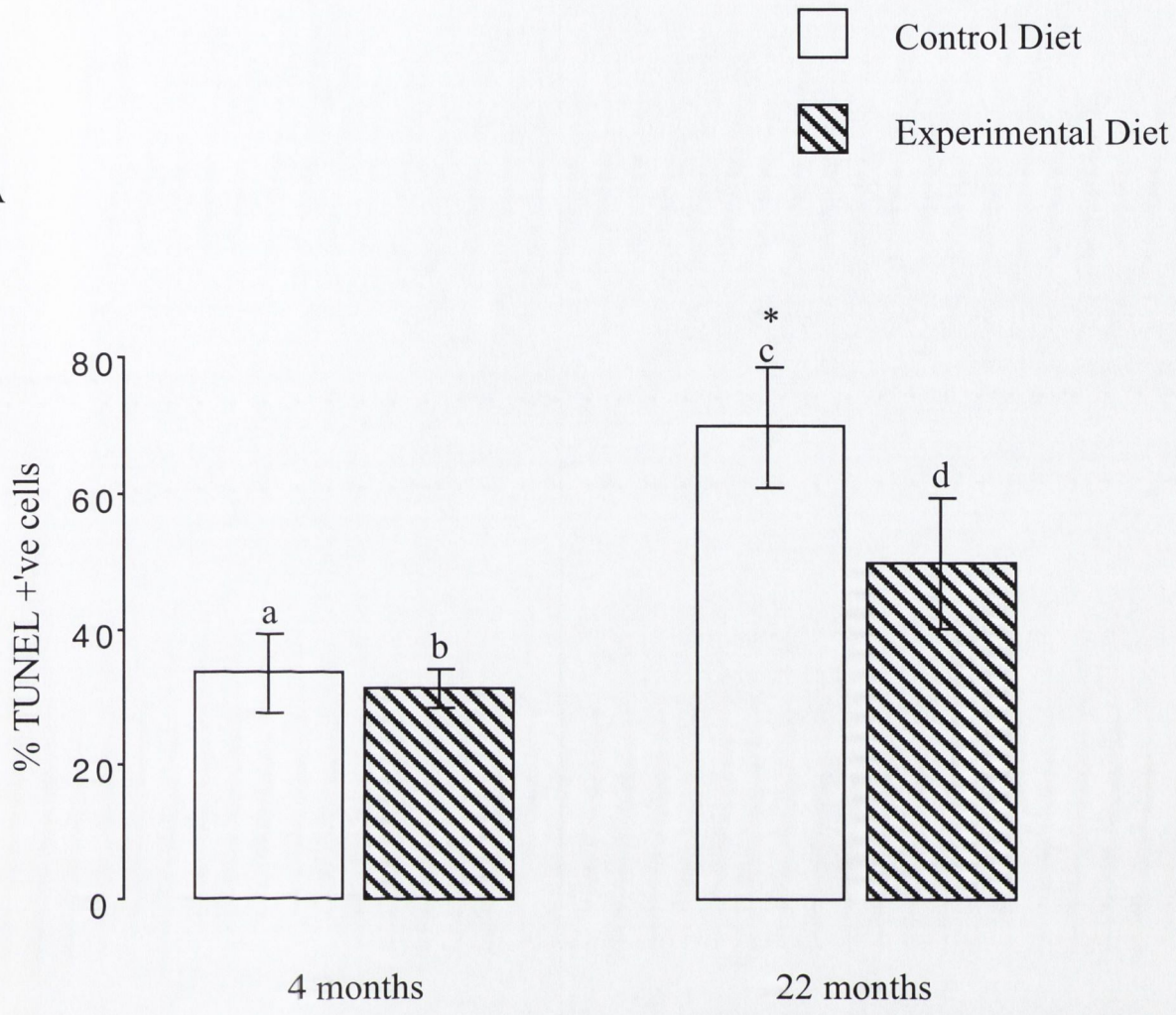

Control Diet
EPA Diet
Arbitrary units
4
2
0
a
b
*
c
d
4 months
22 months

Figure 3.44: Age is associated with an increase in TUNEL positive cortical cells.

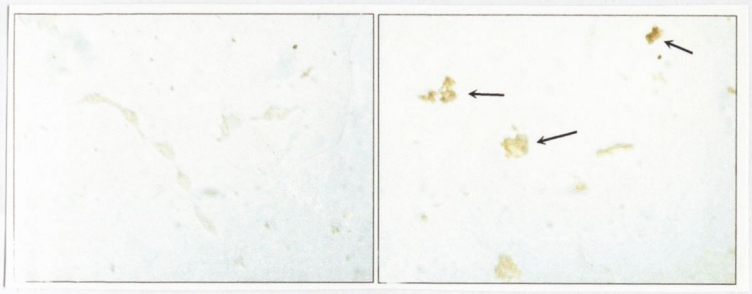
A) The percentage of TUNEL positive cells was significantly increased in cortical cells prepared from aged rats fed the control diet compared with tissue prepared from young rats fed either the control or EPA diet or aged rats fed the EPA diet (n=4 for all groups; *$p<0.05$ *a/b* v *c*, ANOVA). Values are expressed as the percentage of TUNEL positive cells and are the means ± the standard error of the means.

B) Two sample photographs of cortical cells following TUNEL staining. The photograph on the left depicts cells that are negative for TUNEL staining and the photograph on the right depicts cells that are positive for TUNEL staining.

A


Control Diet
Experimental Diet
% TUNEL +'ve cells
80
60
40
20
0
a
b
*
c
d
4 months
22 months

B



ANALYSIS OF CHANGES IN HIPPOCAMPAL TISSUE THAT OCCUR FOLLOWING WHOLE-BODY IRRADIATION AND LPS TREATMENT.

In the final series of experiments insults other than age were assessed for their ability to impair LTP. Whole-body γ-irradiation can result in one of the most devastating forms of damage that can be inflicted in the CNS. A large range of apoptosis-inducing agents may be stimulated and even low doses of irradiation can affect cellular activity. Irradiation has been shown to stimulate IL-1β production (Dinarello, 1996) and p38 activation (Robinson and Cobb, 1996). It has also been shown to increase caspase-3 activation and decrease PARP activity (Ferrer, 1999). It has been reported that the hippocampus is particularly sensitive to irradiation (Tolliver and Pellmar, 1987) and it has been shown that irradiation exerts damaging effects in CA1 and the dentate gyrus (Pellmar and Lepinski, 1993). γ-Irradiation was assessed for its ability to inhibit the maintenance of LTP in rats and biochemical changes were analysed for their possible impact on the inhibition of LTP.

LPS was also assessed for its ability to affect LTP. LPS is an integral part of the cell wall of Gram-negative bacteria. It has been shown previously in this laboratory to impair LTP and this may be due to increases in p38 and JNK activation as well as an increase in ROS production and IL-1β concentration (Vereker *et al.*, 2000b). The ability of EPA to reverse the expected impairment in LTP caused by both γ-irradiation and LPS was assessed. EPA has been shown to reduce UVB-induced immunosuppression in mice (Moison and Beijersbergen Van Henegouwen, 2001). As both irradiation (Ibuki and Goto, 2000) and LPS (Vereker *et al.*, 2000b) have been shown to increase ROS production, the ability of the antioxidant scavengers, vitamins E and C, were also assessed for their ability to reverse the expected impairment in LTP.

The results of an initial study on the effects of γ-irradiation on LTP and subsequent biochemical analyses on the hippocampal tissue obtained from these animals are shown below. The effects of diets enriched in either EPA or the vitamins E and C on γ-irradiation- and LPS-induced impairments in LTP is also shown. (see Appendix I for mean data).

3.45 Long-term potentiation in the dentate gyrus is attenuated in rats that received γ-irradiation.

In the first set of experiments whole-body γ-irradiation was assessed as a model system, which might allow evaluation of changes as a consequence of an increase in ROS production. Two doses of irradiation were used in these studies. The first dose was 10Gy @20Gy/min. Young rats were subjected to whole-body irradiation and allowed to recover for 4 days. LTP was assessed after this time and Figure 3.45 shows the result of these experiments. It shows that delivery of a high frequency train of stimuli to the perforant path (at time point 0) resulted in an immediate increase in the mean percentage change in the EPSP slope in both rats that received the γ-irradiation and those that were sham irradiated.

3.46 γ-Irradiation affects the percentage change in the EPSP slope.

Analysis of the data indicated that γ-irradiated rats were unable to maintain this increase whereas sham irradiated rats maintained LTP for the duration of the test. There was a significant difference in the percentage change of the EPSP slope in the first 2min and in the last 5min of recording post tetanic stimulation between γ and sham irradiated rats (100.8 ± 0.5 and 103.3 ± 2.0 % for sham and γ-irradiated rats respectively in the first 2min post stimulation and 125.5 ± 0.5 and 84.2 ± 1.3 for sham and γ-irradiated rats respectively in the last 5min of recording post stimulation; Figure 3.46).

3.47 γ-Irradiation is associated with an increase in hippocampal reactive oxygen species production.

In order to elucidate the factors that may have caused the impairment in LTP, hippocampal tissue was assessed for changes in a number of measures. ROS production was significantly increased in hippocampal tissue prepared from young rats which were exposed to whole-body irradiation compared with tissue prepared from rats which were sham irradiated (*$p<0.01$ *a* v *b*, Student's t-test for independent means Figure 3.47).

3.48 Lipid peroxidation in the hippocamus is not affected by γ-irradiation.

Earlier experiments (see section 3.5 and 3.18) indicated that an increase in ROS production was coupled with an increase in lipid peroxidation and, therefore, lipid

peroxidation was assessed in hippocampal tissue prepared from sham-irradiated and irradiated rats. Figure 3.48 shows that lipid peroxidation was similar in both groups.

3.49 Activity of interleukin-1β converting enzyme in the hippocampus.

ICE activity was markedly increased in hippocampal tissue prepared from γ-irradiated rats compared with tissue prepared from rats that were sham irradiated however, this difference did not reach statistical significance (Figure 3.49).

3.50 γ-Irradiation is associated with an increase in hippocampal IL-1β concentration.

Figure 3.50 shows that IL-1β concentration was significantly increased in hippocampal tissue prepared from rats that were subjected to γ-irradiation compared with tissue prepared from sham irradiated rats (*$p<0.05$ *a* v *b*, Student's t-test for independent means).

3.51 Correlation between interleukin-1β and long-term potentiation.

Figure 3.51 shows that a significant negative correlation existed between IL-1β concentration in the hippocampus and the mean percentage change in the EPSP slope in the last 5min of the experiment; the r^2 value was 0.65 (*$p<0.005$, Student's t-test for independent means). This implies that the irradiation-induced increase in IL-1β concentration is likely to be a major contributing factor in the impairment in LTP observed in these rats.

3.52 γ-Irradiation is associated with a decrease in protein synthesis in the entorhinal cortex.

As the entorhinal cortex contains the cell bodies of the perforant path granule cell synapses, protein synthesis in the entorhinal cortex was assessed using ^{35}S methionine labelling of proteins. Figure 3.52 shows that protein synthesis was significantly decreased in tissue prepared from rats which were exposed to γ-irradiation compared with tissue prepared from rats that were sham irradiated (*$p<0.05$ *a* v *b*, Student's t-test for independent means).

3.53 LTP is impaired by γ-irradiation but restored by dietary manipulation.

As EPA was successful in reversing some key age-related changes observed in the hippocampus, its ability to reverse the γ-irradiation-related impairment in LTP was assessed. Young rats were fed for 4 weeks on a control diet (oil enriched in monounsaturated fatty acids) or an experimental diet enriched in 1% or 2% (of their daily food intake) EPA. After 4 weeks the rats were exposed to a lower dose of γ-irradiation than previously (10Gy @10Gy/min) and allowed to recover for 4 days before assessing their ability to maintain LTP. Figure 3.53 shows that delivery of a high frequency train of stimuli to perforant path (at time point 0) resulted in an immediate increase in the EPSP slope in both rats that received the γ-irradiation and those that were sham irradiated in all dietary groups.

3.54 γ-Irradiation affects the percentage change in the EPSP slope in the dentate gyrus.

Analysis of the data indicates that there was a significant increase in the mean percentage change in the population EPSP slope in the first 2min post tetanic stimulation (compared with the last 5min pre-stimulation) in the sham irradiated rats that were fed the 1% EPA diet compared with those that were fed either the control or 2% diet (*$p<0.05$ *a/c* v *b*, ANOVA). This significant increase also was seen in the γ-irradiated rats that were fed the control diet compared with those that were fed either EPA diet (*$p<0.05$ *d* v *e/f*, ANOVA; Figure 3.54A). There was a significant decrease in the percentage change in the population EPSP slope in the last 5min of recording post tetanic stimulation (compared with the last 5min pre-stimulation) in sham irradiated rats that were fed the 1% and 2% EPA diet compared with those that were fed the control diet (+$p<0.001$ *g* v *h/j*, ANOVA). This significant decrease was also seen in the γ-irradiated rats fed on the control diet compared with those fed the 1% and 2% EPA and all sham irradiated dietary groups (*$p<0.001$ *k* v *m/n*, ANOVA; Figure 3.54B). The EPA diet was effective in reversing the irradiation-induced impairment in LTP.

3.55 LTP is attenuated by γ-irradiation but restored by dietary vitamins in the dentate gyrus.

The antioxidant scavenger vitamins E and C were assessed for their ability to mimic the polyunsaturated fatty acid diet in restoring the γ-irradiation-related

impairment in LTP. Young rats were fed for 4 weeks on an experimental diet enriched in 250mg/day vitamin E and C or on a control diet (oil enriched in monounsaturated fatty acids) and received the same dose of γ-irradiation as the previous experiment. Figure 3.55 shows that delivery of a high frequency train of stimuli to perforant path granule cells (at time point 0) resulted in an immediate increase in mean EPSP slope in both rats that received the γ-irradiation and those that were sham irradiated in all dietary groups.

3.56 γ-Irradiation affects the percentage change in the EPSP slope in the dentate gyrus, reversal by dietary vitamins.

Analysis of the data indicates that the percentage change in the population EPSP slope in the first 2min post tetanic stimulation (compared with the last 5min pre-stimulation) was significantly increased in the γ-irradiated rats fed the control diet compared with those fed the vitamin diet and both sham irradiated dietary groups (*$p<0.01$ *a/b/d* v *c*, ANOVA Figure 3.56A). The percentage change in the population EPSP slope in the last 5min of recording (compared with the last 5min pre-stimulation) was significantly decreased in the γ-irradiated rats fed on the control diet compared with those fed the vitamin diet and the sham irradiated rats from both dietary groups (*$p<0.001$ *e/f/h* v *g*, ANOVA). There was a significant difference between the sham-irradiated rats fed the control diet and all other groups (*$p<0.001$ *e* v *f/g/h*, ANOVA, Figure 3.56B). The vitamin-enriched diet was effective in reversing the irradiation-induced impairment in LTP.

3.57 LTP is impaired by LPS in the dentate gyrus, but restored by dietary manipulation with EPA.

The insult used in the second set of experiments was lipopolysaccharide (LPS), a integral part of Gram negative bacteria that causes an inflammatory response in the host animal. Young rats were fed for 4 weeks on a control diet (oil enriched in monounsaturated fatty acids) or an experimental diet enriched in 1% or 2% (of their daily food intake) EPA. After the 4 weeks the rats were injected intraperitoneally with either a control buffer (1ml; 0.9% saline) or LPS (1ml; 100μg/ml) and allowed to recover for 3 hours before assessing their ability to maintain LTP. Figure 3.57 shows that delivery of a high frequency train of stimuli to perforant path granule cells (at time

point 0) resulted in an immediate increase in the mean percentage change in the EPSP slope in both rats that received the saline or LPS treatment in all dietary groups.

3.58 LPS affects the percentage change in the EPSP slope in the dentate gyrus.

Analysis of the data shows that the percentage change in the population EPSP slope in the first 2min post tetanic stimulation (compared with the last 5min pre-stimulation) was significantly decreased in the saline treated rats fed the 1% EPA diet compared with those fed the control and 2% EPA diet (+$p<0.05$ *a/c* v *b*, ANOVA). This significant decrease was also seen in the LPS treated rats fed the 2% EPA diet (*$p<0.05$ *d/e* v *f*, ANOVA; Figure 3.58A). The percentage change in the population EPSP slope in the last 5min of recording post tetanic stimulation (compared with the last 5min pre-stimulation) was significantly decreased in LPS treated rats fed the control diet compared with all other treatment and dietary groups (*$p<0.001$ *g/h/j/m/n* v *k*, ANOVA). There was a significant increase seen in saline treated rats fed the 2% EPA diet compared with all other treatment and dietary groups (*$p<0.001$ *g/h/k/m/n* v *j*, ANOVA; Figure 3.58B). The EPA diet was effective in reversing the LPS-induced impairment in LTP.

3.59 LTP is impaired by LPS in the dentate gyrus, but restored by dietary vitamins.

The vitamin diet was also used to try to restore the LPS-related impairment in LTP. The same procedure was used as before. Figure 3.59 shows that delivery of a high frequency train of stimuli to perforant path granule cells (at time point 0) resulted in an immediate increase in the mean percentage change in the EPSP slope in both rats that received the saline or LPS treatment in all dietary groups.

3.60 LPS affects the percentage change in the EPSP slope in the dentate gyrus, reversal by dietary vitamins.

Analysis of the data shows that the percentage change in the EPSP slope in the first 2min post tetanic stimulation (compared with the last 5min pre-stimulation) was significantly increased in saline treated rats fed the vitamin diet compared with those fed the control diet and both LPS treated dietary groups (*$p<0.05$ *a/c/d* v *b*, ANOVA; Figure 3.60A). The percentage change in the population EPSP slope in the last 5min of

recording (compared with the last 5min pre-stimulation) was significantly decreased in LPS treated rats fed the control diet compared with those fed the vitamin diet and both saline treated dietary groups (*p<0.001 *e/f/h* v *g*, ANOVA). There was a significant increase in the percentage change in the EPSP slope in the saline treated rats fed the vitamin diet compared with the saline treated rats fed the control diet and both LPS treated dietary groups (+p<0.001 *e/g/h* v *f*, ANOVA; Figure 3.60B). The vitamin diet was effective in reversing the LPS-induced impairment in LTP.

Figure 3.45: Long-term potentiation in the dentate gyrus is attenuated in rats which received γ-irradiation.

Long-term potentiation (LTP) in the dentate gyrus is significantly attenuated in rats which received a dose of γ-irradiation compared to sham irradiated rats. The mean slope of the population EPSP evoked by test stimuli delivered at 30s intervals before and after tetanic stimulation is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5min immediately prior to tetanic stimulation and values are expressed as the means ± the standard error of the means. Standard errors are included for every tenth response and some are so small in some cases as to be obscured.

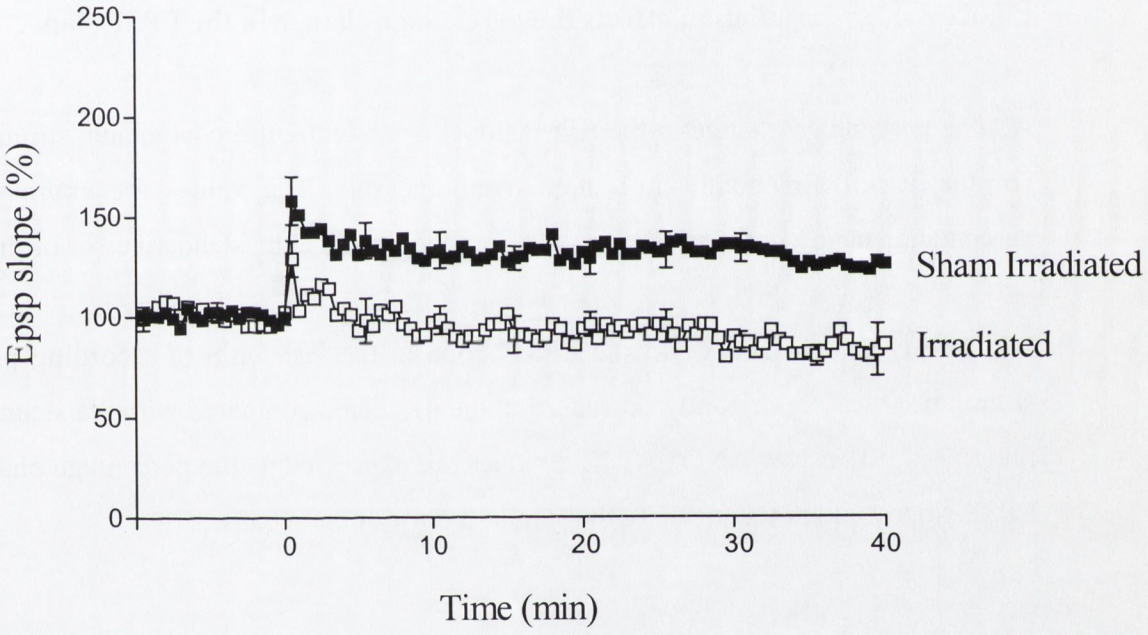
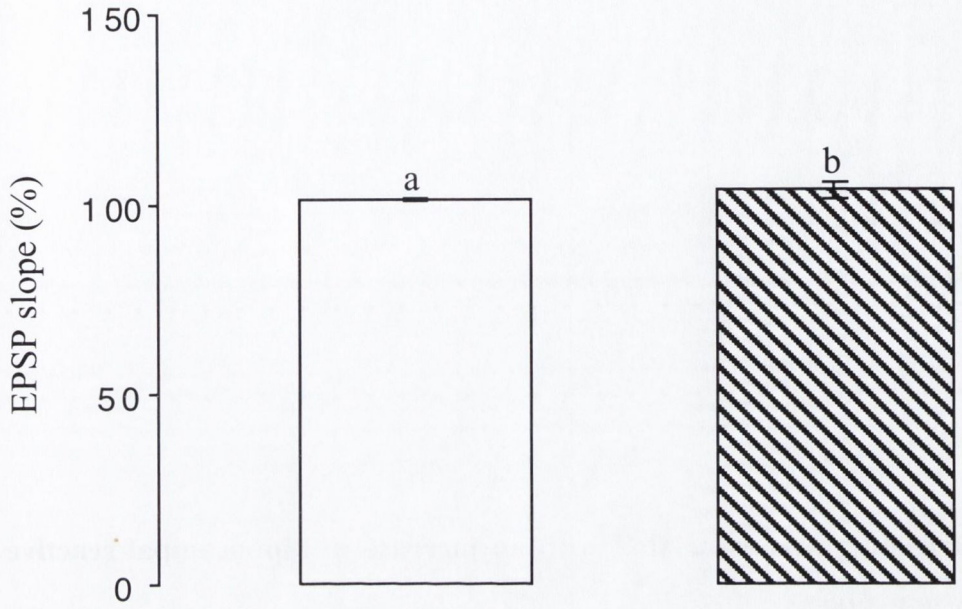

250
200
150
100
50
0
Epsp slope (%)
0
10
20
30
40
Time (min)
Sham Irradiated
Irradiated

Figure 3.46: γ-Irradiation affects the percentage change in the EPSP slope.

A) The percentage change in the EPSP slope in the first 2min post tetanic stimulation was similar in both irradiated and sham irradiated rats. The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

B) The percentage change in the EPSP slope in the last 5min of recording post tetanic stimulation was significantly decreased in the irradiated compared with the sham irradiated rats (*$p<0.001$ *c* v *d*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

A


150
100
50
0
EPSP slope (%)
a
b

B

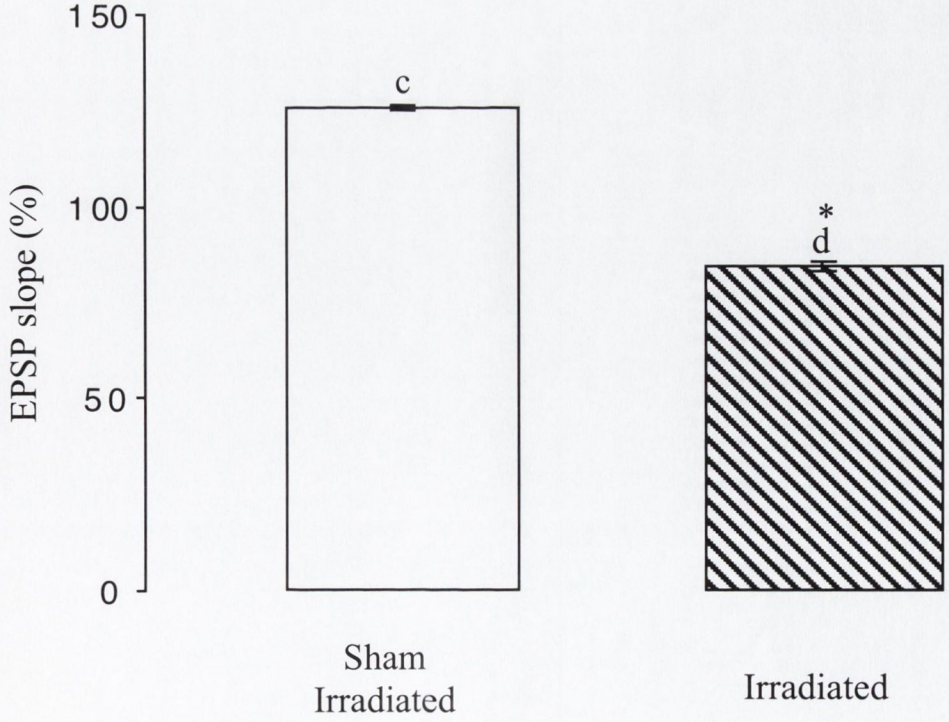

150
100
50
0
EPSP slope (%)
c
*
d
Sham
Irradiated
Irradiated

Figure 3.47: γ-Irradiation is associated with an increase in hippocampal reactive oxygen species production.

Reactive oxygen species (ROS) production was significantly increased in hippocampal tissue prepared from rats which received a dose of γ-irradiation compared with those that were sham irradiated (n=6 for both groups; *$p<0.01$ *a* v *b*, Student's t-test for independent means). Values are expressed as nmol DCF/mg protein and are the means ± the standard error of the means.

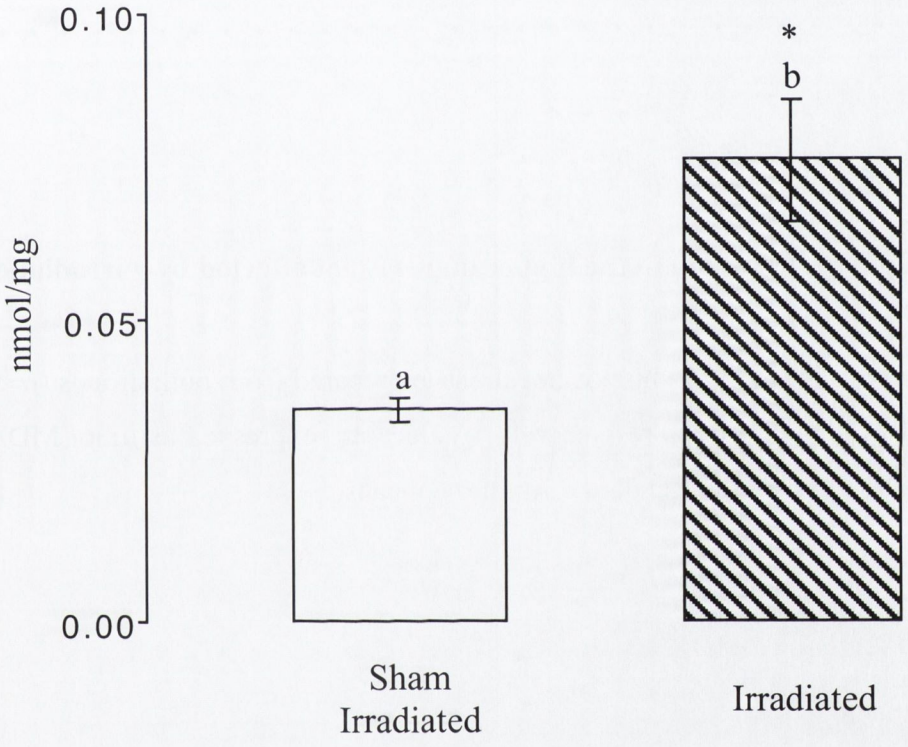

0.10
0.05
0.00
nmol/mg
a
*
b
Sham
Irradiated
Irradiated

Figure 3.48: Lipid peroxidation in the hippocampus is not affected by γ-irradiation.

Lipid peroxidation was similar in hippocampal tissue prepared from both groups (n=5 and 7 for sham and irradiated groups respectively). Values are expressed as μmol MDA/mg protein and are the means ± the standard error of the means.

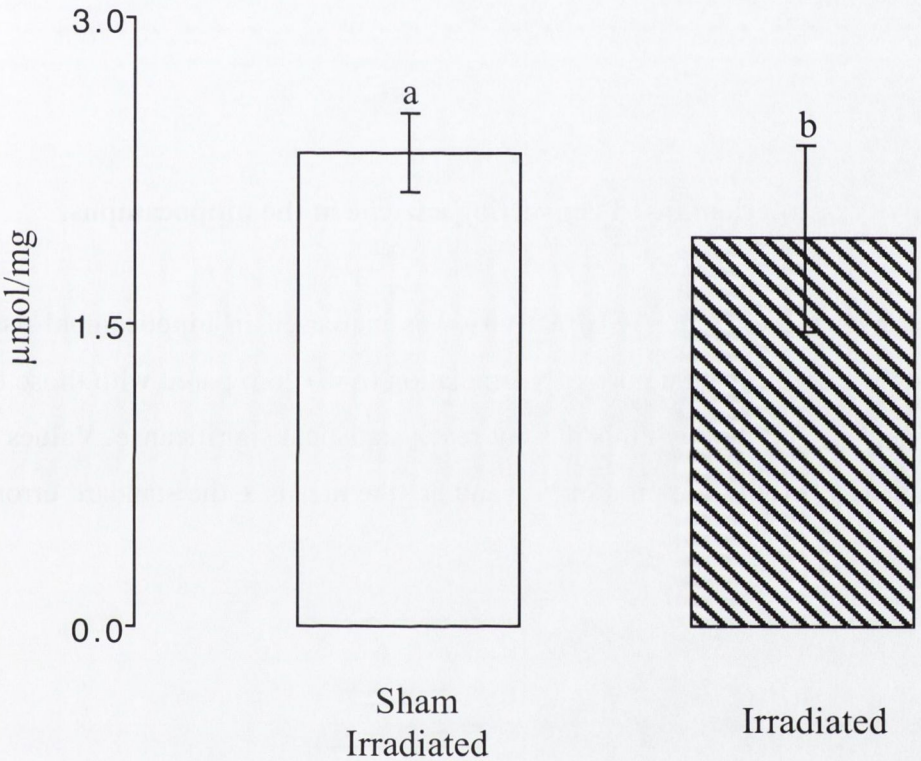

3.0
1.5
0.0
µmol/mg
a
b
Sham Irradiated
Irradiated

Figure 3.49: Activity of interleukin-1β converting enzyme in the hippocampus.

Interleukin-1β converting enzyme (ICE) activity was increased in hippocampal tissue prepared from rats which received a dose of γ-irradiation (n=6) com\pared with those that were sham irradiated (n=5), although this did not reach statistical significance. Values are expressed as nmol ICE activity/mg protein/min and are the means ± the standard error of the means.

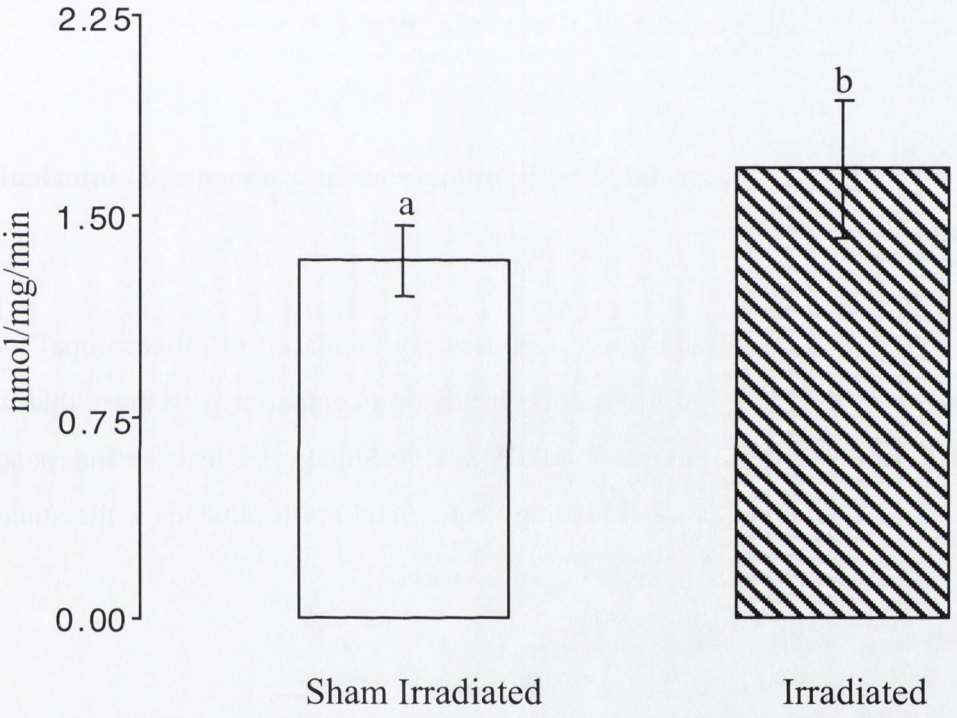

2.25
1.50
0.75
0.00
nmol/mg/min
a
b
Sham Irradiated
Irradiated

Figure 3.50: γ-Irradiation is associated with an increase in hippocampal interleukin-1β concentration.

Interleukin-1β (IL-1β) concentration was significantly increased in hippocampal tissue prepared from rats which received a dose of γ-irradiation compared with those that were sham irradiated (n=6 for both groups; *$p<0.05$ *a* v *b*, Student's t-test for independent means). Values are expressed as ng IL-1β/mg protein and are the means ± the standard error of the means.

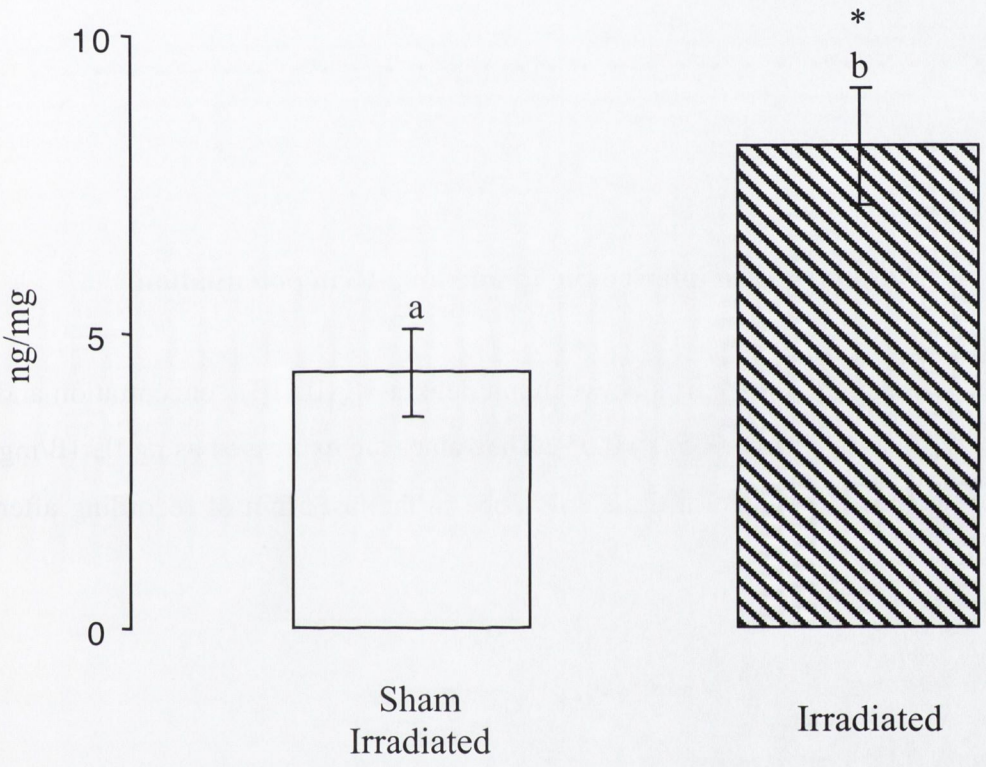
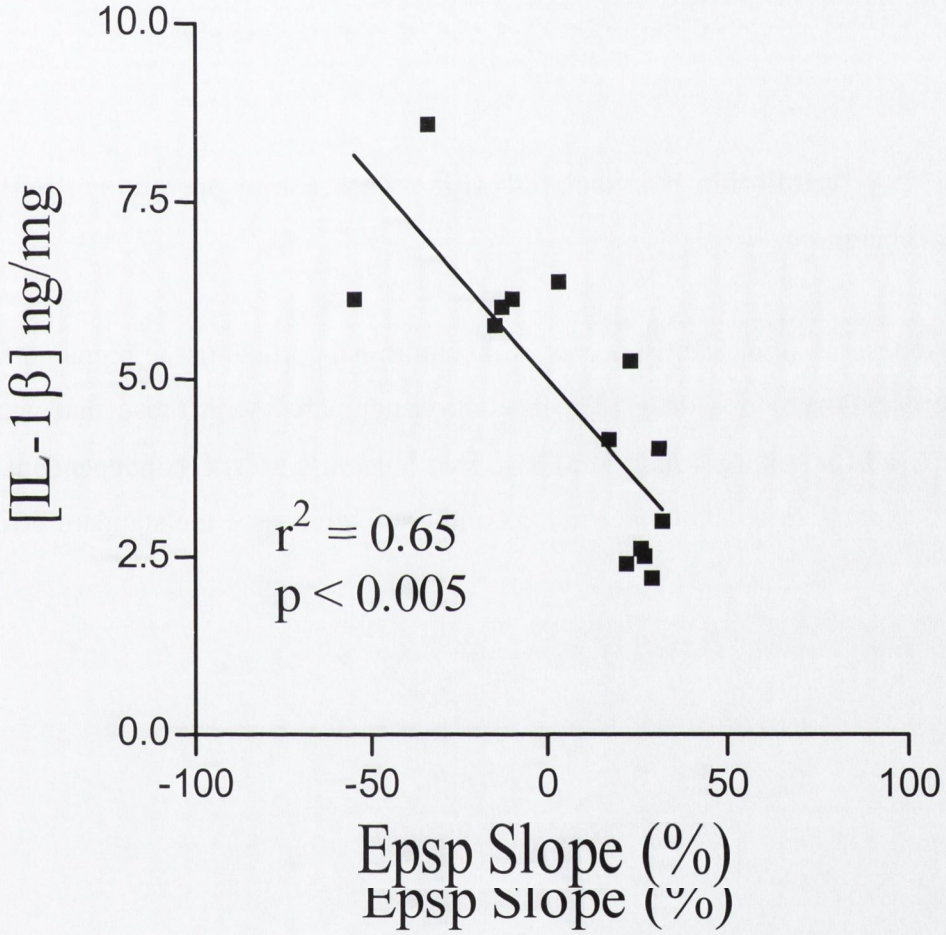

10
5
0
ng/mg
a
*
b
Sham
Irradiated
Irradiated

Figure 3.51: Correlation between interleukin-1β and long-term potentiation.

A significant negative correlation exists between interleukin-1β (IL-1β) concentration and long-term potentiation (LTP; $p<0.005$; $r^2=0.65$). The values are expressed as ng IL-1β/mg protein and the percentage change in the EPSP slope in the last 5min of recording after tetanic stimulation.



Epsp Slope (%)

Figure 3.52: γ-Irradiation is associated with a decrease in protein synthesis in the entorhinal cortex.

Protein synthesis is significantly decreased in entorhinal cortical tissue prepared from rats which were exposed to a dose of γ-irradiation compared with those that were sham irradiated (n=6 for both groups; *$p<0.05$ *a* v *b*, Student's t-test for independent means). Values are expressed as counts per minute and are the means ± the standard error of the means.

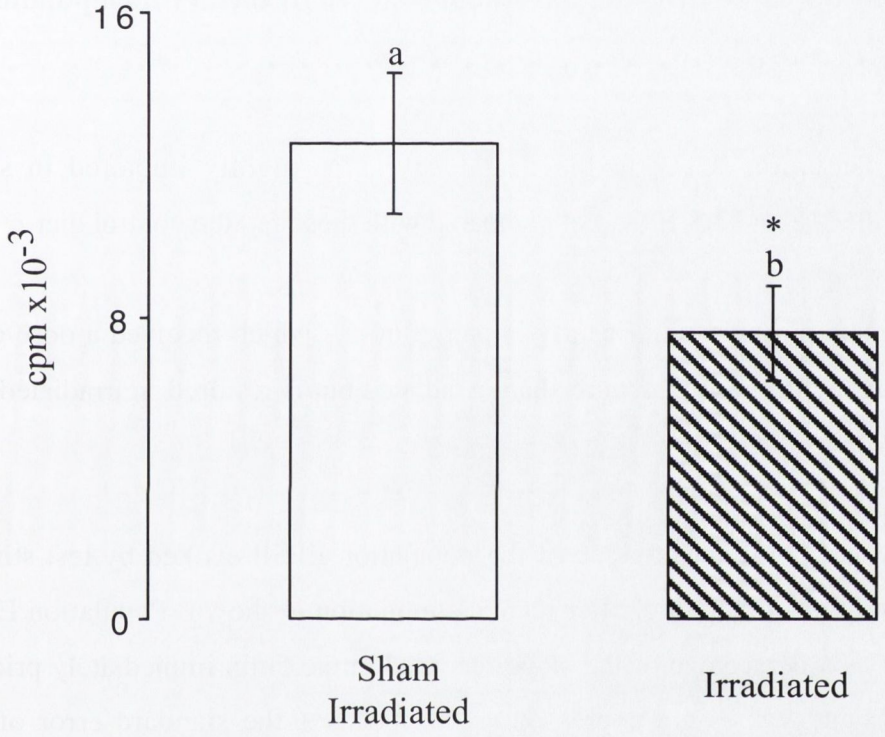

16
8
0
cpm x10-3
a
*
b
Sham
Irradiated
Irradiated

Figure 3.53: LTP is impaired by γ-irradiation but restored by dietary manipulation in the dentate gyrus.

A) Long-term potentiation (LTP) in the dentate gyrus is slightly impaired in sham irradiated rats fed the 1% and 2% EPA diet compared with those fed the control diet.

B) LTP in the dentate gyrus is significantly impaired in rats which received a dose of γ-irradiation compared with those that were sham irradiated but is restored in irradiated rats fed the 1% and 2% EPA diet.

For both figure A and B the mean slope of the population EPSP evoked by test stimuli delivered at 30s intervals before and after tetanic stimulation is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5min immediately prior to tetanic stimulation and values are expressed as the means ± the standard error of the means. Standard errors are included for every tenth response and some are so small in some cases as to be obscured.

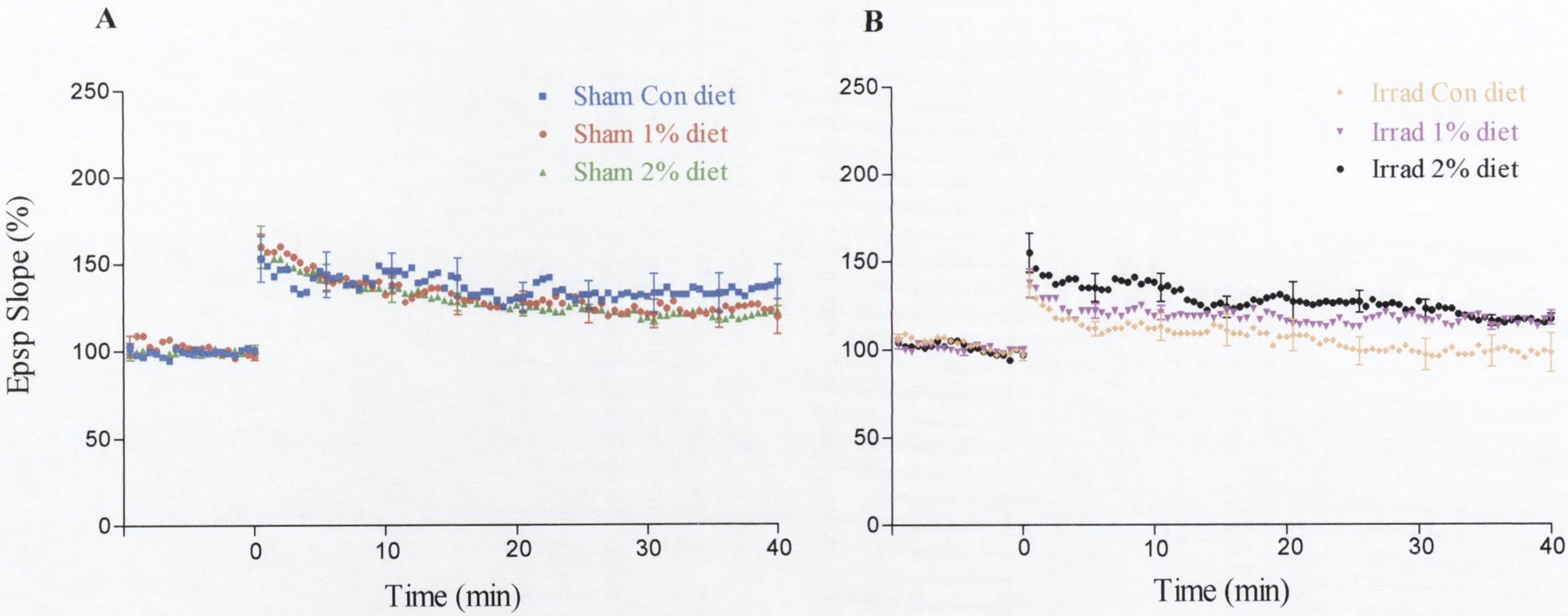

A
B
Epsp Slope (%)
250
200
150
100
50
0
Sham Con diet
Sham 1% diet
Sham 2% diet
Irrad Con diet
Irrad 1% diet
Irrad 2% diet
0
10
20
30
40
Time (min)

Figure 3.54: γ-Irradiation affects the percentage change in the EPSP slope in the dentate gyrus.

A) The percentage change in the EPSP slope in the first 2min post tetanic stimulation was significantly increased in sham irradiated rats fed on the 1% experimental rats and irradiated rats fed the control diet compared with all the other groups (*p<0.05 *a/c/e/f* v *b/d*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

B) The percentage change in the EPSP slope in the last 5min of recording post tetanic stimulation was significantly decreased in the irradiated rats fed the control experimental diets and sham irradiated rats fed the experimental diets compared with the sham irradiated rats fed the control diet (+p<0.001 *g* v *h/j*, ANOVA). There was a significant difference the γ-irradiated rats fed the control diet and all other groups (*p<0.001 *k* v *m/n*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

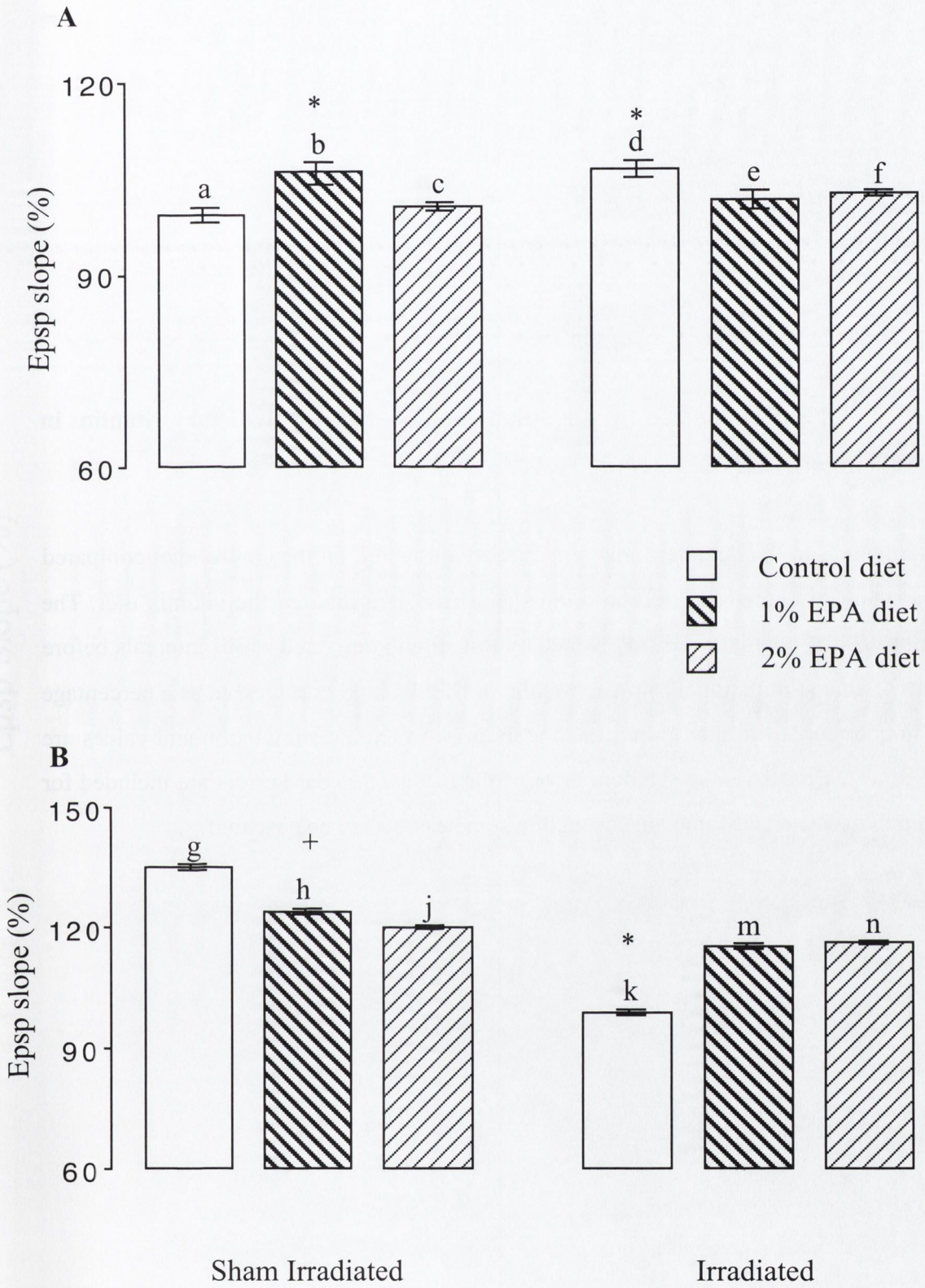

A
120
90
60
Epsp slope (%)
a
*
b
c
*
d
e
f
Control diet
1% EPA diet
2% EPA diet
B
150
120
90
60
Epsp slope (%)
g
+
h
j
*
k
m
n
Sham Irradiated
Irradiated

Figure 3.55: LTP is attenuated by γ-irradiation but restored with dietary vitamins in the dentate gyrus.

LTP is impaired in the dentate gyrus of γ-irradiated rats fed on the control diet compared with the sham irradiated rats fed both diets and irradiated rats fed the vitamin diet. The mean slope of the population EPSP evoked by test stimuli delivered at 30s intervals before and after tetanic stimulation is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5min immediately prior to tetanic stimulation and values are expressed as the means ± the standard error of the means. Standard errors are included for every tenth response and some are so small in some cases as to be obscured.

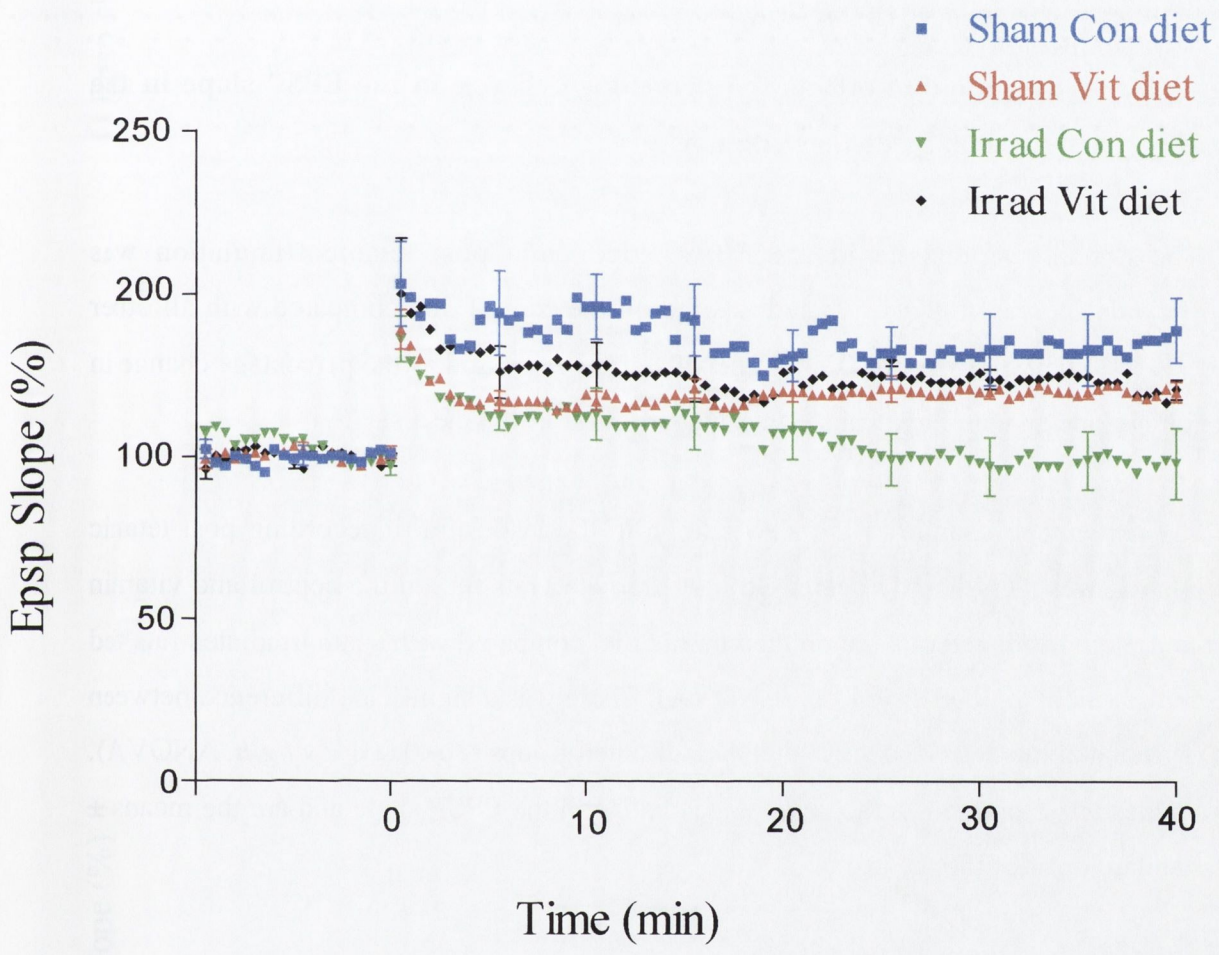

Sham Con diet
Sham Vit diet
Irrad Con diet
Irrad Vit diet
250
200
100
50
0
Epsp Slope (%)
0
10
20
30
40
Time (min)

Figure 3.56: γ-Irradiation affects the percentage change in the EPSP slope in the dentate gyrus, reversal by dietary vitamins.

A) The percentage change in the EPSP slope 2min post tetanic stimulation was significantly increased in γ-irradiated rats fed on the control diet compared with all other groups (*$p<0.01$ *a/b/d* v *c*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

B) The percentage change in the EPSP slope in the last 5min of recording post tetanic stimulation was significantly decreased in γ-irradiated rats fed on the control and vitamin diet and sham irradiated rats fed on the vitamin diet compared with sham irradiated rats fed the control diet ($^{+}p<0.001$ *e/f/h* v *g*, ANOVA). There was a significant difference between the γ-irradiated rats fed the control diet and all other groups (*$p<0.001$ *e* v *f/g/h*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

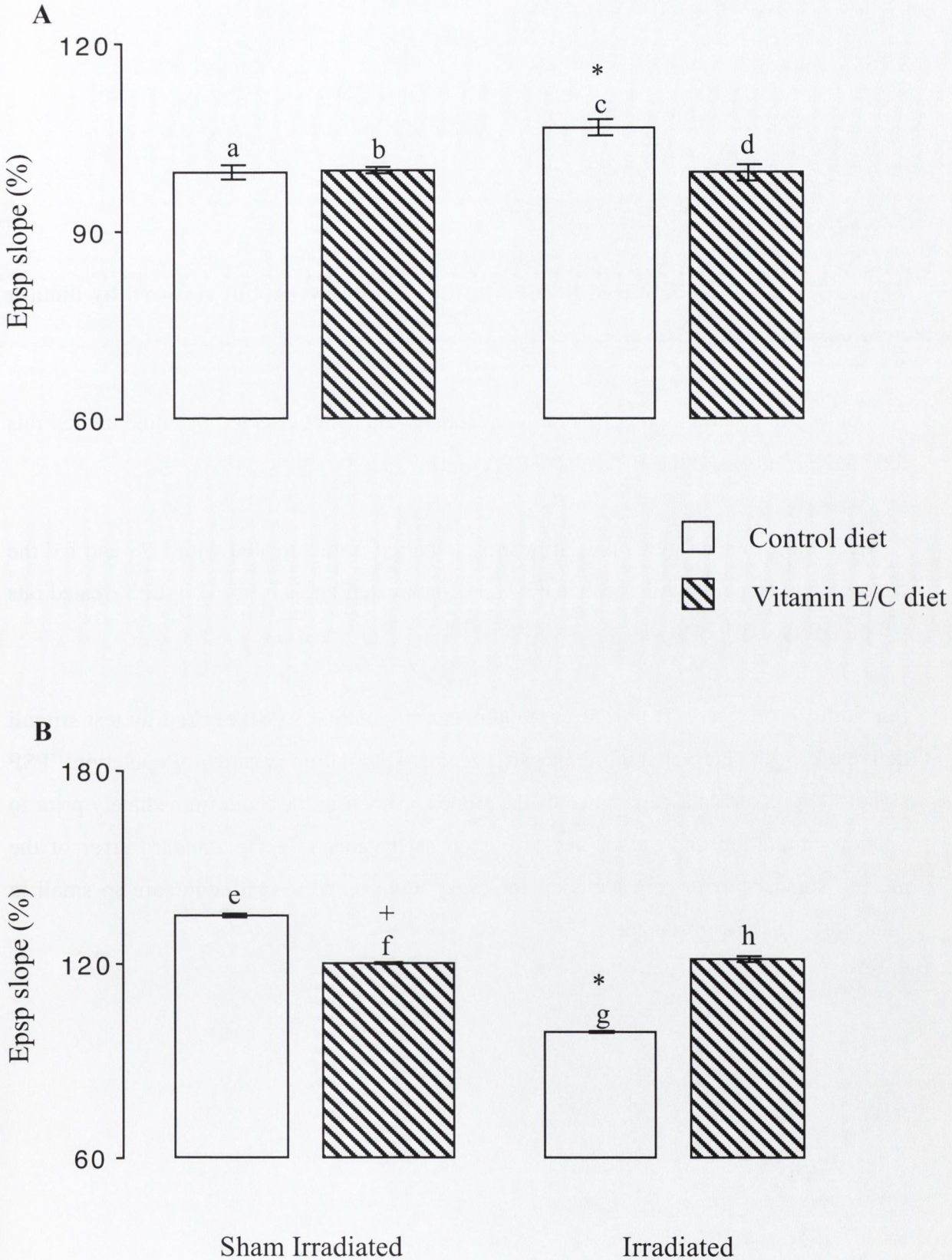

A
120
90
60
Epsp slope (%)
a
b
*
c
d
Control diet
Vitamin E/C diet
B
180
120
60
Epsp slope (%)
e
+
f
*
g
h
Sham Irradiated
Irradiated

Figure 3.57: LTP is impaired by LPS in the dentate gyrus, but restored by dietary manipulation with EPA.

A) Long-term potentiation (LTP) in the dentate gyrus is not effected in saline treated rats fed on the control and the 1% and 2% EPA diet.

B) LTP in the dentate gyrus is significantly impaired in rats treated with LPS and fed the control diet compared with those that were saline treated but is restored in LPS treated rats fed the 1% and 2% EPA diet.

For both figure A and B the mean slope of the population EPSP evoked by test stimuli delivered at 30s intervals before and after tetanic stimulation is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5min immediately prior to tetanic stimulation and values are expressed as the means ± the standard error of the means. Standard errors are included for every tenth response and some are so small in some cases as to be obscured.

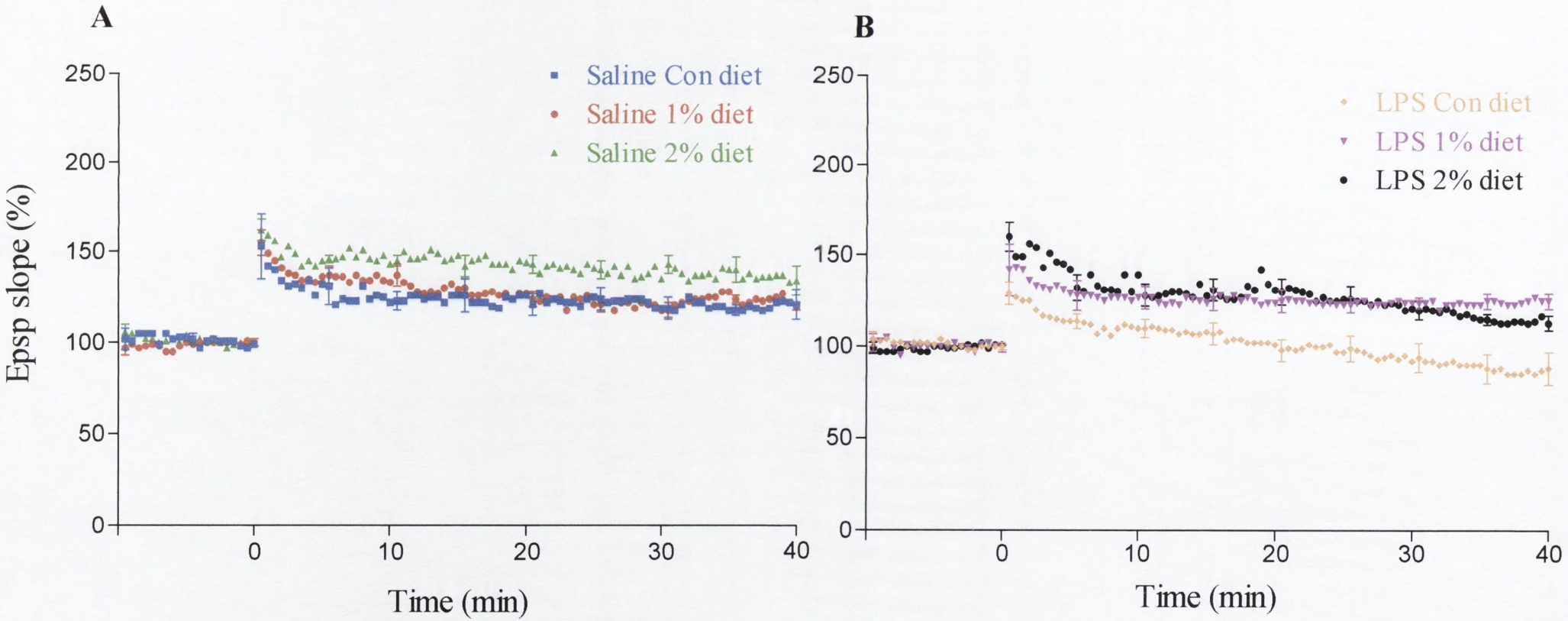

A
B
Saline Con diet
Saline 1% diet
Saline 2% diet
LPS Con diet
LPS 1% diet
LPS 2% diet
Epsp slope (%)
Time (min)
250
200
150
100
50
0
0
10
20
30
40

Figure 3.58: LPS affects the percentage change in the EPSP slope in the dentate gyrus.

A) The percentage change in the EPSP slope in the first 2min post tetanic stimulation was significantly decreased in saline treated rats fed on the 1% EPA diet and LPS treated rats fed the 2% EPA diet compared with all the other groups (*$p<0.05$ *a/c/d/e* v *d/f*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

B) The percentage change in the EPSP slope in the last 5min of recording post tetanic stimulation was significantly decreased in the LPS treated rats fed the control experimental diets compared with all other groups (*$p<0.001$ *g/h/j/m/n* v *k*, ANOVA). There was a significant increase observed in the percentage change in the EPSP slope in the saline treated rats fed the 2% diet compared with all other groups ($^{+}p<0.001$ *g/h/k/m/n* v *j*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

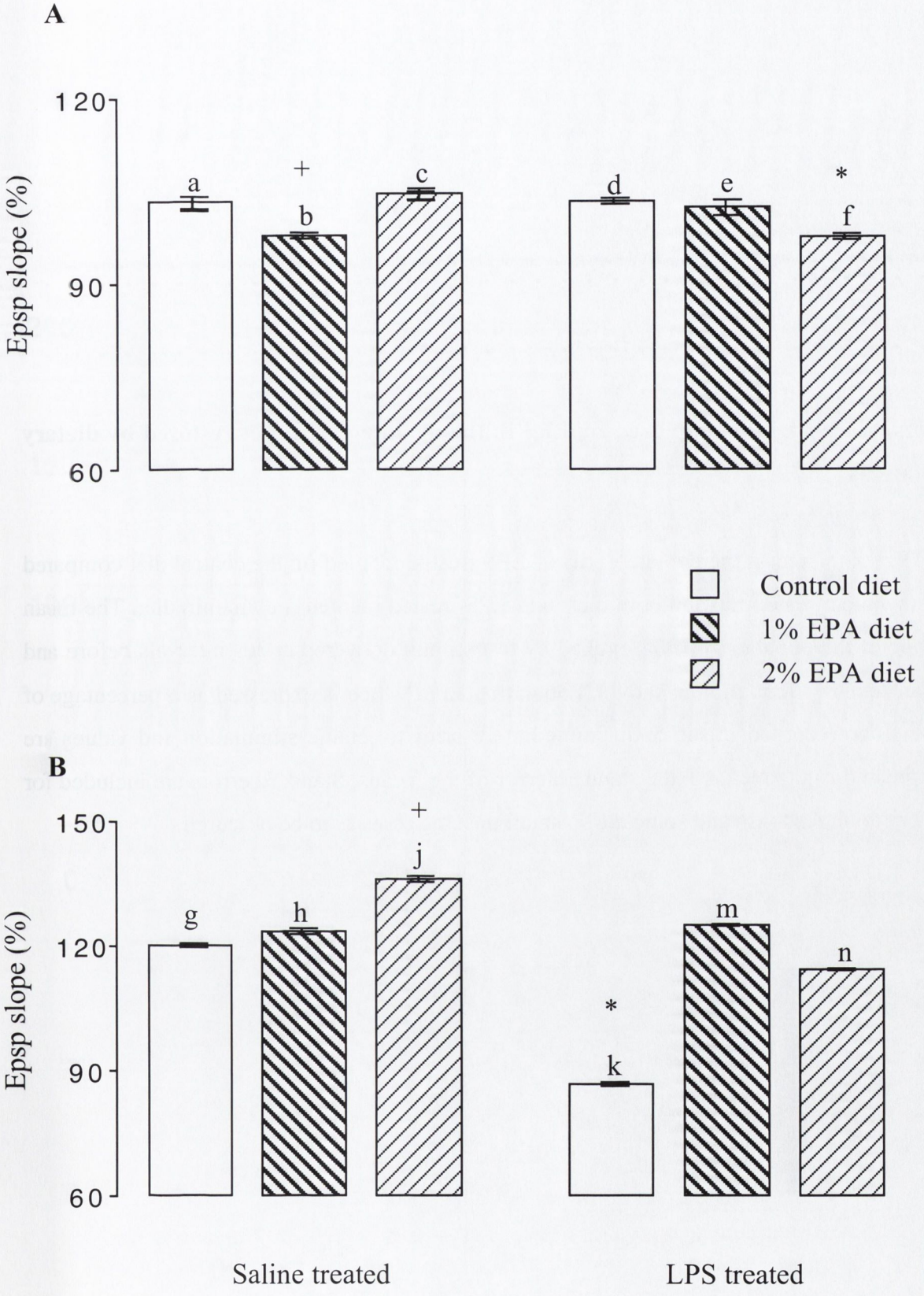

A
120
90
60
Epsp slope (%)
a
b
+
c
d
e
f
*
Control diet
1% EPA diet
2% EPA diet
B
150
120
90
60
Epsp slope (%)
g
h
+
j
*
k
m
n
Saline treated
LPS treated

Figure 3.59: LTP is impaired by LPS in the dentate gyrus, but restored by dietary vitamins.

LTP is impaired in the dentate gyrus of LPS treated rats fed on the control diet compared with saline treated rats fed both diets and LPS treated rats fed the vitamin diet. The mean slope of the population EPSP evoked by test stimuli delivered at 30s intervals before and after tetanic stimulation is shown. Population EPSP slope is expressed as a percentage of the slope recorded in the 5min immediately prior to tetanic stimulation and values are expressed as the means ± the standard error of the means. Standard errors are included for every tenth response and some are so small in some cases as to be obscured.

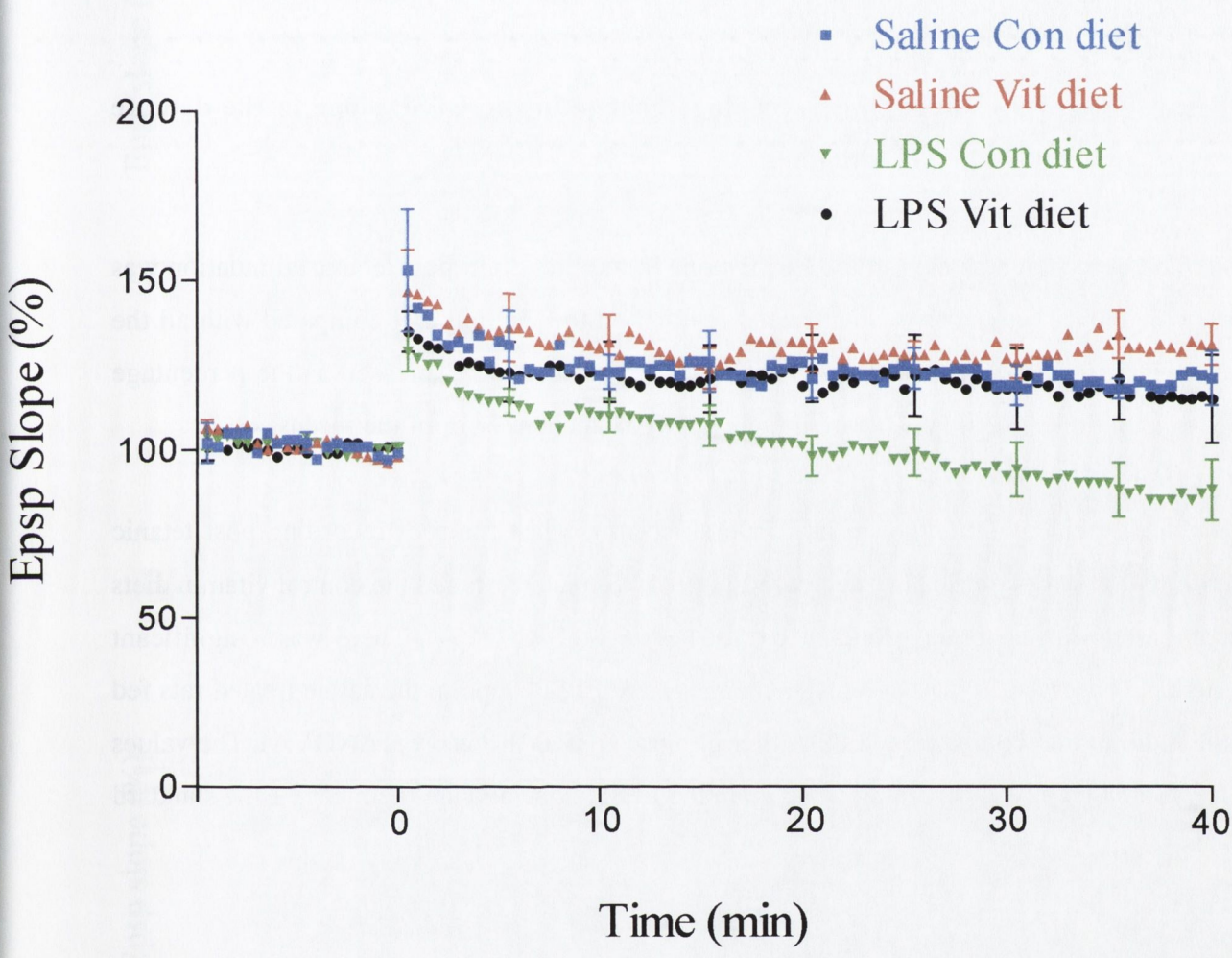
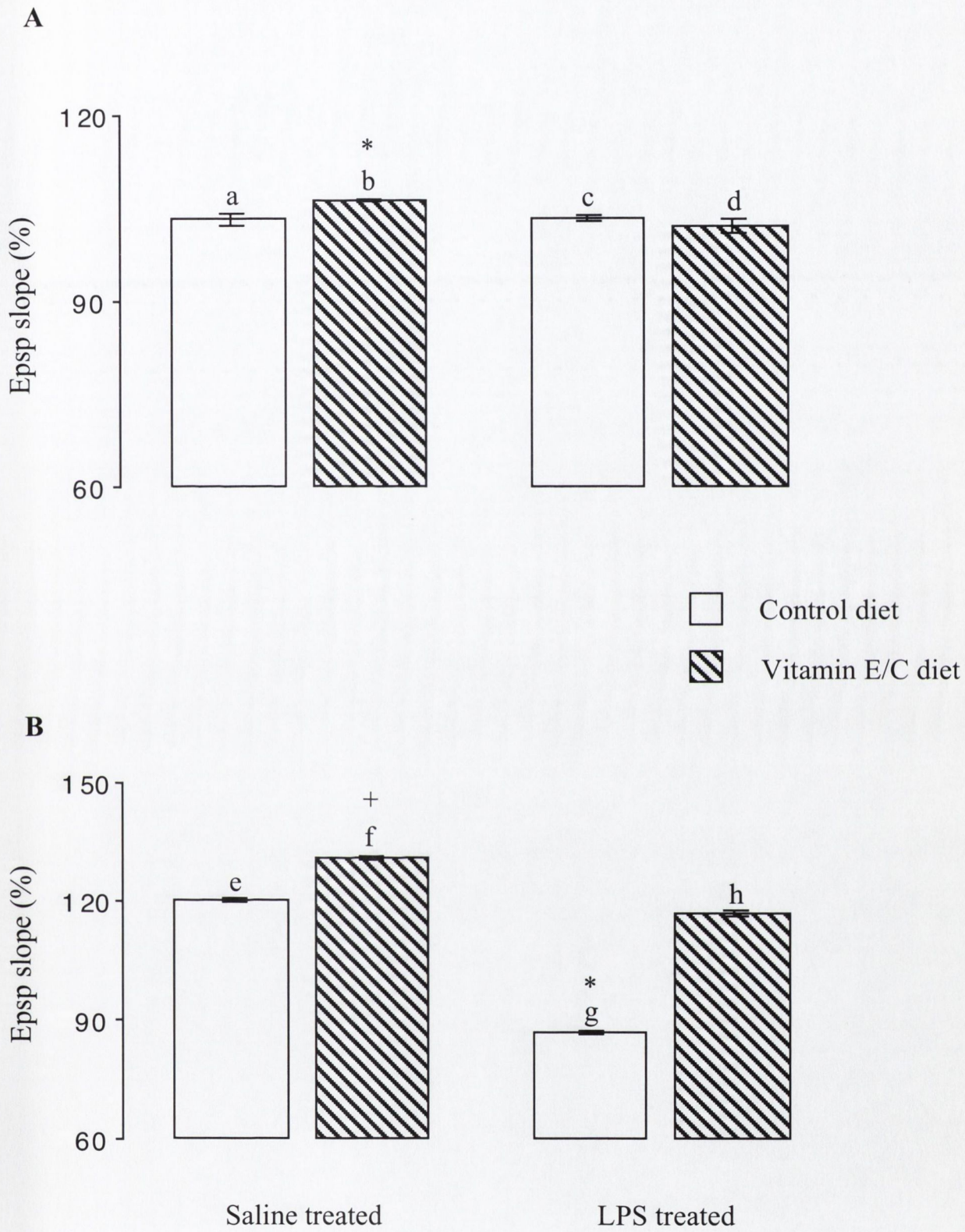

Saline Con diet
Saline Vit diet
LPS Con diet
LPS Vit diet
Epsp Slope (%)
200
150
100
50
0
0
10
20
30
40
Time (min)

Figure 3.60: LPS affects the percentage change in the EPSP slope in the dentate gyrus, reversal by dietary vitamins.

A) The percentage change in the EPSP slope in the first 2min post tetanic stimulation was significantly increased in saline treated rats fed on the vitamin diet compared with all the other groups (*p<0.05 *a/c/d* v *b*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.

B) The percentage change in the EPSP slope in the last 5min of recording post tetanic stimulation was significantly decreased in the LPS treated rats fed the control vitamin diets compared with all other groups (*p<0.001 *e/f/h* v *g*, ANOVA). There was a significant increase observed in the percentage change in the EPSP slope in the saline treated rats fed the vitamin diet compared with all other groups (+p<0.001 *e/g/h* v *f*, ANOVA). The values are expressed as the percentage change in the EPSP slope and are the means ± the standard error of the means.


A
120
90
60
Epsp slope (%)
a
*
b
c
d
Control diet
Vitamin E/C diet
B
150
120
90
60
Epsp slope (%)
e
+
f
*
g
h
Saline treated
LPS treated

Chapter 4:

Discussion

4 Discussion.

The main aim of this thesis was to assess the changes that occur in the brains of aged rats and rats that have been challenged with other stresses such as γ-irradiation and LPS. In all cases an increase in ROS production and IL-1β concentration was observed and, therefore, the role played by such an increase in neuronal stress was examined. The ability of certain putative antioxidants and the scavenger antioxidants vitamin C and E were assessed for their ability to reverse the observed changes in ROS and IL-1β. In order to achieve these aims four separate series of experiments were undertaken and the results of these are discussed in the following pages.

4.1 Analysis of changes in cortical tissue prepared from young and aged rats fed on a diet supplemented with α-lipoic acid.

The objective of the first series of experiments was to assess the age-related changes that occur in the cortex of the rat and to establish whether dietary supplementation with the putative antioxidant α-lipoic acid could reverse the changes observed. The main finding was that dietary supplementation reversed the age-related increases in reactive oxygen species production, interleukin-1β concentration and lipid peroxidation. Although it does not directly address the question, the evidence obtained was consistent with the idea that IL-1β increases ROS; there is some evidence that the action involves an increase in the activity of superoxide dismutase (SOD).

In a review of age-related changes, Harman stated that there is "accumulating evidence that aging is largely due to free radical damage" (Harman, 1981) and it is widely accepted that damage caused by free radicals results in the production of more free radicals. Therefore, it seemed reasonable to predict that ROS might be increased in the brain with age. The results of this study demonstrate that this increase occurs in the aged cortex. However, this increase was not seen in cortical tissue prepared from the aged animals fed on diet enriched in α-lipoic acid. It appeared that a lower concentration of the diet was more effective than a higher one in reversing the age-related increase in ROS. One possible explanation for this might be the more profound increase in the concentration of the n-3 polyunsaturated fatty acid, docosahexaenoic acid (DHA) with the higher concentration diet since it has been shown that when weanling rats were fed a diet enriched in this fatty acid, there was a significant increase in the production of ROS in the hippocampus (McGahon *et al.*, 1999c). It is possible

that the increased free DHA, which can be easily attacked by ROS, leads to a further increase in the production of ROS. Therefore, reducing the concentration of this PUFA in the diet may prevent this effect and, therefore, be more beneficial.

The present data, which indicate an age-related increase in ROS in the cortex, is consistent with data previously obtained in the hippocampus (Murray and Lynch, 1998a) and an increase in ROS has been observed in many disease states such as atherosclerosis, diabetes and cancer (Yu, 1994). The ability of α-lipoic acid to reverse the increase in ROS is not surprising since it is a recognised scavenger of many reactive oxygen and nitrogen species such as singlet oxygen, hydrogen peroxide, hydroxyl radicals, nitric oxide, peroxynitrite and hydrochlorous acid. In addition its reduced form, dihydrolipoic acid, is known to scavenge superoxide and peroxyl radicals (Biewenga *et al.*, 1997; Packer *et al.*, 1997).

Changes in the activity of the antioxidant enzymes SOD, catalase and glutathione peroxidase were analysed to try to explain the age-related increase in ROS. The data show that there was an increase in the activity of SOD in the cortical tissue prepared from the aged rats fed the control diet. A similar increase in SOD activity was seen in the hippocampus of the same aged rats, which concurs with the results of O'Donnell and Lynch (1998) thereby indicating an age-related increase in SOD activity in the cortex prepared from aged rats. The present study shows that there was an age-related decrease in the activity of the other antioxidant enzymes assessed, namely catalase and glutathione peroxidase; the decrease in glutathione peroxidase is consistent with previous findings (O'Donnell and Lynch, 1998). The age-related increase in SOD activity and concomitant decrease in glutathione peroxidase and catalase activity are likely to lead to an increase in the accumulation of H_2O_2. This may explain why there was an increase in ROS production because if there is a reduction in the activities of glutathione peroxidase and catalase, H_2O_2 will not be metabolised into water and molecular oxygen, thereby leading to the accumulation of H_2O_2. Dietary supplementation with lipoic acid reversed these age-related changes, which may also explain the reduction in ROS in the aged animals that received α-lipoic acid.

The findings of this study show that the concentrations of the antioxidant scavengers in the cortex were unaffected by age. This is consistent with results presented by Zhang and colleagues (1993) who reported no change in the concentrations of GSH and vitamin C in the cortex of the gerbil brain with age.

However, this is inconsistent with the findings of Arivazhagan and Panneerselvam (2000) who report a significant age-related decrease in the concentrations of the antioxidant scavengers in the cortex and hippocampus of rats. Therefore, the literature is conflicting. However, the concentrations of vitamin E and GSH in the present study were increased in the cortical tissue prepared from the aged rats fed the 100% α-LPA diet and this correlates more with the findings of Arivazhagan and Panneerselvam (2000) who showed that dietary supplementation with α-lipoic acid reversed the age-related decrease in the antioxidant scavengers. This may be due to the ability of α-lipoic acid to regenerate the endogenous antioxidants suggested by previous studies (Biewenga *et al.*, 1997; Packer *et al.*, 1997; Nickander *et al.*, 1996).

The consequences of the increase in ROS were sought, and the age-related increase in lipid peroxidation that was observed in this study confirms data obtained in previous studies (O'Donnell and Lynch, 1998; McGahon *et al.*, 1999a; Arivazhagan and Panneerselvam, 2000). The findings are also in agreement with the observation that ROS increases lipid peroxidation in several other cell types (Yu, 1994; Packer *et al.*, 1997; Harman, 1981). In the present study, dietary supplementation with α-lipoic acid was shown to reverse the age-related increase in lipid peroxidation. This is consistent with results presented by Arivazhagan and Panneerselvam (2000) who also showed that with α-lipoic acid in the cortex, as well as in the hippocampus, reversed the aged-related increase in lipid peroxidation.

Substrates for lipid peroxidation include the polyunsaturated fatty acids, one of which is AA. The age-related decrease in AA, which accompanied the increase in lipid peroxidation observed in this study, has been documented before in both the hippocampus (McGahon *et al.*, 1999a,b and c; Lynch and Voss, 1994) and cortex of the rat (O'Donnell and Lynch, 1998). However, the significance of the present data is that dietary supplementation with α-lipoic acid reversed this effect in cortical tissue. It seems reasonable to propose that this is due to the fact that α-lipoic acid reversed the age-related increase in ROS. It should be considered that reversal of this change in ROS may also be a consequence of the small concentration of AA that is present in the diet. Similar changes in AA concentration were also seen in hippocampal tissue prepared from these animals, including reversal of the age-related decrease with the α-lipoic acid diet (McGahon *et al.*, 1999b).

The ability of the pro-inflammatory cytokine IL-1β to increase ROS production was assessed in cortical tissue *in vitro*. The data indicate an IL-1β-induced increase of ROS production. It has been shown previously that IL-1β also increases ROS in the hippocampus *in vitro* (Murray and Lynch, 1998a). The finding that the SOD inhibitor, triethylenetetraamine blocked the effect of IL-1β implies that IL-1β increases ROS production via the action of SOD. This idea was supported by the observation that IL-1β significantly increased SOD activity.

IL-1β is activated by interleukin-1β converting enzyme (ICE) and so the effect of age on ICE activity was analysed. The data show that there was a significant age-related increase in ICE activity. The α-LPA diet was effective in reversing this increase suggesting that the antioxidant may act by downregulating the activity of ICE in aged animals. Since α-lipoic acid has been shown to act as an antioxidant in this study, and previously by others (Biewenga *et al.*, 1997, Packer *et al.*, 1997 and Nickander *et al.*, 1996), it seems reasonable to propose that ICE activation may be downstream of ROS production.

A significant age-related increase in IL-1β concentration was found in the cortex of rats fed on the control diet and both 50% and 100% α-LPA diets were effective in reversing this increase. The increase in ICE activity correlates with, and is likely to explain, the increase in the concentration of IL-1β. This increase in IL-1β confirms previous reported data, which indicated that there was an age-related increase in IL-1β in the hippocampus (Murray and Lynch, 1998a) and in the cortex (O'Donnell, *et al.*, 2000) of rats. The lower concentration of α-LPA diet was found to be more effective than the higher concentration and, as suggested before, this may be explained by the increased intake of DHA in this dietary group. It has been shown that incubating hippocampal tissue in the presence of H_2O_2 significantly increased ROS production (O'Donnell *et al.*, 2000) and, therefore, the increase in dietary DHA concentration, which the data show to enhance ROS in weanling rats (McGahon *et al.*, 1999c), may increase ROS which in turn may increase IL-1β. These findings are also consistent with those found in the hippocampus of the present study. IL-1β has also been shown to increase lipid peroxidation in cortical tissue *in vitro* (O'Donnell and Lynch, 1998) and this may be used to explain the age-related increase in lipid peroxidation.

Thus, several related changes occur in the cortex of the aged brain and several of these can be reversed by dietary supplementation with α-lipoic acid. Age is

associated with an increase in ROS production and the evidence indicates that this is due to the age-related increase in IL-1β concentration which has been shown to lead to an increase in the activity of SOD. Age is also associated with a decrease in the activities of catalase and glutathione peroxidase and it is proposed that this decrease, combined with the increase in SOD activity, results in an increase in ROS. The data is consistent with the suggestion that increased ROS production leads to an increase in lipid peroxidation and a decrease in the concentration of AA. This results in a loss of membrane fluidity that is thought to be responsible for the decline in synaptic function which in turn is likely to significantly contribute to the decline in cognitive function associated with age.

α-Lipoic acid was shown to be an effective antioxidant, in the first instance, by its ability to reverse the age-related increase in ROS production. The ability of the diet to reverse the age-related increase in IL-1β concentration, which is likely to be responsible for the increased SOD activity, may be a key factor in this. The dual reduction in ROS accumulation and IL-1β concentration may lead to the reduced lipid peroxidation and thus, effect a reversal in the age-related decrease in the concentration of AA in the cortex of the rat.

4.2 Analysis of changes in hippocampal tissue prepared from aged and young rats fed on a diet supplemented with eicosapentaenoic acid.

Subsequent experiments assessed the ability of a diet enriched in the polyunsaturated fatty acid and putative anti-inflammatory eicosapentaenoic acid (EPA) in reversing the age-related changes observed in the rat brain. One objective of this study was to assess the ability of the EPA diet to reverse age-related changes in LTP. The second objective was to assess age-related changes in certain factors that may cause an impairment in LTP and to assess the ability of the diet to reverse this impairment.

The main findings of the study were that there was an age-related impairment in LTP and the EPA-enriched diet reversed this impairment. The impairment was accompanied by age-related increases in ROS, lipid peroxidation and IL-1β in the hippocampus, which are possible contributing factors in the impairment of LTP. Some, but not all, of these changes were reversed by dietary supplementation.

In the dentate gyrus, LTP was successfully induced and maintained in young rats fed either the control or EPA-enriched diet but aged rats fed on the control diet were unable to maintain LTP in the present study. While it is generally accepted that there is an impairment in the ability of aged animals to sustain LTP, there are conflicting views over whether the induction of LTP is impaired in the aged animal. Some research groups suggest that induction of LTP is unaffected by age but that the rate of decay of LTP is accelerated in aged animals (Barnes, 1979; Deupree *et al.*, 1993). Lynch and Voss (1994) showed that there was an attenuation in the percentage increase in the EPSP slope in aged rats following tetanic stimulation of the perforant path. Other reports have shown that the attenuation may not be significant but does exist (McGahon *et al.*, 1997 and 1999c). This may be due to the impairment in temporal summation of multiple EPSPs during high frequency stimulation (Rosenzweig *et al.*, 1997). The present study does not address the question of whether an age-related impairment in the induction of LTP exists as the recording equipment used was set up for this measurement.

It has previously been shown by Landfield and colleagues (1978) that an age-related impairment in the maintenance of LTP exists in CA1 slices following stimulation of the Schaffer collaterals and this has been repeatedly shown to occur in the dentate gyrus *in vivo* in this laboratory (McGahon *et al.*, 1997; McGahon *et al.*, 1999a,b and c; O'Donnell *et al.*, 2000 and Kelly *et al.*, 2000). This impairment may be due to a number of factors but, importantly, it has been shown previously that it is probably due to an age-related decrease in glutamate release in synaptosomes prepared from either the dentate gyrus or hippocampus (Lynch and Voss, 1994; McGahon *et al.*, 1997; McGahon *et al.*, 1999a,b and c, and Murray *et al.*, 1997). The presynaptic release of glutamate is necessary for the maintenance of LTP. The induction of LTP requires an influx of Ca^{2+} into the postsynaptic terminal and it has also been shown that a decrease in Ca^{2+}-channel numbers and activity is seen in aged rats (Verkhratsky *et al.*, 1994). Protein synthesis has been shown to be necessary for the maintenance of LTP, since the protein synthesis inhibitor anisomycin prevents LTP, and protein synthesis is also reduced in the aged rat (Mullany and Lynch, 1997).

In the present study, aged rats fed on the EPA-enriched diet had their ability to maintain LTP restored. This correlates with findings by McGahon and colleagues (1999a and c) who showed that dietary supplementation with other polyunsaturated

fatty acids such as AA and docosahexaenoic acid (DHA) reversed the age-related impairment in LTP. In the present study, considering the above findings, an explanation for the ability of EPA to reverse the age-related impairment in LTP was sought.

An age-related impairment in LTP is known to be associated with several measures as discussed above. Another of these factors is an increase in ROS production (Murray and Lynch, 1998a) and, therefore, ROS production was assessed in the hippocampus. An age-related increase in ROS production was found and dietary manipulation with EPA only partially reversed this increase. This observation is contrary to results reported by Murray and Lynch (1998b) where a diet enriched in vitamins C and E reversed the age-related increase in ROS production in the hippocampus. However, a similar result, to that reported by Murray and Lynch (1998b), was found when the age-related increase in ROS production was reversed in aged rats fed on a diet enriched in DHA. The inability of the EPA diet to completely reverse the age-related changes may be due to the relatively low concentration of EPA added to the diet. The approximate percentage of EPA added to the laboratory chow was 0.05% of the daily food taken per rat. In a review by Youdim and colleagues (2000) the recommended intake of EPA per day was 0.4% of food consumed per day or around 900mg/day for humans. The dose of 10 to 20mg/rat/day which was used in this study falls short of this recommendation.

Antioxidant enzyme status was assessed in an effort to explain the age-related increase in ROS production. The literature appears to be contradictory in this respect. Data presented by Danh and colleagues (1983) showed that SOD activity was unchanged in the hippocampus prepared from aged rats, but this differs from previous studies carried out in this and other laboratories where SOD activity was increased (O'Donnell *et al.*, 2000 and Carrillo *et al.*, 1992). In the present study SOD activity was similar in both young and aged rats and supports the findings of Danh and colleagues (1983). A significant, positive correlation exists between the activity of SOD and ROS production implying that (in the absence of other changes) with increasing SOD activity there is a theoretical concomitant increase in ROS production. The explanation for the differing results is unclear as the animals used by Danh and colleagues (1983) were of the same species, sex and strain as those used in this laboratory. One plausible explanation for this discrepancy would be the condition of the aged rats. Although they

were of the same age, the conditions of a set of aged rats may differ causing discrepancies between findings.

Catalase activity was similar in the cortex of aged and young rats, as found previously in this laboratory, but not in the hippocampus of aged rats (O'Donnell *et al.*, 2000 and O'Donnell and Lynch, 1998). The present study, in the hippocampus, shows that catalase activity was similar in all groups. An age-related increase in glutathione peroxidase activity was found in the present study and again, this differs from previous findings where no change in activity was seen in the hippocampus of aged rats (O'Donnell *et al.*, 2000). The differing results may be explained again by a possible difference in the condition of the different groups of aged rats. Therefore, the activity of the antioxidant enzymes does not provide an explanation for the age-related increase in ROS production in this study. However, it has been shown previously in this laboratory that there is an age-related decrease in the concentrations of vitamin E and C and GSH in the hippocampus and this may explain the increase in ROS accumulation in the present study (O'Donnell *et al.*, 2000).

Parallel changes in ROS and lipid peroxidation were observed in the cortex and similar changes have been found in the hippocampus of aged rats (Murray and Lynch, 1998b). An age-related increase in lipid peroxidation was found in the hippocampus in this study, which confirms findings of other studies (Arivazhagan and Panneerselvam, 2000; McGahon *et al.*, 1999a). If lipid peroxidation is causally related to ROS accumulation, then the prediction from the ROS data in this study, might be that dietary manipulation with EPA would not affect the age-related increase in lipid peroxidation. In agreement with the prediction the experimental EPA diet was found to be unsuccessful in reversing this increase in lipid peroxidation correlating with the limited ability of the diet to reverse the age-related increase in ROS production. In a study by Moison and Beijersbergen Van Henegouwen (2001) rats fed a diet enriched in EPA also failed to reverse the UVB radiation–induced increase in lipid peroxidation. Thus, the strong link between the ROS production and lipid peroxidation is maintained.

The ability of the EPA diet to reverse the age-related impairment in LTP does not parallel with its lack of ability to reverse the age-related increase in ROS. Therefore, since IL-1β has been shown previously to impair LTP following intracerebroventricular (icv) injection (Vereker *et al.*, 2000a; Murray and Lynch, 1998a) the concentration of the cytokine was assessed in the hippocampus. An age-

related increase in IL-1β was observed in the hippocampus and this result has been reported before in the rat hippocampus (McGahon *et al.*, 1999a and b), cortex (O'Donnell and Lynch, 1998) and dentate gyrus (Murray and Lynch, 1998a). This increase was not seen in tissue prepared from aged rats fed on the EPA diet. Previous studies carried out in this laboratory showed that dietary supplementation with a combination of AA and γ-linolenic acid also reversed the age-related increase in IL-1β (McGahon *et al.*, 1999a). In the AA diet study, the age-related increase in lipid peroxidation was also reversed and parallel changes in IL-1β concentration and oxidative stress were observed which contrasts with data obtained in the present study as the EPA diet had no effect on the age-related increase in lipid peroxidation. Babcock and colleagues (2000) proposed one plausible explanation for how EPA-enriched diets might reverse the age-related increase in IL-1β. The authors propose that substitution of EPA for AA in phospholipids reduces the production of cytokines such as IL-1β. This occurs through the phospholipase A_2-dependent metabolism of AA (which leads to an increase in free AA), which may result in an increase in the cyclooxygenase-dependent conversion of free AA to prostaglandin E_2 (PGE_2). PGE_2 is an eicosanoid, which is capable of producing an increase in cytokines such as IL-1β. Therefore, substitution of EPA for AA reduces free AA, which may in turn reduce PGE_2 production and, hence, IL-1β production (Babcock *et al.*, 2000).

In an effort to understand a possible interaction between IL-1β and ROS, H_2O_2, a known ROS, was shown to increase IL-1β concentration in hippocampal tissue *in vitro*. On the basis of this it might be suggested that the age-related increase in ROS may lead to the increase in IL-1β concentration in hippocampii prepared from aged rats. However, the relationship between IL-1β and ROS is more complex since the converse has also been shown where IL-1β significantly increased ROS production in hippocampal tissue *in vitro* (O'Donnell *et al.*, 2000). It is reasonable to presume, therefore, that a feed-back loop exists between IL-1β and ROS where, when one is increased it leads to the increase of the other.

The mitogen-activated protein kinase, p38 is involved in receiving signals from upstream activators and transferring them downstream to other target proteins. It can be activated by many stimuli including the proinflammatory cytokines, irradiation and oxidative stress (Egan and Weinberg, 1993). In the present study an experiment *in vitro* in hippocampal tissue, IL-1β was shown to increase p38 phosphorylation. H_2O_2 was

also shown to increase p38 phosphorylation *in vitro*. This is consistent with work reported by Badger and colleagues (2000) who showed that the p38 inhibitor SB 242235 blocked the IL-1β induced increase in PGE_2 in both bovine and human chondrocyte cultures. In another study, which highlights the ability of p38 to affect LTP, SB203580, another selective p38 inhibitor, was shown to block the inhibitory effects of IL-1β and β-amyloid on LTP in the dentate gyrus *in vitro* (Coogan et al., 1999; Saleshando and O'Connor, 2000).

Since age is associated with an increase in IL-1β concentration and ROS production, and since both were shown to stimulate p38 activity *in vitro,* the age-related and dietary-related effects on p38 phosphorylation were assessed. An age-related increase in p38 phosphorylation was found in hippocampal homogenate and this confirmed data from a previous study in which p38 phosphorylation was increased *in vivo* as well as *in vitro* (O'Donnell *et al.*, 2000). Dietary supplementation with EPA reversed this age-related effect on p38 and it is suggested that this may be attributed to the ability of the EPA diet to reverse the age-related increase in IL-1β concentration.

Previous studies have shown an age-related decrease in glutamate release in the dentate gyrus (Vereker *et al.*, 2000a; McGahon *et al.*, 1999a, b and c; McGahon *et al.*, 1997) and it has been reported that the age-related impairment in LTP is closely associated with a decrease in glutamate release. Indeed a positive correlation between LTP and glutamate release has been shown (Martin *et al.*, 2001). It is significant that IL-1β exerts an inhibitory effect on glutamate release. Thus, icv injection of IL-1β, which blocks LTP, inhibits LTP-associated increase in glutamate release (Vereker *et al.*, 2000a). The possibility that the IL-1β induced reduction in glutamate release was related to its ability to increase p38 phosphorylation, was, therefore, assessed. In the present study IL-1β was shown to impair glutamate release in dentate gyrus tissue *in vitro* and the p38 inhibitor SB203580 blocked this effect. It was therefore predicted that glutamate release would be decreased in aged rats in parallel with the age-related increases in IL-1β concentration and p38 activity. This prediction was confirmed in this study where an age-related decrease in glutamate release was observed. This confirms previous studies (Vereker *et al.*, 2000a; McGahon *et al.*, 1999a, b and c; McGahon *et al.*, 1997) and it has also been shown that an age-related decrease in other transmitters, such as 5-hydroxytryptamine, exists (Friedman and Wang, 1989). Dietary manipulation with EPA reversed the age-related decrease in glutamate release; this may be secondary

to the ability of the diet to reverse the increases in both IL-1β and p38 in the hippocampus prepared from aged rats. Significantly, dietary supplementation with α-lipoic acid (McGahon *et al.*, 1999b) and the polyunsaturated fatty acids AA and DHA also reversed the age-related decrease in glutamate release (McGahon *et al.*, 1999a and c). These data add weight to the argument that LTP and an increase in glutamate release are closely linked.

The cell bodies of the perforant path synapses are located in the entorhinal cortex, therefore, any changes in these may impact on presynaptic activity in the dentate gyrus and so the activity of p38 was assessed in this region. An age-related increase in p38 activity was found which parallels changes in the hippocampus reported here and in previous studies (O'Donnell *et al.*, 2000). Dietary manipulation with EPA did not appear to be successful in reversing this age-related change. However, it should be acknowledged that the number of observations was low, due to the relatively small amount of tissue that was obtained from the entorhinal cortex. The number of experiments needs to be increased to provide more convincing evidence of age-related and diet-related changes.

TUNEL staining allows identification of cells containing nicked DNA and it is considered to be one indicator of programmed cell death. Cell death (or apoptosis) is associated with DNA fragmentation, chromatin condensation, mitochondrial damage and formation of apoptotic bodies (Thornberry and Lazebnik, 1998). It is widely known that cell death is triggered by insults such as ROS (Harman, 1981), IL-1β (Hu *et al.*, 1997), LPS treatment (Vereker *et al.*, 2000b) and traumatic brain injury (Clark *et al.*, 1999). The percentage of TUNEL positive cells was increased in aged rats fed on control diet and dietary supplementation with EPA reversed this age-related increase in the entorhinal cortex. Unfortunately, the reason for this observation can only be surmised, again because of the lack of tissue. It is possible that EPA exerts its effect by reversing the age-related increase in IL-1β. In a study by Holmin and Mathiesen (2000), intracerebroventricular injection of IL-1β increased the percentage of TUNEL positive cells in Sprague-Dawley rats suggesting that the increase in cell death in the present study may be due to an increase in IL-1β. The increase in cell death in the region of the brain that contains the cell bodies of perforant path-granule cell synapses (i.e. the pathway in which LTP experiments were carried out) might be expected to have a deleterious effect on the ability of aged rats to maintain LTP. The positive

influence of the diet in sparing these cells from death is likely to have an impact on the potential of the aged rats to maintain LTP. Therefore, the capacity of dietary EPA to reverse the age-related impairment in LTP is probably due to its inhibition of the increases observed in IL-1β concentration as described by Babcock and colleagues (2000). This, in turn, may prevent an increase in p38 phosphorylation. This is significant because p38 phosphorylation has been shown to cause cell death. It has also been shown that arsenite-induced apoptosis was mediated through the activation of p38 (Namgung and Xia, 2000).

Based on several experiments from various laboratories, including those in the present study, it can suggested that the ability of the EPA diet to reverse the age-related decrease in glutamate release impacts greatly on the aged rats potential to maintain LTP. Similarly, protecting the cells that play a role in synaptic plasticity from apoptosis will allow the aged rat to maintain LTP. This protection may be afforded by both a reduction in p38 activity and IL-1β concentration. This may, in turn, reverse the decrease in glutamate release observed with ageing.

4.3 Analysis of changes in cortical tissue prepared from aged and young rats fed on a diet supplemented with eicosapentaenoic acid.

Due to the limited amount of tissue available from the hippocampus of these rats, further investigations into the effects that the EPA diet had on the aged brain were carried out in cortical tissue prepared from the same rats. ROS production was assessed initially to observe if the findings in the cortex were similar to those found in the hippocampus. This was confirmed to be the case; an age-related increase in ROS accumulation in the cortex was found and, as observed in the hippocampus, dietary manipulation was unsuccessful in reversing this increase.

Several studies (Murray and Lynch, 1998 a and b; O'Donnell *et al.*, 2000), including this one, have shown that parallel changes in ROS and IL-1β occur in the hippocampus. An age-related increase in IL-1β concentration was found in cortical tissue and this confirms previous studies in the cortex (O'Donnell and Lynch, 1998). Dietary manipulation with EPA was successful in reversing this increase in IL-1β. These data are, therefore, similar to the results found in the hippocampus. The action whereby EPA has the ability to reduce cytokine production was alluded to earlier in the report by Babcock and colleagues (2000).

TNF-α is another cytokine which can be released during inflammation and cell stress. TNF-α mRNA levels were found to be increased in the hippocampus of mice following intraperitoneal injection of the trimethyltin, which is an organometal known to induce neuronal necrosis (Bruccoleri *et al.*, 1998). TNF-α has also been shown to be increased in peritoneal macrophages and spleen cells of aged mice (Han *et al.*, 1995). It was thought possible that concomitant increases in TNF-α and IL-1β may occur in the cortex of aged rats. However, the present study shows that TNF-α concentration is not altered with age or with dietary supplementation. The literature suggests that TNF-α can be both neuroprotective (Tamatani *et al.*, 1999) and neurodegenerative (Loddick and Rothwell, 1999).

As changes in IL-1β concentration and p38 phosphorylation were found to be closely linked in the hippocampus (O'Donnell *et al.*, 2000; see above) p38 activity was assessed in the cortex to establish whether a similar link was specific to the hippocampus or more widespread. The data revealed an age-related increase in p38 phosphorylation. This increase was not observed in tissue prepared from aged rats fed the EPA diet, indicating that in the cortex, like the hippocampus, a coupling of these parameters occurs. This coupling was confirmed by the observation that IL-1β increased p38 phosphorylation in cortical tissue *in vitro,* which parallels data from the hippocampus. Therefore, it can be postulated that dietary manipulation with EPA reverses the age-related increase in p38 phosphorylation in cortical tissue by reducing the concentration of IL-1β.

Features of cell stress that can lead to cell death are often associated with the mitochondria. A mitochondrial transmembrane potential exists ($\Delta\Psi_m$) and disruption of this potential signals an early event in apoptosis (Cortopassi and Wong, 1999). A second related detectable event that occurs is the release of cytochrome c from the mitochondria to the cytosol of the cell (for review see Cortopassi and Wong, 1999). In an attempt to assess the degree of cell stress in the cortex, cytochrome c translocation in aged rats was examined and an age-related increase in this measure was found in cortical tissue prepared from aged rats fed on control diet. However, this increase was reversed by dietary supplementation with EPA.

Apoptotic signals have been shown to increase cytochrome c translocation (Yang and Cortopassi, 1998) and there is evidence to suggest that IL-1β acts as an apoptotic agent in some circumstances (Touzani *et al.*, 1999). It is not clear whether

p38 plays such a role, and to address this question in cortical tissue, samples were incubated with IL-1β and the p38 inhibitor SB203580. It was found that IL-1β increased cytochrome c expression in the cytosol suggesting that it induced translocation. Significantly, its ability induce translocation was blocked by the IL-1β receptor antagonist (IL-1ra). IL-1ra competes with IL-1β for binding sites on the IL-1 type I receptor (for review see Dinarello, 1996) and, therefore, it appears that the interaction of IL-1β with its receptor is required for the translocation of cytochrome c. The IL-1β induced increase in cytochrome c translocation *in vitro* was blocked by the actions of the p38 inhibitor SB203580. These observations suggests that binding of IL-1β to its receptor leads to downstream activation of p38 which then instigates cytochrome c translocation from the mitochondria to the cytosol. The present results are the first to show that p38 directly interacts with mitochondrial proteins such as cytochrome c in the cortex.

This study also showed that incubation *in vitro* with H_2O_2 increased cytochrome c translocation in cortical tissue. It has previously been shown that H_2O_2 increased cytochrome c translocation in Jurkat T cells within 2hr of treatment (Stridh *et al.*, 1998). This is not surprising as oxidative stress is known to disrupt the $\Delta\Psi_m$ (Cortopassi and Wong, 1999). However, in the present study it is more likely that IL-1β has the greater effect on the mitochondria due to the fact that EPA did not reverse the age-related increase in ROS production, while it was effective in reversing the age-related increase in IL-1β concentration.

Two mitochondrial membrane proteins that modulate the release of cytochrome c into the cytosol are Bcl-2, which is thought to be anti-apoptotic, and Bax, which is thought to be pro-apoptotic. Bcl-2 and Bax can homo- or heterodimerise and each can neutralise the other's effect suggesting that their relative concentration in mitochondrial membranes can determine whether or not cell death will occur (Kannan and Jain, 2000). Bcl-2 is localised on the cytoplasmic face of the mitochondria and seems to maintain the membrane integrity of the mitochondria by preventing, directly or indirectly, the release of cytochrome c. Heterodimerisation of Bcl-2 with Bax or large concentrations of the Bax homodimer, can inhibit the ability of Bcl-2 to prevent cytochrome c release (Kannan and Jain, 2000). It was considered that altered expression of Bax and/or Bcl-2 may contribute to the increase in cytochrome c translocation in the present study, therefore, the expression of these proteins was

assessed in cortical tissue prepared from young and aged rats. However, no clear-cut age-related or diet-related change was observed in either protein or in the ratio of one to the other. This was unexpected, as in a report by Savory and colleagues (1999) where aged rabbits were treated intracisternally with aluminium maltolate, which is thought to induce oxidative stress, in hippocampal neurons after 72hr the immunoreactivity for Bcl-2 was low and high for Bax. This finding was not observed in the young rabbits (Savory *et al.*, 1999). It may be a case that other members of the Bcl-2 family, that are known to be both anti- and pro-apoptotic, such as Bad, Bcl-XL and p53, may be responsible for the observed translocation in cytochrome c

One downstream consequence of increased cytochrome c translocation into the cytosol is an increase in caspase-3 activation. This occurs due to the ability of cytochrome c to activate Apoptotic Protease-Activating Factor-1 (APAF-1), which in turn activates the caspase cascade (Kannan and Jain, 2000). Caspase-9, known as an initiator caspase, is activated first and this is followed by activation of caspase-3, known as an effector caspase (Kannan and Jain, 1999; Slee *et al.*, 1999). Caspases are cysteine proteases that cleave target proteins at aspartate residues.

Consistant with the view that cytochrome c can activate the pathway leading to an increased caspase-3 activation, an age-related increase in the enzyme was observed. This result provides further evidence of an age-related activation of a cascade of biochemical events which may ultimately lead to cell death. Dietary supplementation with EPA reversed this age-related increase in caspase-3. In order to assess whether caspase-3 was activated primarily due to an increase in IL-1β, experiments *in vitro* were performed on cortical tissue incubated in the presence and absence of IL-1β. The data from these experiments show that IL-1β increased caspase-3 activity in cortical tissue *in vitro* and this was blocked by the binding of IL-1ra to the receptor (as discussed above) The IL-1β-induced increase in caspase-3 activation was also blocked by the p38 inhibitor SB203580. This implies that IL-1β activates caspase-3 through the phosphorylation of p38. Although the findings were made *in vitro,* the data provides strong circumstantial evidence that the age-related increase in caspase-3 activation observed in this study was activated by IL-1β and relied on the ability of IL-1β to phosphorylate p38. This work concurs with results presented by Zhuang and colleagues (2000) who reported that HL-60 cells were saved from singlet oxygen-induced apoptosis through incubation with SB203580. Incubation of SB203580 also prevented

the increase in caspase-3, which is usually associated with singlet oxygen-induced apoptosis (Zhuang *et al.*, 2000). A recent study by McLaughlin and colleagues (2001) confirmed this finding. They showed that another p38 inhibitor, SB239063, prevented caspase activity in thiol-oxidant-induced apoptosis in forebrain neuronal-enriched cell cultures. The ability of the EPA diet to reverse the age-related increase in IL-1β may, therefore, explain its capacity to reverse the age-related increase in caspase-3 activation.

H_2O_2 was also tested for its ability to increase caspase-3 activation. Its stimulating effect on caspase-3 activation in cortical tissue *in vitro* was also blocked by the p38 inhibitor, SB203580. This finding apparently contradicts the work of Zhuang and colleagues (2000) who reported that singlet oxygen, but not H_20_2, induced caspase-3 activation. However, their work was carried out in HL-60 cells and not rat cortical tissue as was the case in this study. Other work by Stridh and colleagues (1998) showed that H_2O_2 induced caspase-3 activation in Jurkat T cells 3 to 4 hr after treatment. These findings suggest that activation of caspase-3 is modulated by different mechanisms in different cell types.

Poly- (ADP-ribose) polymerase (PARP) is a DNA repair enzyme that plays an important role in determining cell survival. It is known to be a cellular substrate for caspase-3 and has a defined motif DXXD which is the key site for cleavage (Kannan and Jain, 2000). PARP cleavage is considered to be another cell marker that is detectable during apoptosis. In an effort to consolidate the findings which indicate age-related cell death in cortical tissue, cleavage of the 116kD PARP was assessed. The data showed that there was an age-related decrease in the 116kD PARP protein. This implies that either PARP expression is downregulated in aged rats or that it is cleaved. The age-related increase in caspase-3 activity suggests that the latter is more probable. In parallel with the changes in caspase-3 activation, dietary supplementation with EPA was successful in reversing this decrease in PARP. The data point to the likelihood that this may also be due to the effectiveness of dietary manipulation with EPA in reversing the age-related increase in IL-1β. This is consistent with previous findings in this laboratory, which showed that the caspase-1 (or ICE) inhibitor, Ac-YVAD-CMK blocked LPS-induced increase in IL-1β concentration and the LPS-induced decrease in the 116kD PARP protein (Vereker *et al.*, 2000b).

Reduced activation of PARP, increased activation of caspase-3 and increased cytochrome c translocation are strong indicators, particularly when taken together that apoptotic processes are in progress. Therefore, it can be proposed that there is an age-related increase in cell death in the cortex. In an effort to consolidate this, TUNEL staining of cortical cells was assessed The percentage of TUNEL positive cells was increased in cortical cells prepared from aged rats fed on the control diet. This age-related increase was reversed by dietary EPA supplementation. The reason for this is likely to be a consequence of the effect of the diet on IL-1β concentration.

This study has shown that, at least *in vitro,* IL-1β by acting on IL-1RI can increase cytochrome c translocation and caspase-3 activation through the actions of p38. Cytochrome c, as a consequence of its interaction with APAF-1 and subsequent activation of caspase-9, activates caspase-3 which, in turn, can cleave the DNA repair enzyme PARP. The inactivation of PARP is likely to contribute to an increase in cell death. The reversal of the changes observed in these parameters by dietary manipulation with EPA can be linked back to the diet-related reversal of the increase in IL-1β.

4.4 Analysis of changes in hippocampal tissue that occur following whole-body irradiation and LPS treatment.

In the final series of experiments, stresses other than age were assessed for their ability to change some of the same factors assessed in the age studies. The experiments focused on changes in the hippocampus and specifically on LTP induced by γ-irradiation and LPS treatment. Both have been shown previously to affect changes in synaptic plasticity (Bassant and Court, 1978; Vereker *et al.*, 2000b).

In one series of experiments, young rats were subjected to one dose of whole-body γ-irradiation and allowed to recover for 4 days. A dose of 10Gy at 20Gy/min was chosen because a study by Pellmar and Lepinski (1993) showed that guinea pigs irradiated with this dose showed a reduction in both the population spike and synaptic efficacy in CA1 *in vitro*. In the present study, the results showed that LTP in perforant path-granule cell synapses was inhibited following irradiation and that there was a 40% reduction in the mean percentage EPSP slope in the last 5min of recording post-tetanic stimulation in rats which received irradiation compared with rats that were sham-irradiated. This corroborates data obtained previously (Pellmar and Lepinski, 1993) and

shows that irradiation exerted similar effects in CA1 and the dentate gyrus. The authors also reported that 5Gy of ionising irradiation impaired the acquisition of new learning tasks while 10Gy of radiotherapy induced altered firing patterns and modified synaptic potentials in patients (Pellmar and Lepinski, 1993). The present study, together with the others quoted, show that γ-irradiation impairs synaptic plasticity and LTP.

In order to elucidate the factors that may have caused the γ-irradiation induced impairment in LTP, hippocampal tissue was assessed for changes in a number of measures. Firstly, the ability of γ-irradiation to induce an increase in ROS production was assessed as this was suggested to be a significant contributing factor to the effects of irradiation (Ibuki and Goto, 2000). The data show that ROS production was increased in hippocampal tissue prepared from irradiated rats compared with sham-irradiated rats. This result is in agreement with previous studies which showed that low-dose γ-irradiation induced an increase in the superoxide anion in resident peritoneal macrophages and this was found not to be due to an increase in phagocytotic activity (Ibuki and Goto, 2000). It was also reported that γ-irradiation used in prevention of graft-versus-host disease in transfusion patients induced increased ROS production in circulating red blood cells (Anand *et al.*, 1997).

Lipid peroxidation, which often results from increased ROS production, was similar in hippocampal tissue prepared from irradiated, compared with sham-irradiated, rats. This is contradictory to two studies which reported that lipid peroxidation was increased after ionising irradiation (Tolliver and Pellmar, 1987; Varshney and Kale, 1995). However, 0.5Gy of X-ray irradiation was shown to decrease lipid peroxidation in the brain and thymus of Fischer rats (Yamaoka *et al.*, 1991). However, low doses of X-radiation used in the study by Yamaoka and colleagues (1991) do not compare with the high dose used in the present study. The reason for the lack of change in lipid peroxidation in hippocampal tissue following irradiation in the present study may be due to the 4 day recovery period. In a study by Morcillo and colleagues (2000) the γ-irradiation (20Gy)-induced increase in lipid peroxidation peaked at 24hr in the liver of Sprague-Dawley rats, while another study, where the cervico-thoracic spinal chords of F344 rats were irradiated, showed that one week following irradiation there was no change in lipid peroxidation (Gutin *et al.*, 1992). These data indicate time-related changes in lipid peroxidation and it is possible that allowing a 4 day recovery period,

such as in the present study, may have meant that the peak in lipid peroxidation was missed.

IL-1β concentration was increased in the hippocampus of γ-irradiated, compared with sham-irradiated, rats. This confirms work presented by Ibuki and Goto (1999) who showed that IL-1β concentration was increased in the presence of macrophages isolated from rats which received whole-body irradiation (4Gy). An increase in the activity of ICE has been associated previously, with radiation induced apoptosis (Dewey *et al.*, 1995). However, although the increase in IL-1β concentration was accompanied by an increase in ICE in this study the increase did not reach statistical significance. Therefore, the increase in ICE activity cannot be considered to be responsible for the increase in IL-1β concentration. However, few assessments (n=6) were made in this study and the data, which revealed a 25% increase in ICE activity, showed a large standard error of the mean. Therefore, more estimations are required to provide definitive data. Significantly, it has been shown that an increase in ICE mRNA expression has been found previously in macrophages following irradiation both *in vivo* and *in vitro* (Dewey *et al.*, 1995).

To stress the importance of the inhibitory effect of IL-1β on LTP, a correlation between these two factors was assessed using data from both sham- and γ-irradiated rats. A significant negative correlation was found between IL-1β concentration in the hippocampus and the mean percentage change in the EPSP slope in the last 5min of the experiment. A similar correlation was previously shown using data obtained from the studies in which LTP and IL-1β were assessed in the hippocampus of aged and young rats and from stressed and unstressed rats (Murray and Lynch, 1998a).

It was shown previously that pre-treatment with protein synthesis inhibitors, such as cycloheximide and anisomycin, not only block protein synthesis but also block the ability of rats to maintain LTP (Otani *et al.*, 1992; Mullany and Lynch, 1997), thereby stressing the close relationship between LTP and protein synthesis. To obtain further support for the relationship between LTP and protein synthesis the latter was examined in the entorhinal cortex prepared from γ-irradiated and sham-irradiated rats. Protein synthesis was found to be decreased in tissue prepared from the γ-irradiated rats compared with the sham-irradiated rats. This result is similar to results shown previously where a decrease in protein synthesis is seen in aged rats along with a concomitant decrease in the ability to maintain LTP (Mullany and Lynch, 1997).

As EPA was successful in reversing some key age-related changes observed in the hippocampus, its ability to reverse the γ-irradiation-induced impairment in LTP was assessed. Young rats were fed for 4 weeks on a control diet or a diet enriched in 1% or 2% EPA. After the dietary period the rats were sham-irradiated or exposed to 10Gy at 10Gy/min γ-irradiation and allowed to recover for 4 days before assessing their ability to maintain LTP. The results showed that both 1% and 2% EPA diets were successful in reversing the irradiation-induced impairment in LTP. This finding, together with the data indicating a beneficial effect of EPA in ageing, provide strong evidence that dietary manipulation with EPA can limit or prevent at least some stress-induced changes in LTP.

Why EPA had this effect on the γ-irradiation-induced impairment in LTP was not elucidated by biochemical analysis of the tissue obtained from this LTP study. However, the results discussed previously indicated that γ-irradiation increased the concentration of IL-1β while dietary manipulation with EPA has been shown to reverse the age-related increase in IL-1β. Interestingly it has been shown that the UVB radiation-induced immunosuppression (as quantified by contact hypersensitivity response to dinitrofluorobenzene) is prevented in mice fed a diet enriched in EPA (Moison and Beijersbergen Van Henegouwen, 2001).

Dietary manipulation with the antioxidant scavengers vitamins E and C for 4 weeks was also assessed for its ability to reverse the irradiation-induced impairment in LTP. The results show that these vitamins were also successful in reversing the irradiation-induced impairment in LTP in a striking parallel with the effect of dietary manipulation with EPA on the age-related and irradiation-induced impairment in LTP. Previous studies in this laboratory have shown that dietary supplementation with vitamins E and C have reversed the age-related (Murray and Lynch, 1998b) as well as the isolation stress-related impairment in LTP (O'Donnell, 1999).

The mechanism underlying the vitamin-induced reversal of the impairment in LTP remains to be elucidated. However, the age-related increases in both ROS production and IL-1β concentration were shown previously to be reversed by dietary supplementation with vitamins E and C (Murray and Lynch, 1998b) and it might be speculated that a similar effect occurs with irradiation. It is of interest to note that irradiation-induced increase in apoptosis was reduced by dietary supplementation with an antioxidant diet that contained both vitamins E and C (Ushakova *et al.*, 1999). In the

same study, the diet was shown to increase Bcl-2 mRNA expression in splenocytes isolated from irradiated mice compared with mice which were irradiated but did not receive the EPA diet and where only trace amounts of Bcl-2 mRNA expression was detected (Ushakova *et al.*, 1999).

As a further assessment of the effectiveness of dietary manipulation with EPA and the antioxidant vitamins, LPS was assessed for its effect on LTP in rats fed on control and supplemented diets. LPS is an integral part of Gram negative bacteria that causes an inflammatory response in the host animal. It is known to increase both IL-1β concentration and ROS production in hippocampal tissue (Vereker *et al.*, 2000b). After the 4 week dietary period rats were challenged with an intraperitoneal injection of saline or LPS, and after 3hr were assessed for their ability to sustain LTP. As previously shown, LTP was inhibited in LPS-treated rats (Vereker *et al.*, 2000b), but dietary manipulation with EPA (both 1% and 2% diets) and the vitamins E and C reversed the LPS-induced impairment in LTP. The LPS-induced impairment in LTP has been shown to be associated with an increase in both ROS production and IL-1β concentration (Vereker *et al.*, 2000b). Previously discussed results in this thesis have shown that EPA was unsuccessful in reversing an age-related increase in ROS production and, therefore, the ability of EPA to reverse the LPS-induced impairment in LTP is perhaps more likely to be due to its ability to reverse the LPS-induced increase in IL-1β. As the vitamins E and C have been shown to reverse age-related increases in both ROS production and IL-1β concentration, the ability of these vitamins to reverse the LPS-induced impairment in LTP may reflect their capacity to reverse the increase in both these harmful agents.

Thus, stressors like γ-irradiation and LPS cause an impairment in LTP and, like ageing, it is postulated that this may be due to increases in both ROS production and IL-1β concentration in the hippocampus. Diets enriched in EPA and in antioxidants vitamins were successful in reversing the irradiation- and LPS-induced impairment in LTP and it is further postulated that this was probably due to their abilities to reverse the increases in either IL-1β, or ROS, or both.

In both the hippocampus and cortex of aged rats and the hippocampus of γ-irradiated rats, parallel increases in ROS production and IL-1β concentration have been described. The relationship between these two parameters has not been clearly elucidated. IL-1β leads to an increase in ROS and H_2O_2 leads to an increase in IL-1β *in*

vitro. This suggests that a positive feedback loop exists between these 2 agents. In the cortex the IL-1β-induced increase in ROS was found to be through the activity of SOD. In parallel with the findings *in vitro,* an increase in SOD activity (with decreases in the activities of catalase and glutathione peroxidase) were postulated to be responsible for the increase in ROS in aged rats. However, similar results were not found in the hippocampus; no significant changes in activity of the antioxidant enzymes were observed. It is, therefore, suggested that an increase in IL-1β, perhaps by increasing lipid peroxidation, may lead to the increased ROS. It is possible that the observed increase in ICE activity is responsible for the increase in IL-1β concentration.

Both IL-1β and H_2O_2 (as agents which increases ROS) were shown to increase p38 phosphorylation in the cortex and the hippocampus *in vitro*. Blocking the actions of p38 prevented the IL-1β induced decrease in glutamate release in the hippocampus and in the cortex; it also prevented the IL-1β- and ROS-induced translocation of cytochrome c and the activity of caspase-3 *in vitro*. Therefore, it may be speculated that IL-1β and ROS exert their deleterious actions through the phosphorylation of p38. An age-related increase in p38 activity, cytochrome c translocation and caspase-3 activity, probably as a result of increased IL-1β concentration and ROS production, resulted in a increase in the cleavage of PARP. The inability of PARP to perform its task as a DNA repair enzyme leads to an increase in cell death and this was confirmed by the observed increase in TUNEL positive cells in the cortex and entorhinal cortex of aged rats. It is postulated that similar changes occur in tissue prepared from irradiated rats as were found in the cortex of aged rats.

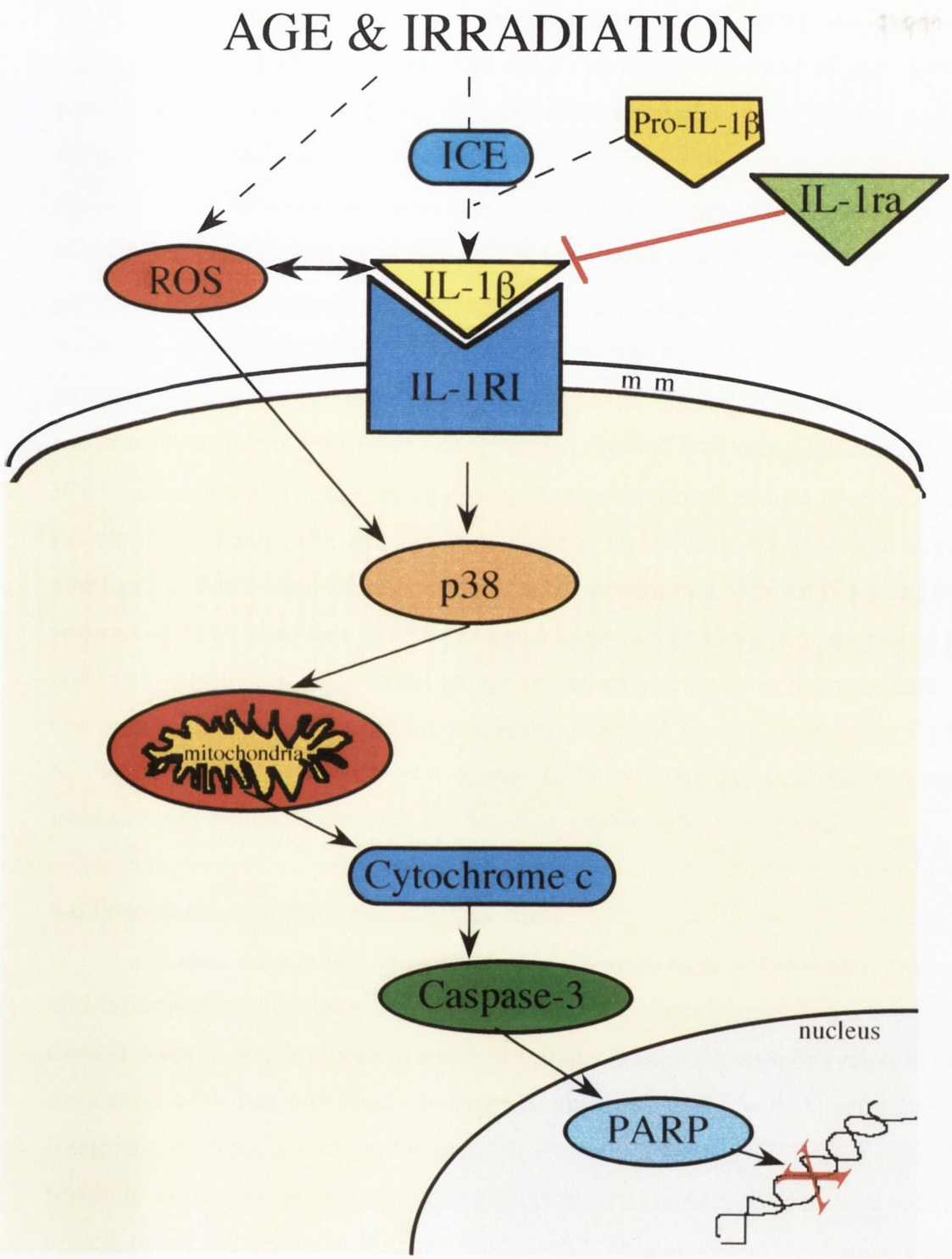
An impairment in LTP in the dentate gyrus was found with age, γ-irradiation and LPS challenge. A possible explanation for this is ROS inflicted damage on the membranes of the perforant path-granule cell synapses or, but most probably in conjunction with, the IL-1β-induced decrease in glutamate release. It is plausible enough to presume that a ROS or IL-1β induced an increase in cell death which may also contribute to the impairment in LTP.

The ability of the putative antioxidant α-lipoic acid to reverse the age-related changes observed in the cortex was probably due to its actions as an antioxidant. In its natural and reduced form it can scavenge most ROS. By reducing ROS production a decrease in the activity in ICE probably occurs, resulting in a reduction in the concentration of IL-1β. The ability of the polyunsaturated fatty acid, EPA, to reverse

the age, γ-irradiation and LPS induced impairment in LTP and the changes observed in the cortex of aged rats is probably due to its ability to reverse the observed increase in IL-1β. Finally, it is also postulated that the actions of the dietary vitamins E and C in reversing the LPS and irradiation-induced impairment in LTP are probably due to their ability to scavenge ROS and thereby reduce the concentration of IL-1β. Figure 4.1 shows a simple schematic depicting one possible pathway whereby age and irradiation may affect cell function.

Figure 4.1 One possible pathway whereby age and irradiation affect cell function.

A simplified schematic showing one possible pathway whereby age and irradiation affect cell function. Age and irradiation have been shown to increase ICE activity and ROS production. ICE, in conjunction with pro-IL-1β and ROS, increases IL-1β concentration. IL-1β binds to the IL-1RI and results in the activation of p38. ROS also increase p38 activity. IL-1ra competes with IL-1β for binding to the IL-1RI. p38 affects the mitochondria, which may lead to an increase in cytochrome c translocation into the cytosol. This, in conjunction with APAF-1 and caspase-9 (not shown here), might lead to an increase in caspase-3 activity. This may inhibit PARP from functioning as a DNA repair enzyme, which in turn may lead to apoptosis.


AGE & IRRADIATION
ICE
Pro-IL-1β
IL-1ra
ROS
IL-1β
IL-1RI
m m
p38
mitochondria
Cytochrome c
Caspase-3
nucleus
PARP

4.5 Future Work:

Several questions arise from the data presented here. Among them is whether similar changes observed in the hippocampus with respect to ageing also occur in the hippocampus of the rats that were subjected to irradiation or LPS challenge. Although parallel changes in IL-1β concentration and ROS accumulation are repeatedly shown, the exact relationship between these two needs to be fully elucidated. Is it the case that the relationship between them is similar in both the cortex and the hippocampus? And is it the case that IL-1β is the trigger or *vice versa*? The data presented, together with a great deal of evidence from the literature, clearly indicate that various stressors lead to an increase in IL-1β. Is it the case that stressors such as age, irradiation and LPS induce an upregulation in the expression of mRNA for both IL-1β and ICE or is it the case that the stressors induce the release of endogenous IL-1β and activation of endogenous pro-ICE? Although the evidence indicates that the age-related alterations in mitochondrial membrane patency, the precise mechanisms underlying the changes requires clarification. For example, further analysis of the relationship between Bcl-2 and Bax is required. Is it the case that there is no age-related change in the expressions of these and other related proteins or might an age-related change occur in the translocation of one or more of these mitochondrial membrane proteins? Finally, the beneficial effects of dietary manipulation with EPA appear to be without question but, the precise mechanism by which these effects are achieved remain to be established.

4.6 Possible clinical implications of this study:

The diets used in this study have been shown to have possible antioxidant and anti-inflammatory properties. This may have important implications in the treatment of disease states in which oxidative stress or inflammation is a cause or symptom of the disease. α-LPA has previously been highlighted as being very beneficial in the treatment of diseases such as Diabetic Neuropathy. Diabetic Neuropathy has been linked to an increase in oxidative stress and clinical trials have shown some beneficial effects of α-LPA treatment (Zeigler *et al.*, 1999). People with other disease states in which oxidative stress is an important factor may also benefit from α-LPA and a recent review has highlighted some of these, which include Parkinson's Disease and AD (Lynch, 2001). Findings in this thesis have shown the advantages of reducing oxidative stress by α-LPA and this may also aid human aging especially with an ever-increasing

aged population. Vitamins, especially vitamin E, have also been shown to be of great advantage to the aged, but vitamin E has also been used in the treatment of AD with some success (Vatassery *et al.*, 1999).

The anti-inflammatory role of fish oils was first investigated in the 1970s and EPA has been used, with some success, in the treatment of Rheumatoid Arthritis (Curtis *et al.*, 2000). It has been suggested that EPA may be beneficial in the treatment of Huntington's Disease, Sepsis, Myalgic Encephalomyelitis and Multiple Sclerosis. It may also be of aid in the treatment of AD (McCarthy, 1999). Again, the use of EPA or other fish oils may be of use to healthy humans in a society of ever-increasing age.

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VIII Appendix I:

Mean data

Table 1. The effect of age and dietary supplementation with α-lipoic acid in the cortex and hippocampus (values are the mean ± the standard error of the mean).

Table 1	Young rats		Aged rats			units
Cortex	Control Diet	100% α-LPA diet	Control diet	50% α-LPA diet	100% α-LPA diet	
ROS	0.35 ± 0.087	0.22 ± 0.036	0.705 ± 0.138	0.462 ± 0.118	0.654 ± 0.23	μmol/mg
SOD	215.1 ± 50.6	441.1 ± 97.7	672.2 ± 59.4	468.4 ± 68.2	453.9 ± 83.0	U/mg
Catalase	0.118 ± 0.058	0.205 ± 0.06	0.128 ± 0.02	N/A	0.262 ± 0.07	U/mg
Gpx	0.408 ± 0.121	0.412 ± 0.077	0.211 ± 0.029	0.294 ± 0.031	0.237 ± 0.045	μmol/mg/min
Vitamin C	0.834 ± 0.256	0.76 ± 0.103	0.585 ± 0.118	0.69 ± 0.176	0.756 ± 0.196	μmol/g
Vitamin E	9.231 ± 1.03	9.174 ± 1.02	9.249 ± 1.47	10.21 ± 1.15	13.483 ± 2.27	pmol/g
GSH	7.13 ± 2.22	12.22 ± 3.15	12.99 ± 3.0	12.39 ± 3.26	16.16 ± 2.22	μmol/g
Lipid peroxidation	32.0 ± 8.4	33.3 ± 10.9	90.7 ± 31.7	49.4 ± 23.9	58.1 ± 16.4	nmol/mg
Arachidonic acid	3.14 ± 0.65	4.14 ± 0.78	1.24 ± 0.3	2.75 ± 0.38	3.42 ± 0.65	μmol/mg
ICE	9.41 ± 2.72	17.38 ± 3.88	24.28 ± 5.24	19.05 ± 5.87	12.13 ± 3.22	pmol/mg/min
IL-1β	9.98 ± 4.23	12.76 ± 3.33	21.64 ± 3.53	12.61 ± 1.76	19.07 ± 4.16	ng/mg
Hippocampus						
SOD	73.04 ± 11.54	84.79 ± 15.83	129.05 ± 17.72	157.39 ± 27.11	93.38 ± 13.08	U/mg
IL-1β	0.26 ± 0.02	0.28 ± 0.03	0.5 ± 0.09	0.42 ± 0.07	0.25 ± 0.03	ng/mg

Table 2a. The effect of age and dietary supplementation with EPA in the hippocampus, cortex and entorhinal cortex (values are the means ± the standard error of the means).

Table 2a	Young rats		Aged rats		units
Hippocampus	Control diet	EPA diet	Control diet	EPA diet	
ROS	0.399 ± 0.043	0.373 ± 0.045	0.505 ± 0.04	0.483 ± 0.047	μmol/mg
SOD	1.11 ± 0.24	0.87 ± 0.18	0.96 ± 0.24	1.03 ± 0.14	U/mg
Catalase	0.286 ± 0.037	0.288 ± 0.055	0.306 ± 0.061	0.251 ± 0.034	U/mg
Gpx	0.025 ± 0.002	0.018 ± 0.003	0.028 ± 0.004	0.029 ± 0.003	μmol/mg/min
Lipid peroxidation	2.2 ± 0.25	1.9 ± 0.38	4.52 ± 0.59	4.44 ± 0.68	μmol/mg
IL-1β	2511 ± 291	1975 ± 235	3349 ± 388	2223 ± 383	pg/mg
p38	10.47 ± 0.92	12.59 ± 1.57	15.67 ± 2.04	11.69 ± 3.0	Arbitrary units
Entorhinal cortex					
p38	9.94 ± 1.94	14.81 ± 2.99	19.66 ± 4.66	18.78 ± 4.67	Arbitrary units
TUNEL	7.18 ± 1.78	5.80 ± 2.12	16.84 ± 1.31	4.25 ± 0.64	% TUNEL +ve cells
Cortex					
ROS	0.088 ± 0.005	0.084 ± 0.006	0.104 ± 0.01	0.104 ± 0.009	nmol/mg
IL-1β	3770 ± 890	4864 ± 754	7081 ± 1013	5083 ± 994	pg/mg
TNFα	40.9 ± 7.9	36.4 ± 4.4	35.4 ± 6.1	44.4 ± 5.7	pg/mg
p38	22.15 ± 4.92	14.35 ± 5.31	36.04 ± 8.29	28.13 ± 8.77	Arbitrary units
Cytochrome c	1.97 ± 0.02	2.18 ± 0.12	2.35 ± 0.12	2.03 ± 0.15	Arbitrary units
Bcl-2	10.4 ± 1.57	7.63 ± 0.75	9.15 ± 0.9	8.73 ± 0.99	Arbitrary units
Bax	9.5 ± 1.07	7.09 ± 1.12	8.0 ± 1.0	8.09 ± 1.74	Arbitrary units
Bcl-2/Bax	2.37 ± 0.49	3.20 ± 0.86	2.89 ± 0.72	2.87 ± 0.66	Arbitrary units
Bax/Bcl-2	0.69 ± 0.18	0.702 ± 0.16	0.65 ± 0.07	0.62 ± 0.11	Arbitrary units
Caspase-3	0.23 ± 0.025	0.166 ± 0.015	0.349 ± 0.046	0.192 ± 0.026	nmol/mg/min
PARP	2.51 ± 0.05	2.33 ± 0.25	1.81 ± 0.17	2.34 ± 0.37	Arbitrary units
TUNEL	33.5 ± 5.81	31.25 ± 2.87	69.75 ± 8.87	49.75 ± 9.58	% TUNEL +ve cells

Table 2b. The effect of age and dietary supplementation with EPA on glutamate release in tetanized (Tet) and untetanised (Untet) tissue prepared from the dentate gyri (values are the mean ± the standard error of the mean).

Table 2b **Hippocampus**	Young rats				Aged rats				Units
	Control Diet		EPA diet		Control Diet		EPA diet		
	Untet	Tet	Untet	Tet	Untet	Tet	Untet	Tet	
Glutamate release	0.15 ± 0.02	0.20 ± 0.03	0.02 ± 0.03	0.25 ± 0.05	0.24 ± 0.05	0.24 ± 0.08	0.15 ± 0.03	0.21 ± 0.02	μmol/mg

Table 2c. The effect of age and dietary supplementation with EPA on LTP in the first 2min (2min) and last 5min of recording (5min) post tetanic stimulation (values are the mean ± the standard error of the mean).

Table 2c **Hippocampus**	Young				Aged				Units
	Control Diet		EPA Diet		Control Diet		EPA Diet		
	2min	5min	2min	5min	2min	5min	2min	5min	
LTP	190.5 ± 4.8	147.7 ± 1.2	184.0 ± 3.2	154.8 ± 0.9	136.5 ± 3.9	111.8 ± 0.8	181.3 ± 3.1	149.1 ± 0.8	EPSP slope (%)

Table 3. The effect of certain experimental conditions *in vitro* on cortical and hippocampal tissue (values are the mean ± the standard error of the mean).* In the case of glutamate release Unstim=Unstimulated tissue and Stim=Stimulated tissue.

Table 3	Control	IL-1β	SB203580	IL-1β+SB203580	IL-1ra	IL-1β+IL-1ra	Units
Cortex							
Cytochrome c	3.213 ± 0.172	4.342 ± 0.339	3.384 ± 0.192	2.963 ± 0.437	2.279 ± 0.184	2.767 ± 0.292	Arbitrary units
Caspase-3	0.513 ± 0.043	0.7 ± 0.07	0.387 ± 0.057	0.527 ± 0.067	0.573 ± 0.08	0.514 ± 0.081	nmoles/mg/min
p38	38.99 ± 2.6	49.05 ± 3.4					Arbitrary units
SOD	3.78 ± 0.15	4.46 ± 0.24					U/mg
Hippocampus							
p38	5.04 ± 1.2	8.82 ± 0.9					Arbitrary units

	Control	H_2O_2	SB203580	H_2O_2+SB203580	Units
Cortex					
Caspase-3	0.360 ± 0.180	0.530 ± 0.044	0.343 ± 0.043	0.373 ± 0.033	nmol/mg/min
Cytochrome c	5.94 ± 0.31	7.14 ± 0.4			Arbitrary units
Hippocampus					
p38	5.01 ± 0.9	9.1 ± 0.3			Arbitrary Units
IL-1β	0.95 ± 0.1	1.21 ± 0.15			ng/mg

Cortex	Control	IL-1β	TETA	IL-1β+TETA	Units
ROS	0.044 ± 0.007	0.071 ± 0.014	0.054 ± 0.008	0.046 ± 0.007	nmol/mg

Hippocampus	Control		IL-1β		IL-1β+SB203580		Units
	Unstim	Stim	Unstim	Stim	Unstim	Stim	
Glutamate release*	0.54 ± 0.06	0.83 ± 0.09	0.81 ± 0.1	0.84 ± 0.22	0.61 ± 0.06	0.85 ± 0.1	μmol/mg

Table 4a. The effect of γ-irradiation on hippocampal and entorhinal cortical tissue (values are the mean ± the standard error of the mean).

Table 4a	Sham-irradiated	Irradiated	Units
Hippocampus			
ROS	0.035 ± 0.002	0.076 ± 0.01	nmol/mg
Lipid peroxidation	2.33 ± 0.2	1.92 ± 0.46	μmol/mg
ICE	1.34 ± 0.13	1.68 ± 0.26	nmol/mg/min
IL-1β	4.31 ± 0.76	8.12 ± 0.99	ng/mg
Entorhinal cortex			
Protein synthesis	12.58 ± 1.86	7.59 ± 1.25	Cpm $x10^{-3}$

Table 4b. The effect of γ-irradiation and dietary supplementation with EPA on LTP in the first 2min (2min) and last 5min of recording (5min) post tetanic stimulation (values are the mean ± the standard error of the mean).

Table 4b	Sham irradiated						Irradiated						Units
Hippocampus	Control Diet		1% Diet		2% Diet		Control Diet		1% Diet		2% Diet		
	2min	5min	2min	5min	2min	5min	2min	5min	2min	5min	2min	5min	
LTP	99.3 ± 1.1	134.9 ± 0.8	106.0 ± 1.7	123.7 ± 0.7	100.5 ±0.7	120.0 ± 0.5	106.5 ± 1.3	98.5 ± 0.5	101.8 ± 1.3	115.4 ± 0.6	102.5 ± 0.5	116.1 ± 0.3	EPSP slope (%)

Table 4c. The effect of γ-irradiation and dietary supplementation with the vitamins E and C on LTP in the first 2min (2min) and last 5min of recording (5min) post tetanic stimulation (values are the mean ± the standard error of the mean).

Table 4c	Sham irradiated				Irradiated				Units
Hippocampus	Control Diet		Vit. E/C Diet		Control Diet		Vit. E/C Diet		
	2min	5min	2min	5min	2min	5min	2min	5min	
LTP	99.3 ± 1.1	134.9 ± 0.8	99.8 ± 0.5	120.2 ± 0.4	106.5 ± 1.3	98.5 ± 0.5	99.3 ± 1.3	121.6 ± 0.9	EPSP slope (%)

Table 5a. The effect of LPS treatment and dietary supplementation with EPA on LTP in the first 2min (2min) and last 5min of recording (5min) post tetanic stimulation (values are the mean ± the standard error of the mean).

Table 5a Hippocampus	Saline treated						LPS treated						Units
	Control Diet		1% Diet		2% Diet		Control Diet		1% Diet		2% Diet		
	2min	5min	2min	5min	2min	5min	2min	5min	2min	5min	2min	5min	
LTP	103.3 ± 1.0	120.0 ± 0.6	97.8 ± 0.5	123.2 ± 0.6	104.5 ± 0.9	136.1 ± 0.6	103.3 ± 0.5	86.5 ± 0.4	102.3 ± 1.3	124.6 ± 0.4	97.5 ± 0.5	114.0 ± 0.4	EPSP slope (%)

Table 5b. The effect of LPS treatment and dietary supplementation with the vitamins E and C on LTP in the first 2min (2min) and last 5min of recording (5min) post tetanic stimulation (values are the mean ± the standard error of the mean).

Table 5b Hippocampus	Saline treated				LPS treated				Units
	Control Diet		Vit. E/C Diet		Control Diet		Vit. E/C Diet		
	2min	5min	2min	5min	2min	5min	2min	5min	
LTP	103.3 ± 1.0	120.0 ± 0.6	106.3 ± 0.3	130.5 ± 0.5	103.3 ± 0.5	86.5 ± 0.4	102.0 ± 1.1	116.5 ± 0.7	EPSP slope (%)

IX Appendix II

Company:	**Address:**
Alexis	Alexis Corporation (UK) Ltd., 3 Moorbridge Court, Bingham, Nottingham NG13 8QG, UK.
Amersham	Amersham International plc, Lincoln Place, Green End, Aylsebury, Buckinghamshire HP20 2TP, UK.
Beeline Healthcare	Beeline Healthcare Ltd., Clondalkin Rd., Clondalkin, Dublin 24, Ireland.
Biosource	Biosource Europe SA, Avenue De L'esperance, Zoning Industriel B-6220, Fleurus, Belgium.
Bio-Rad	Bio-Rad Laboratories Ltd., Bio-Rad House, Maylands Ave., Hemel Hempstead,

	Hertfordshire HP2 7TD, UK.
Calbiochem	Calbiochem/Novabiochem, European Distribution Centre, Boulevard Industrial Park, Nottingham NG9 1BR, UK.
Genzyme Diagnostics	Genzyme Diagnostics, 1 Kendall Square, Cambridge, MA 2139, USA.
Laxdale Research	Laxdale Research Ltd., Kings Park Hse., Laurelhill Buisness Pk., Polmaise Rd., Stirling FK7 9JQ, Scotland.
Lennox	Lennox Laboratory Supplies, John F Kennedy Drive, Naas Rd., Dublin 12, Ireland.
Molecular Probes	Molecular Probes Europe B.V., PoortGebouw, Rijnsburgerweg 10,

	2333 AA Leiden, The Netherlands.
Pierce	Pierce, 3747 N. Meriden Rd., PO Box 117, Rockford, IL 61105, USA.
Promega	Promega Corporation, 2800 Woods Hollow Rd., Madison, Winsconsin, USA.
R&D Systems	R&D Systems Ltd., 4-10 The Quadrant, Barton Lane, Abingdon, Oxford OX14 3YS, UK.
Sartorius	Sartorius AG, 5 Sussex St., Dun Laoghaire, Co. Dublin, Ireland.
Santa Cruz	Santa Cruz Biotechnology, Inc., 2161 Delaware Ave.,

	Santa Cruz, CA 95060 USA.
Sigma	Sigma-Aldrich Company Ltd., Fancy Rd., Poole, Dorset BH12 4QH, UK.

X Appendix III

Solutions used:

Krebs solution
NaCl, 136mM

KCl, 2.54mM

KH_2PO_4, 1.18mM

$MgSO_4.7H_2O$, 1.18mM

$NaHCO_3$, 16mM

Glucose, 10mM

Containing $CaCl_2$, 2mM

Lysis Buffer, pH 7.4
HEPES, 25mM

$MgCl_2$, 5mM

DTT, 5mM

EDTA, 5mM

PMSF, 2mM

Leupeptin, 10μg/ml

Pepstatin, 10μg/ml

Phosphate-Buffered Saline (PBS), pH 7.4
Na_2HPO_4, 80mM

NaH_2PO_4, 20mM

NaCl, 100mM

PBS-Tween 20 (PBS-T)
0.05% Tween 20 solution in PBS

Potassium Phosphate Buffer, pH 7.0
K_2HPO_4, 100mM added to

KH_2PO_4, 100mM

Incubation medium for Gpx assay

Potassium phosphate buffer, 50mM (pH 7)

EDTA, 1mM

NaN_3, 1mM

NADPH, 0.2mM

Glutathione reductase, 1 unit

GSH, 1mM

Cumene hydroperoxide, 1.5mM

Reaction mixture for SOD assay, pH 7.8

Xanthine, 1.8mM

Nitroblue tetrazolium, 2.24mM

Catalase, 40 units

Xanthine oxidase, 7μl/ml

DETAPAC, 1.33mM

dissolved in Phosphate buffer, 50mM

Assay Buffer for Lipid peroxidation

Acetic acid, 3.95ml

added to dH_2O, 15ml (pH 3.5)

added to SDS, 0.213g

and TBA, 0.158g

brought up to 50ml in dH_2O

Incubation Buffer

HEPES, 100mM

DTT, 5mM

HEPES Buffer, pH 7.4

NaCl, 145mM

KCl, 5mM

$MgCl_2$, 1mM

$CaCl_2$, 2mM

$MgSO_4$, 1mM

KH_2PO_4, 1mM

Glucose, 10mM

HEPES, 30mM

SDS-PAGE solutions:

Sample Buffer

Tris-HCl, 0.5M (pH 6.8)

Glycerol, 10% v/v

SDS, 0.05% w/v

β-Marcaptoethanol, 5% v/v

Bromophenol blue, 0.05% w/v

Stacking gel

Arcylamide/Bis-acrylamide (30% stock) 6.5% v/v

Tris-HCl, 0.5M (pH 6.8)

SDS, 1% w/v

Ammonium persulphate, 0.5% w/v

dH_2O

TEMED, 0.1% v/v

Separating gel

Arcylamide/Bis-acrylamide (30% stock) 33% v/v

Tris-HCl, 1.5mM (pH 8.8)

SDS, 1% w/v

Ammonium persulphate, 0.5% w/v

dH_2O

TEMED, 0.1% v/v

Electrode Running Buffer

Tris base, 25mM

Glycine, 200mM

SDS, 17mM

Transfer Buffer, pH8.3

Tris base, 25mM

Glycine, 192mM

MeOH, 20% v/v

SDS, 0.05% w/v

dH_2O

XI List of Publications.

McGahon, B.M., Martin, D.S.D., Horrobin, D.F. and Lynch, M.A. (1999) Age-related changes in synaptic function: analysis of the effect of dietary supplementation with ω-3 fatty acids. *Neuroscience*. **94**, 305-314.

McGahon, B.M., Martin, D.S.D., Horrobin, D.F. and Lynch, M.A. (1999) Age-related changes in LTP and antioxidant defenses are reversed by an α-lipoic acid enriched diet. *Neurobiology of Aging*. **20**, 655-664.

Martin, D.S.D., Towey, M., Horrobin, D.F. and Lynch, M.A. (2000) A diet enriched in α-lipoic acid reverses the age-related compromise in antioxidant defences in rat cortical tissue. *Nutritional Neuroscience*. **3,** 193-206.

Martin, D.S.D., Spencer, P., Horrobin, D.F. and Lynch, M.A. (2001) Long-term potentiation in aged rats is restored when the age-related decrease in polyunsaturated fatty acid concentration is reversed. *Prostagladins, Leukotrienes and Essential Fatty Acids*. (In Press)

Martin, D.S.D., Spencer, P., Horrobin, D.F. and Lynch, M.A. (2000) Eicosapentanoic acid restores the age-related impairment in long-term potentiation in the dentate gyrus. *Society for Neuroscience Abstracts*. **26**, 334.6.

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AGE-RELATED CHANGES IN SYNAPTIC FUNCTION: ANALYSIS OF THE EFFECT OF DIETARY SUPPLEMENTATION WITH ω-3 FATTY ACIDS

B. M. McGAHON,* D. S. D. MARTIN,* D. F. HORROBIN† and M. A. LYNCH*‡

*Department of Physiology, Trinity College, Dublin 2, Ireland

†Laxdale Research Ltd, Stirling, Scotland, U.K.

Abstract—Depolarization-induced transmitter release in synaptosomes prepared from the hippocampus of aged rats is decreased compared with release from young animals. Although the underlying cause of this deficit is not known, some evidence suggests that increased membrane rigidity may contribute to these age-related synaptic changes. One possible consequence of the decreased transmitter release in the hippocampus of aged rats is a reduced ability to sustain long-term potentiation in perforant path–granule cell synapses, a pathway in which maintenance of long-term potentiation and increased glutamate release have been coupled. The observation that there is an age-dependent impairment in long-term potentiation is consistent with this view. If the age-related deficits in release and long-term potentiation are a consequence of increased membrane rigidity, it must be predicted that any manoeuvre which reverses membrane rigidity should reverse these functional deficits. In the present study, we investigated the effect of dietary manipulation of aged rats with ω-3 fatty acids on synaptic function.

The data obtained indicate that an eight-week modified feeding schedule reversed the age-related impairments in long-term potentiation and depolarization-induced glutamate transmitter release. We also report that the concentrations of both docosahexanoic acid and arachidonic acid, two main polyunsaturated fatty acids in neuronal membranes, were decreased in the hippocampus of aged rats, and were restored by dietary manipulation. The data are consistent with the hypothesis that these deficits results from a change in membrane composition. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: long-term potentiation, dentate gyrus, ageing, arachidonic acid, docosahexanoic acid, glutamate release.

Ageing is accompanied by a variety of changes in synaptic function. One of these changes is a deficit in hippocampal long-term potentiation (LTP), which is one of the most impressive forms of synaptic plasticity.[3] While there is some debate with regard to the biological significance of LTP,[17] the bulk of the evidence supports the view that it may represent a biological substrate for learning and/or memory.[23,27,29,30] In this context, it is not surprising that ageing, which is known to be associated with a blunting of cognitive function in rats, is also associated with impaired LTP.[1,19,24,26,32,33]

The underlying cause of the age-related impairment in LTP is not known, although since maintenance of LTP in perforant path–granule cells is closely coupled with, and may even be dependent upon, an increase in glutamate release,[3,7,24,26] one possible cause of the deficit in LTP is the age-related decrease in depolarization-induced transmitter release. Impairments in both LTP and transmitter release have been linked with a decrease in membrane fluidity, which is typified by a decrease in membrane concentration of polyunsaturated fatty acids, such as arachidonic acid.[24,26,32,33] Interpretation of the consequences of an age-related decrease in arachidonic acid concentration is somewhat confounded by the proposed retrograde messenger role of this fatty acid in LTP in perforant path–granule cell synapses.[3,26,32,33] Evidence consistent with this role is the observation that arachidonic acid can induce a form of potentiation[6,10,37] which resembles tetanically induced LTP, at least in some respects.[37] Further support includes evidence that arachidonic acid concentration is increased in a postsynaptic preparation,[9] and in the synaptic cleft following induction of LTP.[22] In addition, arachidonic acid exerts a stimulatory effect on glutamate release under certain circumstances.[16,25,26]

It must be considered that a decrease in the concentration of polyunsaturated fatty acids other than arachidonic acid might also contribute to changes in membrane fluidity; one such polyunsaturated fatty acid is docosahexanoic acid, which is present in high concentrations in brain tissue.

The age-related change in membrane composition, which decreases membrane fluidity,[8,38] might be predicted to affect membrane-associated functions such as transmitter release, which requires fusion of the synaptic vesicle and synaptic plasma membranes. Several other changes in membrane-associated functions, which are likely to play a role in release, are affected by age; examples include calcium handling by cells[20,36] and changes in activity of kinases.[2,31]

If it is proposed that alterations in membrane composition, specifically a decrease in concentration of polyunsaturated fatty acids, are responsible for age-related deficits in glutamate release and LTP, it is reasonable to expect that reversing these alterations should reverse the age-related deficits. We have already assessed the ability of arachidonic acid and its precursor, γ-linolenic acid, to restore age-related impairments in synaptic function in the hippocampus.[26] The data have indicated that dietary supplementation with these fatty acids reverses the age-related decrease in arachidonic acid concentration, the decrease in transmitter release and the impairment in LTP. To address the possibility that this effect can also be achieved by other polyunsaturated fatty acids, groups of aged and young rats were fed for eight weeks on a control diet or a diet enriched in docosahexanoic acid. The ability of rats to

‡To whom correspondence should be addressed. Tel.: +353-1-608 1770; fax: +353-1-679 3545.

E-mail address: lynchma@tcd.ie (M. A. Lynch)

Abbreviations: ACPD, *trans*-1-amino-cyclopentyl-1,3-dicarboxylate; EPSP, excitatory postsynaptic potential; LTP, long-term potentiation; PBS-T, phosphate-buffered saline containing 0.2% Tween-20.

sustain LTP in perforant path–granule cell synapses was assessed, while glutamate release and fatty acid concentrations were analysed in tissue prepared from the dentate gyrus.

EXPERIMENTAL PROCEDURES

Animals

Aged (22 months old) and young (four months old) male Wistar rats (Bantham & Kingman, U.K.) were housed in groups of two to four under a 12-h light schedule, with the ambient temperature controlled between 22 and 23°C. Rats were maintained in the BioResources Unit in Trinity College, Dublin, under veterinary supervision. Every effort was made to reduce discomfort and the minimum number of rats was used. One subgroup of aged animals and one subgroup of young animals were fed for eight weeks on an experimental diet of laboratory chow supplemented with a daily dose of 10 mg docosahexanoic acid (26% w/v in tuna oil; Laxdale Research, U.K.). The other subgroups received standard laboratory chow to which corn oil was added; in this way, the different groups of rats received an isocaloric intake. Food intake was measured for two weeks before the commencement of the experiment. Sufficient diet was prepared for three or four days at a time and daily allowances were stored in airtight containers at −80°C.

In a separate experiment, weanling rats were subdivided into a group which was fed for eight weeks on the experimental diet (10 mg docosahexanoic acid per day; 26% w/v in tuna oil; Laxdale Research, U.K.) and a control group which received standard laboratory chow to which corn oil was added.

Induction of long-term potentiation in the dentate gyrus

All rats were anaesthetized by intraperitoneal injection of urethane. Young animals initially received 1.5 g/kg urethane and a further increment, if required (which did not exceed 0.5 g/kg in any experiment), for induction of deep anaesthesia (the absence of a pedal reflex); the mean doses administered to young rats given the control and experimental diets were 2.07±0.11 and 2.10±0.11 g/kg, respectively. Aged animals received 1.2 g/kg urethane and this was topped up when necessary; the mean doses administered to aged rats given the control and experimental diets were 1.76±0.05 and 1.98±0.09 g/kg, respectively.

LTP was induced in perforant path–granule cell synapses as described previously.[25,26] In brief, rats were placed in a stereotaxic frame, a window of skull was removed, and a bipolar stimulating electrode and a unipolar recording electrode were placed in the perforant path (4.4 mm lateral to lambda) and the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to bregma). The depths of the stimulating and recording electrodes were adjusted to maximize the slope of the excitatory postsynaptic potential (EPSP) and the stimulus intensity was adjusted to the value which was just sufficient to produce a population spike. A stabilization period of up to 30 min followed and then recording commenced; test shocks were given at 30-s intervals for 10 min before and 40 min after three trains of stimuli (250 Hz for 200 ms; inter-train interval: 30 s). Previous experiments indicated that this stimulation paradigm produced saturation of LTP in perforant path–granule cell synapses. The slope in the middle third of the rising phase of the EPSP was taken as the measure of the population EPSP slope in these experiments.

Tissue preparation

At the end of the recording period, rats were killed by decapitation, the dentate gyri were dissected and cross-chopped slices (350 × 350 μm) were prepared using a McIlwain tissue chopper. Samples of control and potentiated dentate gyri were frozen separately in 1 ml Krebs solution (composition of Krebs in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4{\cdot}7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) containing 10% dimethylsulphoxide[15,26] and stored at −80°C until required.

For analysis, slices were thawed rapidly (1.5–2 min) by agitation at 37°C and rinsed four times in excess fresh oxygenated Krebs solution. Fatty acid concentrations and reactive oxygen species production were assessed in samples of homogenate prepared from dentate gyri of aged and young rats and weanling rats fed on either the control or experimental diets. The crude synaptosomal pellet, P_2, was used for analysis of glutamate release; this analysis was not performed in tissue prepared from weanling rats. Synaptosomes were prepared by homogenizing tissue in 0.32 M ice-cold sucrose and centrifuging at 5000 r.p.m. for 5 min; the resulting supernatant was then centrifuged at 15,000 r.p.m. for 15 min.

Assessment of glutamate release

Samples of synaptosomal tissue were resuspended in ice-cold Krebs solution containing 2 mM $CaCl_2$. Tissue was aliquotted on to Millipore filters (0.45 μm) and rinsed under vacuum, and the filtrate was discarded. Tissue was incubated in 250 μl oxygenated Krebs solution at 37°C for 3 min, and filtrate was collected and stored. Release of transmitter was then stimulated by the addition of 40 mM KCl to Krebs solution.

Glutamate was analysed as described previously.[18,34] Glutaraldehyde (0.5% in 100 mM NaH_2PO_4 buffer, pH 4.5; 320 μl) was added to wells in a 96-well plate, incubated for 60 min at 37°C and washed with 100 mM NaH_2PO_4 buffer. Triplicate samples (50 μl) or glutamate standards (50 μl; 50 nM to 10 μM, prepared in 100 mM Na_2HPO_4 buffer, pH 8.0) were added, incubated for 2 h at 37°C and washed in 100 mM Na_2HPO_4 buffer. To bind any unreacted aldehydes, ethanolamine (320 μl; 0.1 M in 100 mM Na_2HPO_4 buffer) was added to each well and incubated for 60 min at 37°C. Plates were washed with phosphate-buffered saline containing 0.2% Tween-20 (PBS-T). To block non-specific binding, donkey serum was added (200 μl; 3% in PBS-T), incubated for 60 min and washed with PBS-T. Anti-glutamate antibody (raised in rabbit; 100 μl; 1:5000 in PBS-T; Sigma, U.K.) was added to each plate, incubated overnight at 4°C and washed with PBS-T. Anti-rabbit horseradish peroxidase-linked secondary antibody (95 μl; 1:10,000 in PBS-T; Amersham, U.K.) was added, incubated for 60 min at room temperature and washed. 3,3′,5,5′-Tetramethylbenzidine liquid substrate was added as chromogen and incubation continued for exactly 60 min at room temperature. H_2SO_4 (4 M; 50 μl) was added to stop the reaction and optical densities were determined at 450 nm using a multiwell plate reader. Values were calculated with reference to the standard curve, corrected for protein[5] and expressed as nmol glutamate/mg protein.

Analysis of fatty acid concentration

Thawed slices prepared from the dentate gyrus were rinsed three times and then homogenized in 200 μl fresh Krebs solution. Fatty acids were extracted into chloroform–methanol (2:1 v/v; 1 ml) by vigorous shaking for 10 min, followed by centrifugation at 1000 × *g* for 5 min to separate the phases. The aqueous layer was discarded and the chloroform phase was evaporated under nitrogen and resuspended in ethanol for analysis. Fatty acid concentrations were analysed as their 2-nitrophenylhydrazine derivatives by reverse-phase high-performance liquid chromatography.[28] Derivatization was achieved by adding 2-nitrophenylhydrazine hydrochloride solution [0.02 M 2-nitrophenylhydrazine hydrochloride in 0.25 M HCl–ethanol (1:1, v/v)] and a solution of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.25 M; in ethanol mixed in equal volume 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. Fatty acid derivatives were concentrated; n-hexane and phosphate buffer (0.033 M, pH 6.4 in 0.5 M HCl) were added, samples were vortex mixed for 30 s and centrifuged for 5 min at 1500 × *g*, and the hexane phase was evaporated to dryness under nitrogen. For high-performance liquid chromatography analysis, samples were resuspended in methanol and injected on to a Microsorb C18 column (maintained in a column oven at 30°C), and fatty acids 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 230 nm. Aliquots of tissue were taken for protein analysis and concentrations were estimated according to standard curves and expressed as nmol/mg protein.

Analysis of reactive oxygen species formation

The formation of reactive oxygen species was assessed in hippocampal tissue (minus dentate gyrus) as described previously.[21] The method relies on oxidation of the non-fluorescent probe, 2′,7′-dichlorofluorescin diacetate, by reactive oxygen species, to the highly fluorescent 2′,7′-dichlorofluorescein. To assess reactive oxygen species production, hippocampal homogenate was resuspended in 1 ml ice-cold 40 mM Tris buffer (pH 7.4). Aliquots (1 ml) of homogenate were incubated with 2′,7′-dichlorofluorescin diacetate (10 μl; final

concentration 5 μM; from a stock solution of 500 μM in methanol) at 37°C for 15 min. To terminate the reaction, the dye-loaded synaptosomes were centrifuged at 13,000 × *g* for 8 min. The pellet was resuspended in 3 ml ice-cold 40 mM Tris buffer (pH 7.4) and fluorescence was monitored at a constant temperature of 37°C at 488 nm excitation (band width 5 nm) and 525 nm emission (band width 20 nm). Reactive oxygen species formation was quantitated from a standard curve of 2′,7′-dichlorofluorescein in methanol (range: 0.05–1 μM). Results are expressed as nmol/mg tissue corrected for protein.

Statistical analysis

A one-way ANOVA was performed to determine whether there were significant differences between conditions. When this analysis indicated significance (at the 0.05 level), post hoc Student Newman–Keuls test analysis was used to determine which conditions were significantly different from each other. In some cases (e.g., when data derived from control tissue were compared with data derived from tissue in which LTP was induced *in vivo*), Student's *t*-test for independent means was used to establish statistical significance.

RESULTS

The average food intake per rat for four-month-old rats was 25 g/rat/day, compared with 18 g/rat/day for 22-month-old rats; these values did not vary for the duration of the experiment. Mean body weights at the end of the experimental period were 442±10.2 and 447±7.1 g in the four-month-old rats fed on the control and experimental diets, and 512±10.5 and 496±9.6 g in the 22-month-old rats fed on the control and experimental diets, respectively.

In the study in which age-related changes in LTP were assessed, the stimulus strength required to induce a spike was similar in the two groups of four-month-old rats (4.14±0.15 and 4.28±0.12 V for those fed on control and experimental diets, respectively). It was also similar in both groups of 22-month-old rats (3.96±0.16 and 3.83±0.12 V for those fed on control and experimental diets, respectively). Similarly, there were no significant differences in the mean EPSP slopes, generated at this stimulus strength, in the different subgroups; values ranged from 0.9 to 1.16 mV/ms.

Figure 1A indicates that there was a marked increase in mean population EPSP slope immediately following tetanic stimulation in four-month-old rats fed on the control or experimental diet. Dietary manipulation did not affect either the early or later response to the tetanus. The mean percentage increases in EPSP slope in the last 5 min of the experiment (compared with the 5 min immediately prior to tetanic stimulation) were 128.88±2.89% and 126.86±3.13% in the control ($n=7$) and experimental ($n=7$) dietary groups, respectively.

Figure 1B indicates that the amplitude of LTP in 22-month-old rats fed on the control diet was attenuated; the mean percentage change in population EPSP slope in the last 5 min of the experiment was 110.35±2.08% ($n=13$), which was significantly lower than the corresponding value observed in four-month-old rats ($P<0.01$, Student's *t*-test for independent means). In contrast, LTP in 22-month-old rats which received the experimental diet was similar to that in four-month-old rats; the mean percentage increase in EPSP slope in the last 5 min of the experiment was 131.67±3.59% ($n=13$). Of the 13 rats fed on the control diet, LTP (i.e. a consistent increase in population EPSP slope of 10% or more in the last 10 min of the experiment) was observed in five but not in the remaining eight rats. Of the 13 aged rats which received the experimental diet, LTP was observed in 11. All four-month-old rats sustained LTP.

Table 1. The age-related decrease in γ-linolenic acid concentration was reversed by dietary manipulation

	Four months	22 months	
	Both diets	Control diet	Experimental diet
α-Linolenic acid	0.28 (0.03)	0.16* (0.02)	0.23 (0.03)
Linoleic acid	1.46 (0.19)	0.96* (0.17)	0.98* (0.19)
γ-Linolenic acid	0.94 (0.10)	0.43* (0.03)	0.42* (0.09)
Palmitic acid	1.23 (0.18)	1.67 (0.31)	0.80 (0.12)
Docosahexanoic acid	1.52 (0.27)	0.62* (0.27)	1.36 (0.38)
Arachidonic acid	0.70 (0.10)	0.35* (0.04)	0.45 (0.07)

Concentrations of linoleic acid, γ-linolenic acid, α-linolenic acid and palmitic acid were similar in both groups of four-month-old rats and therefore the data were pooled. Significant age-related decreases in the concentrations of γ-linolenic acid, α-linolenic acid, linoleic acid, docosahexanoic acid and arachidonic acid were observed in tissue prepared from 22-month-old rats which received the control diet (*$P<0.05$, Student's *t*-test for independent means). Dietary supplementation with docosahexanoic acid fully restored the concentration of docosahexanoic acid and partially restored the concentrations of α-linolenic acid and arachidonic acid to those observed in four-month-old rats. Dietary manipulation did not affect the concentrations of either γ-linolenic acid or linoleic acid. There were no significant changes in the concentration of palmitic acid with age or diet. Data are means (S.E.M.).

Analysis of fatty acids in homogenates prepared from dentate gyri indicated that concentrations were similar in the two four-month-old dietary groups and therefore the data were pooled. Figure 2A shows that the concentration of docosahexanoic acid was significantly decreased in samples prepared from dentate gyri of 22-month-old rats fed on the control diet compared with four-month-old rats ($P<0.05$, ANOVA), but was similar to that in 22-month-old rats fed on the experimental diet. Regression analysis indicated that there was a positive correlation between ability to sustain LTP and docosahexanoic acid concentration, but the correlation failed to reach statistical significance ($P=0.064$; Fig. 2B).

Figure 3A shows that the concentration of arachidonic acid was significantly decreased in samples prepared from dentate gyri of 22-month-old rats fed on the control diet compared with four-month-old rats ($P<0.05$, ANOVA). While the concentration of arachidonic acid was partially restored in 22-month-old rats fed on the experimental diet, this value was still significantly decreased compared with the arachidonic acid concentration in tissue prepared from four-month-old rats. Regression analysis revealed that there was a significant positive correlation between ability to sustain LTP and arachidonic acid concentration ($P=0.012$; Fig. 3B).

Table 1 shows that there were significant age-related decreases in the concentrations of α-linolenic, linoleic and γ-linolenic acids ($P<0.05$, ANOVA), but no significant change was observed in the case of palmitic acid (18:0). Dietary supplementation of aged rats with 26% docosahexanoic acid in tuna oil fully restored the concentrations of docosahexanoic acid (Fig. 2A) and its precursor, α-linolenic acid (Table 1), to those observed in four-month-old rats. However, the age-related decreases in γ-linolenic and linoleic acids were unaffected by dietary manipulation (Table 1).

Endogenous glutamate release was assessed in synaptosomes prepared from the dentate gyrus in each of the experimental groups. Figure 4A shows that unstimulated and KCl-stimulated glutamate release were similar in synaptosomes prepared from untetanized and tetanized dentate gyri of

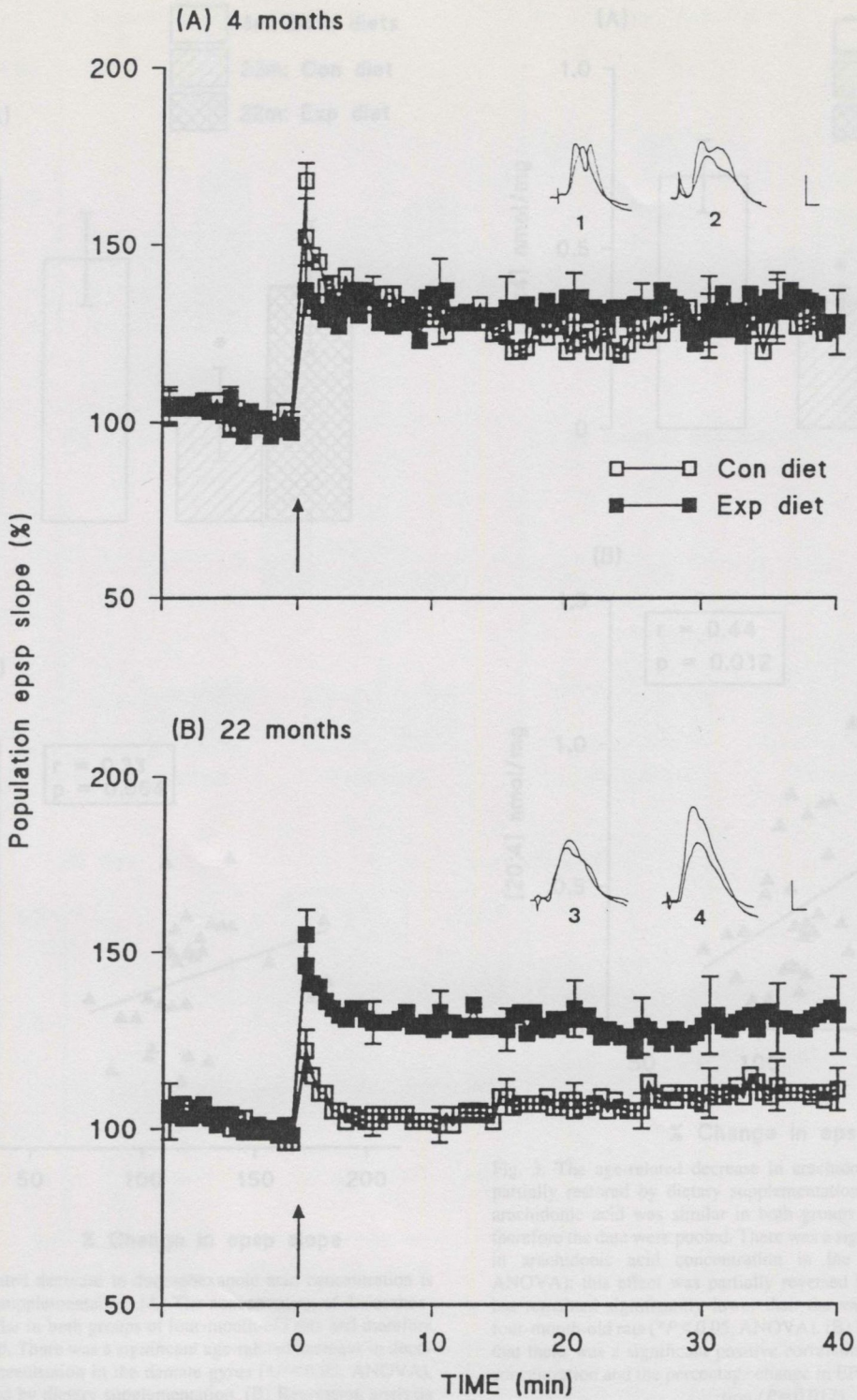

Fig. 1. The age-related impairment in ability to sustain LTP is restored in rats fed on a diet supplemented with docosahexanoic acid. The mean slope of the population EPSP evoked by test stimuli delivered at 30-s intervals before and after tetanic stimulation (arrow) is shown for four- (A) and 22-month-old (B) rats fed on a control diet (open symbols) or an experimental diet (filled symbols). Population EPSP slope is expressed as a percentage of the slope recorded in the 5 min immediately prior to tetanic stimulation and results are presented as means (±S.E.M.). Standard errors are included for every 10th response and are so small as to be obscured in some cases.

four-month-old rats. KCl significantly enhanced release in untetanized tissue ($P<0.05$, ANOVA), but the enhancement was more marked in synaptosomes prepared from dentate gyri in which LTP was sustained ($P<0.01$, ANOVA). Addition of arachidonic acid and *trans*-1-amino-cyclopentyl-1,3-dicarboxylate (ACPD) significantly increased KCl-stimulated release of glutamate in synaptosomes prepared from untetanized tissue, but this effect was occluded in synaptosomes prepared from tetanized tissue (Fig. 4A, B). KCl-stimulated release of glutamate was markedly attenuated in synaptosomes prepared from untetanized dentate gyri of 22-month-old rats fed on the control diet, and there was no evidence of a KCl-induced enhancement of release in synaptosomes prepared from tetanized dentate gyrus

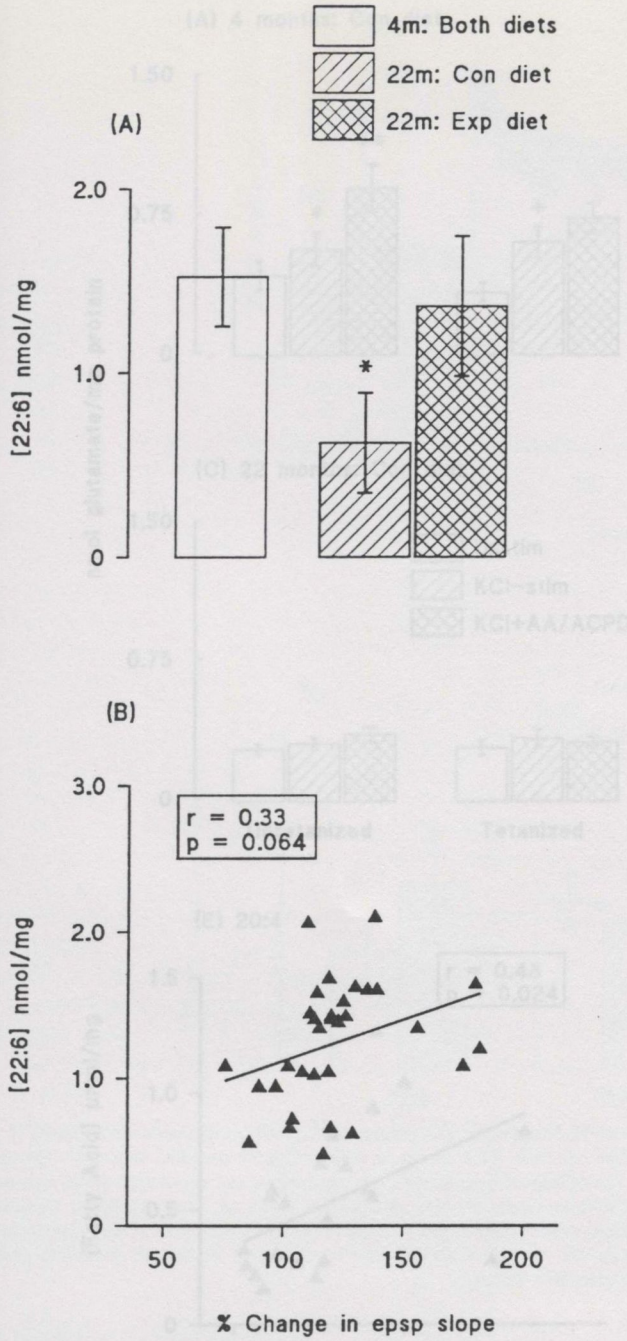

Fig. 2. The age-related decrease in docosahexanoic acid concentration is restored by dietary supplementation. (A) The concentration of docosahexanoic acid was similar in both groups of four-month-old rats and therefore the data were pooled. There was a significant age-related decrease in docosahexanoic acid concentration in the dentate gyrus (*$P<0.05$, ANOVA), but this was reversed by dietary supplementation. (B) Regression analysis revealed that there was a positive correlation between docosahexanoic acid concentration and the percentage change in EPSP slope following tetanization, but this correlation did not reach statistical significance.

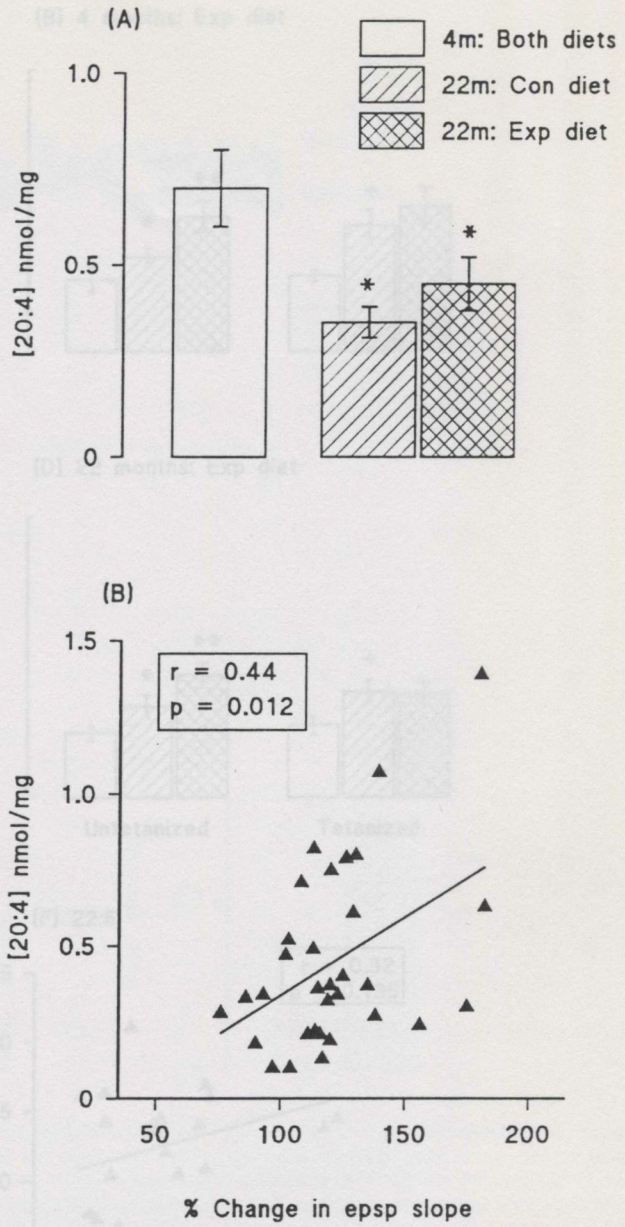

Fig. 3. The age-related decrease in arachidonic acid concentration was partially restored by dietary supplementation. (A) The concentration of arachidonic acid was similar in both groups of four-month-old rats and therefore the data were pooled. There was a significant age-related decrease in arachidonic acid concentration in the dentate gyrus (*$P<0.05$, ANOVA); this effect was partially reversed by dietary supplementation, but remained significantly lower than the value in the dentate gyrus of four-month-old rats (*$P<0.05$, ANOVA). (B) Regression analysis revealed that there was a significant positive correlation between arachidonic acid concentration and the percentage change in EPSP slope following tetanization ($P=0.012$).

(Fig. 4C). Addition of arachidonic acid and ACPD failed to affect KCl-induced release in either untetanized or tetanized tissue. In contrast to the lack of response in tissue prepared from 22-month-old rats fed on the control diet, KCl significantly increased glutamate release in synaptosomes prepared from untetanized dentate gyri of 22-month-old rats fed on the experimental diet ($P<0.05$, ANOVA). A further enhancement of release was observed in synaptosomes prepared from tetanized tissue ($P<0.01$, ANOVA). Addition of arachidonic acid and ACPD to the incubation medium significantly increased KCl-induced release in synaptosomes prepared from untetanized dentate gyri ($P<0.01$, ANOVA), but this effect was occluded in synaptosomes prepared from tetanized tissue (Fig. 4D).

Regression analyses revealed that there was a significant positive correlation between arachidonic acid concentration and KCl-stimulated glutamate release ($P=0.024$; Fig. 4E), while the correlation between docosahexanoic acid concentration and KCl-stimulated glutamate release was not significant ($P=0.135$; Fig. 4F).

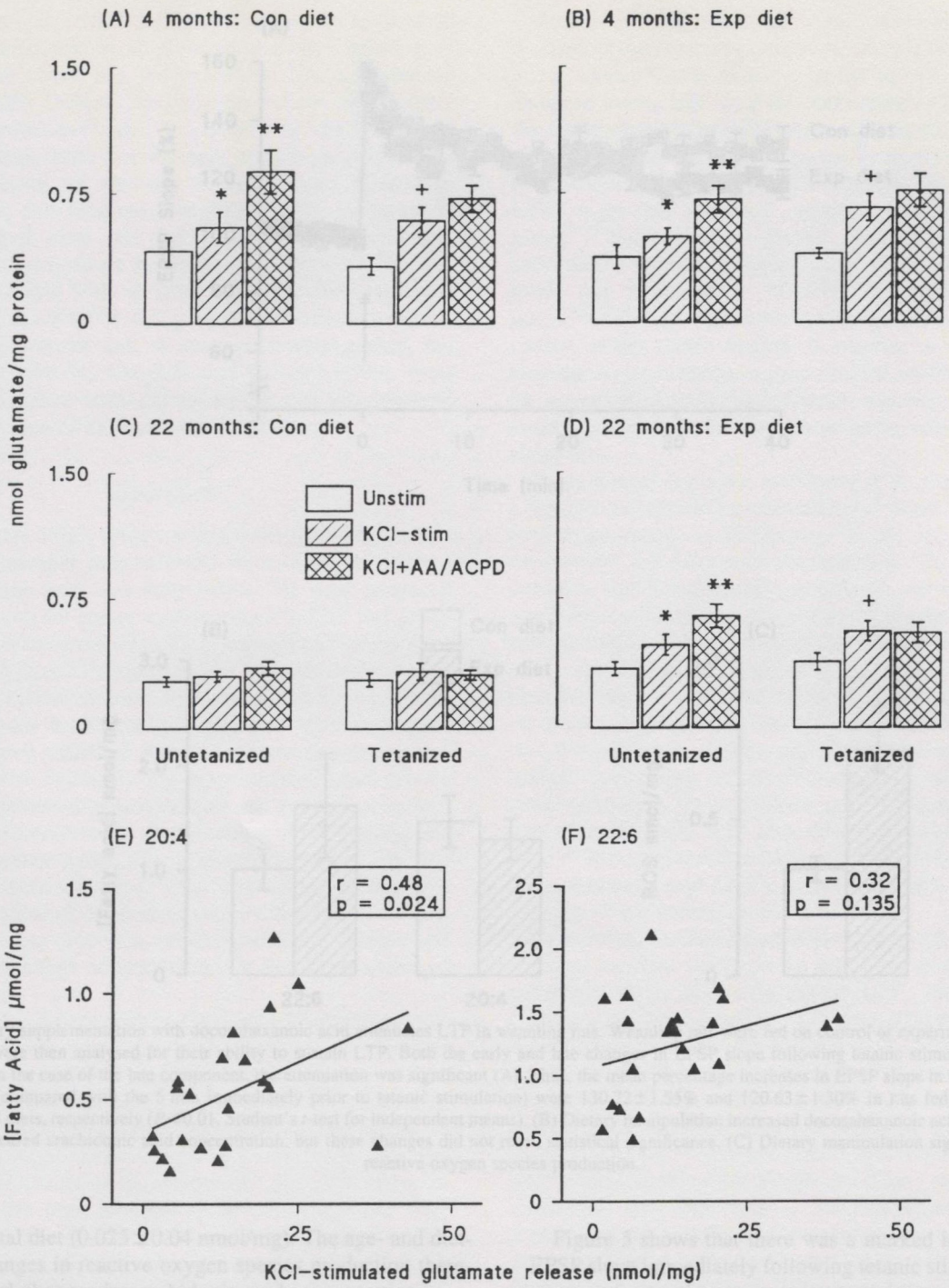

Fig. 4. Dietary manipulation reversed the age-related impairment in glutamate release. KCl-stimulated release of endogenous glutamate was assessed in synaptosomes prepared from dentate gyri of four-(A, B) and 22-month-old (C, D) animals fed on control and experimental diets. KCl (40 mM) increased release to a significantly greater degree in tetanized (**$P<0.01$, ANOVA), compared with untetanized (*$P<0.05$, ANOVA), tissue prepared from four-month-old rats which were fed either on the control or experimental diet, and while arachidonic acid (AA) and ACPD significantly enhanced KCl-stimulated release in untetanized tissue ($^{+}P<0.01$, ANOVA), this effect was not observed in tetanized tissue. KCl failed to increase glutamate release in synaptosomes prepared from either untetanized or tetanized tissue obtained from 22-month-old rats fed on the control diet. In 22-month-old rats fed on the experimental diet, KCl (40 mM) increased release to a significantly greater degree in tetanized tissue (**$P<0.01$, ANOVA), compared with untetanized tissue (*$P<0.05$, ANOVA), while arachidonic acid (AA) and ACPD significantly enhanced KCl-stimulated release in untetanized ($^{+}P<0.01$, ANOVA) but not tetanized tissue. Regression analysis revealed that there was a positive correlation between KCl-stimulated release and concentration of arachidonic acid (E) and docosahexanoic acid (F); the correlation was statistically significant in the case of arachidonic acid but not docosahexanoic acid.

Analysis of reactive oxygen species production in hippocampal tissue prepared from these rats revealed no differences between the two groups of young rats (0.21 ± 0.06 vs 0.20 ± 0.04 nmol/mg in hippocampal tissue prepared from rats fed on the control and experimental diets, respectively). There was an age-related increase in reactive oxygen species production in tissue prepared from aged rats which were fed on the control diet (0.32 ± 0.04 nmol/mg; $P<0.05$, Student's *t*-test; data compared with values from either group of young rats), but this was reversed in aged rats fed on the

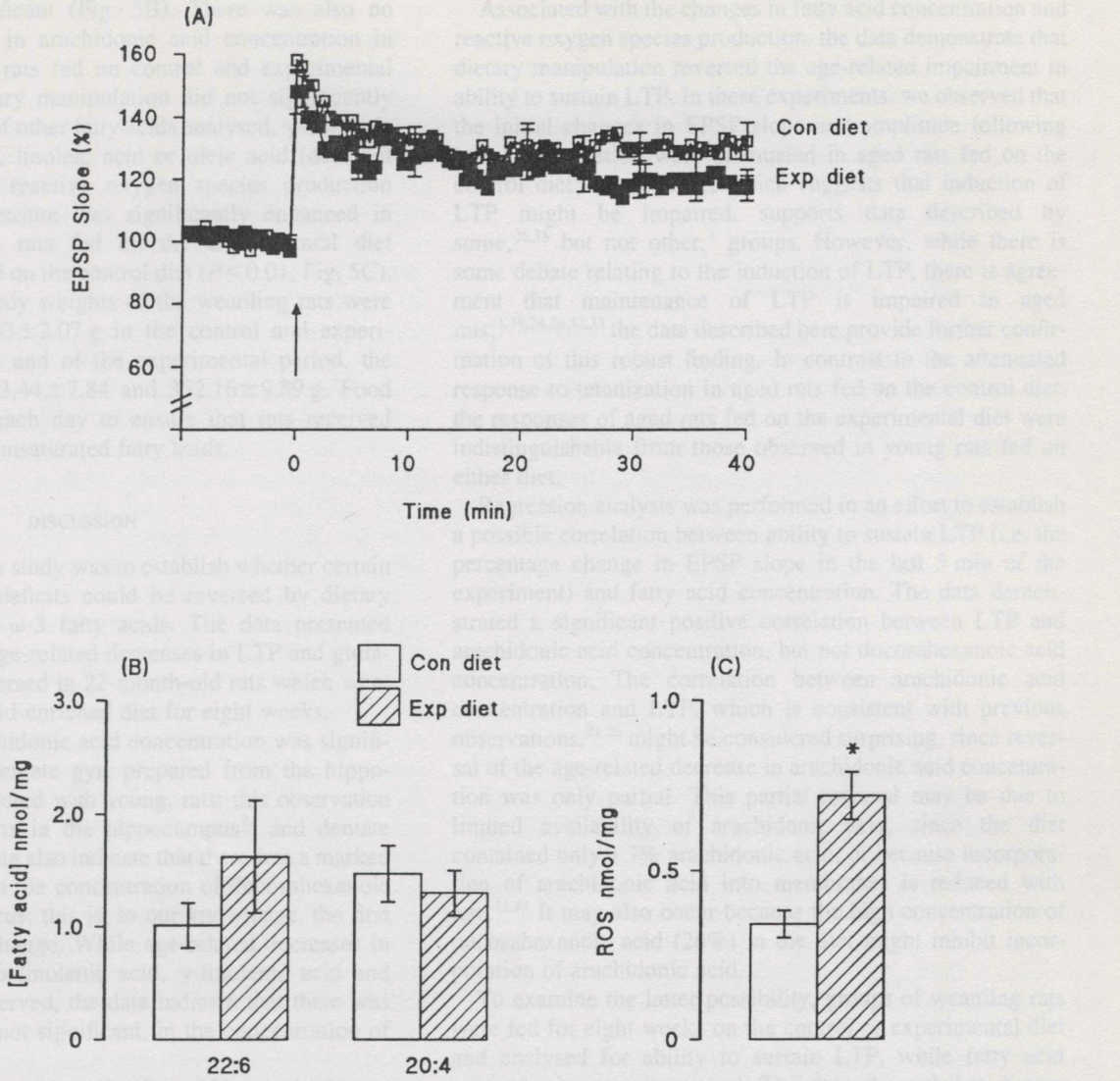

Fig. 5. Dietary supplementation with docosahexanoic acid attenuates LTP in weanling rats. Weanling rats were fed on control or experimental diet for eight weeks and were then analysed for their ability to sustain LTP. Both the early and late changes in EPSP slope following tetanic stimulation (arrow) were attenuated; in the case of the late component, the attenuation was significant (A). Thus, the mean percentage increases in EPSP slope in the last 5 min of the experiment (compared with the 5 min immediately prior to tetanic stimulation) were 130.72±1.55% and 120.63±1.30% in rats fed on the control and experimental diets, respectively ($P<0.01$, Student's t-test for independent means). (B) Dietary manipulation increased docosahexanoic acid concentration and slightly decreased arachidonic acid concentration, but these changes did not reach statistical significance. (C) Dietary manipulation significantly increased reactive oxygen species production.

experimental diet (0.025±0.04 nmol/mg). The age- and diet-related changes in reactive oxygen species production therefore parallel changes in arachidonic acid concentration.

The data presented identify a strong correlation between arachidonic acid concentration and ability to sustain LTP, and also between arachidonic acid concentration and KCl-stimulated release, rather than between docosahexanoic acid and either measure. Although the oil added to the experimental diet contains 1.7% arachidonic acid, dietary manipulation only partially reversed the age-related decrease in arachidonic acid concentration; therefore, it was considered that high concentrations of docosahexanoic acid might prevent incorporation of arachidonic acid. To address this possibility, weanling rats fed on the control or experimental diet for eight weeks were analysed for their ability to sustain LTP, while tissue prepared from dentate gyri of these rats was assessed for concentrations of arachidonic and docosahexanoic acids.

Figure 5 shows that there was a marked increase in mean EPSP slope immediately following tetanic stimulation in both groups of rats; the mean percentage increases in the 2 min immediately following tetanic stimulation (compared with the 5 min immediately prior to tetanic stimulation) were 154.76±2.54% and 145.16±2.90% in rats fed on the control and experimental diets, respectively. Dietary manipulation attenuated the more persistent aspect of LTP; thus, the mean percentage increases in EPSP slope in the last 5 min of the experiment (compared with the 5 min immediately prior to tetanic stimulation) were 130.72±1.55% and 120.63±1.30% in rats fed on the control and experimental diets, respectively. We analysed fatty acid concentrations and reactive species production in tissue prepared from rats fed on the control and experimental diets. The data showed that, while there was an increase in concentration of docosahexanoic acid in rats fed on the experimental diet compared with the control diet, the difference between the two groups was

not statistically significant (Fig. 5B). There was also no significant difference in arachidonic acid concentration in tissue prepared from rats fed on control and experimental diets (Fig. 5B). Dietary manipulation did not significantly affect concentrations of other fatty acids analysed, γ-linolenic acid, α-linolenic acid, linoleic acid or oleic acid (data not shown). Analysis of reactive oxygen species production revealed that this measure was significantly enhanced in tissue prepared from rats fed on the experimental diet compared with rats fed on the control diet ($P<0.01$; Fig. 5C).

The mean initial body weights of the weanling rats were 63.92 ± 2.01 and 64.43 ± 2.07 g in the control and experimental groups; at the end of the experimental period, the mean values were 333.44 ± 7.84 and 352.16 ± 9.89 g. Food intake was adjusted each day to ensure that rats received the full daily dose of unsaturated fatty acids.

DISCUSSION

The objective of this study was to establish whether certain age-related neuronal deficits could be reversed by dietary supplementation with ω-3 fatty acids. The data presented demonstrate that the age-related decreases in LTP and glutamate release were reversed in 22-month-old rats which were fed on an ω-3 fatty acid-enriched diet for eight weeks.

We report that arachidonic acid concentration was significantly decreased in dentate gyri prepared from the hippocampus of aged, compared with young, rats; this observation confirms earlier reports in the hippocampus[24] and dentate gyrus.[26] The present data also indicate that there was a marked age-related decrease in the concentration of docosahexanoic acid in the dentate gyrus; this is, to our knowledge, the first indication of such a change. While age-related decreases in the concentration of α-linolenic acid, γ-linolenic acid and linoleic acid were observed, the data indicate that there was an increase, although not significant, in the concentration of palmitic acid.

Addition of ω-3 fatty acids to the diet of 22-month-old rats completely reversed the age-related decreases in membrane docosahexanoic acid and partially reversed the age-related decrease in arachidonic acid concentration. The restoration of membrane polyunsaturated fatty acid concentrations in aged brain to concentrations found in young brains indicates the ability of brain tissue to incorporate fatty acids, although this process has been reported to be down-regulated with age.[11,14] Since the oil added to the diet investigated in this study contained 26% docosahexanoic acid and only 1.7% arachidonic acid, it is perhaps not surprising that reversal of the age-related decrease in docosahexanoic acid was complete, but was partial in the case of arachidonic acid. Dietary manipulation also reversed the age-related decrease in α-linolenic acid, which is a precursor of docosahexanoic acid.

Data from this laboratory have suggested that the age-related decrease in arachidonic acid concentration is associated with an increase in lipid peroxidation and that this, in turn, is due to an increase in reactive oxygen species production.[32,33] In support of this hypothesis, we report here that reactive oxygen species production was enhanced in hippocampal tissue prepared from aged rats fed on the control diet; the observation that dietary manipulation reversed this effect, in parallel with restoring arachidonic acid concentration, offers further support for this hypothesis.

Associated with the changes in fatty acid concentration and reactive oxygen species production, the data demonstrate that dietary manipulation reversed the age-related impairment in ability to sustain LTP. In these experiments, we observed that the initial changes in EPSP slope and amplitude following tetanic stimulation were attenuated in aged rats fed on the control diet; this finding, which suggests that induction of LTP might be impaired, supports data described by some,[26,35] but not other,[1] groups. However, while there is some debate relating to the induction of LTP, there is agreement that maintenance of LTP is impaired in aged rats;[1,19,24,26,32,33] the data described here provide further confirmation of this robust finding. In contrast to the attenuated response to tetanization in aged rats fed on the control diet, the responses of aged rats fed on the experimental diet were indistinguishable from those observed in young rats fed on either diet.

Regression analysis was performed in an effort to establish a possible correlation between ability to sustain LTP (i.e. the percentage change in EPSP slope in the last 5 min of the experiment) and fatty acid concentration. The data demonstrated a significant positive correlation between LTP and arachidonic acid concentration, but not docosahexanoic acid concentration. The correlation between arachidonic acid concentration and LTP, which is consistent with previous observations,[24,26] might be considered surprising, since reversal of the age-related decrease in arachidonic acid concentration was only partial. This partial reversal may be due to limited availability of arachidonic acid, since the diet contained only 1.7% arachidonic acid, or because incorporation of arachidonic acid into membranes is reduced with age.[11,14] It may also occur because the high concentration of docosahexanoic acid (26%) in the diet might inhibit incorporation of arachidonic acid.

To examine the latter possibility, groups of weanling rats were fed for eight weeks on the control or experimental diet and analysed for ability to sustain LTP, while fatty acid concentration were assessed. The data showed that dietary manipulation increased the concentration of docosahexanoic acid by about 60%, while arachidonic acid concentration was decreased by 10%. A similar increase in docosahexanoic acid in brain tissue has been described following dietary manipulation of adult rats[4,13] and aged rats[13] with fish oil, although in these studies significant decreases in *n*-6 fatty acids, including arachidonic acid, were reported. The fact that an insignificant decrease in arachidonic acid concentration was observed in the present study, compared with a significant change in the other studies, might indicate differences between brain areas, or differences in the specific diet or period of dietary supplementation. Ingestion of high doses of fish oil has been associated with oxidative stress in membranes,[12] a factor recently shown to impact negatively on expression of LTP,[32,33] and in this experiment we observed that reactive oxygen species production was markedly enhanced in tissue prepared from rats which had been fed on the experimental diet. We set out to establish whether a diet enriched in docosahexanoic acid offered to weanling rats affected (i) LTP, (ii) concentrations of arachidonic acid and docosahexanoic acid, and (iii) reactive oxygen species production. The evidence indicates that tetanic stimulation exerted no effect on the early change in the population EPSP slope; a decrease was observed only in the last 10 min of the experiment. This attenuation in LTP was coupled with increased concentration of docosahexanoic

acid, without a change in arachidonic acid. Rats fed on the diet enriched with docosahexanoic acid showed an increase in reactive oxygen species production in the hippocampus, which may be responsible for the compromise in LTP, since such a change has already been associated with an impairment in LTP in aged rats and rats treated with interleukin-1β.[32,33]

Transmitter release relies on several membrane-associated functions, e.g., influx of calcium through voltage-sensitive channels, docking of synaptic vesicles and the fusion of synaptic plasma and synaptic vesicle membranes; therefore, an age-related decrease in membrane fluidity might be expected to impact on glutamate release. The data presented show that synaptosomes prepared from the dentate gyrus of aged rats failed to respond to added KCl, which was in contrast to the stimulatory effect of KCl in tissue prepared from four- and 22-month-old rats fed on the experimental diet. The fact that glutamate release was not stimulated by KCl in tissue prepared from aged rats is surprising, but it confirms earlier findings in which this deficit was also observed.[24,26] The present data also indicate that, while KCl-induced release was further enhanced in tissue prepared from four-month-old rats in which LTP was sustained, this effect was absent in tissue prepared from aged rats fed on the control diet. This finding is consistent with the view that maintenance of LTP is, at least in part, dependent on increased glutamate release.[3,7,25,26] However, it should be recognized that the decreased ability of aged rats to release glutamate might result in decreased depolarization during tetanic stimulation, one consequence of which might be the impairment in maintenance of LTP and the associated compromise in release after 40 min. The finding that dietary manipulation reversed this age-related impairment suggests that membrane composition is an important determinant in the release response to KCl. It may also be an important factor in determining responses to agonists, since arachidonic acid and ACPD interacted to significantly increase KCl-stimulated release in untetanized tissue prepared from young rats and aged rats fed on the experimental, but not the control, diet. The present data also confirm the previous finding that the interaction between arachidonic acid and ACPD was occluded in synaptosomes prepared from tissue in which LTP was induced *in vivo*.[25,26] We have interpreted this finding as being consistent with the hypothesis that this interaction plays a role in increasing glutamate release following induction of LTP.

CONCLUSIONS

We have reported previously that reversing the age-related decrease in membrane arachidonic acid by dietary manipulation with γ-linolenic acid and arachidonic acid reversed the age-related impairments in expression of LTP and the age-related decrease in glutamate release.[26] This study extends these findings by indicating that similar effects can occur by dietary manipulation with other polyunsaturated fatty acids. However, regression analysis revealed that there was a significant positive correlation between arachidonic acid and release, but not between docosahexanoic acid and release. This observation suggests that, in relation to the release response to KCl, there may be a specific requirement for arachidonic acid. Similarly, the correlation between LTP and arachidonic acid, but not docosahexanoic acid, is consistent with the idea of a specific requirement for arachidonic acid in expression of LTP. These observations therefore provide indirect evidence supporting a role for arachidonic acid in LTP, adding to the existing body of evidence.[9,22,24,32,33] Despite these indications of a special role for arachidonic acid in release and in LTP, it must be emphasized that the present data demonstrate that age-related decreases in LTP and transmitter release were reversed when the decrease in docosahexanoic acid was completely reversed, while the decrease in arachidonic acid was only partially reversed. This finding is consistent with the view that membrane fluidity is likely to be a significant factor in determining expression of LTP and maintaining transmitter release in aged rats.

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Age-related changes in LTP and antioxidant defenses are reversed by an α-lipoic acid-enriched diet

Bernadette M. McGahon[a], Donen S.D. Martin[a], David F. Horrobin[b], Marina A. Lynch[a,*]

[a]*Department of Physiology, Trinity College, Dublin, Ireland*
[b]*Laxdale Research Ltd., Stirling, Scotland*

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Abstract

Among the age-related changes identified in rat hippocampus are impairments in LTP and glutamate release. These deficits have been coupled with decreased arachidonic acid concentration. In this study we compared LTP and glutamate release in groups of aged and young rats fed for 8 weeks on a control diet or on a diet enriched in α-lipoic acid. Dietary supplementation in aged rats restored hippocampal arachidonic acid concentration to levels observed in tissue prepared from young rats. We observed that aged rats that received the experimental diet sustained LTP in perforant path-granule cell synapses in a manner indistinguishable from young rats whereas the age-related impairment in glutamate release was reversed in synaptosomes prepared from dentate gyrus obtained from these rats. The evidence presented supports the hypothesis that the α-lipoic acid-enriched diet has antioxidant properties, because the age-related increase in superoxide dismutase activity and decrease in α-tocopherol concentration were reversed. The finding that the age-related increase in interleukin-1 (IL-1)β concentration was also reversed suggests a possible role for this cytokine in ageing. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Long-term potentiation; α-Lipoic acid; Arachidonic acid; Glutamate release; Hippocampus; IL-1

1. Introduction

It is has been shown repeatedly that maintenance of long-term potentiation (LTP) is impaired in aged rats [1,2,12,13,18,21,25,28,29]. In contrast to this agreement the effects of age on induction of LTP remain unclear, with evidence of a deficit reported by some groups [21,25,29] but not others [1,14].

Among the age-related changes that correlate with the impairment in LTP in dentate gyrus is a decrease in arachidonic acid concentration [21,25,28,29], a proposed retrograde messenger in LTP [11,19]. Because arachidonic acid contributes to membrane fluidity, a decrease in its concentration is likely to be accompanied by a decrease in membrane fluidity, and this, in turn, is likely to impact on several membrane-associated functions that play a role in expression of LTP [19]. One of these membrane-associated functions is transmitter release, an increase of which has been coupled with maintenance of LTP in perforant path synapses [6,8,24,25]. Evidence from this laboratory has consistently shown that there is an age-related decrease in KCl-stimulated release of glutamate in synaptosomes prepared from dentate gyrus, whereas an attenuated release response to KCl has been observed in synaptosomes prepared from aged rats in which LTP was compromised [21, 25]. It is significant that the impairment in LTP and the compromise in transmitter release were reversed when arachidonic acid concentration was restored in aged rats fed on a diet supplemented with arachidonic acid and γ-linolenic acid [25]. It is therefore a major objective to establish the underlying cause of the age-related decrease in arachidonic acid concentration.

Recent evidence is consistent with the idea that increased lipid peroxidation is primarily responsible for the decrease in arachidonic acid concentration and that this is likely to be triggered by the proinflammatory cytokine, IL-1β or increased production of reactive oxygen species [28,29]. These proposals are consistent with the findings that (a) these age-related changes are reversed by dietary supplementation with the antioxidant vitamins E and C [28] and

* Corresponding author. Tel.: 1 1-353-1-608-1770; fax: 1 1-353-1-679-3545.
E-mail address: lynchma@tcd.ie (M.A. Lynch)

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(b) that a number of age-related changes in neuronal function have been attributed to accumulation of reactive oxygen species, arising from either increased formation of reactive oxygen species, compromised ability of the aged brain to cope with oxidative stress, or both [10,35,40].

In an effort to establish a causal relationship between age-related changes in synaptic function and age-related changes in handling of oxidative processes in cells, we assessed the ability of a diet, enriched in a-lipoic acid, to reverse age-related changes in a number of parameters. a-Lipoic acid is a naturally-occurring free radical scavenger that has been shown to regenerate endogenous antioxidants like vitamins E and increase formation of glutathione [4]. Consistent with its role as an antioxidant, a-lipoic acid reversed the oxidative stress-induced activation of NFkB [5,33] and the increased lipid peroxidation [30] that are associated with diabetic neuropathy. It has also been shown to reverse the damage induced by ischemia in CA1 pyramidal cells [9] and also the damage that accompanies reperfusion [34].

In this study, groups of aged and young rats were fed either on a control diet or on a diet enriched in a-lipoic acid and assessed for their ability to sustain LTP. Tissue obtained from dentate gyri of these rats was analyzed for fatty acid concentration and assessed for glutamate release. We observed that dietary manipulation reversed age-related impairment in LTP and glutamate release and these changes were accompanied by restoration of fatty acid, IL-1b and a-tocopherol concentrations to values observed in young rats.

2. Experimental procedures

2.1. Animals

Aged and young male Wistar rats (Bantham and Kingman, UK) were housed in groups of 2–4 under a 12-h light schedule, with the ambient temperature controlled between 22 and 23°C. Rats were maintained in the BioResources Unit in Trinity College, Dublin, were checked daily and were subject to veterinary supervision throughout the experiment. Young and aged rats were divided into groups that were fed on 'control' or 'experimental' diet. The composition of the experimental diet was as follows: 20% g-linolenic-lipoic acid diol, 40% tuna oil containing 24% docosahexanoic acid and 1.7% arachidonic acid and 40% microbial oil containing 40% arachidonic acid, 3% g-linolenic acid and 4% dihomo-g-linolenic acid (Laxdale Research, UK). The overall composition of the diet, in terms of polyunsaturated fatty acids, was therefore 20% of g-linolenic-lipoic acid diol, 9.6% of docosahexanoic acid, 16.4% of arachidonic acid, 1.2% of g-linolenic acid and 1.6% of dihomo-g-linolenic acid. One group of aged and one group of young animals were fed for 8 weeks on 100 mg/rat/day experimental diet, referred to hereafter as '100% experimental diet'. A second group of young and aged animals received standard laboratory chow to which corn oil was added to ensure an isocaloric intake for all rats. A third group of aged rats received 50 mg/rat/day of experimental diet (hereafter referred to as '50% experimental diet'). Food intake was measured for 2 weeks before the commencement of the experiment, sufficient diet was prepared for 3 or 4 days, daily allowances were stored in airtight containers at 2 80°C and rats were offered their full daily requirement each day. At the end of the period of dietary manipulation the average ages of the aged and young rats were 22 months and 4 months respectively.

2.2. Induction of long-term potentiation in dentate gyrus

Rats were anesthetized with urethane; young animals initially received 1.5 g/kg of urethane intraperitoneally (i.p.), and a further increment, if required (that did not exceed 0.6 g/kg in any experiment) for induction of deep anesthesia, that was assessed by the absence of a pedal reflex. The mean doses (6 SEM) administered to young rats given the control and experimental diets were 1.92 g/kg (6 0.08) and 1.82 g/kg (6 0.05) respectively. Aged animals received 1.2 g/kg of urethane initially and topped up when necessary; the mean doses administered to aged rats given the control, 50% lipoic acid and 100% lipoic acid diets were 1.44 g/kg (6 0.05), 1.37 g/kg (6 0.04) and 1.48 g/kg (6 0.06), respectively.

LTP was induced in perforant path-granule cell synapses as described previously [24]. Briefly, a bipolar stimulating electrode was placed in the perforant path (4.4 mm lateral to Lambda) and a unipolar recording electrode was placed in the dorsal cell body region of the dentate gyrus (2.5 mm lateral and 3.9 mm posterior to Bregma). The depths of the stimulating and recording electrodes were adjusted to maximize the amplitude of the response; the stimulus intensity was adjusted to the value that was just sufficient to induce a population spike. Test shocks were given at 30 s intervals for 10 min before and 40 min after, 3 trains of stimuli (250 Hz for 200 ms, with a 30 s intertrain interval) that was shown previously to produce saturation of LTP in perforant path-granule cell synapses.

2.3. Tissue preparation

At the end of the recording period, rats were killed by decapitation, the hippocampus proper and the dentate gyri were dissected, and cross-chopped slices (350 mm 3 350 mm) were prepared using a McIlwain tissue chopper. Samples of hippocampus and control and potentiated dentate gyri were frozen separately in 1 ml of Krebs solution (composition of Krebs in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) containing 10% dimethylsulphoxide [15] and stored at 2 80°C until required.

For analysis, slices were thawed rapidly (1.5–2 min) by

agitation at 37°C and rinsed 4 times in excess fresh oxygenated Krebs solution. Fatty acid concentrations were assessed in samples of homogenate prepared from dentate gyrus of aged and young rats, whereas the crude synaptosomal pellet, P_2, prepared as previously described [24] was used for analysis of glutamate release. Homogenate prepared from hippocampal tissue was used for analysis of a-tocopherol and IL-1b concentrations and activity of superoxide dismutase.

2.4. Assessment of glutamate release

Samples of synaptosomal tissue were resuspended in ice-cold Krebs solution containing 2 mM of $CaCl_2$. Tissue was aliquotted onto Millipore filters (0.45 mm), rinsed under vacuum and the filtrate was discarded. Tissue was incubated in 250 ml of oxygenated Krebs solution at 37°C for 3 min and filtrate was collected and stored. Release of transmitter was then stimulated by the addition of 40 mM of KCl to Krebs solution. In some cases, arachidonic acid (1 mM) and ACPD (50 mM) were added to the incubation medium to assess the effect of these agents on KCl-stimulated release.

Glutamate was analyzed as described [26,32]. Briefly, 96-well plates were coated with glutaraldehyde (0.5% in 100 mM of NaH_2PO_4 buffer, pH 4.5; 320 ml) by incubation for 60 min at 37°C. Plates were then washed with 100 mM of NaH_2PO_4 buffer. Triplicate samples (50 ml) or glutamate standards (50 ml; 50 nM to 10 mM prepared in 100 mM of Na_2HPO_4 buffer, pH 8.0) were added, plates were incubated for 2 h at 37°C and washed with 100 mM of NaH_2PO_4 buffer. To bind any unreacted aldehydes, ethanolamine (320 ml; 0.1 M in 100 mM of Na_2HPO_4 buffer) was added to each well and incubated for 60 min at 37°C. Plates were washed with phosphate-buffered saline (PBS) containing Tween-20 (0.2% Tween; PBS-T). To block non-specific binding, samples were incubated with donkey serum for 60 min (200 ml; 3% in PBS-T). Antiglutamate antibody (raised in rabbit; 100 ml; 1:5,000 in PBS-T; Sigma, UK) was added to each plate, incubated overnight at 4°C and washed with PBS-T. Anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (95 ml; 1:10,000 in PBS-T; Amersham, UK) was added, incubated for 60 min at room temperature and plates were again washed with PBS-T. 3,3Q5,5Q-Tetramethylbenzidine (100 ml) liquid substrate was added as chromogen and incubation continued for 60 min at room temperature. H_2SO_4 (4 M; 50 ml) was used to stop the reaction and optical densities were determined at 450 nm using a multiwell plate reader. Values were calculated with reference to the standard curve, corrected for protein [7] and expressed as nmol glutamate/mg protein.

2.5. Analysis of fatty acid concentration

Thawed slices prepared from dentate gyrus were rinsed 3 times and then homogenized in 200 ml of fresh Krebs solution. Fatty acids were extracted into chloroform:methanol (2:1 v/v; 1 ml) by vigorous shaking for 10 min followed by centrifugation at 1000 3 g for 5 min to separate the phases. The aqueous layer was discarded and the chloroform phase was evaporated under nitrogen and resuspended in ethanol for analysis. Fatty acid concentrations were analyzed as their 2-nitrophenylhydrazine (NPH) derivatives by reverse phase HPLC [22]. Derivatization was achieved by adding 2-NPH-HCl solution (0.02 M 2-nitrophenylhydrazine-HCl in 0.25 M HCl-ethanol (1:1, v/v) and EDC solution (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide 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 MeOH: H_2O, 80:20) samples were incubated at 60°C for 15 min and cooled in running water. Fatty acid derivatives were concentrated; n-hexane and phosphate buffer (0.033 M, pH 6.4 in 0.5 M HCl) were added, samples were vortex mixed for 30 s, centrifuged for 5 min at 1500 3 g and the hexane phase evaporated to dryness under nitrogen. For HPLC analysis, samples were resuspended in methanol, injected onto a Microsorb C18 column (maintained in a column oven at 30°C) and fatty acids 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 230 nm. Aliquots of tissue were taken for protein analysis and concentrations were estimated according to standard curves and expressed as nmol/mg protein.

2.6. Analysis of a-tocopherol

a-Tocopherol was analyzed according to the method of Vatassery [38]. Briefly, aliquots of hippocampal homogenate (150 ml) were incubated in the presence of ethanol containing 0.025% butyl-hydroxytoluene (150 ml), 25% ascorbic acid (70 ml) and 10% potassium hydroxide (135 ml) for 30 min at 60°C. Hexane (540 ml) containing 0.025% butyl-hydroxytoluene was added, samples were vortex-mixed for 1 min and centrifuged at 1500 3 g for 6 min. The hexane phase was removed and evaporated to dryness under nitrogen; the recovery of a-tocopherol using this procedure was between 70 and 80%. For HPLC analysis, dried samples were resuspended in methanol (150 ml) containing 0.025% butyl-hydroxytoluene and 30 ml volumes were injected onto an Intersil C18 column. Separation of a-tocopherol was achieved using a mobile phase of 75% acetonitrile: 25% methanol at a flow rate of 1.2 mL/min and samples were detected by uv spectroscopy at 292 nm. a-Tocopherol concentration was estimated by the external standard method and expressed as mg/mg tissue.

2.7. Analysis of superoxide dismutase activity

Superoxide dismutase activity was determined in samples of hippocampal homogenate [36]. Aliquots (800 ml) of incubation buffer (50 mM potassium buffer (pH 7.8) containing 1.8 mM xanthine, 2.24 mM nitroblue tetrazolium

(NBT), 40 units of catalase, 7 ml/ml xanthine oxidase and 1.33 mM diethylenetriaminepentacetic acid) were added to 1.5 ml microfuge tubes containing samples of supernatant (100 ml) at different dilutions (1:2, 1:5, 1:10, 1:20, 1:50 and 1:100) and analyzed by uv spectroscopy at 560 nm. Activity of superoxide dismutase was assessed as the rate of reduction of NBT, that was inhibited with increasing concentrations of protein. One unit of activity was defined as the amount of protein necessary to decrease the rate of the reduction of NBT by 50%. Results were expressed in units of superoxide dismutase activity per mg protein.

2.8. Analysis of IL-1b

IL-1b (IL-1b; Genzyme Diagnostics, USA) concentrations in hippocampal homogenate were analyzed by ELISA as previously described [28]. Briefly, 96-well plates were coated with 100 ml of capture antibody (2.0 mg/ml of final concentration, diluted in 0.1 M of sodium carbonate buffer, pH 9.5; monoclonal hamster anti-mouse IL-1b antibody), incubated overnight at 4°C, washed with PBS containing 0.05% Tween 20 and blocked for 2 h at 37°C with 250 ml of blocking buffer, (PBS, pH 7.3; 0.1 M with 4% bovine serum albumin (BSA)). Aliquots (50 ml) of samples or IL-1b standards (0–1000 pg/ml) were incubated for 1 h at 37°C. Plates were washed and secondary antibody (100 ml; final concentration 0.8 mg/ml in PBS containing 0.05% Tween 20 and 1% BSA; biotinylated polyclonal rabbit anti-mouse IL-1b antibody) was added and incubated for 1 h at 37°C. Detection agent (100 ml of horseradish peroxidase-conjugated streptavidin; 1:1000 dilution in PBS containing 0.05% Tween 20 and 1% BSA) was added, incubated for 15 min at 37°C. Substrate (100 ml; tetramethylbenzidine liquid substrate; Sigma, UK) was added, incubated at room temperature for 10 min and absorbance read at 450 nm. Results were expressed as pg IL-1b/mg tissue.

2.9. Statistical analysis

Data were analyzed, as appropriate, using either the Student's *t*-test for independent means, or by using a one-way analysis of variance (ANOVA) followed by post hoc analysis using the Newmann–Keuls test.

3. Results

The average food intake per rat for young rats was 22 g/rat/day compared with 18 g/rat/day for aged rats; these values did not vary for the duration of the experiment. Mean body weights (6 SEM) at the end of the experimental period were 481 g (6 28.4) and 481 g (6 16.1) in the young rats fed on the control and experimental diets and 525 g (6 15.5), 516 g (6 9.8) and 479 g (6 14.8) in the aged rats fed on the control, 50% and 100% diets respectively.

In these experiments, the stimulus strength used was that which was just sufficient to induce a spike; the mean values (6 SEM; V) were 1.53 (6 0.31) and 4.69 (6 0.19) in young and aged rats respectively. The mean values for EPSP slope (6 SEM; mV/ms) in young rats fed on control and experimental diets were 1.83 (6 0.10) and 1.82 (6 0.08) respectively. In aged rats, the values were significantly lower (p , 0.05; student's *t*-test for independent means); the means were 1.30 (6 0. 03), 1.29 (6 0.07) and 1.63 (6 0.11) for the groups fed on control, 50% and 100% diets respectively. The corresponding values for the mean amplitude of the response (mV) in young rats fed on control and experimental diets were 4.42 (6 0.14) and 4.40 (6 0.13). These values were higher than those recorded from aged rats, that were 3.62 (6 0.07), 4.11 (6 0.27) and 3.37 (6 0.09) for rats fed on control, 50% and 100% experimental diets respectively.

Fig. 1a indicates that there was a marked increase in mean EPSP slope immediately after tetanic stimulation in young rats fed on control or experimental diet; an increase in mean EPSP slope was observed for the duration of the experiment though dietary manipulation attenuated the more persistent EPSP response (see Table 1). Fig. 1b indicates that LTP was compromised in aged rats fed on the control diet, whereas aged rats fed on either experimental diet demonstrated an ability to sustain LTP that was similar to that of young rats; we observed an age-related attenuation in both the early and later response (see Table 1).

Analysis of several fatty acids in homogenate prepared from dentate gyri indicated that there were no significant differences in concentrations between the young rats fed on control and experimental diets (Fig. 2). The data indicate that the concentration of arachidonic acid was markedly decreased in tissue prepared from aged rats fed on the control diet (n 5 11) compared with tissue prepared from young rats (p , 0.05; ANOVA; n 5 8 in both dietary groups). Fig. 2 shows that dietary manipulation with either the 50% diet (n 5 9) or the 100% diet (n 5 11) completely reversed the age-related decrease in arachidonic acid concentration. In contrast to the age- and diet-related changes in arachidonic acid, the concentration of the arachidonic acid precursor, g-linolenic acid was unchanged. Analysis of the ratio of g-linolenic acid to arachidonic acid indicated values of 0.15 (6 0.03) and 0.20 (6 0.06) in the young rats fed on control and experimental diets respectively. The corresponding values were 0.38 (6 0.06), 0.17 (6 0.02) and 0.11 (6 0.02) in aged rats fed on the control, 50% and 100% diets respectively.

The concentration of docosahexanoic acid was markedly decreased in tissue prepared from aged rats fed on the control diet compared with tissue prepared from young rats (p , 0.05; ANOVA). Dietary manipulation partially reversed this decrease, though concentrations were still significantly lower then those in tissue prepared from young rats. In contrast, the concentrations of linoleic and palmitic acids were significantly increased with age (p , 0.05; ANOVA), whereas there was no significant age-, or diet-related change in the concentration of a-linolenic acid. The

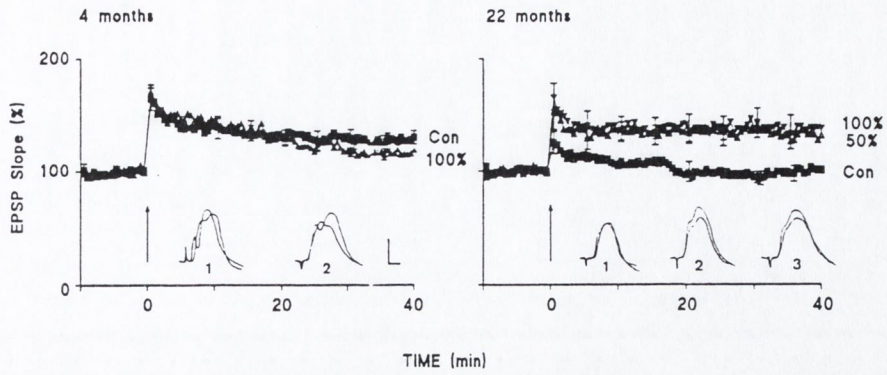

Fig. 1. The age-related impairment in LTP is restored by dietary supplementation. The mean slope of the population EPSP was markedly increased after tetanic stimulation (arrow) in both groups of young rats (n 5 8 in each case). In contrast, there was a marked impairment in maintenance of LTP in aged rats that received the control diet (n 5 11). Dietary manipulation with either diet (n 5 9 for the 50% diet and n 5 11 for the 100% diet) reversed the age-related impairment in LTP. Mean EPSP slopes are expressed as a percentage of the values recorded in the 5 min immediately before tetanic stimulation. Standard errors are included for every tenth response and are so small as to be obscured in some cases. Sample waveforms are included for young rats (left hand panel) fed on the control (1) and experimental (2) diets, and for aged rats (right-hand panel) fed on the control (1), 50% (2) and 100% (3) diets; in each case responses obtained after tetanic stimulation are superimposed on responses obtained before tetanic stimulation. The scale bars represent 1 mV and 2 ms refer to responses for both aged and young rats.

concentrations of palmitic and linoleic acids were also not significantly affected by diet.

Endogenous glutamate release was assessed in synaptosomes prepared from dentate gyrus in each of the experimental groups. Fig. 3a shows that unstimulated glutamate release was similar in synaptosomes prepared from untetanized and tetanized dentate gyri of both groups of young rats. Addition of 40 mM of KCl to the incubation medium significantly enhanced release in untetanized tissue prepared from both groups of young rats (p , 0.05; ANOVA), but the enhancement was more marked in synaptosomes prepared from dentate gyrus in which LTP was sustained (p , 0.01; ANOVA). Addition of arachidonic acid and ACPD significantly increased KCl-stimulated release of glutamate in synaptosomes prepared from untetanized tissue, but this effect was occluded in synaptosomes prepared from tetanized tissue (Fig. 3a and 3b). KCl-stimulated release of glutamate was markedly attenuated in synaptosomes prepared from untetanized dentate gyrus of aged rats fed on the control diet and there was no evidence of a KCl-induced enhancement of release in synaptosomes prepared from tetanized dentate gyrus (Fig. 3c). Addition of arachidonic acid and ACPD failed to affect KCl-induced release in either untetanized or tetanized tissue. In contrast to the lack of response in tissue prepared from aged rats fed on the control diet, KCl significantly increased glutamate release in synaptosomes prepared from untetanized dentate gyrus of aged rats fed on either experimental diet (p , 0.05; ANOVA). A further enhancement of release was observed in synaptosomes prepared from tetanized tissue (p , 0.01; ANOVA). Addition of arachidonic acid and ACPD to the incubation medium significantly increased KCl-induced release in synaptosomes prepared from untetanized dentate gyrus (p , 0.01; ANOVA), but this effect was occluded in synaptosomes prepared from tetanized tissue (Fig. 3d and 3e).

Table 1
Mean percentage change in epsp slope[a]

	0–2 min posttentanus	35–40 min posttetanus
4 months		
Control diet	157.64 (6.34)	127.01 (2.09)
Experimental diet	159.22 (5.09)	116.80 (1.22)
22 months		
Control diet	117.76 (2.99)	98.88 (1.04)
50% diet	145.68* (6.10)	130.26* (2.07)
100% diet	155.33* (5.88)	137.04* (4.66)

[a] Mean percentage changes in epsp slope in two time intervals posttetanus (0–2 min and 35–40 min) in young and aged rats fed on control or experimental diets. The early mean percentage change in epsp slope was similar in both groups of young rats, through dietary manipulation attenuated and later response. LTP was attenuated in aged rats fet on the control diet, but this was not observed in aged rats fed on either the 50% or 100% diet. The asterisks indicate significant differences between values in rats fed on control and experimental diets (p , 0.05; ANOVA).

Because a-lipoic acid has been shown to have antioxidant properties and because antioxidative defenses have been shown to be compromised with age [31], we analyzed age-related changes in 3 parameters in hippocampal tissue prepared from the young and aged rats that received control or experimental diets. The data indicated that dietary manipulation in young rats exerted no effect on a-tocopherol concentration, IL-1b concentration or superoxide dismutase, therefore data from the two groups of young rats are pooled in Fig. 4. We observed that the concentration of a-tocopherol was decreased in the hippocampus of aged rats fed on the control diet, compared with young rats (p , 0.05; ANOVA; Fig. 3a). Dietary manipulation with 100% diet

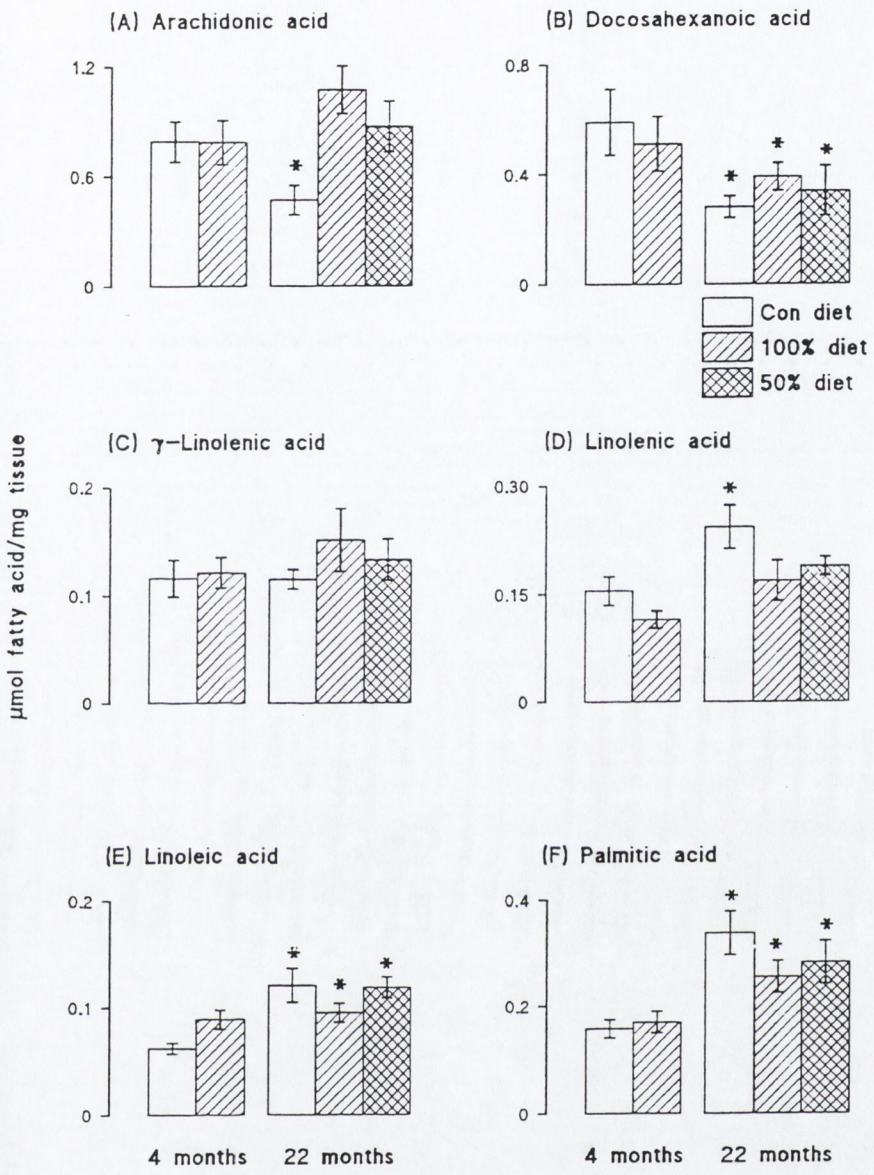

Fig. 2. Dietary manipulation reverses age-related changes in concentrations of arachidonic acid and docosahexanoic acid. Concentrations of arachidonic acid, docosahexanoic acid, linolenic acid, g-linolenic acid, linoleic acid and palmitic acid were similar in both groups of young rats (n 5 8 in each case). The concentrations of arachidonic and docosahexanoic acids were significantly decreased in tissue prepared from dentate gyrus of aged rats that received the control diet (n 5 11), whereas significant increases in the concentrations of linoleic acid and palmitic acid were observed (*p , 0.05; Student's t-test for independent means). Dietary supplementation with either the 50% (n 5 9) or 100% (n 5 11) diets reversed the age-related changes in arachidonic acid and linolenic acid.

partially reversed this effect, but reversal was almost complete in tissue prepared from rats that received the 50% diet.

An age-related increase in hippocampal concentration of IL-1b was also observed (p , 0.05; ANOVA; Fig. 4b). We found that this effect was completely reversed by supplementation with the 100% lipoic acid diet, whereas the 50% diet was less effective. Similarly, the activity of superoxide dismutase was significantly increased in the hippocampus of aged rats (p , 0.05; ANOVA; Fig. 4b); this effect was completely reversed in hippocampal tissue prepared from rats that received the 100% lipoic acid diet. Enzyme activity, however, was similar in tissue prepared from aged rats that received the control diet and those that received the 50% diet.

4. Discussion

The objective of this study was to establish whether certain age-related changes in hippocampal synaptic func-

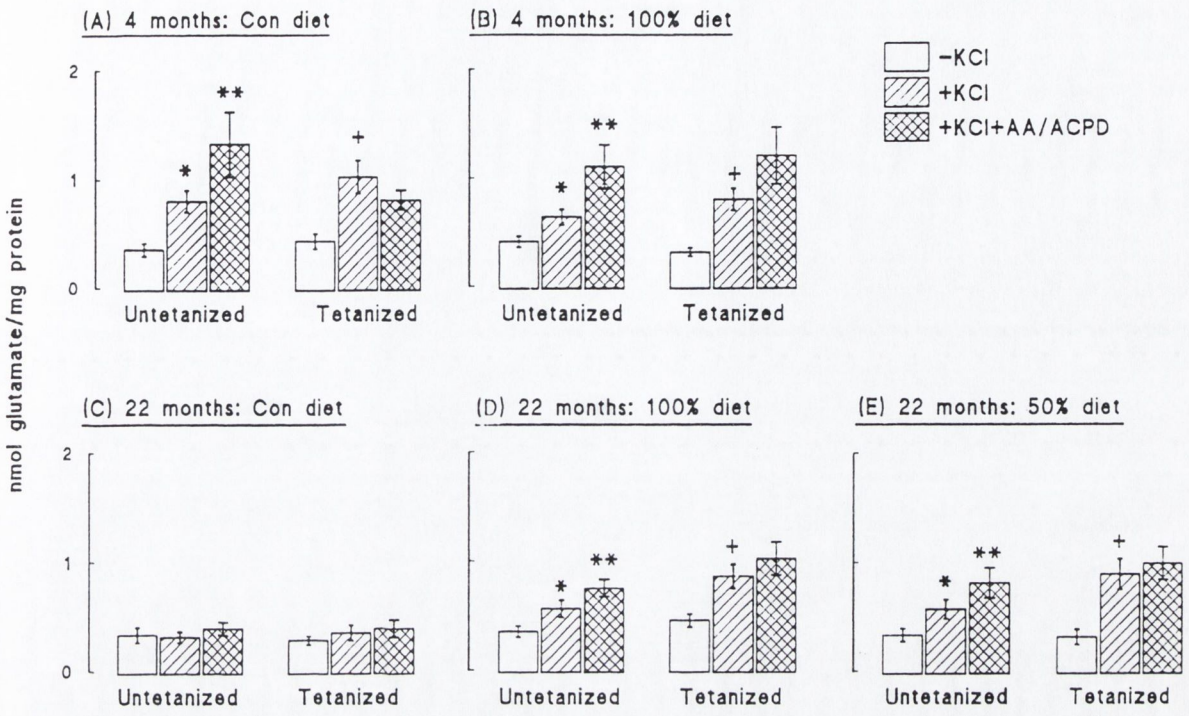

Fig. 3. Dietary manipulation reverses age-related changes in KCl-stimulated glutamate release. KCl (40 mM) increased release to a significantly greater degree in tetanized (**p , 0.01; ANOVA), compared with untetanized (*p , 0.05; ANOVA), tissue prepared from young rats that were fed either diet (n 5 8 in each case); arachidonic acid (AA) and ACPD significantly enhanced KCl-stimulated release in untetanized tissue (1 p , 0.01; ANOVA), but not in tetanized tissue. KCl failed to increased glutamate release in synaptosomes prepared from either untetanized or tetanized tissue obtained from aged rats fed on the control diet (n 5 11). In aged rats fed on either experimental diet (n 5 9 for 50% diet and n 5 11 for 100% diet), KCl (40 mM) increased release to a significantly greater degree in tetanized tissue (**p , 0.01; ANOVA), compared with untetanized tissue (* p , 0.05; ANOVA); arachidonic acid (AA) and ACPD significantly enhanced KCl-stimulated release in untetanized (1 p , 0.01; ANOVA), but not tetanized tissue.

tion might be reversed by supplementation with a diet enriched in a-lipoic acid. The data show that dietary manipulation reversed the age-related impairment in ability of aged rats to sustain LTP in perforant path-granule cell synapses.

In this study we observed that dietary manipulation of aged rats with either experimental diet completely reversed the age-related attenuation in the initial tetanus-induced enhancement of the synaptic response. Similarly, the marked attenuation in the later response, that reflects impaired maintenance of LTP was also completely reversed by dietary manipulation.

We have previously reported that aged rats that received a diet enriched in arachidonic acid and its precursor g-linolenic acid exhibited an ability to sustain LTP that was comparable to young rats [25]; this was accompanied by a reversal of the age-related decrease in arachidonic acid concentration. Analysis of fatty acids in dentate gyrus of the young and aged rats in the present study demonstrated that the age-related decrease in arachidonic acid concentration was also reversed by dietary manipulation with an a-lipoic acid-enriched diet. This may be a direct effect, due to incorporation of arachidonic acid, because the diet contained arachidonic acid (16.4%). It may also be an indirect effect because the antioxidant properties of a-lipoic acid [4] have been shown here to be effective in hippocampal, as well as other, tissues. One reported antioxidant effect of a-lipoic acid is its ability to regenerate vitamin E [4]; this effect of a-lipoic acid may contribute to the observed effects, because we have reported that dietary manipulation with vitamins E and C reverse the age-related impairment in LTP [28]. It is also possible that dietary manipulation affects the activity of D 5-desaturase that catalyzes formation of arachidonic acid from g-linolenic acid. Activity of this enzyme is decreased with age [3,17]. This possibility is consistent with the finding that the ratio of g-linolenic acid to arachidonic acid was increased with age and reversed by dietary manipulation.

The data indicate that there is also an age-related decrease in the concentration of docosahexanoic acid, that was partially, but not completely, reversed by dietary manipulation. This suggests that, although docosahexanoic acid is present in the diet, it is unlikely to be primarily responsible for reversing the age-related decrease in LTP, a finding that is consistent with other observations from this laboratory indicating a lack of correlation between docosahexanoic acid concentration and LTP [27]. The concentrations of a less unsaturated fatty acid, linoleic acid, as well as the saturated fatty acid, palmitic acid, were increased in hippocampal tissue prepared from aged rats compared with

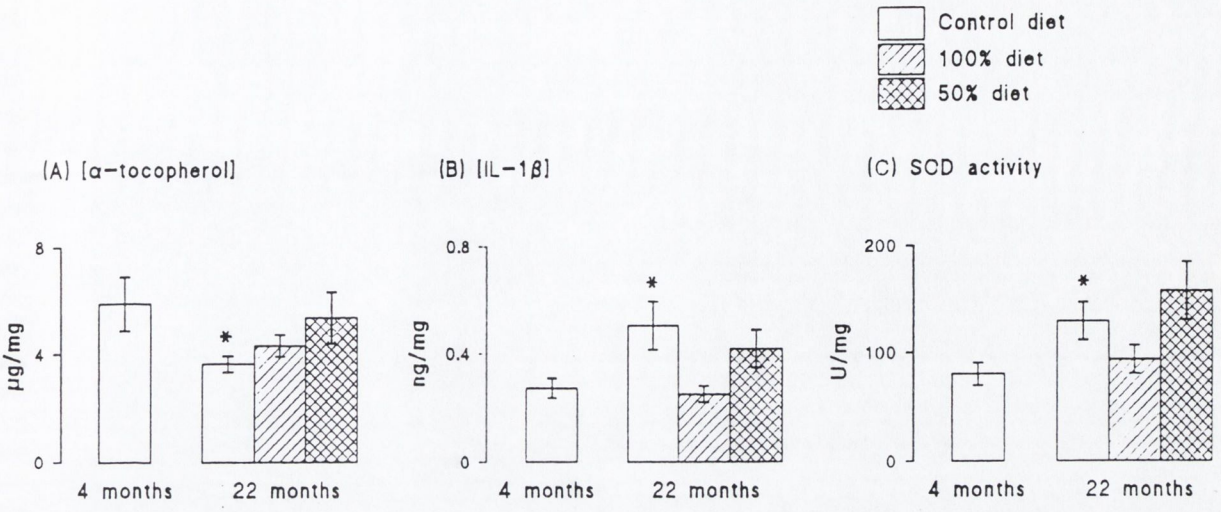

Fig. 4. Dietary supplementation reversed age-related changes in a-tocopherol, IL-1b and superoxide dismutase. The concentrations of vitamin E (a) and IL-1b (b) and the activity of superoxide dismutase (SOD: c) were similar in hippocampal tissue prepared from both groups of young rats and therefore the data are pooled (n 5 16 in total). The age-related decrease in a-tocopherol concentration and the age-related increase in IL-1b concentration in tissue prepared from aged rats fed on the control diet (*p , 0.05: Student's t-test for independent means; n 5 11) were reversed in tissue prepared from aged rats fed on the experimental diets (n 5 9 for 50% diet and n 5 11 for 100% diet). Superoxide dismutase activity was significantly increased in aged rats fed on the control diet (*p , 0.05: Student's t-test for independent means) but dietary manipulation reversed the age-related change.

young rats. These data may indicate that during ageing there is a gradual replacement of highly unsaturated fatty acids with less unsaturated and saturated fatty acids, accounting for the increase in membrane rigidity that has been described in aged tissues [40].

KCl-stimulated release of endogenous glutamate in synaptosomes prepared from either subgroup of young rats, was significantly greater in tetanized tissue compared with untetanized tissue, confirming previous results [8,24], whereas the occlusion of the stimulatory effect arachidonic acid and ACPD enhanced KCl-stimulated release in synaptosomes prepared from tetanized dentate gyrus has also been reported [24]. Dietary manipulation in young rats did not affect these measures. KCl-stimulated release was attenuated in synaptosomes of dentate gyrus prepared from either untetanized or tetanized tissue of aged rats fed on the control diet, whereas arachidonic acid and ACPD were without effect. In marked contrast to these findings, the stimulatory effect of KCl, and the enhancing effect of arachidonic acid and ACPD on KCl-stimulated release, were clearly evident in synaptosomes prepared from untetanized dentate gyrus of aged rats that had been fed on the experimental diets. Similarly, the increase in KCl-stimulated release and the occlusion of the effect of arachidonic acid and ACPD, were both evident in synaptosomes of tetanized dentate gyrus prepared from aged rats that received the dietary supplement. It is likely that the impaired responses are a consequence of decreased membrane fluidity, because fusion of synaptic plasma and synaptic vesicle membranes, that is a necessary step leading to release, is likely to be sensitive to fluidity changes in membrane.

Although an age-related decrease in arachidonic acid (and docosahexanoic acid) is likely to affect membrane fluidity, its putative role as retrograde messenger in LTP [6,39], suggests other actions might be predicted. It has been proposed that arachidonic acid is released from a postsynaptic site [11] into the synaptic cleft [20] and acts presynaptically to increase glutamate release [16,23,24], upon activation of PLCg and PLCb [26]. Thus an age-related decrease in arachidonic acid concentration is likely to limit tetanus-induced liberation of arachidonic acid from membrane phospholipids and therefore contribute to the age-related compromise in glutamate release.

In view of the evidence suggesting that a-lipoic acid exerts antioxidant effects [4], we considered that the beneficial effects of this diet might be a consequence of such action. We report here that there was an age-related decrease in a-tocopherol concentration, that was completely reversed by dietary manipulation with the 50% diet, and partially reversed by the 100% diet. The mechanism underlying this effect remain to be established; possibilities are that a-lipoic acid, acting as a free-radical scavenger [4], prevents accumulation of reactive oxygen species, but it has also been shown to regenerate a-tocopherol [4] and to decrease lipid peroxidation [30], that might limit oxidation of a-tocopherol.

Previous experimental data indicated that the age-related decrease in a-tocopherol concentration was accompanied by an increase in concentration of the proinflammatory cytokine, IL-1b (28), whereas in vitro evidence indicated that IL-1b induced an increase in ROS [28,31]. The present results confirm that there was an age-related increase in IL-1b concentration in hippocampus [28,29]; such a change, that has the capacity to increase ROS, coupled with

decrease in the scavenger a-tocopherol, is consistent with reports off oxidative changes in the aged brain [35,37,40]. We observed that dietary manipulation reversed the age-related increase in IL-1b concentration in hippocampus. We have recently found that IL-1b stimulates activity of superoxide dismutase in vitro and, consistent with this, is the present finding that the age-related increase in IL-1b concentration is accompanied by an increase in superoxide dismutase activity, whereas both effects are absent in tissue prepared from aged rats fed on the experimental diet. The action of the diet on superoxide dismutase activity identifies another potential antioxidant effect of a-lipoic acid because an increase in activity of superoxide dismutase in the absence of parallel changes in glutathione peroxidase or catalase, might allow accumulation of hydrogen peroxide that, in the presence of metal ions, leads to formation of hydroxyl radicals.

The present findings do not identify the primary action of the a-lipoic acid-rich diet but are consistent with the view that it exerts an antioxidant effect. The data also indicate parallel decreases in a-tocopherol and arachidonic acid concentrations that are coupled with increased IL-1b concentration and increased superoxide dismutase activity in the aged brain. A causal relationship between these parameters is suggested by the diet-induced reversal of these age-related effects.

The primary finding of this study is that supplementation with a diet enriched in a-lipoic acid reverses several age-related changes in synaptic function in rat hippocampus. Thus age-related impairments in LTP and glutamate release were reversed adding to the previous evidence that these measures are tightly coupled. The data are consistent with the proposal that these compromises in synaptic function might be a consequence of age-related decreases in membrane fluidity and antioxidative defenses, possibly triggered by increased IL-1b concentration.

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A Diet Enriched in α-Lipoic Acid Reverses the Age-Related Compromise in Antioxidant Defences in Rat Cortical Tissue

D.S.D. MARTIN[a], M. TOWEY[a], D.F. HORROBIN[b] and M.A. LYNCH[a,*]

[a]*Department of Physiology, Trinity College, Dublin 2, Ireland;* [b]*Laxdale Research Ltd., Stirling, Scotland, UK*

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In this study we set out to establish whether age-related changes in reactive oxygen species production in cortical tissue might be modulated by a diet enriched in the proposed antioxidant, α-lipoic acid. Twenty-two month-old and 4 month-old rats were fed on control diet or α-lipoic acid-enriched diet for 2 months. We report that cortical tissue prepared from rats fed on control diet revealed an age-related increase in reactive oxygen species production, which was accompanied by an increase in activity of superoxide dismutase activity and decreases in activities of glutathione peroxidase and catalase. These changes were attenuated in rats fed on the experimental diet. There was an age-related increase in the concentration of interleukin-1β (IL-1β), which was reversed by dietary manipulation. IL-1β increased reactive oxygen species production *in vitro*, suggesting that it may also exert this effect *in vivo*. We observed an age-related increase in lipid peroxidation accompanied by a decrease in arachidonic acid concentration and suggest that these changes are a consequence of increased reactive oxygen species production. Dietary manipulation with α-lipoic acid reverses the age-related changes in activities of superoxide dismutase, catalase and glutathione peroxidase and accumulation of reactive oxygen species production adding support to the hypothesis that α-lipoic acid exerts antioxidant effects.

Keywords: Ageing, α-Lipoic acid, Reactive oxygen species, Interleukin-1β, Antioxidant defences, Diet

INTRODUCTION

It has been suggested that some of the functional deficits associated with ageing are due to an age-related decrease in membrane fluidity (Zs-Nagy, 1994) one potential cause of which is a decrease in the concentration of the polyunsaturated fatty acid, arachidonic acid. In the hippocampus, decreased arachidonic acid concentration is accompanied by deficits in long-term potentiation (Lynch and Voss, 1994; McGahon *et al.*, 1997; Murray and Lynch, 1998a,b; McGahon *et al.*, 2000a,b) and glutamate release (Lynch and Voss, 1994; McGahon *et al.*, 1997; 2000a,b). It is significant that the impairment in LTP and the compromise in transmitter release were reversed when arachidonic acid concentration was restored in aged rats fed on a diet supplemented

*Corresponding author. Tel.: +353 1 608 1770. Fax: +353 1 679 3545. E-mail: lynchma@tcd.ie.

with arachidonic acid and its precursor γ-linolenic acid (McGahon *et al.*, 1997; 2000a). It is therefore a major objective to establish the underlying cause of the age-related decrease in arachidonic acid concentration.

It is likely that the decrease in concentration of arachidonic acid (and other polyunsaturated fatty acids) is due to increased lipid peroxidation, which in turn is triggered by an accumulation of reactive oxygen species (Zs-Nagy, 1994; Murray and Lynch, 1998a,b). The findings of recent studies have indicated that the proinflammatory cytokine, interleukin-1β (IL-1β) might play a role in generation of reactive oxygen species in hippocampal tissue (Murray and Lynch, 1998a; Murray *et al.*, 1999), while further evidence in support of this suggestion was obtained in a recent study which indicated that dietary manipulation with the antioxidant vitamins E and C reversed the age-related increase in lipid peroxidation and decrease in arachidonic acid concentration in cortical tissue (O'Donnell and Lynch, 1998). This suggests that enhancing the concentration of scavengers inhibits reactive oxygen species accumulation and therefore overcomes some of the changes associated with ageing.

In an effort to further probe the putative relationship between age-related changes in arachidonic acid concentration and age-related changes in handling of oxidative processes in cells, we assessed the ability of a diet enriched in α-lipoic acid to reverse age-related changes in reactive oxygen species production, lipid peroxidation and arachidonic acid concentration in cortical tissue. α-Lipoic acid is a naturally-occurring free radical scavenger which has been shown to increase formation of glutathione and regenerate antioxidants like vitamin E (Bienwenga *et al.*, 1997), to reverse oxidative stress-induced activation of NFκB associated with diabetic neuropathy (Bierhaus *et al.*, 1997; Packer, 1998) and to reverse the increased lipid peroxidation associated with diabetic neuropathy (Nickander *et al.*, 1996).

We observed that dietary manipulation reversed the age-related changes in reactive oxygen species accumulation, lipid peroxidation and arachidonic acid concentration to values observed in 4 month-old rats and propose that in cortical tissue, the trigger leading to these changes may be an increase in concentration of IL-1β.

METHODS

Animals

Male Wistar rats (Bantham and Kingman, UK; aged 22 and 4 months at the end of the experimental period) were housed in groups of 2–4 under a 12-h light schedule, with the ambient temperature controlled between 22°C and 23°C. Rats were under veterinary supervision, checked daily and maintained in the BioResources Unit in Trinity College, Dublin. Rats were randomly divided into groups which were fed on either the 'control' or 'experimental' diet. The composition of the lipid component of the experimental diet was as follows: 20% γ-linolenic–lipoic acid diol (racemic mixture), 40% tuna oil containing 24% docosahexanoic acid and 1.7% arachidonic acid and 40% microbial oil containing 40% arachidonic acid, 3% γ-linolenic acid and 4% dihomo-γ-linolenic acid (Laxdale Research, UK). The overall composition of the diet, in terms of polyunsaturated fatty acids, was therefore 20 mg γ-linolenic–lipoic acid diol, 9.6 mg docosahexanoic acid, 16.4 mg arachidonic acid, 1.2 mg γ-linolenic acid and 1.6 mg dihomo-γ-linolenic acid. One group of young and one group of older animals were fed for 8 weeks on laboratory chow supplemented with 100 mg experimental lipid/rat/day, referred to hereafter as '100% experimental diet', while two corresponding groups of animals received standard laboratory chow to which corn oil was added to ensure an isocaloric intake for all rats. A third group of older rats received laboratory chow supplemented with 50 mg experimental lipid/rat/day (hereafter referred to as '50% experimental diet'). Food intake was measured for 2 weeks before the

commencement of the experiment, sufficient diet was prepared for 3 or 4 days, daily allowances were stored in airtight containers at −80°C and rats were offered their full daily requirement each day. At the end of the eight-week period of dietary manipulation the average ages of the rats were 22 and 4 months.

Rats were anaesthetized with urethane (1.5 g/kg), analysed for their ability to sustain long-term potentiation in hippocampus (not reported here; see McGahon *et al.*, 2000b) and at the end of a 1 h recording period were killed by decapitation. Cortices were removed and divided into portions from which homogenate or slices were prepared. Some tissue was homogenized in either Krebs solution containing 2 mM $CaCl_2$, or in 5% trichloroacetic acid (TCA) for later analysis of ascorbic acid and glutathione. Aliquots of homogenate were transferred to microfuge tubes and stored at −80°C until required for analysis. The remaining tissue was cross-chopped (350 μm × 350 μm) using a McIlwain tissue chopper and stored at −80°C in Krebs solution containing DMSO (10%; Haan and Bowen, 1981) until required for analysis. In some experiments, synaptosomes were used and these were prepared from the frozen slices. The composition of the Krebs solution was (in mM) NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4 \cdot 7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13.

Analysis of Reactive Oxygen Species

The formation of reactive oxygen species was assessed in the impure synaptosomal preparation, P_2, as previously described (Lebel and Bondy, 1990). The method relies on oxidation of the non-fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), by reactive oxygen species, to the highly-fluorescent 2′,7-dichlorofluorescein (DCF). To assess reactive oxygen species production, synaptosomes were prepared as previously described (McGahon *et al.*, 1997) from cortical slices obtained from 22 and 4 month-old rats treated with either diet. The resultant pellet was resuspended in 1 ml ice-cold 40 mM Tris buffer (pH 7.4). In some experiments, aliquots of synaptosomal preparations from 4 month-old untreated rats were incubated for 15 min in the absence or presence of IL-1β (1 ng/ml) to which either the superoxide dismutase inhibitor, triethylenetetraamine (TETA; 100 μM) or vehicle (Krebs solution) was added. In all experiments, aliquots (1 ml) of homogenate were incubated with DCFH-DA (10 μl; final concentration 5 μM; from a stock solution of 500 μM in methanol) at 37°C for 15 min. To terminate the reaction, the dye-loaded synaptosomes were centrifuged at 13000 × *g* for 8 min. The pellet was resuspended in 3 ml ice-cold 40 mM Tris buffer, pH 7.4 and fluorescence was monitored at a constant temperature of 37°C at 488 nm excitation (bandwidth 5 nm) and 525 nm emission (bandwidth 20 nm). Reactive oxygen species formation was quantified from a standard curve of DCF in methanol (range 0.05–1 μM). Results were expressed as nmol/mg tissue corrected for protein (Bradford, 1976).

Analysis of Vitamin C Concentration

Vitamin C concentrations were determined as previously described (Omaye *et al.*, 1979). Samples of supernatant (100 μl) were added to a 2,4-dinitrophenylhydrazine/thiourea/copper (DTC) solution (50 mM thiourea, 2 mM copper sulphate and 150 mM dinitrophenylhydrazine in 9 N H_2SO_4; 20 μl) and incubated for 3 h at 37°C. Ice-cold H_2SO_4 (65%; 150 μl) was added to stop the reaction, samples were vortex-mixed, incubated at room temperature for 30 min and aliquots (100 μl) were transferred to 96-well plates for assessment by UV spectroscopy at 545 nm. The 96-well plates were loaded and read within 5 min and for this reason time-related changes in stability of vitamin C were not assessed. Results were expressed as μmol/g tissue with reference to a standard curve prepared in 5% TCA and were of the same order as those reported for whole brain (Omaye *et al.*, 1979).

Analysis of Vitamin E

Vitamin E was analysed by HPLC (Vatassery *et al.*, 1994). Aliquots of homogenate (150 µl) were incubated in the presence of ethanol containing 0.025% butyl-hydroxytoluene (150 µl), 25% ascorbic acid (70 µl) and 10% potassium hydroxide (135 µl) for 30 min at 60°C. Hexane (540 µl) containing 0.025% butyl-hydroxytoluene was added, samples were vortex-mixed for 1 min and centrifuged at 1500 rpm for 6 min. The hexane phase was removed and evaporated to dryness under nitrogen; the recovery of vitamin E using this procedure was between 70% and 80%. (We did not correct for recovery). For HPLC analysis, dried samples were resuspended in methanol (150 µl) containing 0.025% butyl-hydroxytoluene and 30 µl volumes were injected onto an Intersil C18 column. Separation of α-tocopherol was achieved using a mobile phase of 75% acetonitrile : 25% methanol at a flow rate of 1.2 ml/min and samples were detected by UV spectroscopy at 292 nm. Vitamin E concentration was estimated by the external standard method and expressed as µg/g tissue.

Analysis of Glutathione

Total glutathione was measured as described previously (Tietze, 1969). Briefly, aliquots of supernatant prepared from homogenate (30 µl) were incubated for 1 min on ice with sample buffer (100 mM potassium phosphate containing 5 mM EDTA, pH 7.5; 450 µl), in the presence of glutathione reductase (100 µl; 5 U/ml) and 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (50 µl). NADPH (2.4 mM; 100 µl) was added and the absorbance monitored for 2 min at 412 nm. Results were expressed as µmol GSH/g tissue.

Analysis of Superoxide Dismutase Activity

Superoxide dismutase activity was determined according to the method described by Spitz and Oberley (1989). Aliquots (800 µl) of incubation buffer (50 mM potassium buffer (pH 7.8) containing 1.8 mM xanthine, 2.24 mM nitroblue tetrazolium (NBT), 40 units of catalase, 7 µl/ml xanthine oxidase and 1.33 mM diethylenetriaminepentacetic acid) were added to 1.5 ml microfuge tubes containing samples of supernatant (100 µl) at different dilutions (1 : 2, 1 : 5, 1 : 10, 1 : 20, 1 : 50 and 1 : 100) and analysed by UV spectroscopy at 560 nm. In some experiments samples were prepared following incubation of tissue in the presence of IL-1β (1 ng/ml) for 15 min at 37°C. Activity of superoxide dismutase was assessed as the rate of reduction of NBT, which was inhibited with increasing concentrations of protein. One unit of activity was defined as the amount of protein necessary to decrease the rate of the reduction of NBT by 50%. Results were expressed as units of superoxide dismutase activity per mg protein (U/mg).

Analysis of Catalase Activity

Catalase activity was determined as previously described (Cohen *et al.*, 1996); we had insufficient tissue from 22 month-old rats treated with 50% experimental diet for this analysis, therefore results from aged and 4 month-old rats fed on control and 100% diet only are presented. Duplicate aliquots of supernatant (50 µl) were added to microfuge tubes containing buffer (10 mM potassium phosphate, pH 7.0; 800 µl) and kept on ice. The reaction was initiated by the addition of ice-cold H_2O_2 (60 mM; 100 µl), samples were mixed and incubated on ice for 2 or 10 min after which time aliquots (100 µl) were removed and quenched by addition to H_2SO_4 (0.6 N; 4 ml) and 10 mM $FeSO_4$ (1 ml) at room temperature. Colour was developed at room temperature by addition of 2.5 M potassium thiocyanate (400 µl). Aliquots (200 µl) were transferred immediately to 96-well plates, absorbance was read at 492 nm and results were expressed in terms of the first order reaction rate constant (k), corrected for protein using the formula: enzyme units = k/protein = (ln($A1$/$A2$)/t)/protein, where $A1$ and $A2$ represented the

absorbance at the two selected time points (i.e. 2 and 10 min), and *t*, the time difference between the two points (i.e. 8 min). Results are expressed as enzyme units per gram of protein.

Analysis of Glutathione Peroxidase Activity

Glutathione peroxidase activity was measured according to the method of Lawrence and Burk (1976). Samples of supernatant (100 µl) were added to incubation buffer (50 mM potassium phosphate (pH 7), containing 1 mM EDTA, 1 mM sodium azide, 0.2 mM NADPH, 1 unit of glutathione reductase, 1 mM GSH glutathione and 1.5 mM cumene hydroperoxide; 800 µl) and incubated for 5 min at room temperature. The reaction was initiated by addition of cumene hydroperoxide (15 mM; 100 µl). Absorbance was recorded at 340 nm for 5 min; activity was calculated from the slope (i.e. change in absorbance with time) and the results were expressed as µmoles NADPH oxidised/mg/min.

Analysis of IL-1β Concentration

We used an ELISA for analysis of interleukin-1β (Murray and Lynch, 1998a; IL-1β; Genzyme Diagnostics, USA). Briefly, 96-well plates were coated with 100 µl capture antibody (2.0 µg/ml final concentration, diluted in 0.1 M sodium carbonate buffer, pH 9.5; monoclonal hamster anti-mouse IL-1β antibody) and incubated overnight at 4°C. Wells were washed several times with phosphate buffered saline (PBS) containing 0.05% Tween 20 and then blocked for 2 h at 37°C with 250 µl blocking buffer, (PBS, pH 7.3; 0.1 M with 4% bovine serum albumin). Blocking buffer was aspirated and aliquots (50 µl) of samples or IL-1β standards (0–1000 pg/ml) were added to each well and incubated for 1 h at 37°C. Plates were washed, secondary antibody (100 µl; final concentration 0.8 µg/ml in PBS containing 0.05% Tween 20 and 1% bovine serum albumin; biotinylated polyclonal rabbit anti-mouse IL-1β antibody) was added to each well and incubation continued for 1 h at 37°C. The plates were washed and 100 µl detection agent (horseradish peroxidase conjugated streptavidin; 1 : 1000 dilution in PBS containing 0.05% Tween 20 and 1% bovine serum albumin) was added to each well, incubation continued for a further 15 min at 37°C and plates were again washed. Aliquots of substrate (100 µl; tetramethylbenzidine (TMB) liquid substrate; Sigma, UK) were added and the samples were incubated at room temperature for 10 min. Absorbance was read at 450 nm within 30 min. Results were expressed as pg IL-1β/mg tissue.

Analysis of IL-1β Converting Enzyme (ICE) Activity

Cleavage of the ICE substrate (DEVD, Alexis Corporation, USA) to its fluorescent product was used as a measure of ICE activity. Slices of tissue were washed three times in fresh oxygenated Krebs solution and then homogenized in lysis buffer (400 µl; 25 nM HEPES, 5 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA, 2 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin; pH 7.4). Samples were subjected to 4 freeze–thaw cycles and then centrifuged at 15000 rpm for 20 min at 4°C. Samples of supernatant (90 µl) were added to 500 µM ICE substrate (10 µl; DEVD peptide) and incubated at 37°C for 60 min. Incubation buffer (900 µl; 100 mM HEPES containing 10 mM DTT; pH 7.4) was added and the samples were transferred to cuvettes to measure fluorescence at excitation and emission wavelengths of 400 and 505 nm respectively. ICE activity was expressed as pmol fluorescent product/mg/min.

Analysis of Lipid Peroxidation

Lipid peroxidation was determined by a previously described method in which analysis of thiobarbiturate reactive substances (TBARS) was made (Dexter *et al.*, 1989). Aliquots (10 µl) of homogenate were incubated at 37°C for 60 min, at which time 8.1% (wt/vol) SDS (30 µl), 20% acetic acid (pH 3.5 with NaOH; 225 µl) and 0.8%

(wt/vol) thiobarbituric acid (225 µl) were added. The volume was adjusted with H_2O to 600 µl, the samples incubated for a further 60 min at 95°C, cooled at room temperature and absorbance assessed at 532 nm with reference to a standard curve made using 1,1,3,3-tetramethoxypropane. TBARS were expressed as nmol MDA/mg.

Analysis of Arachidonic Acid Concentration

Arachidonic acid concentration was assessed as previously described (Miwa *et al.*, 1986). Aliquots (150 µl) of cortical homogenate were added to chloroform : methanol (2 : 1 v/v; 1 ml) and fatty acids were extracted by vigorous shaking for 10 min followed by centrifugation at 1000*g* for 5 min to separate the phases. The aqueous layer was discarded and the chloroform phase was evaporated under nitrogen and resuspended in ethanol for analysis. Arachidonic acid was analysed as its 2-nitrophenylhydrazine (NPH) derivative by reverse phase HPLC. Fatty acids were derivatized by adding 2-NPH–HCl solution (0.02 M 2-nitrophenylhydrazine–HCl in 40 mM HCl–ethanol (3 : 1, v/v)) and EDC solution (1-ethyl-3-(3-dimethylaminopropyl)carbo-diimide hydrochloride; 0.25 M 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 MeOH : H_2O, 80 : 20) samples were incubated at 60°C for 15 min and cooled in running water. Fatty acid derivatives were concentrated; *n*-hexane and phosphate buffer (0.033 M, pH 6.4 in 0.5 M HCl) were added, samples were vortex mixed for 30 s, centrifuged for 5 min at 1500*g* and the hexane phase evaporated to dryness under nitrogen. For HPLC analysis, samples were resuspended in methanol, injected onto a Hypersil BDS C18 column and fatty acid derivatives were separated in isocratic mode with a mobile phase of 85% acetonitrile : 15% water (maintained at pH 4.5 with HCl) and detected by UV spectroscopy at 230 nm. Arachidonic acid concentration, expressed as µmol/mg, was estimated using the external standard method and the values obtained were similar to those previously reported by us (McGahon *et al.*, 1997; 1999a,b,c).

Statistical Analysis

Data were analysed, as appropriate, using either the Student's *t*-test for independent means, or by using a one-way analysis of variance (ANOVA) followed by *post hoc* analysis using the Newmann–Keuls test.

RESULTS

Body weight and food intake were assessed for the duration of the experiment. Mean body weights for the 4 month-old rats fed on control and experimental diets were 481 g (±28.4) and 481 g (±16.1) respectively on the last day of the experiment, while their average daily food intake was 22 g per rat. Mean body weights for the 22 month-old rats fed on control diet and, 50% and 100% experimental diets were 525 (±15.5), 516 (±9.8) and 479 g (±14.8) respectively, while their average daily food intake was 18 g per rat.

Reactive oxygen species production was assessed in cortical synaptosomes prepared from these groups of rats. Values were similar in both groups of 4 month-old rats and therefore the data are pooled. There was a marked increase in synaptosomes prepared from 22 month-old rats which were fed on the control diet ($p < 0.05$; ANOVA; Figure 1). This effect was not evident in 22 month-old rats which were fed on either the 50% or 100% diet; in these rats the mean value was not significantly different from that in 4 month-old rats. However it must be acknowledged that the lack of a significant effect on the rats fed with the 100% diet is probably due to the large SEM values.

In an effort to establish the underlying cause of the age-related increase in reactive oxygen species production, we analysed activities of the enzymes responsible for metabolism of these molecules,

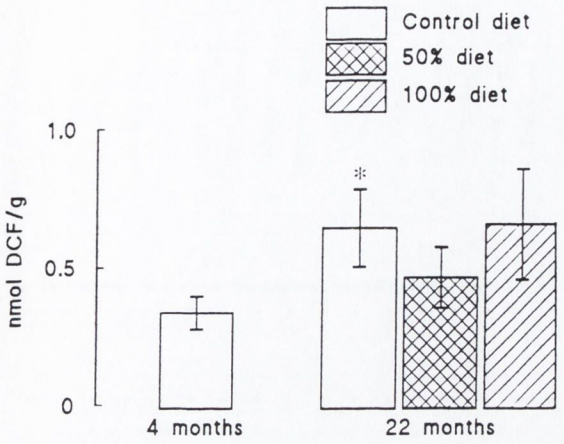

FIGURE 1 Reactive oxygen species production was significantly increased in cortical tissue prepared from aged rats (*$p < 0.05$; ANOVA). Dietary manipulation with either 50% or 100% experimental diet prevented this change; thus there was no significant difference between young rats and aged rats which received experimental diet. Data from young rats fed on control and experimental diets were similar and therefore are pooled. Values are a mean of between 5 and 11 estimations.

superoxide dismutase, catalase and glutathione peroxidase. Values in all cases were similar in 4 month-old rats irrespective of diet, therefore values are pooled. Figure 2A shows that activity of superoxide dismutase was markedly increased in cortical tissue prepared from 22 month-old rats which were fed on the control diet ($p < 0.05$; ANOVA), while enzyme activity was similar in tissue prepared from 4 month-old rats and in tissue prepared from 22 month-old rats fed on either experimental diet. Activities of both catalase and glutathione peroxidase were decreased in tissue prepared from 22 month-old rats which were fed on the control diet compared with tissue prepared from 4 month-old rats ($p < 0.05$; ANOVA; Figure 2B and C). In the case of catalase, we assessed enzyme activity only in tissue prepared from 22 month-old rats fed on 100% diet and in this group we observed that activity was similar to that in 4 month-old rats. In the case of glutathione peroxidase, dietary manipulation with 50% (though not 100% experimental diet) partially reversed the age-related increase in enzyme activity. Thus while superoxide dismutase activity was increased with age, there were no parallel increases, but rather decreases, in activities of catalase and glutathione peroxidase.

Analysis of scavengers, vitamins E and C and glutathione revealed no significant differences in concentrations in cortical tissue prepared from 4 month-old rats and 22 month-old rats which received control diet. Dietary supplementation with 50% diet did not affect scavenger concentrations, but 100% diet increased both the concentrations of vitamin E and glutathione; in the latter case the increase was significant (Figure 2D–F; $p < 0.05$; ANOVA).

We have previously observed that increased reactive oxygen species production in hippocampal synaptosomes prepared from 22 month-old rats was accompanied by increased IL-1β concentration (Murray and Lynch, 1998a), and that IL-1β could increase reactive oxygen species production in hippocampal tissue *in vitro* (Murray and Lynch, 1998a). For this reason we analysed IL-1β concentration and ICE activity in cortical tissue prepared from these rats. Figure 3A shows that IL-1β concentration was enhanced in cortical homogenate prepared from 22 month-old rats fed on the control diet compared with homogenate prepared from 4 month-old rats ($p < 0.05$; ANOVA). Supplementation with diet enriched in α-lipoic acid reversed the age-related increase. The data shown in Figure 3B indicates that these changes in IL-1β concentration were paralleled by changes in ICE activity. Thus we observed that there was a statistically significant age-related increase in ICE activity ($p < 0.05$; ANOVA) which was reversed by dietary manipulation.

We observed that IL-1β significantly increased reactive oxygen species production in cortical synaptosomes *in vitro* ($p < 0.05$; Student's *t*-test for paired values). Coincubation in the presence of TETA inhibited the IL-1β-induced effect, while TETA alone exerted no effect on reactive oxygen species production (Figure 4A). We also observed that IL-1β significantly increased SOD activity (Figure 4B; $p < 0.05$; Student's *t*-test for paired means) suggesting that the increase in reactive

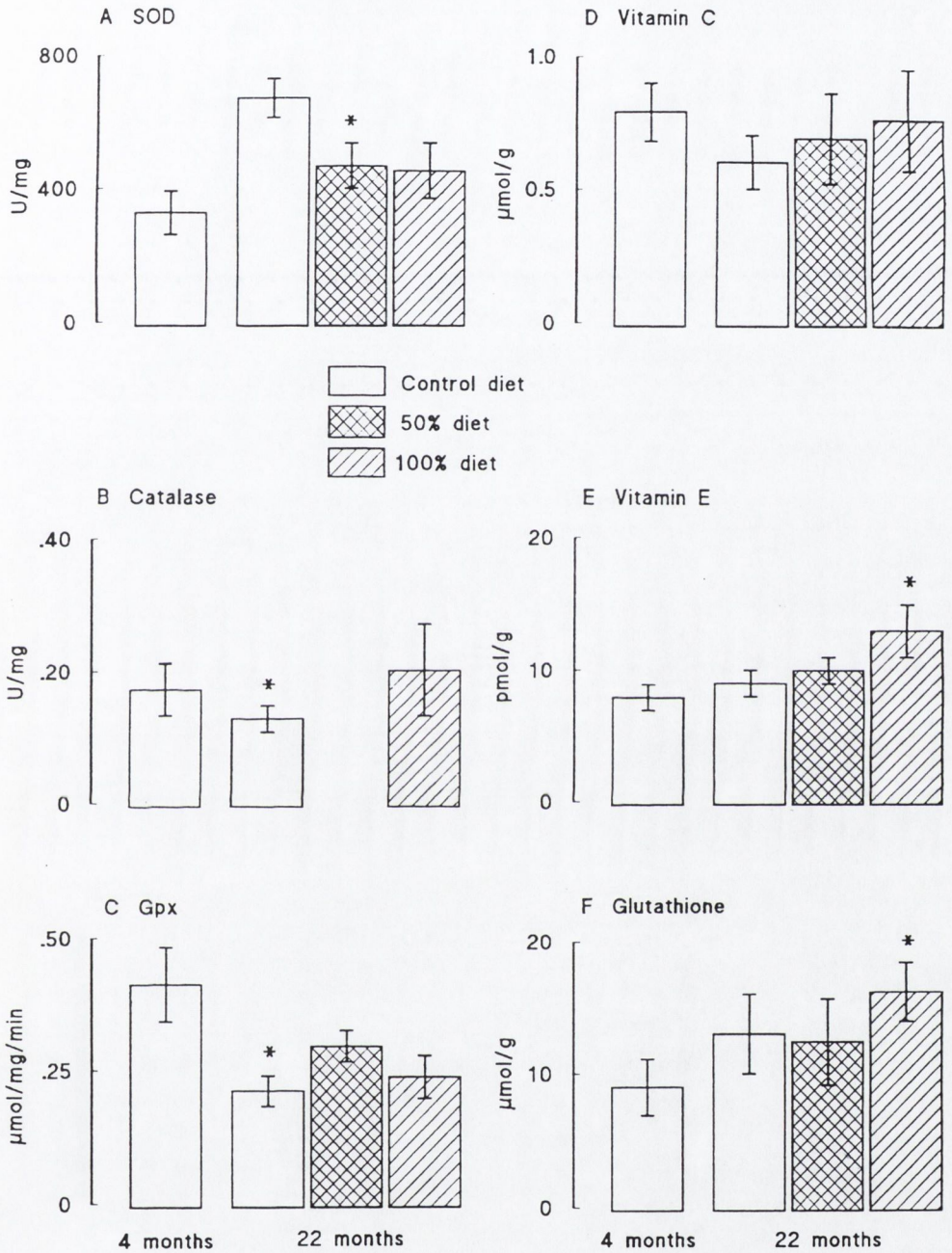

FIGURE 2 Activity of superoxide dismutase (SOD; A) was significantly enhanced and activities of catalase (B) and glutathione peroxidase (Gpx; C) significantly reduced in aged rats fed on control diet compared with young rats fed on either diet ($^*p < 0.05$; ANOVA). Dietary manipulation reversed the changes in superoxide dismutase and catalase, thus there was no significant difference between young rats and aged rats which received experimental diet. In the case of glutathione peroxidase, 50% diet partially reversed the age-related changes while the 100% diet was not effective. There were no age-related changes in the concentrations of vitamins C (D) or E (E) and glutathione (F). Dietary manipulation with 100% diet increased the concentration of glutathione ($^*p < 0.05$; ANOVA), but did not affect concentrations of vitamins E or C. Data from young rats fed on control and experimental diets were similar and therefore are pooled. Values are a mean of between 5 and 11 estimations.

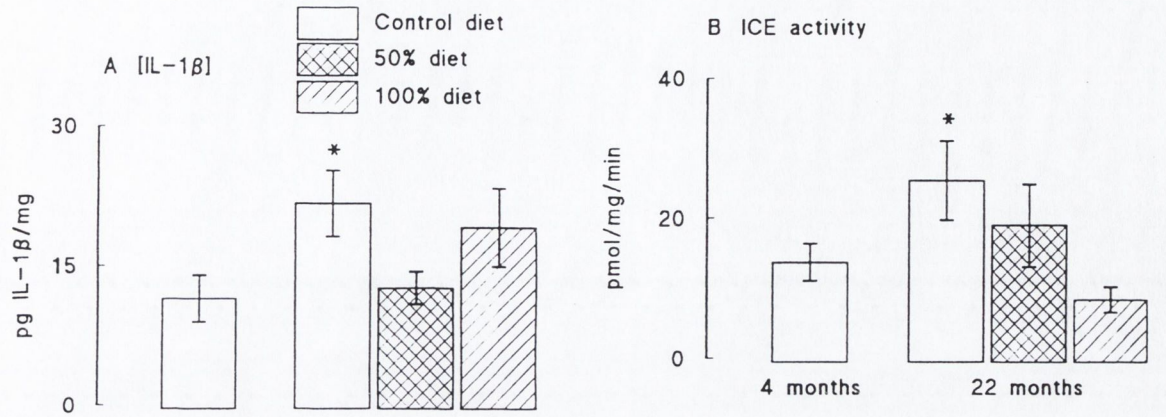

FIGURE 3 IL-1β concentration (A) and ICE activity (B) were significantly enhanced in tissue prepared from aged rats fed on the control diet, compared with tissue prepared from young rats (A: $^*p < 0.05$; ANOVA). Dietary manipulation with either the 50% or 100% diet reversed these changes. Data from young rats fed on control and experimental diets were similar and therefore are pooled. Values are a mean of between 5 and 11 estimations.

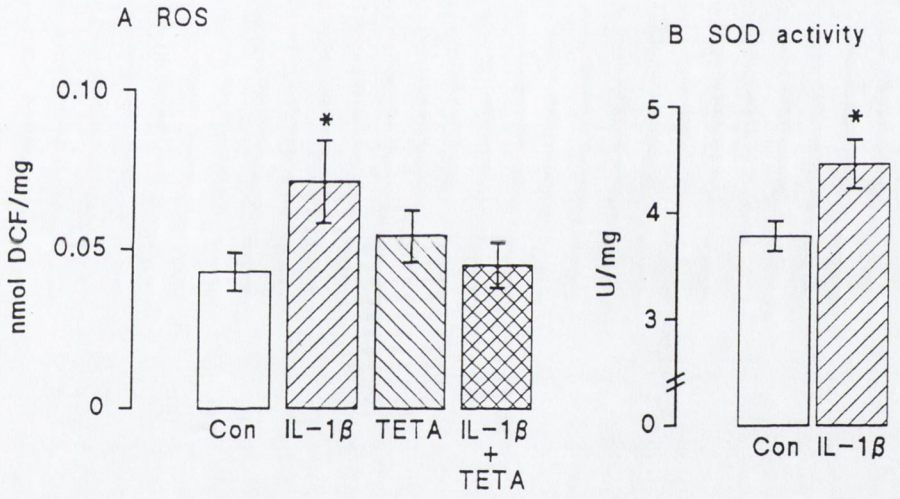

FIGURE 4 IL-1β (1 ng/ml) significantly enhanced reactive oxygen species (ROS) production ($^*p < 0.05$; Student's *t*-test for paired values). This effect was inhibited by TETA (100 μM), which alone exerted no significant effect. IL-1β (1 ng/ml) also significantly enhanced superoxide dismutase (SOD) activity ($^*p < 0.05$; Student's *t*-test for paired values). In these experiments cortical tissue was prepared from young rats and the values given are the mean of 6 estimations.

oxygen species production might arise from increased activity of this enzyme.

Lipid peroxidation was similar in 4 month-old rats fed on either control or experimental diet and therefore data from these two groups were pooled. Figure 5A shows that lipid peroxidation was significantly enhanced in cortical tissue prepared from 22 month-old rats fed on the control diet ($p < 0.05$; ANOVA). In contrast, the data indicated that lipid peroxidation was similar in tissue prepared from 4 and 22 month-old rats fed on either experimental diet (Figure 5A). In parallel with the age-related increase in lipid peroxidation, we observed an age-related decrease in arachidonic acid concentration; thus the concentration of the fatty acid was significantly decreased in tissue prepared from 22 month-old rats fed on the control diet compared with the concentration in tissue prepared from 4 month-old rats (Figure 5B; $p < 0.05$; ANOVA). In contrast,

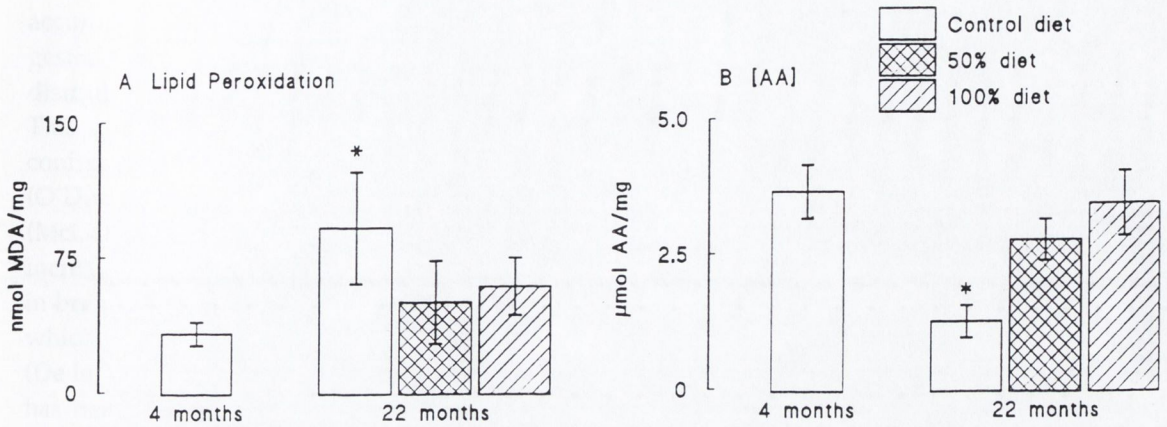

FIGURE 5 Lipid peroxidation (A) was significantly enhanced and arachidonic acid concentration ([AA]; B) was significantly reduced in tissue prepared from aged rats fed on the control diet compared with tissue prepared from young rats on either diet ($^*p < 0.05$; ANOVA). Dietary manipulation with either the 50% or 100% diet prevented this change, therefore there was no significant difference in either measure in tissue prepared from aged rats fed on the experimental diet compared with tissue prepared from young rats. Data from young rats fed on control and experimental diets were similar and therefore are pooled. Values are a mean of between 5 and 11 estimations.

arachidonic acid concentration was similar in tissue prepared from 4 and 22 month-old rats fed on α-lipoic acid enriched diet.

DISCUSSION

The objective of this study was to establish whether age-related changes in cortical tissue might be reversed by supplementation with a diet enriched in α-lipoic acid; attention was focused, in particular, on the possibility that the proposed antioxidant capacity of α-lipoic acid might be of significance. We present data which indicate that dietary manipulation reversed the age-related increases in reactive oxygen species production and IL-1β concentration, which we suggest lead to an age-related increase in lipid peroxidation and the subsequent decrease in arachidonic acid concentration.

Our initial observation was that reactive oxygen species accumulation in cortical tissue prepared from rats fed on the control diet increased with age; this change was not observed in tissue prepared from 22 month-old rats which were fed on a diet enriched in α-lipoic acid. The age-related increase in reactive oxygen species production reported here mirrors that previously observed in hippocampus (Murray and Lynch, 1998a; Murray *et al.*, 1999; Lynch, 1998), while the reversal of this effect by dietary manipulation is consistent with the suggestion that α-lipoic acid exerts antioxidant actions (Nicklander *et al.*, 1996; Bienwenga *et al.*, 1997; Bierhaus *et al.*, 1997; Packer, 1998). Although DCFH is sensitive to reactive oxygen species, it is also oxidized by other oxidants including NO. For this reason the data presented here may not reflect changes in reactive oxygen species exclusively. The mechanism underlying this effect remains to be established though one possibility is that α-lipoic acid, acting as a free-radical scavenger (Bienwenga *et al.*, 1997), prevents accumulation of reactive oxygen species. However it has also been shown to regenerate α-tocopherol (Bienwenga *et al.*, 1997) and this is consistent with the present observation that vitamin E concentration was significantly enhanced in 22 month-old rats which were fed on the 100% experimental diet.

Assessment of the main components of the antioxidative defence system, in an effort to identify the underlying cause of the increased

accumulation of reactive oxygen species, suggested that the age-related increase in superoxide dismutase activity might play a significant role. This change in superoxide dismutase activity confirms previous observations in both cortical (O'Donnell and Lynch, 1998) and hippocampal (McGahon *et al.*, 2000b) tissue. It is of interest that increased Cu–Zn superoxide dismutase activity in brain tissue is a feature of Down's Syndrome, which is associated with accelerated ageing (De la Torre *et al.*, 1996), while a similar increase has been reported to occur in the degenerative disorder, amyotrophic lateral sclerosis (Gurney *et al.*, 1996). It might be argued that these findings, combined with the present observations, suggest a link between increased superoxide dismutase activity and degeneration and/or ageing.

We observed that activities of glutathione peroxidase and catalase were decreased in cortical tissue prepared from 22 month-old rats; this contrasts with earlier studies in which these enzymes were reported to be unchanged with age (Geremia *et al.*, 1990; Vertechy *et al.*, 1993; O'Donnell and Lynch, 1998). There is no obvious explanation for the differences in these findings, though we cannot rule out the possibility that it is due to the fact that tissue was prepared from different cortical regions. The most significant aspect of the present findings is that the age-related increase in superoxide dismutase activity was not accompanied by parallel increases in activities of glutathione peroxidase and catalase; this imbalance of enzymatic activity will lead to an accumulation in hydrogen peroxide, which, in turn, is likely to result in increased production of reactive oxygen species. This is consistent with the finding that there is an increase in formation of hydroxyl ions in both cortex and hippocampus of the aged gerbil (Zhang *et al.*, 1993). The proposed causal link between the increase in superoxide dismutase activity and the increase in accumulation of reactive oxygen species is supported by the observation that both changes are reversed in tissue prepared from 22 month-old rats which were fed on the α-lipoic acid enriched diet. There was no evidence of age-related changes in concentrations of vitamins E or C or glutathione in this study, therefore it seems reasonable to conclude that the observed age-related increase in accumulation in reactive oxygen species cannot be attributable to changes in concentration of these scavengers. However, dietary manipulation resulted in increases in concentrations of vitamin E and glutathione, enhancing antioxidative capacity and adding to the evidence which indicates an antioxidative effect of α-lipoic acid (Cao and Phillis, 1995; Nickander *et al.*, 1996; Panigrahi *et al.*, 1996; Beinwenga *et al.*, 1997; Bierhaus *et al.*, 1997; Packer, 1998).

An important question arising from these observations is that of the identity of the trigger which induces the increase in reactive oxygen species production. It has been shown previously that IL-1β stimulates reactive oxygen species production in peripheral tissue (Sumoski *et al.*, 1989), while recent findings from this laboratory have indicated that the age-related increase in reactive oxygen species production in hippocampus was accompanied by increased IL-1β concentration and that the cytokine induced production of reactive oxygen species *in vitro* (Murray and Lynch, 1998a). Thus we proposed that IL-1β might increase reactive oxygen species production and that this might be achieved by stimulating activity of superoxide dismutase. We undertook a series of *in vitro* experiments to assess this possibility and observed that IL-1β increased reactive oxygen species production *in vitro* but that this effect was inhibited in the presence of TETA, an inhibitor of superoxide dismutase activity (De Man *et al.*, 1996), suggesting the involvement of superoxide dismutase. This was confirmed directly by demonstrating an IL-1β-induced stimulation of superoxide dismutase activity. A similar IL-1β-induced activation of superoxide dismutase has been described in rat pancreatic islets (Borg *et al.*, 1992).

The data presented demonstrate that the age-related increase in IL-1β concentration in cortical tissue was accompanied by an increase in activity

of ICE. This is, to our knowledge, the first report of concomitant increases in enzyme activity and cytokine concentration in neuronal tissue and suggests that the increase in IL-1β concentration may be a consequence of increased ICE activity. This suggestion is supported by the findings that dietary supplementation with α-lipoic acid reversed both the age-related increase in both ICE activity and IL-1β concentration in cortical tissue. It is significant that the age-related increase in IL-1β concentration in hippocampus is reversed by dietary manipulation with α-lipoic acid (McGahon *et al.*, 1999b), which parallels the effect of diet on IL-1β in cortex observed in the present study. In a recent study, we observed that dietary manipulation with arachidonic acid and its precursor, γ-linolenic acid, also reversed the age-related increase in IL-1β concentration in hippocampus (McGahon *et al.*, 1999a). Evidence from that study indicated that this diet also exerted some antioxidant effects, for example we found that it reversed the age-related increases in superoxide dismutase activity and lipid peroxidation in hippocampus. These data, and those of the present study, provide support for the hypothesis that increased IL-1β concentration is closely coupled with increased activity of superoxide dismutase and increased lipid peroxidation. The age-related increase in lipid peroxidation and the ability of dietary manipulation to reverse this increase was accompanied by changes in arachidonic acid concentration. Thus there was an age-related decrease in arachidonic acid concentration as previously reported by us (McGahon *et al.*, 1997; 1999; 2000a,b) and this was reversed by dietary manipulation. These observations suggest that one substrate for lipid peroxidation was arachidonic acid.

On the basis of the present findings, we propose that antioxidative capacity is impaired in cortical tissue prepared from 22 month-old rats and that this is reversed by supplementation with a diet enriched in α-lipoic acid. (We must acknowledge, however, that the diet also contained arachidonic acid and docosahexanoic acid which have been shown to reverse some age-related neuronal deficits (McGahon *et al.*, 1997; 1999; 2000a) and γ-linolenic acid, which has been shown, like the antioxidant BM15.0639, to reverse nerve conduction and perfusion deficits in diabetic rats (Cameron and Cotter, 1996)). Such age-related changes have been reported by several groups (for review see Reiter, 1995). Specific age-related changes observed in this study include an increase in activity of superoxide dismutase coupled with decreased activities of catalase and glutathione peroxidase. Evidence suggests that the increase in activity of superoxide dismutase may be stimulated by the age-related increase in IL-1β concentration. We propose that one consequence of these changes is an increase in reactive oxygen species production and that this, in turn, stimulates lipid peroxidation, leading to a decrease in concentrations of polyunsaturated fatty acids, particularly arachidonic acid. These observations are summarized in a flow diagram (Figure 6), which must be recognized as being an oversimplification of the immensely complex reactions which contribute to age-related deficits. However we suggest that the decrease in arachidonic acid concentration result arising from this series of changes contribute to an increased membrane rigidity and will impact on membrane associated functions, which may explain many of the deficits in neuronal function observed with age. The findings of the present study indicate that

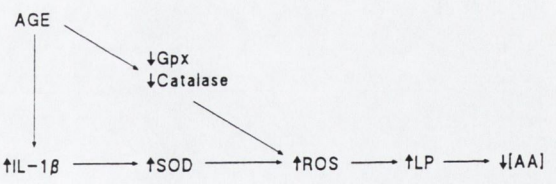

FIGURE 6 This scheme proposes that age is associated with decreased activities of catalase and glutathione peroxidase (Gpx), while the age-related increase in IL-1β concentration stimulates an increase in superoxide dismutase (SOD) activity. It is proposed that these changes lead to a compromise in antioxidative defenses and therefore an accumulation of reactive oxygen species (ROS) occurs. Among the consequences of increased reactive oxygen species production are an increase in lipid peroxidation (LP) and a decrease in arachidonic acid (AA) concentration.

supplementation with a diet enriched in α-lipoic acid can prevent many of these changes and, in the hippocampus at least, has been shown to reverse the impairments in LTP and glutamate release.

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Long-term potentiation in aged rats is restored when the age-related decrease in polyunsaturated fatty acid concentration is reversed.

DSD Martin, P. Spencer, DF Horrobin* and MA Lynch,
Department of Physiology,
Trinity College,
Dublin 2
Ireland

and

*Laxdale Research Ltd.,
Stirling,
Scotland
UK

Corresponding author: MA Lynch
Phone (353)-1-608 1770
Fax (353)-1-679 3545
email lynchma@tcd.ie

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SUMMARY

Several age-related changes have been identified in rat hippocampus; among these are deficits in glutamate release and long-term potentiation in dentate gyrus. These deficits correlate with a decrease in the concentration of arachidonic acid in hippocampus. In this study, the effects of dietary supplementation for 8 weeks with ω-6 or ω-3 fatty acids were assessed in groups of aged and young rats. The data presented indicate that dietary supplementation in aged rats restored the concentrations of arachidonic acid and docosahexanoic acid in hippocampal preparations to those observed in tissue prepared from young rats. In parallel, aged rats which received the experimental diets sustained long-term potentiation in a manner indistinguishable from young rats. The evidence presented supports the view that an age-related increase in reactive oxygen species production is linked with the decrease in polyunsaturated fatty acids and that a diet enriched in eicosapentanoic acid has antioxidant properties which may play a key role in reversal of the observed age-related deficits.

Key Words: Long-term potentiation, hippocampus, arachidonic acid, docosahexanoic acid, glutamate release, reactive oxygen species.

INTRODUCTION

It been repeatedly reported that cognitive function is compromised in aged animals. Consistent with this observation, is the finding that maintenance of hippocampal long-term potentiation (LTP), a putative model of learning and/or memory, is impaired in aged rats.[1-9] In parallel with this impairment are several indicators of compromised synaptic function, for example age-related changes in calcium handling by cells[10,11] and changes in activity of kinases[12,13], including the mitogen-activated protein kinase, ERK, which we have shown to modulate transmitter release.[14] While the underlying cause of the age-related impairment in LTP is not known, one possible contributory factor is the decrease in depolarization-induced glutamate release, since maintenance of LTP in perforant path-granule cells is closely coupled with, and may even be dependent upon, an increase in glutamate release.[7,15-19] Significantly the age-related decreases in both LTP and transmitter release in dentate gyrus are accompanied by a decrease in membrane fluidity, which is typified by a decrease in membrane concentration of polyunsaturated fatty acids, like arachidonic acid[6-9, 17-20]. This decrease in fluidity is likely to impact on transmitter release since this process requires fusion of the synaptic vesicle and synaptic plasma membranes. We have proposed that the decrease in polyunsaturated fatty acids is a consequence of increased reactive oxygen species production in hippocampus,[8,21] leading to increased lipid peroxidation, substrates for which are the polyunsaturated fatty acids.[8,20-24] This proposal is supported by the finding that dietary supplementation with antioxidant vitamins E and C reversed the age-related change increase in reactive oxygen species production and the age-related decrease in concentrations of polyunsaturated fatty acids; coupled with these changes we observed that LTP was restored to levels similar to those observed in young rats.[8] Recent studies have revealed that dietary

supplementation with polyunsaturated fatty acids also reverse some age-related deficits.[17-24] Here we compare the effectiveness of a diet enriched in the ω-6 fatty acids, gamma-linolenic acid and arachidonic acid with a diet enriched in the ω-3 fatty acid, eicosapentanoic acid. We report that supplementation with either diet reversed the age-related decrease in both arachidonic acid and docosahexanoic acid and also the age-related decrease in LTP.

METHODS AND MATERIALS

Animals

Groups of young and aged male Wistar rats (Bantham and Kingman, UK) were used in these experiments. Rats were housed in groups of 2 - 4 under a 12-hour light schedule, with the ambient temperature controlled between 22 and 23°C. Subgroups of aged and young animals were fed on a diet of laboratory chow supplemented with a daily dose of 10mg arachidonic acid and 12mg gamma-linolenic acid in a 20% solution of purified egg phosphatidylethanolamine (hereafter referred to as "GLA/AA diet"; Laxdale Pharmaceuticals, UK) or eicosapentanoic acid (hereafter referred to as "EPA diet"; Laxdale Pharmaceuticals, UK) for 8 weeks. The other subgroups were fed on a diet of laboratory chow to which corn oil was added so that an isocaloric diet was fed to all subgroups. To ensure that animals received their full daily dose of control or experimental diet, food intake was measured daily for 2 weeks before the commencement of the experiment and rats were offered 100% of their normal daily intake for the following 8 weeks. Sufficient diet was prepared for 3 days at a time and daily allowances were stored in airtight containers at -80°C. At the end of the period of dietary manipulation the average ages of the aged and young rats were 22 months and 4 months respectively.

Induction of long-term potentiation in dentate gyrus

Rats were anaesthetized with urethane; young animals initially received 1.5g/kg urethane intraperitoneally, and a further increment, if required (which did not exceed 0.6g/kg in any experiment) for induction of deep anaesthesia, which was assessed by the absence of a pedal reflex. Aged animals received 1.2g/kg urethane initially and topped up when necessary.

LTP was induced in perforant path-granule cell synapses as described previously.[7] Briefly, a bipolar stimulating electrode was placed in the perforant path (4.4mm lateral to Lambda) and a unipolar recording electrode was placed in the dorsal cell body region of the dentate gyrus (2.5mm lateral and 3.9mm posterior to Bregma). The depths of the stimulating and recording electrodes were adjusted to maximize the amplitude of the response; the stimulus intensity was adjusted to the value which was just sufficient to induce a population spike. Test shocks were given at 30 sec intervals for 10 min before and 40 min after, 3 trains of stimuli (250 Hz for 200 msec, with a 30 sec intertrain interval) which was shown previously to produce saturation of LTP in perforant path-granule cell synapses.

Tissue preparation

At the end of the recording period, rats were killed by decapitation, the hippocampus proper and the dentate gyri were dissected, and cross-chopped slices (350μm x 350μm) were prepared using a McIlwain tissue chopper. Samples of hippocampus and control and potentiated dentate gyri were frozen separately in 1ml Krebs solution (composition of Krebs in mM: NaCl 136, KCl 2.54, KH_2PO_4 1.18, $MgSO_4.7H_2O$ 1.18, $NaHCO_3$ 16, glucose 10, $CaCl_2$ 1.13) containing 10% dimethylsulphoxide[25] and stored at -80°C until required.

For analysis, slices were thawed rapidly (1.5-2 min) by agitation at 37°C and rinsed

4 times in excess fresh oxygenated Krebs solution. Fatty acid concentrations were assessed in samples of homogenate prepared from dentate gyrus of aged and young rats, while the crude synaptosomal pellet, P_2, prepared as previously described[7] was used for analysis of glutamate release. Homogenate prepared from hippocampal tissue was used for analysis of the activities of superoxide dismutase, glutathione peroxidase and catalase and also for assessment of reactive oxygen species production.

Assessment of glutamate release

Samples of synaptosomal tissue were resuspended in ice-cold Krebs solution containing 2mM $CaCl_2$. Tissue was aliquotted onto Millipore filters (0.45μm), rinsed under vacuum and the filtrate was discarded. Tissue was incubated in 250μl oxygenated Krebs solution at 37°C for 3min and filtrate was collected and stored. Release of transmitter was then stimulated by the addition of 40mM KCl to Krebs solution as previously described.[15]

Glutamate concentration was analysed by the method used in previous experiments.[15,26]. Briefly, 96-well plates were coated with glutaraldehyde (0.5% in 100mM NaH_2PO_4 buffer, pH 4.5; 320μl) by incubation for 60 min at 37°C. Plates were then washed with 100mM NaH_2PO_4 buffer. Triplicate samples (50μl) or glutamate standards (50μl; 50nM to 10μM prepared in 100mM Na_2HPO_4 buffer, pH 8.0) were added, plates were incubated for 2 hours at 37°C and washed with 100mM NaH_2PO_4 buffer. To bind any unreacted aldehydes, ethanolamine (320μl; 0.1M in 100mM Na_2HPO_4 buffer) was added to each well and incubated for 60 min at 37°C. Plates were washed with phosphate buffered saline containing Tween-20 (0.2% Tween; PBS-T). To block non-specific binding, samples were incubated with donkey serum for 60min (200μl; 3% in PBS-T). Antiglutamate antibody (raised in rabbit; 100μl; 1:5,000 in PBS-

T; Sigma, UK) was added to each plate, incubated overnight at 4°C and washed with PBS-T. Anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (95µl; 1:10,000 in PBS-T; Amersham, UK) was added, incubated for 60 min at room temperature and plates were again washed with PBS-T. 3,3',5,5'-Tetramethylbenzidine (100µl) liquid substrate was added as chromogen and incubation continued for 60 min at room temperature. H_2SO_4 (4M; 50µl) was used to stop the reaction and optical densities were determined at 450nm using a multiwell plate reader. Values were calculated with reference to the standard curve, corrected for protein[27] and expressed as nmol glutamate/mg protein.

Analysis of hippocampal concentration of arachidonic acid and docosapentanoic acid

Thawed slices prepared from dentate gyrus were rinsed 3 times and then homogenized in 200µl fresh Krebs solution. Fatty acids were extracted into chloroform:methanol (2:1 v/v; 1ml) by vigorous shaking for 10 min followed by centrifugation at 1000g for 5 min to separate the phases. The aqueous layer was discarded and the chloroform phase was evaporated under nitrogen and resuspended in ethanol for analysis. Fatty acid concentrations were analyzed as their 2-nitrophenylhydrazine (NPH) derivatives by reverse phase HPLC.[28] Derivatization was achieved by adding 2-NPH-HCl solution (0.02M 2-nitrophenylhydrazine-HCl in 0.25M HCl-ethanol (1:1, v/v) and EDC solution (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide 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 MeOH:H_2O, 80:20) samples were incubated at 60°C for 15 min and cooled in running water. Fatty acid derivatives were concentrated; n-hexane and phosphate buffer (0.033M, pH 6.4 in 0.5M HCl) were added, samples were vortex mixed for 30

sec, centrifuged for 5 min at 1500g and the hexane phase evaporated to dryness under nitrogen. For HPLC analysis, samples were resuspended in methanol, injected onto a Microsorb C18 column (maintained in a column oven at 30°C) and fatty acids 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. Aliquots of tissue were taken for protein analysis and concentrations were estimated according to standard curves and expressed as nmol/mg protein.

Analysis of reactive oxygen species

The formation of reactive oxygen species was assessed in the impure synaptosomal preparation, P_2, as previously described.[29] The method relies on oxidation of the non-fluorescent probe, 2'7'-dichlorofluorescin diacetate (DCFH-DA), by reactive oxygen species, to the highly-fluorescent 2',7-dichlorofluorescein (DCF). To assess reactive oxygen species production, synaptosomes were prepared as previously described[7] from cortical slices obtained from 22 and 4 month-old rats treated with either diet. The resultant pellet was resuspended in 1 ml ice-cold 40mM Tris buffer (pH 7.4) and aliquots (1ml) of homogenate were incubated with DCFH-DA (10µl; final concentration 5µM; from a stock solution of 500µM in methanol) at 37°C for 15 min. To terminate the reaction, the dye-loaded synaptosomes were centrifuged at 13,000 x g for 8 min. The pellet was resuspended in 3 ml ice-cold 40mM Tris buffer, pH 7.4 and fluorescence was monitored at a constant temperature of 37°C at 488nm excitation (band width 5nm) and 525nm emission (band width 20nm). Reactive oxygen species formation was quantified from a standard curve of DCF in methanol (range 0.05 to 1µM). Results were expressed as nmol/mg tissue corrected for protein.[27]

Analysis of superoxide dismutase activity

Superoxide dismutase activity was determined in samples of hippocampal homogenate.[30] Aliquots (800µl) of incubation buffer (50mM potassium buffer (pH 7.8) containing 1.8mM xanthine, 2.24mM nitroblue tetrazolium (NBT), 40 units of catalase, 7µl/ml xanthine oxidase and 1.33mM diethylenetriaminepentacetic acid) were added to 1.5ml microfuge tubes containing samples of supernatant (100µl) at different dilutions (1:2, 1:5, 1:10, 1:20, 1:50 and 1:100) and analysed by uv spectroscopy at 560nm. Activity of superoxide dismutase was assessed as the rate of reduction of NBT, which was inhibited with increasing concentrations of protein. One unit of activity was defined as the amount of protein necessary to decrease the rate of the reduction of NBT by 50%. Results were expressed in units of superoxide dismutase activity per mg protein.

Analysis of catalase activity

Catalase activity was determined as previously described.[31] Briefly, duplicate aliquots of supernatant (50µl) were added to microfuge tubes containing buffer (10mM potassium phosphate, pH 7.0; 800µl) tubes and kept on ice. The reaction was initiated by the addition of ice-cold H_2O_2 (60mM; 100µl), samples were mixed and incubated on ice for 2 or 10 min after which time aliquots (100µl) were removed and quenched by addition to H_2SO_4 (0.6N; 4ml) and 10mM $FeSO_4$ (1ml) at room temperature. Colour was developed at room temperature by addition of 2.5M potassium thiocyanate (400µl). Aliquots (200µl) were transferred immediately to 96-well plates, absorbance was read at 492nm and results were expressed in terms of the first order reaction rate constant (k), corrected for protein using the formula: enzyme units = k/protein= [*ln* (A1/A2/t]/protein, where A1 and A2 represented the absorbance at the two selected time points (i.e. 2 and 10 min), and t, the time difference between

the two points (i.e. 8 minutes). Results are expressed as enzyme units per gram of protein.

Analysis of glutathione peroxidase activity

Glutathione peroxidase activity was measured according to the method of Lawrence and Burk.[32] Samples of supernatant (100µl) were added to incubation buffer (50mM potassium phosphate (pH 7), containing 1mM EDTA, 1mM sodium azide, 0.2mM NADPH, 1 unit of glutathione reductase, 1mM GSH glutathione and 1.5mM cumene hydroperoxide; 800µl) and incubated for 5 minutes at room temperature. The reaction was initiated by addition of cumene hydroperoxide (15mM; 100µl). Absorbance was recorded at 340nm for 5 minutes; activity was calculated from the slope (i.e. change in absorbance with time) and the results were expressed as µmoles NADPH oxidised/mg/min.

RESULTS

Figure 1 shows that delivery of tetanic stimulation to the perforant path of young rats led to an immediate and sustained increase in the mean population epsp slope in perforant path-granule cell synapses. The mean percentage increase in the 2 min immediately following the tetanus, compared with the mean of the values in the 5 min immediately prior to tetanic stimulation, was 146.1% (± 4.9), while the mean value in the last 5 min of the experiment was 129.4% (± 5.0). In contrast to this robust potentiation of the response in young rats, the corresponding mean percentage changes in population epsp slope in the 2min following tetanic stimulation and in the last 5 min of the experiment in aged rats were 120.0% (± 3.9) and 112.7% (± 6.9).

Coupled with the age-related impairment in LTP, we observed a deficit in KCl-stimulated glutamate release. Figure 2 indicates (in a scattergram and a histogram)

that KCl (40mM) significantly increases release of glutamate from synaptosomes prepared from dentate gyrus of young rats ($p < 0.05$; student t-test for paired values), but that this effect was absent in preparations obtained from aged rats. The figure also illustrates a significant correlation between KCl-stimulated release and change in epsp slope following tetanic stimulation ($p < 0.025$).

In addition to the coupled deficits in LTP and glutamate release in dentate gyrus of aged rats, we observed that ageing was also associated with a decrease in the concentration of both docosahexanoic acid and arachidonic acid ($p < 0.05$ in both cases; student's t-test for independent means; Figure 1B and 1C). We argued that, if the decrease in fatty acids was a key factor in the functional impairments observed in aged rats, then dietary supplementation with either ω-3 or ω-6 fatty acids might reverse the age-related changes. Figure 3 shows that dietary manipulation with arachidonic acid and GLA reversed the age-related decrease in LTP and that this was accompanied by reversal of the age-related decreases in the concentrations of both docosahexanoic acid and arachidonic acid (Figure 4). Dietary manipulation did not exert any significant effect in young rats (compare Figures 3A and 3B), but while aged rats fed on the control diet exhibited an impairment in LTP (Figure 3C; $p < 0.01$ compared with either dietary group of young rats; ANOVA), the aged rats which received the diet supplemented with GLA and arachidonic acid (Figure 3D) exhibited LTP which was similar to that in the young rats.

The data indicate that dietary supplementation with EPA also reversed the age-related impairment in LTP (Figure 5). While the changes in mean epsp slope following tetanic stimulation were similar in both groups of young rats and in aged rats which received the experimental diet (compare Figure 5C with Figures 5A and B), aged rats which received the control diet exhibited a deficit in LTP ($p < 0.01$

compared with all other groups; ANOVA). In parallel with this observation, we found that dietary manipulation with EPA also reversed the age-related decreases in both docosahexanoic acid and arachidonic acid; thus while the concentration of both fatty acids was significantly decreased in hippocampus of aged rats fed on the control diet ($p < 0.05$; student's t-test for paired values), values in the hippocampus of aged rats fed on the experimental diet were similar those observed in young rats (Figure 6). Figure 7 indicates that there was a significant positive correlation between arachidonic acid concentration and the ability of rats to sustain LTP (as indicated by the mean percentage change in epsp slope in the last 5 min of the experiment; $p < 0.025$; B), but that the correlation between docosahexanoic acid concentration and epsp slope (A) was not statistically significant.

Further analysis revealed that reactive oxygen species production was significantly increased in hippocampus of aged rats fed on the control diet compared with the value observed in young rats ($p < 0.05$; student's t-test for independent means; Figure 8A), and although reactive oxygen species production was only slightly reduced in hippocampus of aged rats fed on the EPA-supplemented diet, this value did not significantly differ from the value observed in young rats. Thus dietary manipulation at least partly reversed the age-related decrease in reactive oxygen species production. Although no significant age-related or diet-related changes were found in activities of superoxide dismutase, glutathione peroxidase or catalase (see Table 1), Figure 8B indicates that there was a significant positive correlation between reactive oxygen species production and the ratio between activity of superoxide dismutase and the product of glutathione peroxidase and catalase (Figure 8B; $p < 0.05$).

DISCUSSION

The objective of this study was to compare the beneficial effects of feeding aged rats a diet enriched with ω-6 fatty acids with a diet enriched with ω-3 fatty acids. We report that the impairment in LTP in perforant path-granule cell synapses in aged rats was coupled with decreased concentrations of arachidonic acid and docosahexanoic acid and that dietary manipulation with ω-6 or ω-3 fatty acids reversed the age-related decrease in fatty acids and restored the ability of aged rats to sustain LTP.

It has been repeatedly found that maintenance of LTP is impaired with age[1-9] and the present findings support this large body of evidence. The question of whether induction of LTP is impaired with age remains to be resolved, with some reports indicating a deficit[17-20,33] and others reporting no clearcut change.[1,2,34] Here we observed that the early response of granule cell synapses to tetanic stimulation of the perforant path is attenuated in aged rats compared with young rats. This age-related decrease in response to tetanic stimulation was coupled with a decrease in the concentrations of arachidonic acid and docosahexanoic acid and this is consistent with previous evidence which reported a decrease in membrane fluidity with age.[35]

Release of transmitter requires interaction between proteins in the synaptic vesicle membrane and the synaptic plasma membrane, which ultimately leads to fusion of the two membranes allowing exocytosis to occur. It seems reasonable to propose that a change in membrane fluidity, for example as a consequence of a change in membrane composition, would impact on exocytosis. Evidence supporting this idea are presented here; thus in parallel with the age-related decreases in arachidonic and docosahexanoic acids, we observed an age-related decrease in KCl-stimulated glutamate release in dentate gyrus. Several studies in this laboratory have found that KCl-stimulated glutamate release is attenuated with age,[6,7,14,17-20] while other groups

have reported that release of glutamate,[36] acetylcholine[37] and 5-hydroxytryptamine[38] are also downregulated with age. Since maintenance of LTP in perforant path-granule cell synapses is closely coupled with an increase in glutamate release, it is possible that the age-related decrease in release observed here significantly contributes to the impairment in LTP.

If it is argued that decreased membrane fluidity, consequent upon decreased polyunsaturated fatty acid concentration, contributes significantly to the age-related impairment in LTP, then it might be predicted that restoring the membrane composition to that found in the young rat should overcome the age-related deficit in LTP. Consistent with this argument is the finding that when membrane concentration of arachidonic acid and docosahexanoic acid are increased in aged rats by dietary supplementation, the age-related decrease in LTP is reversed. This effect of arachidonic acid has been reported previously[7,20] and we have also found that dietary manipulation with docosahexanoic acid[19] and the antioxidant, α-lipoic acid[18] had the ability to reverse the age-related deficit in LTP. However here we assessed the ability of a novel diet, enriched in eicosapentanoic acid, to reverse the age-related decreases in arachidonic and docosahexanoic acids in parallel with LTP. We report that this diet was as effective as the diet enriched in ω-6 fatty acids; both the age-related decreases in polyunsaturated fatty acids and LTP were reversed. These data add support to the idea that membrane lipid composition is critically important in modulating synaptic function and that one of the consequences of a decrease in polyunsaturated fatty acid concentration is a compromise in synaptic plasticity. However, as we previously reported when a smaller number of observations were analysed, hippocampal concentration of arachidonic acid correlates significantly with the ability of rats to sustain LTP, while a non-significant correlation existed between

the concentration of docosahexanoic acid and LTP.

Reactive oxygen species accumulation increases in cortical tissue with age[9,21,22] and the results of the present experiment indicate that a similar change occurs in the hippocampus. It seems likely that this increase is a consequence of changes in the concentrations of antioxidant scavengers and also in the activities of antioxidant enzymes.[21] Although we did not observed any significant change in activities of superoxide dismutase, glutathione peroxidase or catalase in the present experiments, we found that the enhanced accumulation of reactive oxygen species was positively correlated with the ratio between activities of superoxide dismutase and the product of glutathione peroxidase and catalase. Vitamin E concentration was not assessed in these experiments, but previous findings have indicated that it decreases with age and that it is correlated with reactive oxygen species accumulation.[9] It is likely that the age-related changes in polyunsaturated fatty acids observed here are a consequence of the increase in reactive oxygen species production and this is supported by the finding that lipid peroxidation increased in parallel with reactive oxygen species accumulation in the present study (data not shown).

While reactive oxygen species accumulation was significantly increased in hippocampus of aged rats fed on the control diet, compared with young rats, the value observed in hippocampus prepared from aged rats fed on the EPA-enriched diet was not. This suggests that EPA possesses antioxidant properties, which was previously suggested by the finding that EPA administration decreased peroxidation of low density lipoproteins in dialysis patients.[39] Interestingly previous studies have indicated that a diet enriched in ω-6 fatty acids also exhibited antioxidant properties in hippocampus.[20]

The primary finding of this study is that dietary manipulation with an EPA-rich diet

reverses the age-related decreases in concentrations of arachidonic acid and docosahexanoic acid and overcomes the age-related impairment in LTP. The evidence points to a particular role for arachidonic acid since a significant positive correlation was shown to exist between concentration of arachidonic acid and the ability of rats to respond to tetanic stimulation. Our findings are consistent with the hypothesis that a compromise in antioxidative defences in the hippocampus contributes to these age-related changes.

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TABLE 1

Enzyme	**Young (Pooled)**	**Aged (Control diet)**	**Aged (EPA diet)**
SOD (U/mg)	0.98 (0.14)	0.96 (0.2)	1.03 (0.13)

Gpx (μmol//mg/min)	0.022 (0.001)	0.028 (0.004)	0.029 (0.003)
Catalase (U/mg)	0.28 (0.03)	0.31 (0.06)	0.25 (0.03)

Activities of superoxide dismutase (SOD), glutathione peroxidase (Gpx) and catalase (cat) were assessed as described in the text. No differences in values were obtained between dietary groups in the 4 month-old rats and data are pooled. No age-related changes in activity of any of the 3 enzymes was observed.

FIGURE LEGENDS

Figure 1. The age-related decrease in LTP is coupled with age-related decreases in hippocampal concentrations of arachidonic acid and docosahexanoic acid. (A) Tetanic stimulation (arrow) induced an immediate and sustained increase in the mean slope of the population epsp in 4 month old rats, but both the early and later responses to the stimuli were attenuated in 22 month-old rats. (B) and (C) Hippocampal concentrations of both arachidonic acid (AA) and docosahexanoic acid (DHA) were significantly decreased in aged, compared with young, rats ($p < 0.05$; student's t-test for independent means).

Figure 2. KCl-stimulated glutamate release is decreased with age. Addition of KCl (40mM) to synaptosomes prepared from dentate gyrus of young (4 month-old) rats significantly enhanced glutamate release as indicated by the scattergram and histogram. However the effect of KCl was markedly diminished in synaptosomes prepared from dentate gyrus of aged rats. The data indicate that there was a significant positive correlation between glutamate concentration (KCl-stimulated

glutamate release) and the slope of the population epsp in the last 5 min of the experiment ($p < 0.025$).

Figure 3. The mean slope of the population epsp was markedly increased after tetanic stimulation (arrow) in both groups of young rats (n = 8 in each case). In contrast, there was a marked impairment in maintenance of LTP in aged rats which received the control diet (n = 11). Dietary manipulation with GLA/AA (n = 9) reversed the age-related impairment in LTP. Mean epsp slopes (± SEM) are presented in 5 min blocks and are expressed as a percentage of the values recorded in the 5 minutes immediately prior to tetanic stimulation.

Figure 4. Dietary manipulation reverses age-related changes in concentrations of arachidonic acid and docosahexanoic acid.

The mean concentrations of docosahexanoic acid (DHA; A) and arachidonic acid (AA; B) were similar in both groups of young rats (n = 8 in each case) and therefore data are pooled. The concentrations of both fatty acids were significantly decreased in tissue prepared from dentate gyrus of aged rats which received the control diet (n = 11; $*p < 0.05$; student's t-test for independent means, while dietary supplementation with a diet enriched in GLA and AA reversed the age-related changes so that the concentrations of both fatty acids were similar in young rats and aged rats which received the experimental diet.

Figure 5. The mean slope of the population epsp was markedly increased after tetanic stimulation (arrow) in both groups of young rats (n = 8 in each case). In contrast, there was a marked impairment in maintenance of LTP in aged rats which received the control diet (n = 11). Dietary manipulation with eicosapentanoic acid (EPA; n = 9) reversed the age-related impairment in LTP. Mean epsp slopes (± SEM) are presented in 5 min blocks and are expressed as a percentage of the values recorded

in the 5 minutes immediately prior to tetanic stimulation.

Figure 6. Dietary manipulation reverses age-related changes in concentrations of arachidonic acid and docosahexanoic acid.

The mean concentrations of docosahexanoic acid (DHA; A) and arachidonic acid (AA; B) were similar in both groups of young rats (n = 8 in each case) and therefore data are pooled. The concentrations of both fatty acids were significantly decreased in tissue prepared from dentate gyrus of aged rats which received the control diet (n = 11; *$p < 0.05$; student's t-test for independent means, while dietary supplementation with a diet enriched in EPA reversed the age-related changes so that the concentrations of both fatty acids were similar in young rats and aged rats which received the experimental diet.

Figure 7. The change in epsp slope following tetanic stimulation correlates with arachidonic acid, but not docosahexanoic acid.

There was a significant positive correlation between arachidonic acid concentration in hippocampus and the ability of rats to sustain LTP (as indicated by the mean percentage change in epsp slope in the last 5 min of the experiment; $p < 0.025$; B), but that the correlation between docosahexanoic acid concentration and epsp slope (A) was not statistically significant.

Figure 8. Reactive oxygen species production (upper panel) was similar in hippocampus prepared from both groups of young rats and therefore the data are pooled. However it was significantly increased in hippocampus of aged rats fed on the control diet ($p < 0.05$; student's t-test for independent means), but this significance was lost in in hippocampus of aged rats fed on the EPA-supplemented diet. A significant positive correlation was observed between reactive oxygen species

production (ROS) and the ratio between activity of superoxide dismutase and the product of glutathione peroxidase and catalase ($p < 0.05$).

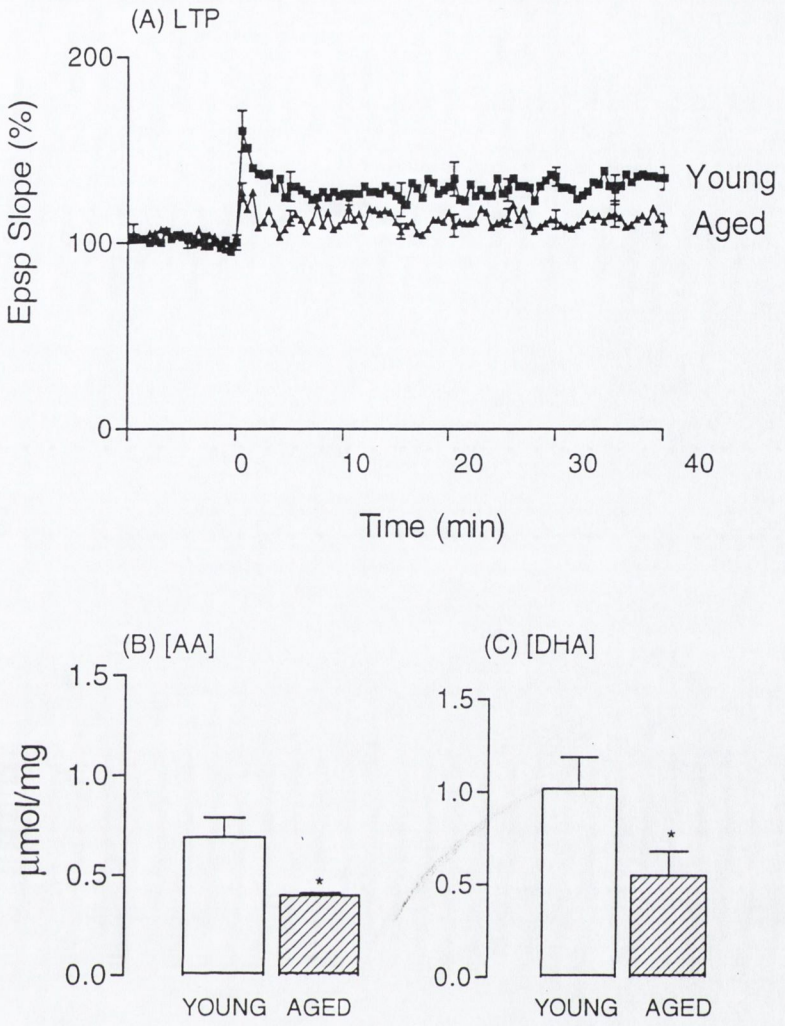
(A) LTP
200
100
0
Epsp Slope (%)
0
10
20
30
40
Time (min)
Young
Aged
(B) [AA]
(C) [DHA]
µmol/mg
1.5
1.0
0.5
0.0
YOUNG
AGED
*

Figure1

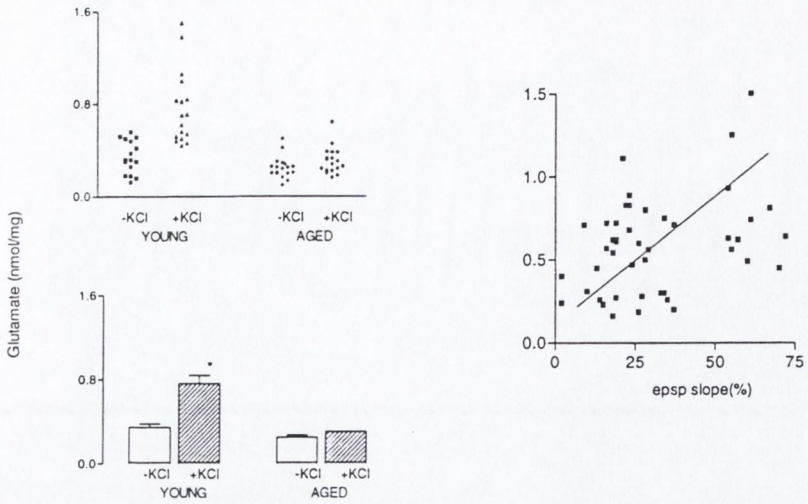
Glutamate (nmol/mg)
1.6
0.8
0.0
-KCl +KCl
YOUNG
-KCl +KCl
AGED
1.6
0.8
0.0
-KCl +KCl
YOUNG
-KCl +KCl
AGED
1.5
1.0
0.5
0.0
0
25
50
75
epsp slope(%)

Figure2

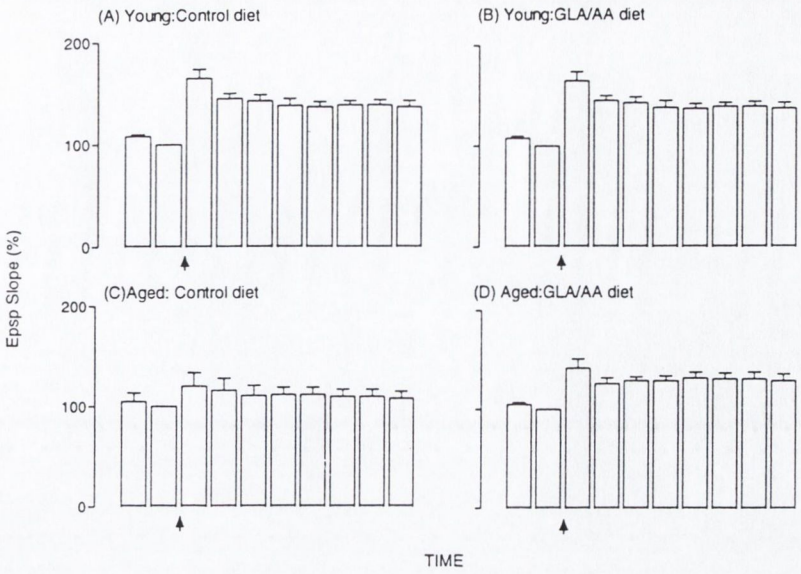
(A) Young:Control diet
(B) Young:GLA/AA diet
(C)Aged: Control diet
(D) Aged:GLA/AA diet
200
100
0
Epsp Slope (%)
TIME

Figure3

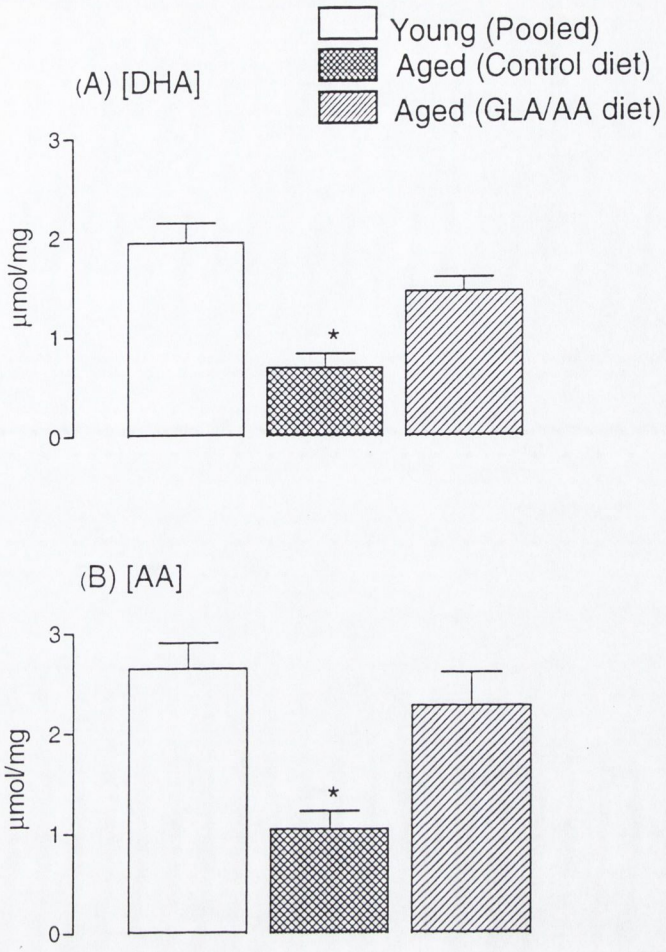
Young (Pooled)
Aged (Control diet)
Aged (GLA/AA diet)
(A) [DHA]
µmol/mg
3
2
1
0
*
(B) [AA]
µmol/mg
3
2
1
0
*

Figure4

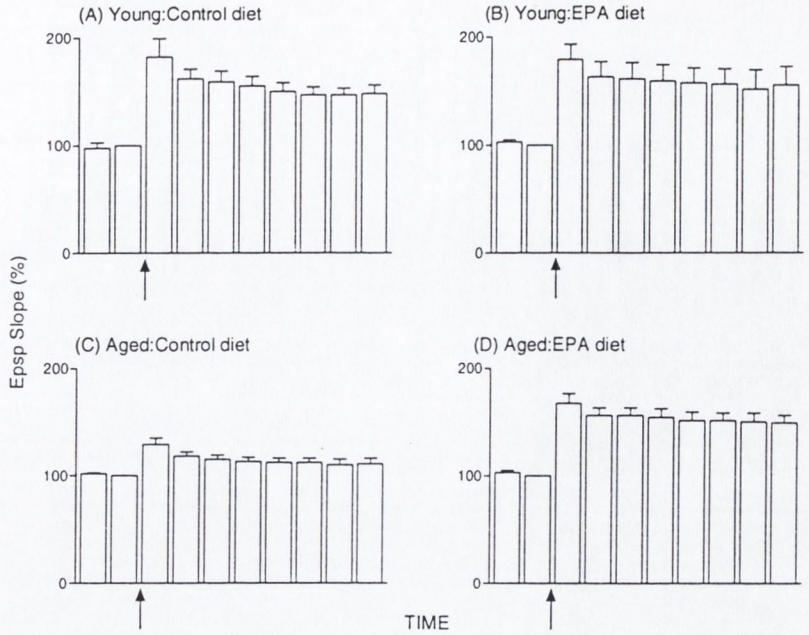
(A) Young:Control diet
(B) Young:EPA diet
(C) Aged:Control diet
(D) Aged:EPA diet
200
100
0
Epsp Slope (%)
TIME

Figure5

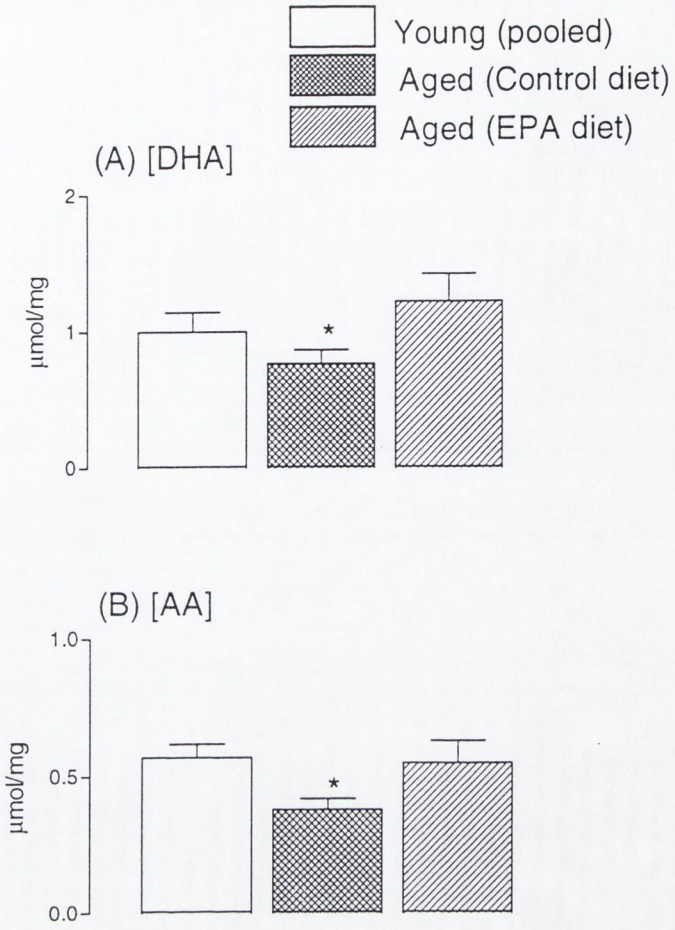
Young (pooled)
Aged (Control diet)
Aged (EPA diet)
(A) [DHA]
µmol/mg
2
1
0
*
(B) [AA]
µmol/mg
1.0
0.5
0.0
*

Figure6

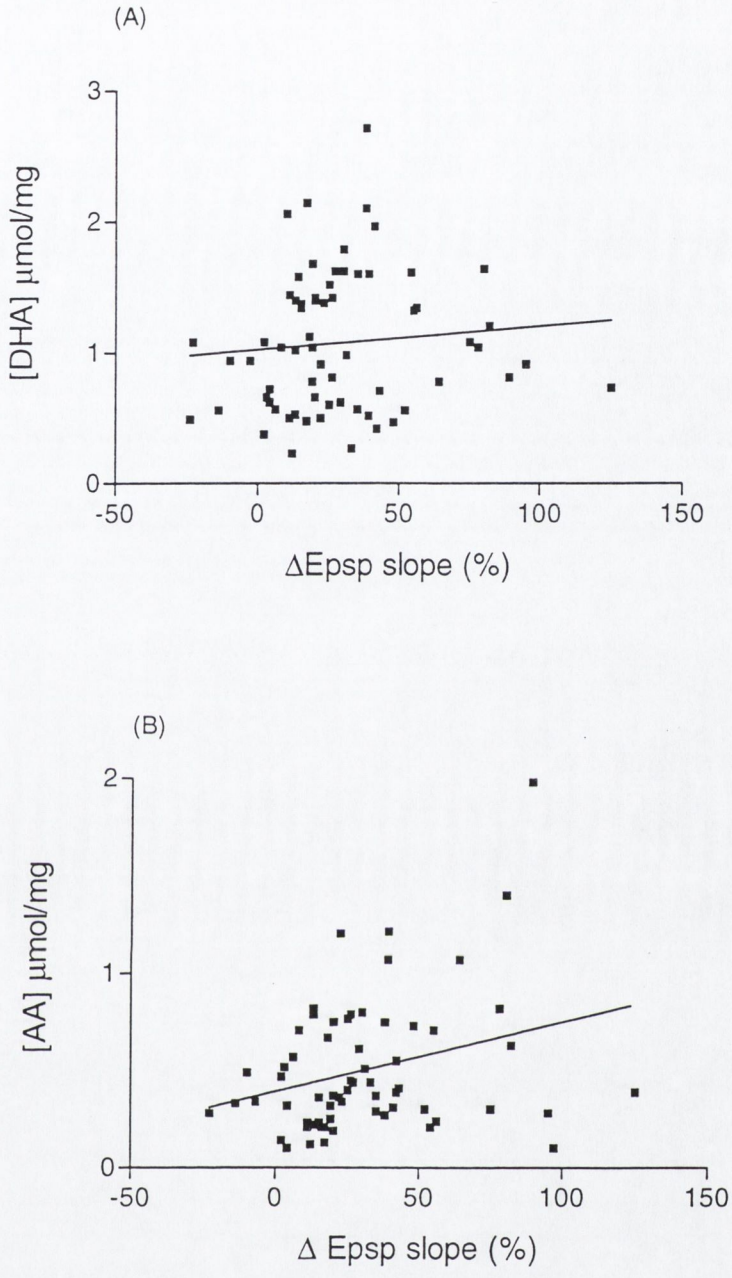
(A)
[DHA] µmol/mg
3
2
1
0
-50
0
50
100
150
ΔEpsp slope (%)
(B)
[AA] µmol/mg
2
1
0
-50
0
50
100
150
Δ Epsp slope (%)

Figure7

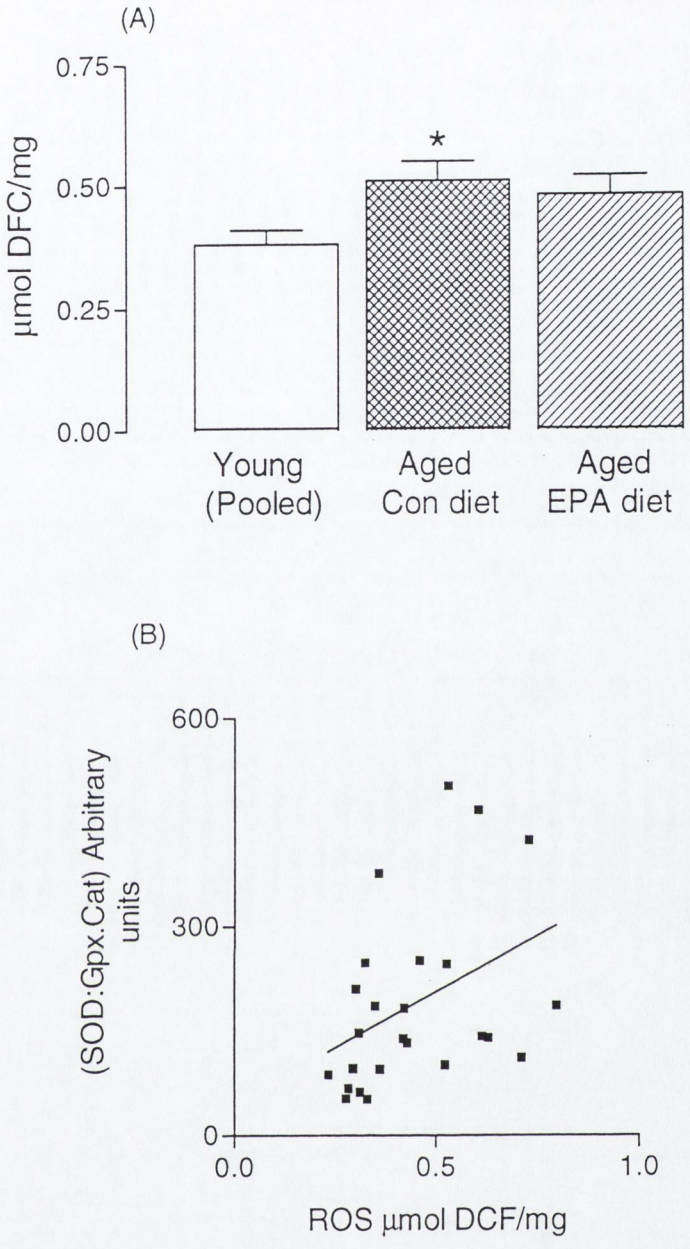
(A)
0.75
0.50
0.25
0.00
µmol DFC/mg
*
Young
(Pooled)
Aged
Con diet
Aged
EPA diet
(B)
600
300
0
(SOD:Gpx.Cat) Arbitrary
units
0.0
0.5
1.0
ROS µmol DCF/mg

Figure8

SOCIETY FOR NEUROSCIENCE ABSTRACT.

EICOSAPENTANOIC ACID RESTORES THE AGE-RELATED IMPAIRMENT IN LONG-TERM POTENTIATION IN THE DENTATE GYRUS.

D.S.D. Martin [1], P. Spencer [1], D. Horrobin [2], M.A. Lynch [1].

[1]*Dept. of Physiology, Trinity College, Dublin 2, Ireland.* [2]*Laxdale Research Ltd., Stirling, Scotland, UK.*

It has been shown that there is an age-related impairment in long-term potentiation (LTP) in perforant path-granule cell synapses which is associated with a decrease in the concentration of polyunsaturated fatty acids in the membrane. In this study young (4 month) and aged (24 month) rats were fed for eight weeks on a control diet or a diet enriched with eicosapentanoic acid (20mg/rat/day). At the end of this period rats were anaesthetised with urethane (1.5mg/kg i.p.) and placed in a stereotaxic frame and stimulating and recording electrodes were placed in the perforant path (4.4mm lateral to Lambda) and the granule cells of the dentate gyrus (2.5 mm lateral and 3.9mm posterior to Bregma) respectively. The mean percentage increase in the epsp slope in the last 5 minutes of the experiment (40-45 min post-tetanus) were 143.39 ± 3.40 and 144.23 ± 5.98 in the young rats and 105.97 ± 2.54 and 149.33 ± 3.98 in the aged rats fed on the control and experimental diet respectively. Reactive oxygen species (ROS) production was significantly increased in the hippocampus prepared from the aged rats fed the control diet compared with young rats (0.50 ± 0.04 vs. 0.38 ± 0.03 μmolesDCF/mg); dietary manipulation prevented this significant increase. This increase in ROS was coupled with an increase in superoxide dismutase activity without concomitant increases in catalase and glutathione peroxidase activity and there was a significant negative correlation between the ratio of these enzyme activities and LTP. Evidence for a role for the cytokine interleukin-1ß will be presented.

(Supported by BioResearch Ireland)