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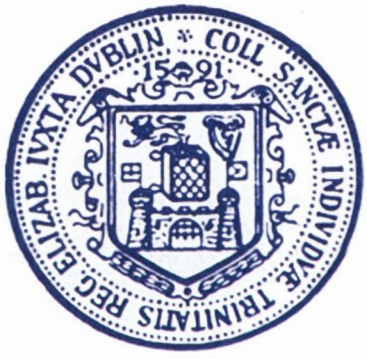
An investigation of age-related changes in the brain and the neuroprotective effects of the omega-3 fatty acids docosapentaenoic acid and eicosapentaenoic acid



Laura Catherine Kelly

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

2009

I Declaration

This thesis is submitted by the undersigned for the degree of Doctor in Philosophy at the University of Dublin. I declare that this thesis is entirely my own work with the following exceptions; certain results were produced in collaboration with Dr. Aileen Lynch, Dr. Thelma Cowley and Dr. Anne-Marie Miller and the behavioural testing was carried out by Dr. Andrea Della Chiesa and these are acknowledged within the thesis. This work has not been submitted previously for a degree at this or any other University. I give my permission to the library to lend or copy this thesis upon request.

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II Summary

The data presented here indicate that the n-3 polyunsaturated fatty acid docosapentaenoic acid (DPA), a metabolite of eicosapentaenoic acid (EPA), possesses neuroprotective and anti-inflammatory properties. Previous evidence has shown that EPA can have a neuroprotective effect and has the ability to attenuate age-related changes. EPA has been demonstrated to prevent the age-related deficit in LTP, attenuate the age-related increase in interleukin (IL)-1β and can increase IL-4 levels, which are known to decline with age. The data presented in this study indicate that oral administration of DPA and EPA for a period of 8 weeks modulated changes induced by age in the rat cortex and hippocampus including the age-related impairment in long-term potentiation (LTP) in the hippocampus.

The evidence indicates that caspase 3 activity was increased in cortical tissue prepared from aged rats, which is indicative of cell stress. *In vitro* analysis suggests a role for sphingomyelinase in caspase 3 activation induced by lipopolysaccharide (LPS), as the LPS-induced increase in caspase 3 activity in neurons was blocked by inhibition of sphingomyelinase. Significantly, EPA and DPA attenuated the effect of LPS on activities of sphingomyelinase and caspase 3, revealing a neuroprotective effect. There was an age-related increase in membrane sphingomyelinase activity which was not present in EPA- or DPA-treated animals.

Furthermore, both DPA and EPA attenuated the age-associated increase in major histocompatibility complex class II (MHCII) mRNA. These data indicate that DPA and EPA can act to modulate microglial cell activation. In conclusion, the findings demonstrate that EPA and DPA exert effects in the cortex and hippocampus, on both microglia and neurons and possess anti-inflammatory and neuroprotective properties.

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I would also like to thank my parents for their continued support and for always being there for me. I would like to thank my friends as well.

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V List of figures

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VII Abbreviations

ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate
ANOVA	Analysis of variance
Ara-c	Arabino-furanoside
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	*Cornu ammonis*
CaMKII	Calcium/calmodulin-dependent protein kinase II
Ca^{2+}	Calcium ions
CD68 / 86	Cluster of differentiation 68 / 86
cDNA	Copy deoxyribonucleic acid
°C	Degrees Celcius
cFLIP	Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein
CTFB	Complete transcription factor buffer
CX_3CL_1	Fractalkine
CX_3CR_1	Fractalkine receptor
dH_2O	Deionised water
DHA	Docosahexaenoic acid
DISC	Death inducing signal complex
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DPA	Docosapentaenoic acid
DR	Death receptor
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate

EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
EPSP	Excitatory postsynaptic potential
ERK	Extracellular signal-regulated kinase
Fas	Cell death receptor ligand
FBS	Fetal Bovine Serum
FADD	Fas-associated protein with death domain
FAN	Factor associated with neutral-sphingomyelinase activation
FLICE	FADD-like IL-1β-converting enzyme
GPR	G-protein coupled receptor
h	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethane sulphonic acid
HETE	Hydroxyeicosatetraenoic
HFS	High frequency stimulation
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
HPETE	Hydroperoxyeicostetraenoic
Hz	Hertz
ICAM	Intercellular adhesion molecule
IFNγ	Interferon-γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
ip	Intraperitoneal
JNK	c-Jun N-terminal kinase
K^+	Potassium ions
LPS	Lipopolysaccharide
LT	Leukotriene
LTP	Long-term potentiation

M	Molar
MAP kinases	Mitogen activated protein kinases
M-CSF	Macrophage colony stimulating factor
mg	Milligram
Mg^{2+}	Magnesium ions
MHC II	Major Histocompatibility Complex class II
min	Minute
ml	Millilitre
mm	Millimeter
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Na^{+}	Sodium ions
NBM	Neurobasal medium
NFκB	nuclear factor κB
NGS	Normal goat serum
NMDA	N-methyl-D-aspartate
8-OHdG	8-hydroxy-2'-deoxyguanosine
O_2	Oxygen
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEA-15PED	phosphoprotein enriched in astrocytes-15kDa/phosphoprotein enriched in diabetes
pg	Picograms
PG	Prostaglandin
pH	Potential of hydrogen
PK	Protein kinase
PLA_2	Phospholipase A_2
PMSF	Phenylmethylsulphonyl fluoride
pNA	p-nitroaniline
PPAR	peroxisome proliferator-activated receptor

PPRE	PPAR response elements
PUFA	Polyunsaturated fatty acid
QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RT-	Reverse transcriptase
RXR	Retinoid X receptor
s	Second
Taq Polymerase	*Thermus aquaticus* DNA polymerase
TBS	Tris buffered saline
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor α
TRADD	TNF-R1-associated death domain
TRAF	TNF receptor-associated factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TX	Thromboxane
UV	Ultraviolet
µg	Microgram
µl	Microlitre
µM	Micromolar

Chapter 1 Introduction

1 Introduction

1.1 The brain: the main cell types

The cells of the brain can be broadly divided into two categories; neurons and glia. Initial robust differentiation of neurons and glia begins as soon as the neural tube has grown into an undeveloped brain and spinal cord. Neurons are primarily responsible for signal transduction and communication. There are ten to twenty times as many glial cells as there are neurons and it has been estimated that the mature human brain contains as many as 100 billion neurons, generated over the course of mere months, originating from a small population of precursor cells, in the ventricular zone, the most internal cell layer around the lumen of the neural tube. In humans, it is estimated that approximately 250,000 neurons per minute may be produced at the height of cell proliferation. Apart from special cases, all neuronal cells of the adult brain are generated before the end of gestation. After this time, few, if any, neurons can be added to replace cells lost with age or as a consequence of injury in most brain areas.

Glial cells were once thought to be merely supporting cells, but it is now clear that these cells have many functions. The word glia originates from the Greek word translated as "glue". This term was used because in the nineteenth century it was thought that these non-neuron cells supported the central nervous system and, by some means, held it together. Glia can be subdivided into three distinct categories; microglia, the macrophage-like immune cells of the brain, oligodendrocytes, which function by laying down myelin around axons, important in modulating the speed of action potential conduction, and astrocytes; the name of these cells is derived from their star-like appearance as they have processes extending from the cell body. Astrocytes have many functions and are particularly important in maintaining a suitable environment for neuronal signalling.

1.2 The hippocampus

1.2.1 Historical perspective

The term "hippocampus" originates from the Greek *hippokampos*, *hippos* meaning horse and *kampos*, which translates to sea monster. This term was given to the brain region by the 16th century anatomist Arantius (Arantius, 1587) due to its three-dimensional shape resembling a sea horse. Later, Winslow (Winslow, 1732) considered the hippocampus to resemble a ram's horn and this led to de Garengeot (Garengeot, 1742) naming the hippocampus "Ammon's horn" with reference to the Greek name of an Egyptian oracle deity. In 1934, Lorente de Nó referred to the hippocampus proper as the "*cornu ammonis*", a Latin translation of the horn of Ammon (Lorente de Nó, 1934) and this is used today synonymously with the hippocampus.

1.2.2 Anatomy of the hippocampus

The hippocampal formation, situated in each hemisphere of the brain beneath the cortex, consists of the dentate gyrus, the hippocampus and the subiculum. Granule cells are the dominant cell type of the dentate gyrus of the hippocampus and the dendrites of these cells extend from the granule layer to the molecular layer, where projections from the entorhinal cortex are received. The hippocampus (hippocampus proper; *cornu ammonis*) comprises three regions; CA1, CA2 and CA3. There are three key afferent pathways in the hippocampus which form the trisynaptic loop; the perforant path sends excitatory impulses from the entorhinal cortex to the granule cells of the dentate gyrus (see Figure 1.1). The granule cells synapse with the pyramidal cells of the CA3, by means of the mossy fibre pathway, and the axons of pyramidal cells of the CA3 form the Schaffer collateral pathway and synapse with pyramidal cells in CA1.

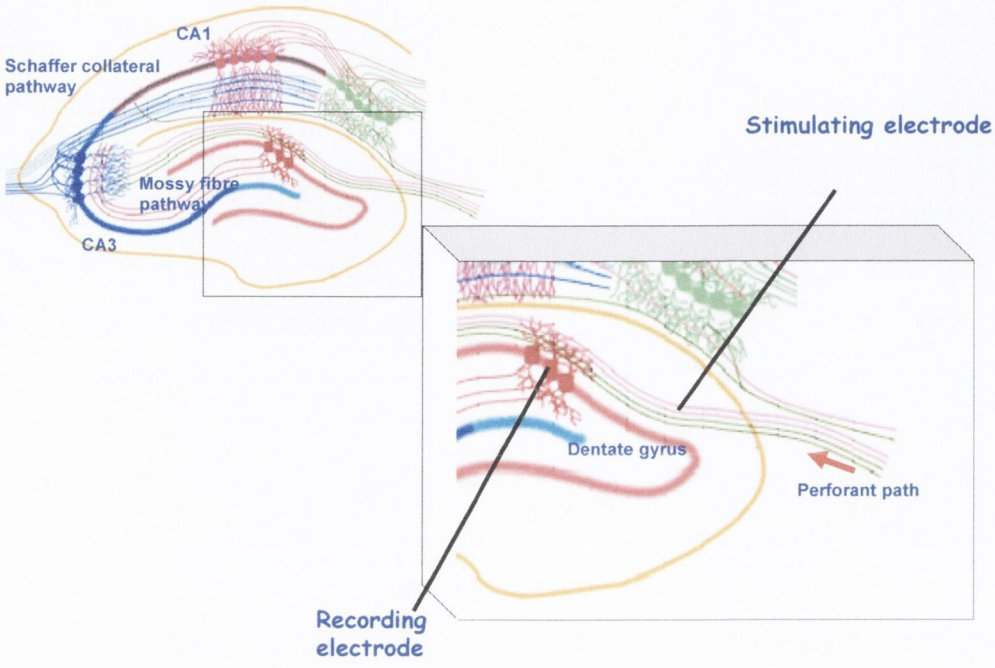


Figure 1.1 Long-term potentiation and hippocampal structure

The perforant fibre pathway projects from the entorhinal cortex and forms excitatory connections with the granule cells of the dentate gyrus.

1.2.3 The hippocampus and memory

The hippocampus is required for the storage of memory(Kandel *et al.*, 2000). It became apparent in the 1950's that the temporal lobe was highly important for the storage of memory. A study was undertaken investigating patients which had undergone bilateral removal of the hippocampus and nearby regions in the temporal lobe as a treatment for epilepsy. One patient (H.M.) suffered from bilateral temporal lobe seizures which were untreatable and the hippocampal formation, amygdala and some of the temporal cortex were removed bilaterally to alleviate symptoms; this resulted in anterograde amnesia and a severe memory deficit, highlighting the role of these areas in memory (Milner, 1966). Further to this, monkeys with lesion of the medial temporal lobe were shown to have marked memory impairment (Mishkin, 1978) but the memory impairment was not as great as when the lesion involved the hippocampus (Murray & Mishkin, 1998). The role of the hippocampus in memory has been confirmed using magnetic resonance imaging and positron emission tomography by monitoring blood flow and consumption of oxygen in the hippocampus while subjects take part in learning tasks (Squire *et al.*, 1992).

1.3 Long-term potentiation

1.3.1 LTP

Long-term potentiation (LTP) is a form of synaptic plasticity which is a putative cellular model for learning and memory in the rat brain. LTP can be described as a sustained increase in synaptic firing and can be induced by the delivery of high frequency stimulation to the major afferent pathways. Of particular relevance to this study is the perforant path. A wealth of evidence indicates that high frequency stimulation of the perforant path, which arises from neurons of the entorhinal cortex and synapses with the granule cells of the dentate gyrus, leads to induction of LTP in perforant path granule cell synapses. This is typified by a persistent increase in the slope and amplitude of the excitatory post-synaptic potential (EPSP, see Figure 1.2).

In 1973, Bliss and Lomo first reported that a succession of high frequency stimulation, applied to any of the three main afferent pathways, in the anaesthesised rabbit, can increase the amplitude of EPSPs (Bliss & Lomo, 1973).

1.3.2 LTP properties

LTP has three important properties – input specificity, associativity (Levy & Steward, 1979) and cooperativity (Bliss & Collingridge, 1993). Input specificity is a term used to describe the fact that when LTP is induced at a particular set of synapses, other synapses close to this site that are not activated do not exhibit LTP (Andersen *et al.*, 1977; Lynch *et al.*, 1977). Associativity describes the fact that LTP can be stimulated in a set of synapses by sub-threshold low frequency stimulation if the activation of these synapses is temporally simultaneous with a stimulus capable of inducing LTP at an adjacent set of synapses (Levy & Steward, 1979). Finally, cooperativity refers to the fact that, in order to induce LTP via high frequency stimulation, a certain number of fibres need to be activated (Bliss & Collingridge, 1993).

Induction of classical high frequency stimulation-induced LTP in the hippocampus is controlled by activation of the glutamate receptors. Glutamate receptors can be classified as either ionotropic receptors, which act as direct gates of ion channels, or metabotropic receptors, which act indirectly on ion channels via second messenger signalling (Kandel *et al.*, 2000). The three main subtypes of ionotropic glutamate receptors are N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and kainate receptors, the names of which come from their respective selective synthetic receptor agonists. The majority of hippocampal neurons possess NMDA, AMPA and kainate receptors and it is the non-NMDA receptors which are responsible for the production of the early phase of the EPSP. These receptors gate ion channels with a lower conductance than NDMA receptors, permeable to Na^+ and K^+ ions. The late phase of the EPSP is

brought about via NMDA receptor-associated ion channels which allow entry of Na^+, K^+ and Ca^{2+}.

NMDA receptor-associated channels are ligand- and voltage-dependent. Membrane depolarisation results in repulsion of Mg^{2+} from its binding site in the NMDA receptor-associated channel (Collingridge *et al.*, 1983) and the consequential entry of Ca^{2+} into the post synaptic area.

Aside from the mossy fibre pathway, NMDA receptor activation is essential for induction of LTP in the hippocampus. This has been shown using the specific NDMA receptor antagonist AP5 (Collingridge *et al.*, 1983) and by blockage of NMDA-associated channels in the CA1 *in vitro* and in the dentate gyrus *in vivo* using MK801 (Coan *et al.*, 1987) which prevented LTP induction. A form of LTP has been described in the CA1 region which is independent of NMDA receptors, but in which the increase in postsynaptic Ca^{2+} occurs via the activation of voltage-dependent Ca^{2+} channels (Lynch & Baudry, 1984).

These findings indicate that Ca^{2+} influx into cells is essential for induction of LTP (Bortolotto *et al.*, 1994), and the maintenance of LTP is dependent on the increase in Ca^{2+} ions in the post-synaptic cell driving signalling events which lead to increased synthesis of proteins, morphological changes and greater synaptic effectiveness (Zucker, 1999). Consistent with the important role of Ca^{2+} entry is the finding that intracellular injection of ethylene glycol tetraacetic acid (EGTA), a Ca^{2+} chelator, can inhibit LTP induction (Lynch *et al.*, 1983). In addition to NMDA receptor activation, a role for metabotropic glutamate receptors in modulating LTP has been identified (McGuinness *et al.*, 1991; Bortolotto *et al.*, 1994; O'Connor *et al.*, 1995).

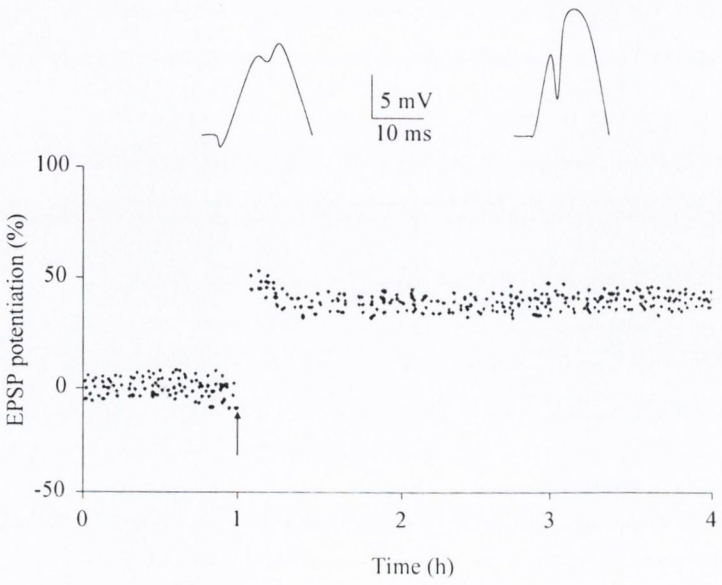


Figure 1.2 Long-term potentiation

An example for long-term potentiation (LTP) recorded *in vivo*. The graph plots the slope of the rising phase of the evoked response, the population EPSP, which was recorded from the cell body in response to constant test stimuli before and after tetanus of 200Hz, 200msec which was given at the time indicated by the arrow. Adapted from Bliss and Collingridge (1993).

1.3.3 LTP and stress

Stress can be defined as a disturbance of homeostasis, physiologically and psychologically, resulting in secretion of corticosteroids from the adrenal cortex (Lynch, 2004). The hippocampus has a high concentration of glucocorticoid receptors and so is highly susceptible to stress and stress hormones and therefore LTP is reduced by a wide range of stressors including irradiation (Tolliver & Pellmar, 1987; Lonergan *et al.*, 2002), amyloid-β (Lynch *et al.*, 2007), oxidative stress (Vereker *et al.*, 2001) and lipopolysaccharide, LPS (Vereker *et al.*, 2000a; Lonergan *et al.*, 2004). The pro-inflammatory cytokine interleukin (IL)-1β has been shown to impair the expression of LTP in perforant path-granule cell synapses (O'Donnell *et al.*, 2000) and increased IL-1β concentration and activation of IL-1β-triggered signalling pathways in the hippocampus have been correlated with an inability to sustain LTP (Murray & Lynch, 1998b; Lynch & Lynch, 2002; Martin *et al.*, 2002a).

1.4 Ageing

Ageing may be described as an accumulation of detrimental changes that occur throughout cells and tissues of the body which, over time, progressively impair function and ultimately result in death (Harman, 2001). Therefore, a decreased resistance to stress has been associated with age. There are four key aspects to take into account when examining causes of ageing. These are developmental, environmental and genetic factors, and disease. At a cellular level, stress physiologically causes increased hormone secretion including the corticosteroids (Yau *et al.*, 2002) and it has been demonstrated that glucocorticoids can acutely impair learning, memory and LTP and this has been somewhat attributed to the high number of glucocorticoid receptors in the hippocampus (McEwen, 1994). There is also an age-associated increase in plasma corticosterone and glucocorticoids in the circulation (He *et al.*, 2008) and these are not the only hormones which are altered with age or other forms of stress; others include noradrenaline, adrenaline and vasopressin which can all impact on hippocampal function.

1.5 Theories of ageing

Haldane (Haldane, 1941) first put forward the theory that late-acting detrimental genetic mutations could build up over time (evolutionary time) through genetic drift in populations and this resulted in age-related mortality. This was later formalised by Sir Peter Medawar whereby he theorised that ageing was an accumulation of mutations. In 1957, it was proposed by Williams (Williams, 1957) that a subset of genes were selected due to offering a reproductive advantage in early adulthood, but that these same genes could be destructive later on in life, post-reproduction (Bowles, 2000). Among the factors which contribute to the deterioration in function with age are increases in production of reactive oxygen and nitrogen species, increase in mitochondrial damage, increased cholesterol and hormone deficiency.

The exact mechanisms of ageing which contribute to the associated deficits in plasticity have yet to be elucidated but there are two main theories of ageing and these are (1) the membrane hypothesis and (2) the free radical theory.

1.5.1 Membrane hypothesis

Membrane fluidity is the physical state of the fatty acyl chains of the membrane bilayer and the rates of motion of molecular elements within the membrane (Youdim *et al.*, 2000). It has been reported that the changing of just one single double bond can cause effects upon the physical properties of a membrane (Cohen & Zubenko, 1985; Mason *et al.*, 1997). Membrane fluidity decreases with age due to increased cholesterol (Yehuda *et al.*, 2002) and sphingomyelin (Giusto *et al.*, 1992) combined with diminished levels of polyunsaturated fatty acids (PUFA) in the membrane (Lynch & Voss, 1994). Cholesterol is a determining factor in membrane fluidity/rigidity as it can regulate the movement of phospholipids (Yeagle, 1989). It has been demonstrated that cholesterol is particularly concentrated in the

hippocampus, where the cholesterol:phospholipid ratio (Viani *et al.*, 1991) is greatest and this may be a reason for the vulnerability of the hippocampus to changes that occur in the ageing process.

Usually, phospholipids, a major component of the membrane comprise a saturated fatty acid at the 1-acyl position and an unsaturated fatty acid at the 2-acyl position, which is often arachidonic acid or docosahexaenoic acid (DHA), particularly in the hippocampus (Gatti *et al.*, 1986; Youdim *et al.*, 2000). The degree of unsaturation of fatty acids in the brain is generally greater than other organs; this led to the development of the theory that changes in phospholipid metabolism can have a profound effect on the hippocampus.

There is less arachidonic acid in membranes of the aged brain (Terracina *et al.*, 1992) and this has been correlated with decreased membrane fluidity. Secondly, reactive oxygen species accumulation with age has been reported and this can lead to peroxidation of lipids which also impacts negatively on membrane fluidity. These factors contribute to increased membrane rigidity, which has been linked with an age-related impairment in spatial learning and a reduced ability to sustain LTP (Lynch & Voss, 1994). Lipid composition of the membrane is crucial in determining how the membrane functions in terms of signal transduction and transmitter release. The more rigid the membrane becomes, the more difficult it is for proteins to interact appropriately, as movement becomes limited and, for example, receptor – ligand interactions or ion channel functioning may be impaired. Impairments in signal transduction and transmitter release have been described in the hippocampus of aged rats and these changes are linked with impairment in LTP (Mullany *et al.*, 1996).

NMDA receptor binding is also impaired in the aged brain and associated NMDA receptor signaling is downregulated (Bonhaus *et al.*, 1990; Tamaru *et al.*, 1991; Ingram *et al.*, 1992); these changes provide evidence for the membrane hypothesis of ageing. The impairment of NMDA receptor binding and signaling cascade points to calcium dysregulation and an offset of calcium homeostasis that

possibly exists in the aged brain. Calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), both enzymes highly sensitive to calcium, are known to be decreased with age (Mullany *et al.*, 1996). These are key enzymes in the expression phase of LTP.

1.5.2 Free radical theory of ageing

It has been long proposed that reactive oxygen species build up over a lifetime due to metabolism and that this accumulation, over time, exerts a negative effect on cell function (Harman, 1956, 1981). It can cause harm by random oxidation of proteins, which can result in protein denaturation, triggering protein degradation by activating proteases (Stadtman & Berlett, 1997). Free radicals can cause membrane damage (Grinna, 1977), involving a decrease in membrane fluidity, by interactions with lipids of the phospholipid bilayer (Murray & Lynch, 1998b). There are, however, anti-oxidant defences; enzymes such as superoxide dismutase (McCord & Fridovich, 1969) and scavengers like glutathione and vitamins C and E play a role in preventing accumulation of reactive oxygen species but it has been reported that the age-related increase in reactive oxygen species accumulation is associated with alterations in these anti-oxidant defence systems (Gabbita *et al.*, 1997; Sastre *et al.*, 2000). Specifically there is an increase in superoxide dismutase coupled with a decrease in glutathione peroxidase and a decrease in the concentration of vitamin E (Murray & Lynch, 1998a; O'Donnell *et al.*, 2000).

1.6 Long-term potentiation and age

Ageing is associated with impairments in learning and memory (Barnes *et al.*, 1980) and the effects can be, at least in part, attributed to changes in hippocampal morphology and hippocampal function. Geinisman and colleagues have shown that ageing leads to a decrease in synapse number and synapse function in the hippocampus (Geinisman *et al.*, 1992). It has also been reported that Ca^{2+} regulation is modified with age (Landfield & Pitler, 1984; Campbell *et al.*, 1996). It may be that

these changes and a change in NMDA-receptor mediated responses bring about the detected reduction in synaptic plasticity with age and the associated impairment in cognition (Jarrard, 1993). Age-related impairments in LTP have been demonstrated (De Toledo-Morrell & Morrell, 1985; Lynch & Voss, 1994; O'Donnell *et al.*, 2000; Lynch *et al.*, 2007) and linked with reduced ability to process spatial information (Barnes *et al.*, 1980). Mullany and Lynch (1997) have shown that the synaptic vesicle protein synaptophysin is decreased with age (Mullany & Lynch, 1997); this protein is involved in synaptic transmitter release and a change in synaptophysin expression may be a factor contributing to the age-related decrease in transmitter release.

1.7 Inflammation

The cardinal signs of inflammation are *rubor, tumor, calor* and *dolor*, which translates from Latin as redness, swelling, heat and pain. Inflammation is a protective pathogenic microorganisms, toxins or allergens (Medzhitov & Janeway, 2002). Briefly, macrophages act by surrounding and engulfing the foreign matter, blood capillaries dilate so as to allow movement of cells to the region of inflammation, additional leukocytes are recruited through chemical messengers such as cytokines and these recruited cells aid in the fight against infection. Stresses, including infection, can initiate cytokine release and it is important to note that, as is the case for the majority of biological mediators, low levels can have a protective effect whereas sustained, persistent high levels can have a negative impact. There is also a critical balance maintained between levels of pro- and anti-inflammatory cytokines and disturbance of this balance can contribute to increased inflammatory stress, for example, the pro-inflammatory cytokine IL-1β is significantly increased with age (Murray & Lynch, 1998b) and there is a concomitant decrease in anti-inflammatory cytokines, for example IL-4 (Maher *et al.*, 2005). It has been proposed that this cytokine imbalance may have a key role in the age-related increase in inflammatory stress.

1.8 Cytokines

Cytokines are chemical mediators that act primarily to modulate the systemic host response to infection/disease and to control local inflammatory changes in the central nervous system. The term "cytokine" comes from the Greek words *kytos*, which means hollow, and *kinein*, meaning "to move". Cytokines function to modulate cells during development and under normal and pathological conditions (Dinarello, 1990; Meager, 1998). In contrast to hormones, cytokines signal to re-establish normal function in the tissue or organ in which they were generated. When there is an acute challenge to tissues, concentrations of cytokines enter the systemic circulation and this can result in sickness behaviour, pyrexia, cachexia and imbalances in hormones (Waage *et al.*, 1989; Slifka & Whitton, 2000). Cytokines bind with specific cell surface receptors to modulate biological effects and most have the ability to induce varied effects in many cell types, making elucidation of the functions of specific cytokines in normal physiology difficult (Foster, 2001). The effect of the cytokine also depends on the microenvironment of the cell (Sun *et al.*, 1999), for example, the developmental stage of the cell and the local cytokine and hormonal concentration (Hasbold *et al.*, 1999). The main cell source of cytokines is activated microglia and a feedback mechanism exists so that the balance between pro- and anti-inflammatory cytokines is crucial in the control of induction and regulation of activation of microglia and their subsequent responses.

Increased levels of the pro-inflammatory cytokine, IL-1β, has been reported to be increased in the hippocampus of rats exposed to stressors, for example, γ-irradiation (Lonergan *et al.*, 2002; Lynch *et al.*, 2003), behavioural stress (Vereker *et al.*, 2001), in rats injected with LPS (Lonergan *et al.*, 2004) or amyloid-β (Lynch *et al.*, 2007) and also in aged rats (Murray *et al.*, 1997; Murray & Lynch, 1998b; Martin *et al.*, 2002a). Indeed, elevated levels of IL-1β may be a contributory factor in the stress-associated increase in corticosteroid hormones. This suggestion is supported by evidence that IL-1β, injected intrahippocampally, induces activation of the hypothalamo-pituitary axis (Melik Parsadaniantz *et al.*, 1999). Significantly, IL-1β

decreases LTP in all of the major pathways of the hippocampus (Katsuki *et al.*, 1990; Bellinger *et al.*, 1993; Murray & Lynch, 1998b) while IL-1β impairs spatial learning in mice (Gibertini *et al.*, 1995).

Increased hippocampal IL-1β concentration induced by isolation stress increases superoxide dismutase activity which suggests that IL-1β plays a role in the age-related accumulation of reactive oxygen species. Isolation stress has been demonstrated to impair LTP in perforant path–granule cell synapses *in vivo* (Murray & Lynch, 1998b) while the age-related increase in IL-1β is correlated with the age-associated impairment in LTP (Landfield *et al.*, 1979; Landfield & Eldridge, 1994; Bodnoff *et al.*, 1995; Murray & Lynch, 1998b).

IL-4 is an important anti-inflammatory cytokine and has been identified as having protective roles. It inhibits the LPS- and amyloid-β-induced activation of microglia (Szczepanik *et al.*, 2001; Iribarren *et al.*, 2003). Significantly, IL-4 has been shown to be decreased in the aged brain (Maher *et al.*, 2005) and the evidence suggests that it plays a role in controlling IL-1β production. Several reports have indicated that IL-4 has the ability to antagonise IL-1β-induced changes (te Velde *et al.*, 1990; Vannier *et al.*, 1992; Kliewer *et al.*, 1997; Nolan *et al.*, 2005) and it has been shown that IL-4 decreases IL-1β mRNA and protein in the hippocampus *in vivo* and in cultured cells prepared from cortex (Nolan *et al.*, 2005; Lynch *et al.*, 2007).

1.9 Activated microglia

Microglia are the resident immune cells of the central nervous system. These cells perform roles in immune surveillance and function in the inflammatory response. Microglial cells are capable of altering from a resting ramified state, to an activated state with the ability to secrete cytokines such as IL-1β and tumour necrosis factor (TNF)α (Davies *et al.*, 1999; Yu *et al.*, 2002; Li *et al.*, 2003). Activated cells are also capable of phagocytosis and presenting antigen (Chen *et al.*, 2002). In their activated state, these cells can be characterised by up-regulated expression of cell

surface markers such as major histocompatibility complex class (MHC) II and CD11b (Bellinger *et al.*, 1993; Rock *et al.*, 2004). Although the primary role of activated microglia is in the inflammatory response, they have also been shown to trigger neuronal damage (Jeohn *et al.*, 1998; Downen *et al.*, 1999; Xu *et al.*, 1999). Increased microglial activation has been associated with neurodegenerative diseases including Alzheimer's disease (Dong & Benveniste, 2001; Tan *et al.*, 2002), Creutzfeld-Jacob disease (Giulian *et al.*, 1986), human immunodeficiency virus (Gonzalez-Scarano & Baltuch, 1999) although it is not clear whether they are a cause or consequence of the neurodegenerative changes.

1.10 Reactive oxygen species

Atoms can exist in the body with an unpaired electron; these radicals can be highly reactive. The most common radicals result from reduction of O_2 to H_2O during oxidative phosphorylation in mitochondria when ATP is produced; these are referred to as reactive oxygen species. In a normal physiological state, there is a balance between reactive oxygen species formation and protection from these by antioxidant enzymes, the most common of which are copper/zinc superoxide dismutase, manganese superoxide dismutase, glutathione peroxidase and catalase. In addition to enzymes, anti-oxidant scavengers act to defend the body from these free radicals; scavengers include vitamins C and E and glutathione. Oxidative stress occurs when this balance is disturbed and free radical production increases without adequate anti-oxidative defences. This can result in oxidative damage to RNA, DNA, proteins and lipids, which, in turn, can cause deterioration of cell function which can result in further damage.

The brain, relative to other organs, has high energy requirements and uses approximately one fifth of consumed O_2. These factors, along with the high concentration of PUFAs and lipids and limited antioxidant capacity compared to other organs, make the brain especially susceptible to oxidative stress and damage.

Coupled with these features, there is a relatively high level of redox active metals present, such as iron (Lovell & Markesbery, 2007). Studies have shown that the superoxide radical O_2^- is generated by approximately 2% of oxygen in the process of oxidative phosphorylation (Halliwell & Gutteridge, 1989), the superoxide radical can be acted upon by manganese superoxide dismutase and the resultant H_2O_2 is changed to water by either glutathione peroxidase or catalase. When redox active metals are close by, these cause the conversion of H_2O_2 to the highly reactive hydroxide ion, OH^- (Fenton or Haber-Weiss reactions; (Moreira *et al.*, 2005)). It is also known that oxidative stress increases over a lifetime and this gradual build-up of levels of reactive oxygen species can result in unsolicited reactions leading to cell damage, with the induction of signalling pathways which may result in deterioration of cell function.

1.11 Astrocytes and neuroinflammation

Astrocytes are the most numerous of the glial cells. They play a vital role in metabolic support of neurons but in addition they can regulate neuroinflammation. A number of studies have indicated that activated astrocytes may have neuroprotective actions. Following stress, proliferation of astrocytes is common and this can be detected through increased glial fibrillary acidic protein expression. Activated astrocytes act to define regions damaged by insult and limit the movement of leukocytes, containing the inflammatory response. However, astrocytes respond to insult by increasing production of cytokines including pro-inflammatory cytokines and the possibility that they contribute to damage cannot be ruled out.

1.12 Age and neuroinflammation

In addition to morphological changes, increased oxidative stress in cells, synaptic loss (Geinisman *et al.*, 1992), cell loss and decreases in membrane fluidity and transmitter release, inflammatory changes occur in the aged brain (Ye & Johnson, 1999; Godbout *et al.*, 2005). Age is associated with increased activation of microglia

which can result in increased expression of cytokines, chemokines and cell surface markers. It is known that the aged brain is more susceptible to injury, in humans (Nakayama *et al.*, 1994) and in animals (Godbout *et al.*, 2005) and it has been suggested that this is a consequence of the underlying neuroinflammation. Several neurodegenerative diseases are age-associated and are linked with chronic inflammation, for example Alzheimer's disease and Parkinson's disease (McGeer & McGeer, 2004), but it remains to be established whether the neurodegeneration is triggered by the inflammatory changes.

1.13 Age and microglial activation

There is a good deal of evidence indicating that microglial activation is increased in the aged brain. For example, a greater number of microglial cells were found to be present in the hippocampus of aged mice, compared with young (Chen *et al.*, 2008) while MHCII expression is upregulated with age (Godbout *et al.*, 2005; Loane *et al.*, 2007), providing evidence that there is an increase in the number of microglia present in the aged brain and that there is an increase in activated microglia.

One definition of activation in immunological terms, as applied to macrophages, is an increased capacity to destroy microbial pathogens, tumour cells and normal tissues by phagocytosis (Nathan, 1986; MacMicking *et al.*, 1997; Nathan & Muller, 2001) and there is evidence of increased phagocytic activity of microglia in the aged brain (Sheng *et al.*, 1998), including from this laboratory (O'Reilly *et al.*, unpublished). However, the trigger leading to activation of microglia is not known, although one possibility is interferon (IFN)γ, which is increased in the hippocampus of aged rats (Griffin *et al.*, 2006; Maher *et al.*, 2006; Clarke *et al.*, 2008) and which is a potent activator of microglia *in vitro* and *in vivo* (Panek & Benveniste, 1995; Grau *et al.*, 1997; Nguyen & Benveniste, 2002). Another possible mechanism by which microglial activation may be modulated is via a change in interaction between these cells and adjacent cells, for example neurons. It has been shown that CD200 receptor

(CD200R) and CD200 ligand, a type 1 membrane glycoprotein (Jenmalm *et al.*, 2006), interact to maintain microglia in a resting state (Hoek *et al.*, 2000). CD200 is expressed on a range of cell types, including neurons, T cells and B cells (Barclay *et al.*, 2002; Lyons *et al.*, 2007), with tissue distribution in mice similar to that found in humans (Wright *et al.*, 2001). CD200R is only expressed on cells of the myeloid lineage, including macrophages and microglia. The intracellular part of CD200 is small and it is improbable that ligation leads to signalling within neurons (Gwyer *et al.*, 2006), it is through interaction with its receptor that CD200 ligand exerts its effects, maintaining microglial cells in an inactive quiescent state, by triggering a negative signal. CD200 protein has been reported to be decreased in the aged brain, contributing to the age-related increase in microglial activation (Lyons *et al.*, 2007) and the age-associated increase in pro-inflammatory cytokines (Murray *et al.*, 1997; Murray & Lynch, 1998b; Martin *et al.*, 2002a). CD200 was first shown to attenuate production of TNFα and IFNγ in a mouse model of arthritis (Gorczynski *et al.*, 2001).

1.14 LPS: a model to study neuroinflammation

LPS is a major component of the cell wall of Gram-negative bacteria. The identification of this bacterial toxin by the immune system induces both innate and adaptive immune responses and results in the expression of inflammatory mediators, induction of pro-inflammatory cytokines and even cell death. LPS leads to the activation of microglia *in vitro*, resulting in the release of pro-inflammatory cytokines including IL-1β in the hippocampus (Lynch *et al.*, 2004). The use of LPS is acknowledged as an effective approach in the investigation of inflammatory changes in the brain, as it also reliably induces an increase in microglial activation and in concentration of the pro-inflammatory cytokine IL-1β in the hippocampus following intraperitoneal administration (Lonergan *et al.*, 2004).

LPS has the ability to exert effects on both neurons and glia. It can act directly on glia by binding to the serum protein, LPS-binding protein, and the soluble or anchored protein CD14. This complex can then bind to the cell plasma membrane

Toll-like receptor (TLR) 4, known to be expressed on microglia, the resident immune cells of the central nervous system. Activation of TLR4 induces downstream signalling cascades including activation of the transcription factor nuclear factor kappa B (NFκB). NFκB is a necessary participant in the process of regulation of inflammatory responses (Palsson-McDermott & O'Neill, 2004). Along with IL-1β, other pro-inflammatory cytokines, like IL-6 and TNFα are released from microglia in response to LPS.

1.15 LPS and microglial activation

Although most authors report that LPS injection markedly increases microglial activation and pro-inflammatory cytokine production in the brains of young rats (Godbout *et al.*, 2005; Tanaka *et al.*, 2006; Henry *et al.*, 2008), others have reported that LPS treatment caused no significant change in microglial cell morphology (Cunningham *et al.*, 2005). The effects of LPS on cytokine production and microglial activation have been shown to be exaggerated in the brain of aged mice and this has been attributed to the pre-existing age-related neuroinflammation. It has been shown recently that LPS administered to the periphery results in hyperactivity of microglial cells in the aged mouse brain, as evidenced by increased induction of IL-1β and increased MHCII expression on microglia (Henry *et al.*, 2008). However, differential changes have been described; for example, ageing did not exacerbate the effects of LPS in terms of mRNA expression of cytokines (Chen *et al.*, 2008). Interestingly, Godbout and colleagues report that the aged animals lost more weight following peripheral LPS injection, compared with adult mice (Godbout *et al.*, 2005). Evidence shows that polyunsaturated fatty acids (PUFAs) can modulate microglial cell activation. EPA has been shown to attenuate increases in MHCII and CD40 and the age-related increase in IL-1β, of which microglia are a likely source (Lynch *et al.*, 2007).

1.16 Lipids and their structure

Lipids have traditionally been defined as a class of compounds which have a high level of solubility in organic solvents and can be thought of as "fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" (Christie, 1989). Lipids are capable of adopting complicated structures, ranging from simple lipids to complex and are essential structural components of cell membranes, as their bilayer forms a barrier between the intra- and extra-cellular compartments.

1.17 Fatty acids

In general, fatty acids are composed of a hydrocarbon chain with a reactive carboxyl group capable of forming ester links. Fatty acids can have chain lengths varying from 2 to 36 carbon atoms; animal tissues commonly contain fatty acids of chain length from 14 to 22 carbons. In general, the 1-acyl position in a phospholipid is esterified with a saturated fatty acid and the 2-acyl position with a PUFA.

1.17.1 Nomenclature of fatty acids

The systemic names for fatty acids are determined by the number of carbon atoms in the acyl chain and number of double bonds (Youdim *et al.*, 2000). The position of the double bonds is identified by numbering the carbon atom on which the double bond occurs, beginning at the carboxyl carbon. The shorthand for this identification can be written as X:Y, where X is the number of carbon atoms in the acyl chain and Y is the number of double bonds. Fatty acids can be classified into families and denoted as (n-x) or (ω-x) where x is the number of carbon atoms from the last double bond to the methyl ($-CH_3$) group and n or n is the chain length of the fatty acid. In this way, fatty acids can be termed as n-3, n-6 and n-9, that is, the distinction between n-3, n-6 and n-9 fatty acids is derived from the location of the first double bond, starting at the methyl end of the fatty acid.

1.17.2 Biosynthesis and sources of essential fatty acids

Saturated fatty acids can be desaturated, a process that can occur within the endoplasmic reticulum. This has been identified in animals; however, mammals lack the desaturases necessary to introduce double bonds beyond the C9 position in the acyl chain. Consequently, the fatty acids linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), the two compounds from which the n-6 and n-3 families of PUFAs are synthesized, are incapable of being synthesized within mammalian cells, owing to a lack of the enzymes, Δ12 desaturase and Δ15 desaturase. Since humans are incapable of producing these fatty acids, dietary intake is relied upon. In 1929, Burr and Burr reported that linoleic acid and α-linolenic acid were essential fatty acids for mammals (Burr & Burr, 1929). Omega-9 fatty acids are not classed as essential fatty acids due to the fact that humans are capable of synthesizing these.

The biological function(s) of n-6 and n-3 fatty acids include roles in maintenance of the fluidity of cell membranes and maintenance of structural integrity (Stillwell *et al.*, 1993) which is partly due to the fact that PUFAs can interact with proteins (Salem & Niebylski, 1995). The PUFA composition of cell membranes has been shown to affect the effectiveness of membrane transporters and enzymes (Okuyama, 1992; Goldberg & Zidovetzki, 1997; Pearce *et al.*, 1997). PUFAs also play a part in modulating enzyme activity and are substrates for enzymes, for example phospholipase A_2 (PLA_2), which leads to the production of second messengers (Martin, 1998). PUFAs are precursors for eicosanoids, diverse mediators of inflammation, capable of being metabolised to several types of lipid mediators including prostaglandins, leukotrienes and thromboxanes (Schror, 1993). Each mediator then acts to regulate inflammation, immune response and tissue repair.

While long chain PUFAs can be formed from dietary linoleic acid and α-linolenic acid, the concentration of the long chain PUFA produced in this way is not sufficient for the body's requirements. For this reason, long chain PUFAs are obtained from the diet directly. As desaturation enzymes are present in mammals,

linoleic acid can be acted upon to form γ-linolenic acid (GLA, 18:3n-6) and a number of other compounds. With the use of enzymes from the same family, α-linolenic acid can be converted to eicosapentaenoic acid (EPA, 20:5n-3; Figure 1.3 and Figure 1.4) which can subsequently be converted to docosapentaenoic acid (DPA, 22:5n-3; Figure 1.3 and Figure 1.4) by a reaction involving the enzyme elongase. DPA can then be further metabolised to form docosahexanoic acid (DHA, 22:6n-3; see Figure 1.3; (Sprecher, 2000)) through a series of desaturation and elongation reactions (Sprecher, 1989; Voss *et al.*, 1991). As n-3 and n-6 families compete for the elongases and desaturases (Blond & Lemarchal, 1984), dietary intake affects the production of PUFA. It has been shown that Δ5 and Δ6 desaturases exist in liver microsomes of the rat (Brenner, 1969) and that the conversion of linoleic acid and α-linolenic acid to 20- and 22-carbon forms occurs by desaturation in liver and brain of the rat (Sinclair, 1975).

1.17.3 Essential fatty acids in the diet

Recommended intake of n-3 from the American Heart Association is at least two servings of fish a week, however, the n-3 content of fish is highly variable ranging from negligible levels detected in some fish to as high as 4g n-3 PUFA /100g of fish (Weaver *et al.*, 2008). Recommendations of daily intake for patients with cardiovascular disease is 1g EPA + DHA and it is even higher at 2-4g EPA + DHA for hypertriglyceridemia sufferers (Lichtenstein *et al.*, 2006). In reviews by Bjerve, 1991, the general recommendation of essential fatty acids is between 3-6% total fat. For α-linolenic acid, the parent compound from which EPA, DPA and DHA are synthesized, 0.5 -1% total fat is required. Optimum dietary intake of EPA has been estimated at 900mg/day EPA (Bjerve, 1991). It has been reported that high amounts of ingested essential fatty acids end up as constituents of phospholipids in the phospholipid bilayer of cell membranes (Carlier *et al.*, 1991).

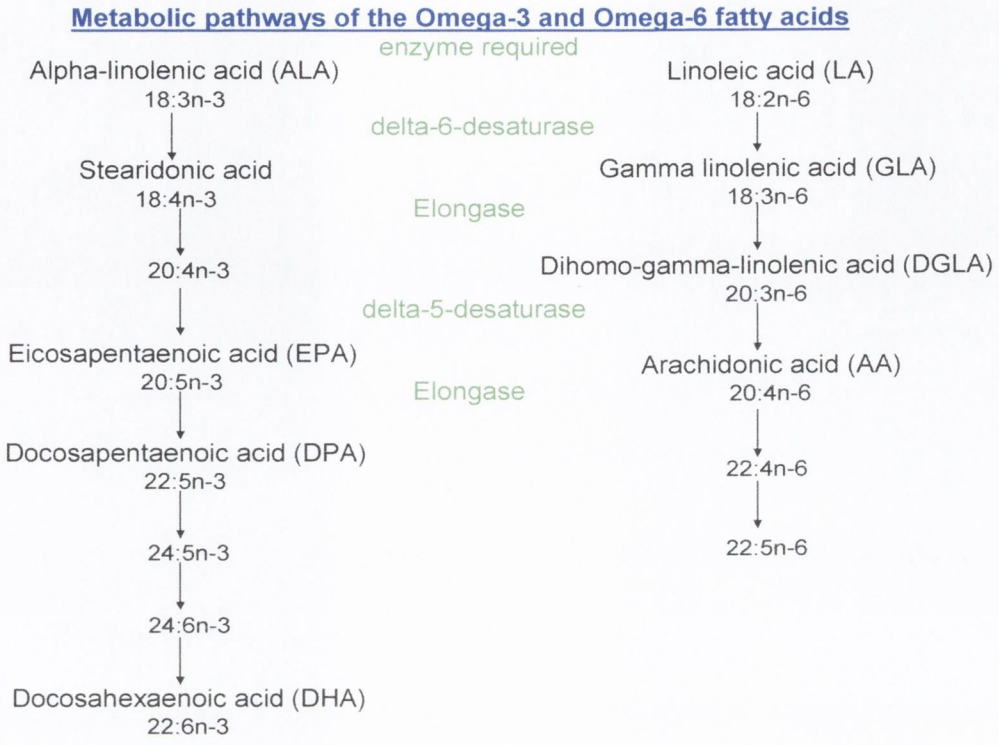
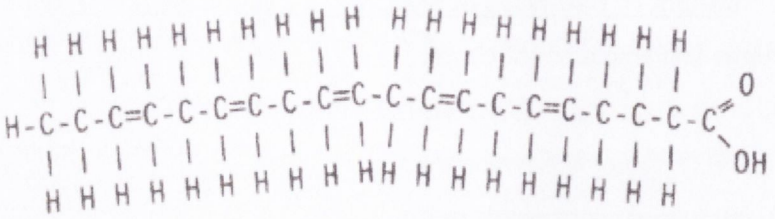
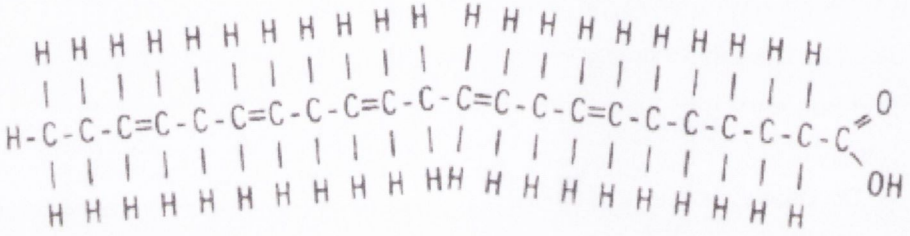


Figure 1.3 Metabolic pathways of n-3 and n-6 fatty acids



Eicosapentaenoic acid (EPA)
c20:5n-3



Docosapentaenoic acid (DPA)
c22:5n-3

Figure 1.4 **Structures of eicosapentaenoic acid and docosapentaenoic acid**

A number of studies have shown that, in the last few decades, fundamental modifications have occurred in diet in terms of the type and quantity of essential fatty acids consumed and the levels of antioxidants contained in foods (Simopoulos, 1991, 1999). Lifestyles in industrialised societies of today are typically associated with increased intake of energy coupled with a reduction in energy expenditure. In dietary terms, there is an increase in n-6 and trans fatty acids coupled with decreased n-3 PUFA intake, lower intake of complex carbohydrates and fibre along with fruit and vegetables, decreased protein and calcium, in conjunction with less antioxidants (Eaton & Konner, 1985; Leaf & Weber, 1987; Simopoulos, 1998, 1999).

Increased trans fatty acids, generated through partial hydrogenation of oils, has been reported to be harmful (Simopoulos, 1995). These fats can affect desaturation and elongation of n-3 and n-6 PUFAs as these families compete for the same enzymes. Accordingly, changes in Western diet, especially over the past century, have led to increased incidence of chronic illnesses, for instance, obesity, diabetes, arthritis, hypertension, atherosclerosis, autoimmune diseases, and breast (Shannon *et al.*, 2007), colon (Hughes-Fulford *et al.*, 2005) and prostate (Hedelin *et al.*, 2007) cancers.

1.17.4 Omega-6/omega-3 ratio

The balance between intake of n-3 and n-6 PUFA is important when you consider that the two main sources of EPA and DHA are fish and poultry (Raper *et al.*, 1992). Studies have been undertaken in order to determine the optimal n-3: n-6 ratio (Yehuda & Carasso, 1993). It has been suggested that the impact of essential fatty acids on brain function comes to light when there is a deficiency in PUFA intake (Kaplan & Greenwood, 1998).

The ideal dietary ratio of 1:1 n-6:n-3 (Raper *et al.*, 1992) intake is considered to have been in place for millions of years (Simopoulos, 2008) due to the presence of

n-3 in fish, meat, wild flora, eggs, nuts and berries (van Vliet & Katan, 1990; Zeghichi *et al.*, 2003; Simopoulos, 2004). Western diets now consist of a ratio closer to 15:1 or 20:1 (Eaton & Konner, 1985; Simopoulos, 1991) with sources of n-6 including margarine, bread, biscuits and chocolate products (Sioen *et al.*, 2007). This can be attributed to economic status, food technology and changes in the agriculture business (Simopoulos, 2008). Intake of cod-liver oil, a good source of n-3 PUFA, has been declining and consequently intake of EPA and DHA has been reduced. Today, it is important to note that comparisons between diets from around the world show epidemiologically that the ratio of n-6:n-3 can be correlated with percentage of cardiovascular disease related deaths (Weber, 1989), while optimal brain function is also related to adequate intake of PUFAs in the appropriate ratio (Kaplan & Greenwood, 1998).

The composition of fatty acids in brain membrane can be altered by diet (Bourre *et al.*, 1990; Fernstrom, 1999) and high concentrations of long chain PUFAs have been shown to have the greatest effect on PUFA levels in the brain. Indeed, a decline in n-3 PUFAs can be reversed within weeks (Galli *et al.*, 1971; Connor *et al.*, 1990). For example, in one study, n-3-deficient chicks were given a control diet based upon corn oil or an experimental diet supplemented with DHA and the experimental diet restored the concentration of DHA in brain and retina (Anderson & Connor, 1994).

1.18 Brain PUFA composition

It has come to light that PUFAs have important roles within the central nervous system, initially, perhaps, through the recognition that these lipids are in such high concentration in neural and retinal tissues. General behavioural patterns of rats and mice fed varying amounts of n-3 and n-6 PUFAs have shown that diets high in n-3, compared with n-6, have more beneficial outcomes in terms of brightness-discrimination learning ability and retinal function (Okuyama, 1992), while PUFA intake is associated with the suppression of carcinogenesis, allergic hyper-reactivity,

thrombotic tendency, apoplexy, hypertension and ageing in animals (Okuyama, 1992; Kaplan & Greenwood, 1998; Mozaffarian *et al.*, 2004).

Although arachidonic acid is an important component of brain lipids, the main PUFA constituent of brain is DHA (Youdim *et al.*, 2000), with a particularly high concentration in synaptosomes (Viani *et al.*, 1991; McGee *et al.*, 1994), mitochondria (Willumsen *et al.*, 1996), microsomes (Srinivasarao *et al.*, 1997) and nerve growth cones (Martin & Bazan, 1992). DHA can affect membrane fluidity, have effects upon proteins vital for cell functions and can modulate synaptic transmission (Salem *et al.*, 1986).

Studies have suggested that astrocytes work together with endothelial cells to synthesize and release PUFA, thus playing a role in the maintenance of the PUFA-rich environment that exists in the brain (Moore, 2001). Moore and colleagues (1991) incubated primary rat neuronal cultures with 1-14C 18:3n-3 or 1-14C 20:5n-3 and investigated the presence of their elongation and desaturation products. The results from these experiments revealed that neuron cultures could not generate substantial quantities of Δ4 desaturase products. The neuron cultures did not prove to be very capable of desaturation at any of the stages of the n-3 PUFA pathway but were capable of elongation steps (Moore *et al.*, 1991). However, the study did reveal that astrocytes could perform all of the elongation and desaturation steps (Moore *et al.*, 1991). Interestingly, most of the long-chain PUFAs generated by astrocytes were liberated into the extracellular fluid and the evidence indicated that neurons quickly accumulated these products. This set of experiments suggested that astrocytes are important in supporting neurons by forming long-chain PUFAs and making these available for neurons. Cerebral endothelial cells can also aid in the enrichment of the brain with PUFAs (Moore *et al.*, 1991). These endothelial cells can increase n-3 PUFA in the basolateral compartment (extracellular space) and this increase is further enhanced when these cells are cultured with astrocytes (Moore *et al.*, 1991). This points to roles for astrocytes and endothelial cells working together to synthesize and make long chain PUFAs available for neurons.

1.19 Protective effects of n-3 PUFA

The first indications that n-3 PUFA were beneficial for health came from epidemiological studies on the Greenland Inuit and comparisons with gender and age matched groups in Denmark. It was revealed that the Greenland Inuit, who consume a diet rich in seafood, containing large quantities of n-3 PUFA, had a much lower incidence of chronic inflammatory disorders such as asthma, type I diabetes mellitus, myocardial infarction and autoimmune disease compared with the matched Danish groups (Kromann & Green, 1980). Since this first epidemiological analysis, increasing numbers of favourable positive health effects of n-3 PUFA have come to light including beneficial effects on inflammatory bowel disease and rheumatoid arthritis (Simopoulos, 2002). Clinical trials assessing anti-inflammatory effects of fish oil dietary supplementation have shown to have positive effects on a range of diseases involving inflammation and autoimmune diseases including Crohn's disease, ulcerative colitis and migraines (Calder, 2008). In addition, there is the mounting evidence that a diet high in PUFA can lower plasma lipid levels and, in this way, reduce disease of the cardiovascular system (Nieuwenhuys & Hornstra, 1998).

Similarly, LPS-induced IL-6, IL-1β, TNFα and a number of other inflammatory markers were investigated in clinically normal dogs following dietary supplementation with EPA and DHA for a twelve week period. Serum concentration of IL-1β, IL-6 and prostaglandin E_2 (PGE_2) were all significantly increased after LPS administration, but these increases were attenuated in serum obtained from dogs which had received EPA and DHA, compared with dogs which were orally administered a sunflower oil-based diet (LeBlanc *et al.*, 2008). Interestingly, the LPS-induced increase in TNFα was unaffected by the n-3 PUFA supplementation (LeBlanc *et al.*, 2008). These studies provide information that highlights that PUFAs act differentially on IL-1β, IL-6 and TNFα and this was supported by data from another study in which no effect of EPA on TNFα and IFNγ were reported (Maes *et al.*, 2007).

Further evidence of an anti-inflammatory action for EPA and DHA was obtained from a study in which mice were fed diets supplemented with EPA and DHA and cultures of splenocytes prepared from these animals were challenged with the T cell mitogen concanavalin A which increases ceramide (Jolly *et al.*, 1996). The data showed that dietary EPA and DHA attenuated the production of ceramide induced by the concanavalin A (McMurray *et al.*, 2000).

Weldon and colleagues investigated the effect of pure EPA and DHA on cytokine expression in human THP-1 monocyte-derived macrophages. They reported that pre-incubation of these cells with EPA or DHA significantly attenuated LPS-induced increases in IL-1β, IL-6 and TNFα production and mRNA expression and decreased the LPS-induced increase in NFκB activation. DHA was reported to be more effective than EPA at attenuating these LPS-induced inflammatory changes (Weldon *et al.*, 2007).

1.20 Neuroprotective effects of n-3 PUFA

The anti-inflammatory effects of PUFAs have recently been shown to extend to brain where treatment of aged rats with EPA decreased activation of microglia and the concentration of inflammatory cytokines in the hippocampus (Lynch *et al.*, 2007). Since the evidence has indicated that LTP is decreased when hippocampal IL-1β concentration in increased as described in section 1.3.3, it was not surprising to find that the age-related deficit in LTP was reversed by treatment of rats with DHA (McGahon *et al.*, 1999a) or EPA (Lynch *et al.*, 2007). EPA has also been demonstrated to increase IL-4 (Kavanagh *et al.*, 2004) which is downregulated in the aged brain (Maher *et al.*, 2005).

In terms of behaviour mediation, the first study to make evident the influence of dietary fat on behaviour was that by Yehuda *et al.*, 1986, without a deficiency in essential fatty acid. In this study, the effects of dietary fat in rats were examined in terms of effect on pain threshold, thermoregulation and motor activity. Rats which

received a diet enriched with PUFA (soybean oil in this study) were more capable of regulating their body temperature in a cooler ambient temperature following an amphetamine challenge, aimed to induce hypothermia, and pain threshold was increased by 3 weeks supplementation with soybean oil, compared with control diet (Yehuda *et al.*, 1986). An analogous study the following year by the same group corroborated these findings (Yehuda & Carasso, 1987). The effects of dietary fat composition on cognitive performance have also been examined. The first study, using the Morris water maze (Morris, 1984), was a spatial memory test, in which animals are compelled to learn where a platform submerged under water is located in a pool (Coscina *et al.*, 1986; Yehuda & Carasso, 1987). Rats fed the PUFA soybean diet were shown to perform better at this task in comparison to a lard diet (Coscina *et al.*, 1986; Yehuda & Carasso, 1987).

Dietary fat intake and performance in neurocognitive tests has been investigated using numerous other behavioural tests sensitive to various aspects of learning and memory, reliant upon different regions of the brain for best possible performance and changes in performance have been linked with ageing. A reduced ability of animals which received lard-based diets, compared with PUFA oral administration, to perform at Olton's radial arm maze (spatial memory task), a variable interval delayed alternation task (investigating temporal memory) and Hebb-Williams maze (spatial learning task) were reported (Greenwood & Winocur, 1990) and the inability of rats to learn in specific mazes in the Hebb-Williams task and Olton's task has been correlated with learning and memory impairment in the hippocampus (Olton, 1983; Winocur & Moscovitch, 1990; Kesner, 1991).

In addition to its effects on age-related changes, EPA has been shown in our laboratory to attenuate the increase in hippocampal IL-1β induced by LPS- (Lonergan *et al.*, 2004) or by exposure to whole body γ-irradiation (Lynch *et al.*, 2003). Consistently, it has been reported that mice which received a dietary supplementation of EPA for a period of five weeks exhibited a diminished response to an

intraperitoneal injection of LPS in terms of plasma concentrations of TNFα. IL-6, IL-10 and IL-1β (Sadeghi *et al.*, 1999).

Another approach in the investigation of the effects of n-3 PUFA on neuronal function has been the direct application of n-3 PUFA to neurons in which modulatory effects on Na^+ and Ca^{2+} currents in CA1 hippocampal neurons have been described (Vreugdenhil *et al.*, 1996). In this study, isolated hippocampal CA1 neurons were treated extracellularly with EPA and this application resulted in a shift of the voltage-dependence of inactivation of Na^+ and Ca^{2+} currents to hyperpolarising potentials, in a dose-dependent manner (Vreugdenhil *et al.*, 1996). It was suggested that EPA may exert anti-convulsive effects *in vivo*, as the effect increased the speed of inactivation and slowed down recovery from this inactivation.

DHA and EPA have been shown to decrease activities of PKC, PKA, extra-cellular signal regulated kinase (ERK) and CaMKII in the brain (Mirnikjoo *et al.*, 2001). These authors reported that LTP induction was impaired following perfusion of EPA and DHA onto hippocampal slices *in vitro*, and suggested that this was a consequence of decreased enzyme activity (Mirnikjoo *et al.*, 2001). Interestingly, a single intracerebroventricular injection of DHA was shown to inhibit LTP in the CA1 but not in the dentate gyrus (Itokazu *et al.*, 2000). While no inhibitory effects of dietary EPA or DHA on LTP in the dentate gyrus have been reported (McGahon *et al.*, 1999a; Martin *et al.*, 2002b; Lynch *et al.*, 2007). These data suggest that the effects of fatty acids on LTP are dependent on dose, mode of administration and area studied. In addition to its effect on LTP, DHA has been shown to inhibit long-term depression (Young *et al.*, 1998).

Deprivation of PUFAs in the diet and impacts on brain lipid membrane composition (Delion *et al.*, 1997) have been shown to decrease learning ability of rats (Gamoh *et al.*, 1999). Conversely, increasing n-3 fatty acids intake in the diet has been shown to improve ability in learning and memory tasks in young rats (Suzuki *et al.*, 1998). Nishikawa and colleagues suggested that beneficial effects of n-3 fatty acids may occur as a consequence of physical changes in the membrane and thereby

influencing the release of neurotransmitters and altering receptor and ion channel functions (Nishikawa *et al.*, 1994). This is consistent with evidence from McGahon and colleagues (1999), who reported that DHA reversed the age-related decrease in glutamate release in hippocampus (McGahon *et al.*, 1999a).

Oxidative stress plays an important role in ageing and in neurodegenerative disease, and ageing is a risk factor for many neurodegenerative diseases. Factors which affect levels of oxidative stress in individuals include genetic mutations and environmental factors such as exposure to irradiation (Lonergan *et al.*, 2002) or toxins, for example LPS (Nolan *et al.*, 2003). Inflammation can also result in reactive oxygen species production (Nolan *et al.*, 2003) which can lead to neuronal death and which play a role in cell death associated with Parkinson's disease (Tansey *et al.*, 2008) and Alzheimer's disease (von Bernhardi, 2007). Hence, it is important to investigate ways to alleviate oxidative stress and one of the aims of this study was to establish whether oral administration of EPA and DPA might exert an effect upon oxidative stress in the cortex and hippocampus of aged rats. An age-related increase in peroxidation of lipids has been described (Youdim *et al.*, 2000) and there is some evidence that this extends to the hippocampus (Petursdottir *et al.*, 2007; Hwang *et al.*, 2008) where a neuroprotective effect of PUFAs has been suggested (McGahon *et al.*, 1999b). A neuroprotective effect of DHA has also been described *in vitro*.

Enrichment of Neuro 2A cells with DHA significantly reduced DNA fragmentation induced via serum starvation (Kim *et al.*, 2000). This effect was in contrast to a shorter experimental time period of 5h, after which no positive effect of DHA was observed. A time period of 24h enrichment with DHA was deemed sufficient to show protection of cells from apoptosis and 48h incubation with DHA offered even further protection, with less serum starvation-induced DNA fragmentation reported (Kim *et al.*, 2000). Not only was this protective effect of DHA revealed through a decrease in DNA fragmentation, but also there was a significant decrease in caspase 3 activity, assessed by colorimetric assay, in cells which were enriched with DHA, for 24h or more, and then serum deprived for up to 48h,

compared with serum-deprived controls (Kim *et al.*, 2000). There was a concomitant decrease in Hoechst staining as well. Interestingly, there was a need for DHA to be present in the media before the serum starvation period, otherwise the protective effect was not observed and this is in agreement with the findings of Kishida and colleagues (1998).

1.21 Sphingolipids and signalling

It has recently been shown that sphingolipids are major signalling molecules (Hannun, 1994; Huwiler *et al.*, 2000; Yabu *et al.*, 2008). They are a major class of lipids located in plasma membranes, Golgi membranes, endosomes and lysosomes (Merrill *et al.*, 1997; Huwiler *et al.*, 2000). Ceramide, an important second messenger, is sphingolipid-derived and is generated when sphingomyelin is acted upon and hydrolysed by the enzyme sphingomyelinase (see Figure 1.5). Sphingomyelin and ceramide are involved in the regulation of cell proliferation, differentiation and survival and sphingomyelin is essential for cell division and differentiation during early development (Longo *et al.*, 1997; Falcone *et al.*, 2004).

At low concentrations, sphingomyelin and ceramide function to ensure cell survival, while higher concentrations induce cell dysfunction or death with an abundance of evidence linking ceramide and apoptosis (Gulbins *et al.*, 2000; Vedin *et al.*, 2008). Specifically, low concentration of C2-ceramide promotes survival of cultured embryonic rat neurons, whereas high concentration induces apoptosis (Goodman & Mattson, 1996). It has been shown that ceramide leads to production of reactive oxygen species in rat adrenal pheochromocytoma cells and that this contributed to their susceptibility to damage (Denisova *et al.*, 1999; Denisova *et al.*, 2001).

Acid sphingomyelinase is a lysosomal enzyme and a deficiency results in Niemann-Pick disease in humans. Neutral sphingomyelinase is associated with the plasma membrane and is located predominantly intracellularly at the plasma

membrane at regions of cell-cell contact (Marchesini & Hannun, 2004). This enzyme is activated in response to stimuli including TNFα, Fas ligand and vitamin D (Dbaibo *et al.*, 1997) and its regulation may require arachidonic acid and PLA_2, whereas ceramide and sphingomyelin can modulate PLA_2 activity (Ballou *et al.*, 1996). Sphingomyelinase activity is also modulated by the cellular redox state and glutathione negatively regulates sphingomyelinase activity (Yang *et al.*, 1997). Increases in the membrane lipids sphingomyelin and sphingosine-1-phosphate, coupled with a decrease in glutathione may be an explanation for heightened susceptibility to oxidative stress (Youdim *et al.*, 2000; Crivello *et al.*, 2005).

Mammalian brain-specific Mg^{2+}-dependent neutral sphingomyelinase has emerged as being of particular importance in terms of ceramide production stimulated by stress (Irmler *et al.*, 1997). Cells which over-express this sphingomyelinase have been shown to have reduced sphingomyelin and raised ceramide levels (Marchesini *et al.*, 2003). Neutral sphingomyelinase, expressed predominantly in neurons throughout the central nervous system, has been linked to growth and differentiation, as sphingomyelinase$^{-/-}$ mice, devoid of neutral sphingomyelinase have been reported to develop dwarfism and delayed onset of puberty (Stoffel *et al.*, 2005).

Stimulation of intestinal epithelial cells with LPS has been shown to induce IL-8 via the activation of sphingomyelinase and production of ceramide (Sakata *et al.*, 2007b), linking sphingomyelinase with inflammatory bowel disease, such as ulcerative colitis and Crohn's disease. Activation of sphingomyelinase may be a key step in mediating LPS effects, as incubation of intestinal epithelial cells in the presence of a sphingomyelinase inhibitor prevented the LPS-induced increase in NFκB activation (Sakata *et al.*, 2007a). Consistently, oral administration of the sphingomyelinase inhibitor SMA-7 to mice with dextran sulphate sodium-induced colitis significantly reduced colonic injury (Sakata *et al.*, 2007a). LPS has also been shown to activate sphingomyelinase in macrophages (Sakata *et al.*, 2007a) and in immature (but not mature) dendritic cells where the subsequent production of ceramide was coupled with evidence of apoptosis (Falcone *et al.*, 2004).

In 2001, Cutler and Mattson proposed a molecular cascade in the activation of acid and neutral sphingomyelinase. The activation of the p55 TNF receptor causes the cytoplasmic domain of p55 to bind to a novel protein called FAN which couples to neutral sphingomyelinase. Activation of acid sphingomyelinase is proposed to rely on the binding of recruited proteins TRADD, TRAF2 and FADD to the death domain of p55 which results in receptor endocytosis and activation of sphingomyelinase within endosomes. It was proposed that the resulting ceramide formed stimulated apoptosis by a mitochondrial-dependent pathway (Cutler & Mattson, 2001).

Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) can induce an apoptotic cascade by interacting with Fas ligand and death receptor (DR) 4/DR5 respectively. Following ligand-receptor binding, the Fas-associated death domain is engaged (Ashkenazi, 2002) and acts to recruit caspase 8 (Kischkel *et al.*, 1995) which leads to activation of caspase 3 (Thornberry *et al.*, 1997) Caspase 3 acts as an executioner caspase, leading to DNA fragmentation and apoptosis (Li *et al.*, 1997).

There is some evidence to suggest that there are differences in the regulation of sphingomyelin and ceramide between neurons and glia. Neurons have been reported to have high sphingomyelinase activity *in vitro* accompanied by low sphingomyelin synthase (Kilkus *et al.*, 2008); it was shown that neurons convert exogenous sphingomyelin to ceramide but the reverse action was much slower. Distinct from these results, primary cultured oligodendrocytes exhibited slow conversion of sphingomyelin to ceramide, suggesting low sphingomyelinase activity, and prompt conversion of ceramide to sphingomyelin, suggestive of high levels of activity of sphingomyelin synthase in oligodendrocytes (Kilkus *et al.*, 2008).

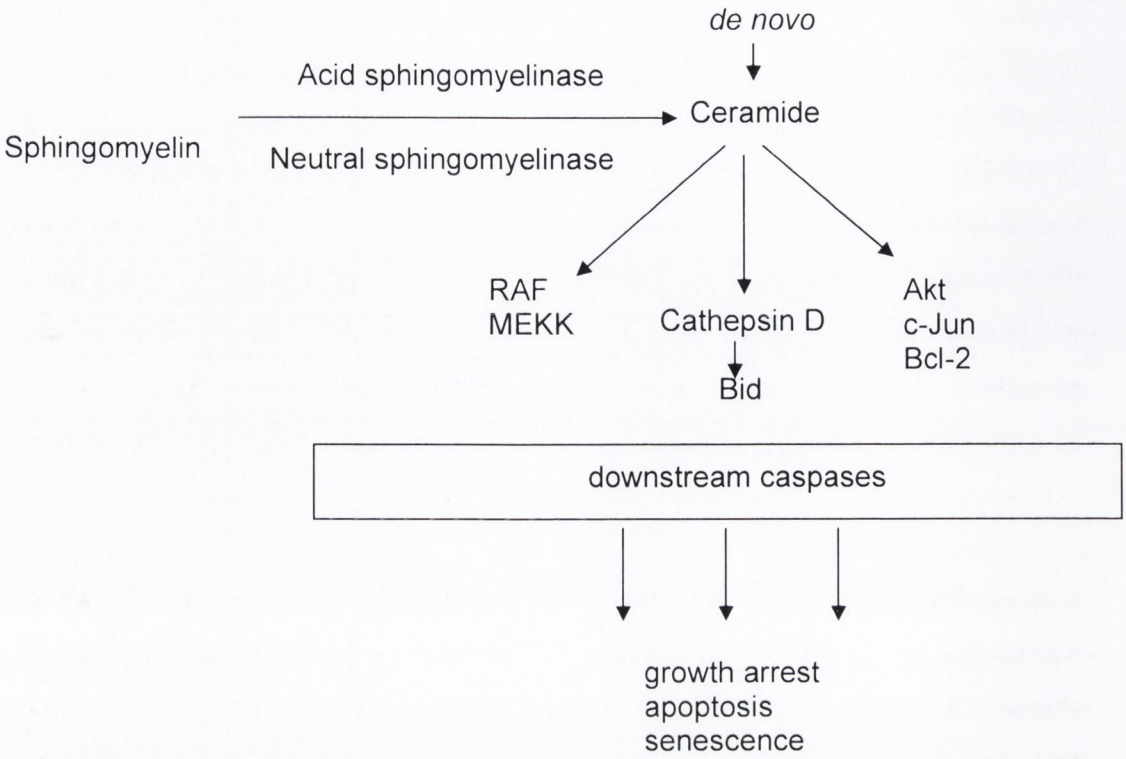


Figure 1.5 Actions of sphingomyelinase and ceramide

Adapted from Ogretmen and Hannun, 2004 (Ogretmen & Hannun, 2004).

1.22 Eicosanoids

Eicosanoids are a group of chemical messengers that have roles in the immune response (Youdim *et al.*, 2000). When stimulated, membrane lipids can be changed to form biologically active lipid mediators which can then serve as either intracellular or extracellular signals. As precursors for eicosanoids, PUFA are capable of generating prostaglandins, thromboxanes, leukotrienes, intermediate hydroperoxyeicostetraenoic (HPETE) and hydroxyeicosatetraenoic (HETE) acids, and this process begins by hydrolysis of fatty acids from the 2-acyl position of phospholipids, which is generally by the action of PLA_2 (Smith, 1992).

Prostaglandins and leukotrienes have been shown to act via transmembrane receptors affecting the cell which produced them or nearby cells and thereby affecting cell function (Smith, 1992; Brooks & Summers, 1996). EPA and DHA are precursors for the 3-series of prostanoids and the 5-series of leukotrienes (Das, 2006) but the corresponding metabolites of arachidonic acid have been shown to be more potent in terms of inflammatory properties.

1.23 Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPAR)s are part of a subfamily of the nuclear hormone receptor family. There are 3 isoforms of PPAR; PPARα, originally described in mouse liver (Issemann & Green, 1990), PPARβ/δ (Dreyer *et al.*, 1993) and PPARγ (Dreyer *et al.*, 1993), which heterodimerise with 9-cis-retinoic acid retinoid X receptors (Kliewer *et al.*, 1992; Kliewer *et al.*, 1994), and become activated transcription factors (Mangelsdorf *et al.*, 1990). PPARs are expressed in mice (Issemann & Green, 1990; Zhu *et al.*, 1993; Kliewer *et al.*, 1994), rats (Gottlicher *et al.*, 1992) and humans (Schmidt *et al.*, 1992; Sher *et al.*, 1993; Mukherjee *et al.*, 1997). PPARγ is principally expressed in adipose tissue and has been shown to be important for the conversion of pre-adipocytes to adipocytes (Spiegelman & Flier, 1996). It is also expressed in the brain, monocytes,

macrophages (Ricote *et al.*, 1998), large intestine, spleen (Kliewer *et al.*, 1994; Braissant *et al.*, 1996) and heart. PPARs and PPARγ in particular, have been reported to be activated by fatty acids, for example DHA and anti-diabetic thiazolidinediones (Keller *et al.*, 1993; Forman *et al.*, 1997; Kliewer *et al.*, 1997; Krey *et al.*, 1997), see Figure 1.6. Li and colleagues have shown that both EPA and DHA down-regulate LPS-induced activation of NF-κB through a PPARγ-dependent pathway in HK-2 cells. Their data indicates that EPA and DHA may be having a beneficial anti-inflammatory effect through a mechanism involving PPARγ activation (Li *et al.*, 2005).

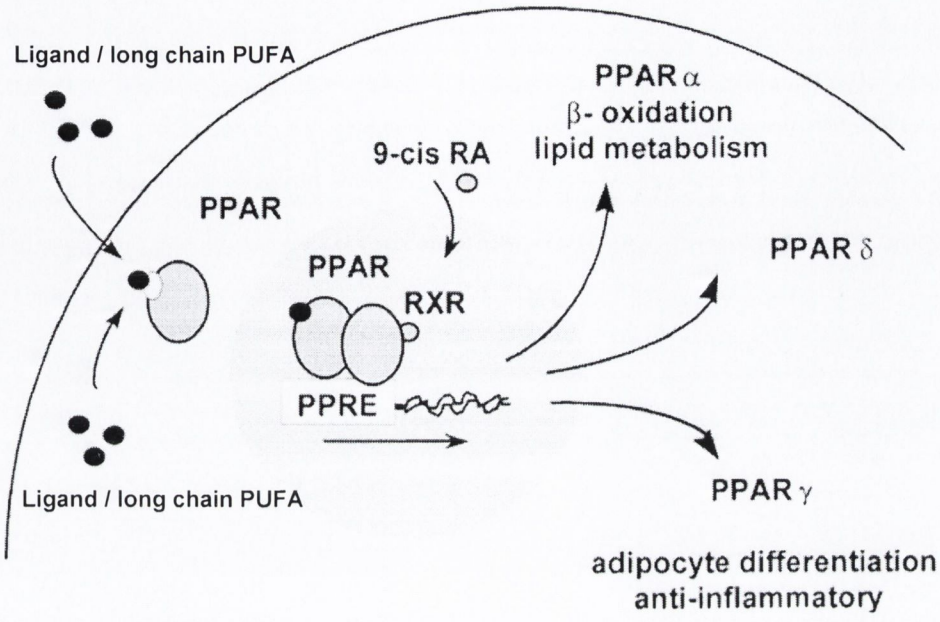


Figure 1.6 Activation of PPAR

PPAR receptors may be activated by PUFA, resulting in accumulation in the nucleus where PPAR can heterodimerise with retinoid X receptors (RXR). This complex can bind to DNA sequences known as PPAR response elements (PPRE) and this leads to transcription leading to anti-inflammatory effects in the case of PPARγ. Adapted from Bishop-Bailey, 2000 (Bishop-Bailey, 2000).

2 Methods

2.1 Animals

2.1.1 Housing of animals

The young animals used in this study were male Wistar rats aged between 2 and 3 months and weighed between 250g and 350g. The aged animals were between 22 and 24 months and weighed between 550g and 650g. Rats were supplied by Bantham & Kingman (Hull, UK). Animals were housed in groups of 3 per cage and were maintained under a 12h light-dark cycle in the Bio Resources Unit, Trinity College, Dublin. Food and water was available *ad libitum*. Ambient temperature was controlled between 22 and 23°C and animals were maintained under veterinary supervision for the duration of all experiments. All animal experimentation was performed under a licence granted by the Minister for Health and Children (Ireland) under the Cruelty to Animals Act, 1876 and the European Community Directive, 86/609/EC.

2.1.2 Polyunsaturated fatty acid treatment

Young and aged rats were treated for 56 days with either EPA or DPA. The composition of the EPA preparation was 20:5n-3 ethyl-eicosapentaenoic acid and its purity was greater than 95%; 0.2% dl-tocopherol was included as an antioxidant (Amarin Neuroscience, UK). The DPA preparation was 22:5n-3 docosapentaenoic acid and its purity was greater than 70% (Amarin Neuroscience, UK). Food intake was measured for 1 week prior to the beginning of the experimental treatment to determine daily food intake. Rats were randomly assigned, within age group, to groups which received normal laboratory chow (controls; Red Mills, Ireland) or normal laboratory chow supplemented with either 200mg/kg/day EPA or 200mg/kg/day DPA (see Figure 2.1). Monounsaturated fatty acid (own brand vegetable oil, Dunnes Stores, Ireland) was added for control animals to ensure

isocaloric value. Percentage purity was taken into account when working out dose per day. Treatments were prepared freshly each day and animals were offered their full daily requirement.

2.1.3 Lipopolysaccharide administration

In another study, young and aged rats were anaesthetised by intraperitoneal (ip) injection of urethane (1.5g/kg; 33% w/v). Deep anaesthesia was verified by the absence of a pedal reflex and if additional urethane was required a top-up was administered to a maximum dose of 2.5g/kg urethane. Rats were given either sterile 0.9% w/v saline or lipopolysaccharide (LPS; 100±g/kg in sterile 0.9% w/v saline) by ip injection. LPS was from *Escherichia coli* serotype 0111:B4 (Sigma, UK).

2.2 Five day Morris water maze trial

Rats were tested using a Morris water maze which consisted of a black circular pool of 2m diameter, 35cm deep and contained water at 20 ± 1°C to a depth of 31cm. A hidden platform was placed in one quadrant of the pool, submerged 2cm below the water surface, invisible at water level. The location of the platform was kept constant for the entire experiment. Visual cues were arranged in the testing room. Animals received four trials per day for 5 days where they were required to search for and find the hidden platform. Each rat entered the maze at a start position and was allowed 60s per trial to find the platform. If the platform was not successfully reached within this time frame, the rat was guided to the platform, where the rat was kept for 15s. A computerized digital tracking system was used to record the swim paths and escape latencies for each rat for each trial (EthoVision, The Netherlands). This experiment was carried out by Dr. Andrea Della Chiesa.

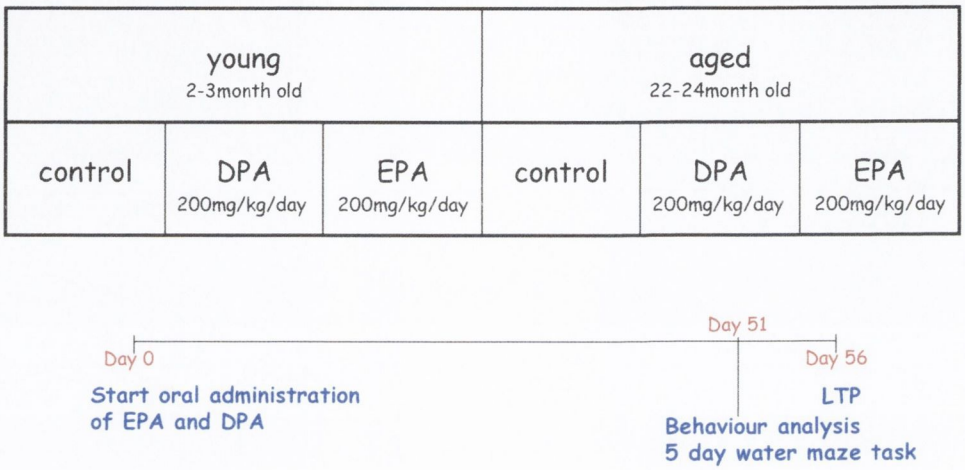


Figure 2.1 Study design for analysis of the effect of EPA and DPA on age-related changes in the brain

2.3 Induction of long term potentiation *in vivo*

2.3.1 Preparation of rats

Rats were anaesthesised as described in Section 2.1.3. The head was positioned in a head holder in a stereotaxic frame (ASI instruments, USA). An incision was made at the midline with a scalpel to expose the skull. The periosteum was cleared and, in order to position the electrodes, a dental drill was used to create two holes in the skull. The recording chamber was made up of a stereotaxic unit on a laboratory bench. A Faraday cage surrounded the setup to block external interference from the environment. All instruments within the cage were grounded to eliminate 50Hz cycle noise.

2.3.2 Electrode implantation

A bipolar stimulating electrode (Clark Electromedical, UK) was positioned on the surface of the brain, 4.4mm lateral to lambda. A unipolar recording electrode was placed at 2.5mm lateral and 3.9mm posterior to bregma. An earth electrode was positioned away from the recording and stimulating electrodes. The stimulating and recording electrodes were then slowly and carefully lowered so the stimulating electrode was placed in the perforant path (angular bundle) and the recording electrode in the granule cell layer (inner molecular layer) of the dentate gyrus. Electrode movement continued incrementally until the typical waveform distinguishing perforant path granule cell synapse response was evident. The depth of the electrodes was modified in order to produce a maximal response. The response was evident by stimulation with a pulse (4V pulse, for a duration of 0.1ms with a 2ms delay, at a frequency of 0.1Hz) and this was accomplished by passing a single square wave of current generated by a constant isolation unit (Isoflex, UK) to the bipolar stimulating electrode. The response induced was detected by the recording electrode and displayed on the computer screen using the software (Spike 2, version 5). The final depth was between 2.5mm and 3.5mm, and between 2.5 and 3mm, for the

recording and stimulating electrodes respectively. Test stimuli were delivered at intervals of 30s.

2.3.3 EPSP recordings

The population field EPSP was employed as a measure of excitatory synaptic transmission in the dentate gyrus. EPSPs were recorded by passing a single square wave of current at low frequency (0.033Hz, 0.1s, with a 2ms delay) to the stimulating electrode, produced by a constant isolation unit (IsoFlex, UK). The induced response was transmitted through a pre-amplifier (DAM 50; differential amplifier; gain 75, World precision Instruments, USA) to an analogue-to-digital converter (Micro 1401, Cambridge Electronic Design, UK), which interfaced with the converter via specially written software (Spike 2, version 5). This was modified in order to be able to control the generation of the square wave pulses and the recording of evoked potentials. The field EPSPs were displayed on-screen, saved and analysed on completion of the experiments. The slope of the EPSP was used as an indicator of excitatory synaptic transmission. After a stabilisation period, test shocks at 1/30s were recorded for a 10min control period. This was important in order to ascertain baseline recordings. After 10min, high frequency stimulation (HFS) was delivered to the perforant path (3 trains of stimuli at 250Hz for 200ms) at 30s intervals. Recording at the original test shock frequency continued for a 40min time period. In the analysis, post-HFS recordings were expressed as a percentage of the baseline recordings preceding tetanus. This experimental work was carried out with the help of Dr. Thelma Cowley.

2.4 Preparation of tissue from *in vivo* studies

2.4.1 Initial preparation of tissue

Rats were sacrificed by decapitation. Brains were rapidly removed and placed on a glass Petri dish mounted on ice for dissection of the hippocampus and cortex. The hippocampus and cortex were dissected free and each separate tissue was

immediately sliced to a thickness of 350μm, turned 90° and sliced again, with the use of a McIlwain tissue chopper. Portions of tissue were flash frozen in liquid nitrogen and stored at -80°C for subsequent mRNA extraction and analysis. Other portions of tissue were placed in Eppendorf tubes containing Krebs-$CaCl_2$ (NaCl 136mM, KCl 2.54mM, KH_2PO_4 1.18mM, $MgSO_4$ 1.18mM, $NaHCO_3$ 16mM, glucose 10mM, $CaCl_2$ 2mM, pH 7.4), washed with Krebs-$CaCl_2$ and stored at -80°C in Krebs-$CaCl_2$ containing 10% dimethyl sulphoxide until required for later analysis.

2.4.2 Separation of tissue into cytosolic and membrane fractions

Cytosolic, membrane and nuclear proteins were separated into fractions from hippocampal and cortical tissue using a ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem®), as per the manufacturer's instructions. Briefly, flash frozen tissue, between 25 and 50mg per sample, was ground on ice using a glass homogeniser and 0.5ml ice-cold extraction buffer I, which contained 2.5μl protease inhibitor cocktail, was added to each tube. The tissue was resuspended by gentle flicking of the tube and samples were incubated for 10min at 4°C with gentle agitation. Samples were centrifuged (760*g* at 4°C for 10min) and the supernatant obtained was carefully transferred to a new Eppendorf tube (fraction 1; cytosolic protein fraction). The pellet was resuspended in 0.5ml ice-cold extraction buffer II with 2.5μl protease inhibitor cocktail by flicking the tube. The samples were incubated for 30min at 4°C with gentle agitation and centrifuged (5,600*g* at 4°C for 10min). The supernatant (fraction 2; membrane and membrane organelle proteins) was removed to a clean Eppendorf tube. The remaining pellet was resuspended in 250μl extraction buffer III containing 2.5μl protease inhibitor cocktail and 0.750μl benzonase® nuclease, incubated for 10min at 4°C with gentle agitation and centrifuged at 7,000*g* for 10min to pellet insoluble material. The resultant supernatant (fraction 3; nuclear fraction) was carefully transferred to a new Eppendorf tube and all fractions obtained were stored at -80°C for later analysis.

2.5 Preparation of cultured cells

2.5.1 Aseptic technique and preparation

Aseptic technique was adhered to for all *in vitro* cell culture experiments. All pipette tips, tubes, dissection instruments and deionised H_2O were wrapped with aluminium foil and autoclave tape (Sigma, UK) and autoclaved at 121°C for 20min, subsequently sprayed with 70% ethanol and placed into a laminar flow hood. All instruments used for dissection were exposed to a germicidal lamp within a laminar flow hood, which emits ultraviolet (UV) radiation at 253.7nm to ensure sterility. Media and solutions used in cell culture experiments were filtered into sterile 50ml falcon tubes (Sarstedt, Germany). Cell culture work was carried out in a sterile environment within a laminar flow hood (AGB Scientific, Ireland) and disposable latex gloves, sprayed frequently with 70% ethanol, were worn at all times.

2.5.2 Preparation of sterile coverslips

Glass coverslips (13mm in diameter; Chance Propper, UK) were placed in 70% ethanol for 24h and then exposed to UV light overnight. Coverslips were coated with poly-L-lysine (40μg/ml in sterile H_2O; Sigma, UK) for 1h at 37°C. This coating step was to provide an appropriate surface to which neuronal cells would adhere. The coated coverslips were allowed to dry within a laminar flow hood.

2.5.3 Preparation of cortical neurons

Cortical neuronal cells were cultured from the cortices of one-day old Wistar rats (BioResources Unit, Trinity College, Dublin). Under sterile conditions, in a laminar flow hood, rats were decapitated, the skull was exposed by cutting the skin from the neck to the top of the head and the skull was removed to expose the brain. The cortex was rapidly dissected and placed in sterile phosphate-buffered saline (PBS; Sigma, UK). The cortex was chopped using a sterile disposable scalpel

(Schwann-Mann, UK) and incubated in warmed trypsin in PBS (1mg/10ml; Sigma, UK) for 25min at 37°C. The chopped cortices were incubated in PBS containing soybean trypsin inhibitor (1mg/10ml; Sigma, UK), DNase (0.07mg/ml; Sigma, UK) and $MgSO_4$ (0.03M; Sigma, UK) and exposed to a more concentrated soybean trypsin inhibitor (3mg/10ml; Sigma, UK) solution, with DNase (0.2mg/ml; Sigma, UK) and $MgSO_4$ (0.1M; Sigma, UK). The preparation was triturated using a Pasteur pipette and filtered through a sterile mesh cell strainer (BD Biosciences, UK). The filtrate was centrifuged at 2,000*g* for 3min at room temperature. The resulting pellet was resuspended in pre-warmed neurobasal medium (NBM; Gibco, UK) which was supplemented with heat-inactivated horse serum (HS; 10% w/v; Sigma, UK), penicillin (100µl/ml; Gibco, UK), streptomycin (100µl/ml; Gibco, UK) and glutamax (2mM; Sigma, UK) and the anti-oxidant B27 (1% w/v; Gibco, UK). The resuspended cells were plated (1.5 x 10^6 cells/ml) in 24-well plates containing glass coverslips which were pre-coated with poly-L-lysine. Plated cells were placed in a humified incubator containing 5% CO_2 and 95% air at a temperature of 37°C for 2h, to allow for the cells to adhere. After this time, each well was flooded with pre-warmed, supplemented (as above) NBM and cells were incubated at 37°C. On day 3, the media was changed and replaced by supplemented NBM (as above) with the addition of cytosine arabino-furanoside (ara–c; 5ng/ml; Sigma, UK) for 24h. After this time, the ara-c-containing media was removed and, from this point on, cells were incubated in NBM containing HS, penicillin, streptomycin and glutamax for 5 or 6 days.

2.5.4 Preparation of cortical mixed glia

Mixed glial cells were cultured from the cortices of one-day old Wistar rats (BioResources Unit, Trinity College, Dublin). Under sterile conditions, in a laminar flow hood, rats were decapitated, the skull was exposed by cutting the skin from the neck to the top of the head and the skull was removed to expose the brain. The cortex was rapidly dissected and placed in sterile PBS (Sigma, UK). The cortex was chopped using a sterile disposable scalpel and incubated in warmed Dulbecco's Modified Eagle Medium (DMEM; Gibco, UK) which was supplemented with fetal

bovine serum (FBS; 10% w/v; Sigma, UK), penicillin (100µl/ml; Gibco, UK), streptomycin (100µl/ml; Gibco, UK) and glutamax (2mM; Sigma, UK). The preparation was triturated using a Pasteur pipette and filtered through a sterile mesh cell strainer (BD Biosciences, UK). The filtrate was centrifuged at 2,000*g* for 3min at room temperature. The resulting pellet was resuspended in pre-warmed DMEM. The resuspended cells were plated (1.5 x 10^6 cells/ml) in 24-well plates which were pre-coated with poly-L-lysine. Plated cells were placed in a humified incubator containing 5% CO_2 and 95% air at a temperature of 37°C for 2h, to allow the cells to adhere. After this time, each well was flooded with pre-warmed, supplemented (as above) DMEM and cells were incubated for 10 days until ready for treatment. Media was changed every 3 days.

2.5.5 Preparation of astrocytes

Glial cells were cultured from the cortices of one-day old Wistar rats (BioResources Unit, Trinity College, Dublin). Under sterile conditions, in a laminar flow hood, rats were decapitated, the skull was exposed by cutting the skin from the neck to the top of the head and the skull was removed to expose the brain. The cortex was rapidly dissected and placed in sterile PBS (Sigma, UK). The cortex was chopped using a sterile disposable scalpel and incubated in warmed DMEM (Gibco, UK) which was supplemented with FBS (10% w/v; Sigma, UK), penicillin (100µl/ml; Gibco, UK), streptomycin (100µl/ml; Gibco, UK) and glutamax (2mM; Sigma, UK) for 25min at 37°C. Following incubation, the preparation was triturated using a Pasteur pipette and filtered through a sterile mesh cell strainer (BD Biosciences, UK). The filtrate was centrifuged at 2,000*g* for 3min at room temperature. The resulting pellet was resuspended in pre-warmed DMEM. The resuspended cells were plated (1.5 x 10^6 cells/ml) in T25 flasks. Plated cells were placed in a humified incubator containing 5% CO_2 and 95% air at a temperature of 37°C for 2h, to allow the cells to adhere. After this time, each flask was flooded with pre-warmed, supplemented (as above) DMEM. The following day, media was removed and replaced with supplemented DMEM containing an additional

supplement of macrophage-colony stimulating factor (M-CSF; 20ng/ml; R&D Systems, UK). The media was replaced again 7 days later and, after 13 days, flasks were removed from the humidified chamber, the neck and cap of each flask was wrapped tightly using parafilm and flasks were shaken on an orbital platform shaker at 110rpm for 2h at room temperature. After 2h the flasks were returned to the laminar flow hood, individually tapped approximately 30 times and the contents poured into a new 50ml sterile falcon tube for preparation of microglial cells. The remaining cells in the flask were incubated with 1ml trypsin-EDTA for 15min at 37°C. The flasks were then tapped and this cell suspension was collected, centrifuged at 2,000rpm for 5min at 20°C. The resulting pellet was resuspended in 1ml DMEM, a cell count was performed and cells were plated at a density of 1×10^5 cells/ml onto 24-well plates. Plated cells were placed in a humified incubator containing 5% CO_2 and 95% air at a temperature of 37°C for 2h, to allow for the cells to adhere. After this time, each well was flooded with pre-warmed, supplemented DMEM and cells were incubated at 37°C until treatment the following day.

2.5.6 Treatment of cultured cells

Cells were pre-treated for 24h with EPA (50μM) or DPA (50μM), or with DMEM or NBM media (controls). After pre-treatment, cells were treated with LPS (100ng/ml; Sigma, UK) or ceramide (100μM; Sigma, UK) for a further 24h and a combination of LPS/ceramide and EPA or DPA at the same concentrations.

2.5.7 Cell viability assay

In some experiments, cell viability was assessed using a Cell Titer 96® Aqueous One solution reagent cell proliferation assay (Promega, USA) as per the manufacturer's instructions. Briefly, 40μl of kit reagent was added to cultured cells (containing 200μl media with treatment) and the samples were incubated for 1h at 37°C. After this time, samples of supernatant (100μl) were pipetted into wells of a 96-well plate (Sarstedt, Germany) and absorbances were read at 492nm.

2.5.8 Harvesting of cells

In some experiments, supernatant was removed and stored at -80°C for later analysis. Cultured cells were washed once with PBS and harvested with either a harvesting buffer (50mM HEPES, pH 7.4 with 1mM EDTA) or lysis buffer (10mM Tris-HCl, 50mM NaCl, 10mM $Na_4P_2O_7.10H_2O$, 50mM NaF, with 1% IGEPAL, pH 7.4 and 1mM Na_3VO_4, 1mM PMSF and protease inhibitor cocktail (Sigma, UK)). Harvesting buffer, or lysis buffer, (65µl) was added per well and cells were harvested using the rubber end of a sterile 1ml syringe insert and the cell samples collected in Eppendorf tubes for later analysis.

2.6 Analysis of cytokines and chemokines

2.6.1 Preparation of samples

Whole-cell homogenates were prepared from cortical and hippocampal tissue for ELISA. Samples were washed three times in Krebs-$CaCl_2$ (136mM NaCl, 2.54mM KCl, 1.18mM KH_2PO_4, 1.18mM $MgSO_4$, 16mM $NaHCO_3$, 10mM glucose, 2mM $CaCl_2$, pH 7.4) and homogenised in 600µl Krebs-$CaCl_2$ using a polytron (Kinematica, Switzerland). Protein concentrations in samples were determined by bicinchonic acid (BCA) assay (Pierce, UK), with bovine serum albumin (BSA) used as a standard.

2.6.2 Protein quantification using BCA assay

Standards (0-2,000µg/ml bovine serum albumin; BSA) were prepared from a stock of BSA (2mg/ml) provided in the kit, diluted in appropriate diluent (Krebs-$CaCl_2$ for tissue prepared in Krebs-$CaCl_2$, harvesting buffer for samples prepared in this way). Working reagent was prepared by the mixing of reagent A and reagent B in a ratio of 50:1. Standards and samples (25µl) were added in triplicate to a 96-well plate (Sarstedt, Germany). Working reagent was added to each well (200µl/well) and

the plate was incubated for 30min at 37°C. After this time, the plate was allowed to equilibrate to room temperature and absorbance was read at 570nm (Labsystems Multiwell RC, UK). Protein concentrations were assessed by regression analysis (GraphPad Prism, USA) and expressed as mg protein/ml. Samples were equalised for protein concentration before further analysis.

2.6.3 Protein quantification using BioRad assay

The concentrations of protein in samples were quantified by the method of Bradford (Bradford, 1976). All samples were analysed in triplicate. A standard curve was prepared using 1000µg/ml BSA stock solution diluted in ddH_2O ranging from 15.625µg/ml to 1000µg/ml and a blank of only ddH_2O was also included. Standards and samples (10µl) were pipetted into a 96-well plate (microtest plate; Sarstedt, Germany) to which Bio-Rad dye reagent (200µl; 1:5 dilution in ddH_2O) was added. Absorbances were measured at 595nm (Labsystems Multiwell RC, UK) and protein concentrations were assessed by regression analysis (GraphPad Prism, USA) and expressed as mg protein/ml. Samples were equalised for protein concentration before undergoing further analysis.

2.6.4 Analysis of IL-1β, IL-6, TNFα and fractalkine concentrations

Concentrations of IL-1β, IL-6, TNFα and fractalkine were assessed by Enzyme-Linked Immunosorbent Assay (ELISA). In all cases, 96-well plates (Nunc Immuno, Denmark) were coated and incubated with capture antibody (100µl; see Table 2.1 for specific details). Plates were washed 3 times with PBS (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4, pH 7.3) containing 0.05% Tween 20 (Sigma, UK; PBS-Tween), and blocking buffer (300µl; PBS containing 1% BSA for IL-1β and fractalkine; 200µl; PBS containing 10% FBS for IL-6 and TNFα) was added. Samples were incubated for 1h at room temperature to prevent non-specific binding. Following washing 3 times with PBS-Tween, specific standards (100µl, see Table 2.1 for specific details) and samples (100µl) were added in triplicate. Plates

were incubated for 2h at room temperature on a platform shaker (STR6, Stuart Scientific). Plates were washed 3 times with PBS-Tween and coated with detection antibody (100µl; see Table 2.1 for specific details) in blocking buffer (with 2% normal goat serum (NGS; Vector laboratories, UK) in the case of IL-1β). Incubation continued for 2h at room temperature for IL-1β and fractalkine and 1h at room temperature for IL-6 and TNFα. Plates were washed again (3 times, in PBS-Tween) and incubated with horse-radish peroxidase (HRP)-conjugated streptavidin (100µl, 1:200 dilution for IL-1β and fractalkine for 20min; 1:250 dilution for IL-6 and TNFα for 30min) at room temperature. Plates were washed with PBS-Tween, substrate solution was added to each well (100µl; 1:1 dilution of reagent A (H_2O_2) and reagent B (tetramethylbenzidine (TMB); R&D Systems, USA) for IL-1β and fractalkine; 100µl TMB (Sigma, UK) for IL-6 and TNFα) and plates were incubated in the dark for 20-30min or until colour was visible in the wells. This enzyme reaction was stopped by the addition of 1M H_2SO_4 (50µl/well) and a colour change from blue to yellow indicated acidification. The absorbances were recorded at 450nm in a 96-well plate reader (Labsystem Multiwell RC, UK). A standard curve was constructed by plotting of the standards against their absorption. The concentrations of cytokine or chemokine present in samples were calculated with reference to the standard curve and expressed as pg cytokine or chemokine/mg protein, except for *in vitro* supernatant analysis, which was expressed as pg cytokine/ml supernatant.

ELISA kit	Capture antibody and conditions	Standard	Detection antibody
IL-1β ELISA (R&D Systems, Duo Set, USA)	0.8μg/ml anti-rat IL-1β monoclonal antibody in PBS overnight at room temperature	Rat recombinant IL-1β; 0-2,000pg/ml	350ng/ml biotinylated a rat IL-1β
IL-6 ELISA (BD OptEIA, BD Biosciences, USA)	4μg/ml anti-rat IL-6 monoclonal antibody in coating buffer (0.1M sodium carbonate pH 9.5 in PBS) overnight at 4°C	Rat recombinant IL-6; 0-4,000pg/ml	4μg/ml biotinylated ant IL-6
TNFα ELISA (BD OptEIA, BD Biosciences, USA)	4μg/ml anti-rat TNFα monoclonal antibody in coating buffer (0.1M sodium carbonate pH 9.5 in PBS) overnight at 4°C	Rat recombinant TNFα; 0-2,000pg/ml	4μg/ml biotinylated ant TNFα
fractalkine ELISA (R&D Systems, Duo Set, USA)	0.8μg/ml anti-rat fractalkine monoclonal antibody in PBS overnight at room temperature	Rat recombinant fractalkine; 0-12,500pg/ml	100ng/ml biotinylated rat fractalkine

Table 2.1 Analysis of cytokine and chemokine concentration by ELISA

2.7 DNA isolation, digestion and analysis of oxidative stress

2.7.1 Sample preparation for DNA isolation

DNA was isolated from flash frozen hippocampal and cortical tissue. Tissue (approximately 40mg) was homogenised in TriReagent (Sigma, UK) using a polytron and incubated for 5min at room temperature. The homogenate was centrifuged at 12,000*g* for 10min at 4°C to remove insoluble material. The supernatant was transferred to a new sterile Eppendorf tube and allowed to stand for a further 5min at room temperature. Chloroform (Sigma, UK) was added (200µl/ml of TriReagent); the tube was shaken vigorously for 15s and allowed to stand for 12min at room temperature. Samples were centrifuged for 15min at 4°C which yielded 3 phases:

1. a colourless upper aqueous phase (RNA)
2. an interphase (DNA)
3. a red organic phase (protein)

The aqueous phase was transferred to a new tube and isopropanol (500µl) was added, mixed and the sample was allowed to stand for 7min at room temperature. RNA was precipitated to a pellet upon centrifugation at 12,000*g* for 10min at 4°C. Supernatant was discarded and the RNA pellet was washed by the addition of 75% ethanol (1ml). Samples were vortexed, centrifuged at 7,600*g* for 5min at 4°C and 75% ethanol was removed from above the resulting pellet. The pellet was subsequently resuspended in 75% ethanol and stored at -20°C.

2.7.2 DNA isolation

Any remaining aqueous phase overlying the interphase was carefully removed and discarded and 100% ethanol (300µl) was added. Samples were mixed by inverting the tubes and allowed to stand for 3min at room temperature. Samples were centrifuged at 2,000*g* for 5min at 4°C, the supernatant was removed and 0.1M sodium

citrate, 10% ethanol solution (1ml) was added. Samples were incubated for 30min at room temperature, mixed occasionally and centrifuged at 2,000*g* for 5min at 4°C. Supernatant was carefully removed and 0.1M sodium citrate, 10% ethanol solution (1ml) was added to wash the pellet one more time. The pellet was resuspended in 75% ethanol (1.5ml) and allowed to stand for 15min at room temperature. Ethanol was removed following centrifugation at 2,000*g* for 5min at room temperature.

2.7.3 DNA solubilisation and quantification

The DNA pellet was air-dried within a laminar flow hood for approximately 10min, dissolved in 8mM NaOH (600µl) and centrifuged at 12,000*g* for 10min. Supernatant was transferred to a new sterile Eppendorf tube, noting that a viscous supernatant was indicative of high molecular weight DNA. The solution was adjusted to pH 7.5 using 0.1M HEPES (159µl). EDTA was added to a final concentration of 1mM. DNA concentration was quantified and its purity assessed using a nanoDrop – spectrophotometer ND-1000 V 3.5 (NanoDrop Technologies Inc. USA). Using a nucleic acid programme, the nanodrop spectrophotometer was initialised with the placing of 1µl DNase/RNase free water onto the pedestal. Another 1µl of water was used as a blank. Sample DNA content was quantified by placing 1µl sample onto the pedestal and measuring the absorbance. Each sample was measured in duplicate. Samples were equalised for DNA concentration and stored at -20°C or DNA digestion was carried out on the same day.

2.7.4 DNA digestion

DNA-containing samples (200µl) were transferred to a new sterile Eppendorf tube and 0.5M sodium acetate (10µl), 0.05M $ZnCl_2$ (15µl), and 2 units nuclease P1 (dissolved in H_2O, Sigma; 10µl) were added. Samples were incubated for 30min at 37°C and 0.4M Tris-HCl (80µl) was added. Samples were stored at -20°C until analysis of 8-hydroxy-2'-deoxyguanosine (8-OHdG) by ELISA the following day.

2.7.5 8-hydroxy-2'-deoxyguanosine assay

The oxidative DNA adduct 8-OHdG was quantitatively measured by competitive ELISA (New 8-OHdG Check, Gentaur, Belgium). Briefly, standards (50µl, 0.007813-200ng/ml) and digested cortical and hippocampal DNA samples (50µl/well) were added in duplicate to 96-well plates (Gentaur, Belgium), pre-coated with 8-OHdG. Monoclonal primary antibody specific for 8-OHdG (50µl) was added, plates were sealed and incubated overnight at 4°C. Samples were washed 3 times with PBS washing solution, and HRP-conjugated secondary antibody (100µl) was added. Plates were sealed and incubated for 1h at 37°C. Samples were washed again (3 times, with PBS washing solution) and enzyme substrate solution (100µl) was added to each well. Plates were incubated in the dark at room temperature for 15min or until colour was visible in the wells. The enzyme reaction was terminated by the addition of 1M phosphoric acid (100µl) to each well and a colour change from blue to yellow indicated acidification. The absorbances were read at 450nm in a 96-well plate reader (Labsystem Multiwell RC, UK). The amount of 8-OHdG present in digested DNA samples was calculated with reference to the standard curve by plotting absorbance versus the log of the concentration of standard and expressed as 8-OHdG µg/pg.

2.8 Analysis of mRNA

2.8.1 mRNA extraction from tissue

Flash frozen hippocampal tissue was homogenised in cell lysis mastermix (353.5µl; Nucleospin RNA II, Macherey-Nagel, Germany) using a polytron (Kinematica, Switzerland). Samples of lysate were filtered with the use of a NucleoSpin Filter, collected in an Eppendorf tube and centrifuged at 11,000*g* for 1min. Ethanol (70%, 350µl; Sigma, UK) was added to the filtrate. Samples were mixed and loaded onto NucleoSpin RNA II columns. Tubes were centrifuged at 8,000*g* for 30s in order to allow binding of RNA to the column. The silica membrane

was desalted by the addition of membrane desalting buffer (350µl) and centrifuged at 11,000*g* for 1min. This step was performed to dry the membrane. DNA was digested by the addition of DNase reaction mixture (95µl) to the column and this was incubated at room temperature for 15min. The silica membrane was washed and dried. RNA was eluted twice, first by adding RNase free H_2O and centrifuging (11,000*g* for 1min) and secondly by re-eluting the sample through the column.

2.8.2 Spectrophotometric analysis of RNA

Following isolation of RNA, its concentration was quantified using a nanoDrop – spectrophotometer ND-1000 V 3.5 (NanoDrop Technologies Inc. USA). Using a nucleic acid programme, the nanodrop spectrophotometer was initialised by placing 1µl DNase/RNase free water onto the pedestal. Another 1µl of water was used as a blank. Sample RNA content was quantified by placing 1µl sample onto the pedestal and measuring the absorbance. Each sample was measured in duplicate. Samples were stored at -80°C until required for cDNA synthesis.

2.8.3 Reverse transcription

Total mRNA (1µg) was reverse transcribed into cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Germany) according to the protocol provided by the manufacturer. In summary, isolated RNA (3µg) was placed into fresh tubes containing the appropriate volume of nuclease-free H_2O to make a 25µl volume. A 2X mastermix containing 10X reverse transcription buffer, 25X dNTPs, 10X random primer multi-scribe reverse transcriptase (50U/µl) was prepared. Mastermix (25µl) was added to the RNA and nuclease free H_2O. Tubes were incubated for 10min at 25°C followed by 2h at 37°C on a thermocycler (PTC-200, Peltier Thermal Cycler, MJ Research, Biosciences Ireland). The cDNA which resulted from this procedure was stored at -20°C until required for real-time polymerase chain reaction (PCR).

2.8.4 Quantitative polymerase chain reaction for IL-1β, CD200L, MHCII, CD11b, CD68, CD36 and β-actin

Real-time PCR primers and probes were from "TaqMan® Gene Expression Assays" for the rat genes listed in Table 2.2 (Applied Biosystems, Germany). Real-time PCR was performed on Applied Biosystems ABI Prism 7300 Sequence Detection System v1.3.1 in 96-well format and a reaction volume of 25µl per well was used. cDNA samples (200pg/well) were mixed with TaqMan Universal PCR Mastermix (Applied Biosystems, Germany) along with the respective target gene assay. The appropriate β-actin RNA was used as a reference (rat β-actin, #4352340E; Applied Biosystems, Germany). Each sample was measured in duplicate in a single RT-PCR run. Forty cycles were run with the following conditions: 2min at 50°C, 10min at 95°C and for each cycle 15s at 95 °C for denaturation and 1min at 60°C for transcription. Subsequent analysis of gene expression values was carried out using the efficiency-corrected comparative CT method, by the determination of target gene expression relative to β-actin endogenous control expression and relative to the control sample.

Gene name	Species	Taqman® Gene Expression Assay no.	GenBank accession no.
IL-1β	Rat	Rn00580432_m1	NM_031512.1
CD200	Rat	Rn00580478_m1	NM_031518.1
CD36	Rat	Rn01442639_m1	NM_031561.2
CD68	Rat	Rn01495631_g1	NM_001031638.1
CD11b	Rat	Rn00709342_m1	NM_012711.1
MHCII	Rat	Rn01768597_m1	NM_198741.1

Table 2.2 Genes used in real-time PCR experiments with their respective Taqman® Gene Expression Assay numbers and GenBank accession numbers

2.9 Analysis of enzyme activity

2.9.1 Analysis of sphingomyelinase activity

Sphingomyelinase enzyme activity was measured using an Amplex® Red sphingomyelinase assay kit (Molecular Probes, Invitrogen, USA). Prior to assay, samples were diluted in reaction buffer (0.1M Tris-HCl, 10mM $MgCl_2$, pH 7.4) and kept on ice. Positive controls for the assay were 0.04U/ml sphingomyelinase and 10μM H_2O_2, prepared in reaction buffer and reaction buffer alone served as a negative control. Diluted samples and controls (100μl) were pipetted, in triplicate, into separate wells of a microplate. A working solution of 100μM Amplex® Red was prepared containing 2U/ml HRP, 0.2U/ml choline oxidase, 8U/ml alkaline phosphatase and 0.5mM sphingomyelin in reaction buffer. The reactions were started by the addition of 100μl working solution, samples were incubated at 37°C for 30min, protected from light with tin foil. Fluorescence was measured at multiple time points to follow the kinetics of the reaction, using a fluorescence microplate reader with excitation in the range of 530-560nm and emission detection at 590nm. Data were corrected for background fluorescence by subtraction of the values derived from the negative control which contained no sphingomyelinase. Activity was expressed as fluorescence units /mg protein.

2.9.2 Analysis of caspase 3 activity

Caspase 3 activity was assessed by a colorimetric assay kit (Biomol QuantiZyme™ Assay System, USA). Assay buffer (50mM HEPES, pH 7.4, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 1mM EDTA, 10% glycerol) was added to wells in a 96-well microplate as follows: sample wells (25μl), positive control wells (25μl), assay buffer only wells (100μl) and assay buffer with substrate wells (50μl). The plate was incubated for 10min at 37°C to equilibrate to assay temperature. Samples, in triplicate, were loaded into the appropriate wells (25μl) and human recombinant caspase-3 enzyme (50U/well) was used as a positive control. P-nitroaniline (pNA;

50μM, 100μl) was added to empty wells as a calibration standard. The reaction was started by the addition of 50μl pNA substrate (Ac-DEVD-pNA; 200μM final concentration). Plates were read at and absorbance of 405nm in a microplate reader and data recorded at 1min intervals for 15min. An average value was determined for the blank (assay buffer plus substrate) wells and this was subtracted from all sample and control wells. Activity was expressed as pmol caspase 3/min, corrected for protein concentration.

2.9.3 Analysis of caspase 8 activity

Caspase 8 activity was assessed by a colorimetric assay kit (Biomol QuantiZyme™ Assay System, USA). Assay buffer (50mM HEPES, pH 7.4, 100mM NaCl, 0.1% CHAPS, 10mM DTT, 1mM EDTA, 10% glycerol) was added to wells in a 96-well microplate as follows: sample wells (25μl), positive control wells (25μl), assay buffer only wells (100μl) and assay buffer with substrate wells (50μl). The plate was incubated for 10min at 37°C to equilibrate to assay temperature. Samples, in triplicate, were then loaded into the appropriate wells (25μl) and human recombinant caspase-8 enzyme (50U/well) was used as a positive control. P-nitroaniline (pNA; 50μM, 100μl) was added to empty wells as a calibration standard. The reaction was started by the addition of 50μl pNA substrate (Ac-DEVD-pNA; 200μM final concentration). Plates were read at 405nm in a microplate reader and data recorded at 1min intervals for 15min. An average value was determined for the blank (assay buffer plus substrate) wells and this was subtracted from all sample and control wells. Activity was expressed as pmol caspase 8/min, corrected for protein concentration.

2.9.4 Analysis of PPARγ

PPARγ binding activity was measured using a colorimetric assay (Cayman Chemical Company, USA). Nuclear fractions isolated from hippocampal tissue were equalised for protein concentration (see Section 2.6.3). Complete transcription factor

buffer (CTFB) was prepared containing ddH_2O, transcription factor binding assay buffer, transcription factor reagent A and 300mM dithiothreitol (DTT), volumes as per the kit manufacturer's instructions. CTFB was added to wells (100µl for blank and non-specific binding wells, 80µl to competitive dsDNA wells and 90µl for sample and clarified cell lysate PPARγ positive control wells). A positive control and samples were added (10µl/well) in duplicate. Competitive dsDNA (10µl) was added in duplicate to the designated wells and 10µl control nuclear extract. The plate was sealed and incubated overnight at 4°C without agitation. All wells were washed 5 times with the wash buffer provided and primary antibody (100µl/well, 1:100 dilution) diluted in antibody binding buffer was added to all wells except blank wells. The plate was sealed and incubated for 1h at room temperature. The plate was washed 5 times and secondary antibody was added (100µl/well, 1:100 dilution) diluted in antibody binding buffer to all wells except blank wells. Incubation continued for 1h at room temperature. The plate was again washed 5 times with wash buffer, developing solution (100µl/well) was added and the plate was incubated for 15-45 min in the dark at room temperature and, on this occasion, samples were placed on a rock and roller to ensure agitation (STR6, Stuart Scientific). When a medium-dark blue colour was noted to have developed in the wells, the reaction was brought to an end by the addition of stop solution and a colour change from blue to yellow was observed, indicative of acidification. The absorbances were recorded at 450nm in a 96-well plate reader (Labsystem Multiwell RC, UK) within 5min of terminating the reaction. PPARγ binding activity was determined in samples by subtraction of the blank and values were corrected for protein.

2.10 Statistical analysis

Data are expressed as means ± standard error of the means. A Student's *t* test for independent means, or a one-way or two-way analysis of variance (AN0VA) was performed where appropriate to determine whether significant differences existed. When this analysis indicated significance (at a significance level of at least α=0.05), post hoc student Newmann-Keuls test analysis was used to determine which conditions were significantly different from each other (GraphPad Prism, USA). Mean data and standard errors of the mean are shown for all results in table format in Appendix I.

Chapter 3 Introduction and methods

3

3.1 Introduction

Ageing is a process characterized by a general decline of physiological capacities. It is associated with a deterioration of function in the rat brain (Wati *et al.*, 2006; Gemma *et al.*, 2007) where there is a deficit in LTP in the hippocampus (Lynch *et al.*, 2007; Clarke *et al.*, 2008), a form of synaptic plasticity which is a putative cellular model for learning and memory. There are multiple factors which lead to this reduction in LTP and evidence from our laboratory suggests it is associated with age-related neuroinflammation (Griffin *et al.*, 2006; Loane *et al.*, 2007; Minogue *et al.*, 2007).

Caspase activation can be used as an indicator of cell stress and the role of caspases in the decline of cell function and apoptosis has been described (Green, 1998; Green & Kroemer, 2004; Bredesen *et al.*, 2006). An age-related increase in caspase 3 has been described (Martin *et al.*, 2002a) and there is an associated age-related decrease in synaptophysin (Mullany & Lynch, 1997); these changes are suggestive of synaptic loss and perhaps cell loss. Neuroinflammation can be induced by LPS (Lee *et al.*, 2004) which is associated with a deficit in LTP (Vereker *et al.*, 2000a; Lynch *et al.*, 2004). Interestingly, increased caspase 3 activity has been reported in striatum of LPS-treated rats (Choi *et al.*, 2007) and this was evident in NeuN positive cells, indicating that it was neuronal (Choi *et al.*, 2007). The trigger for the pathway leading to caspase 3 activation following LPS treatment has yet to be elucidated.

It is recognised that the lipid ceramide can exert effects on mitochondrial function, disrupt the mitochondrial respiratory chain and trigger cell death (Pettus *et al.*, 2002) and it has been shown that the age-related increase in caspase 3 in cortical tissue is linked with an increase in cytosolic cytochrome *c* (Martin *et al.*, 2002a). This increase in cytosolic cytochrome *c* is likely to be due to alterations in mitochondrial

membrane permeability (Cortopassi & Wong, 1999; Martin *et al.*, 2002a) and has been described as a relatively early event in apoptotic cell death (Martin *et al.*, 2002a).

Various stimuli can lead to disruption of mitochondrial membrane permeability and a key stimulus may be oxidative stress (Cortopassi & Wong, 1999) which results from enhanced reactive oxygen species accumulation. Mitochondria are the principal sites for reactive oxygen species generation and therefore changes to mitochondrial function with age can contribute significantly to cell oxidative stress. Generation of H_2O_2 has been shown to be increased in mitochondria of aged rats (Petrosillo *et al.*, 2008). As suggested by the free radical theory of ageing, reactive oxygen species are produced during oxidation and then lead to random cellular damage which has a cumulative effect on organ ageing (Harman, 1956). A spiral of damaging effects can occur because free radical accumulation has been shown to have damaging effects on brain mitochondrial function (Miquel & Fleming, 1986; Sastre *et al.*, 2000; Sharman & Bondy, 2001; Floyd & Hensley, 2002) and this, in turn, contributes to other alterations in the brain with age (Floyd & Hensley, 2002; Lin & Beal, 2006).

Sphingolipids are a class of lipids which are made up of a sphingoid base and a straight chain of 18-20 carbon atoms, usually attached to a long-chain saturated fatty acid, generally palmitate, through an amide bond. Sphingomyelin is an important component of plasma membranes and is mostly found in the outer leaflet of the membrane (Vaena de Avalos *et al.*, 2004). Sphingomyelin can be hydrolysed by sphingomyelinase to form ceramide. Ceramide triggers cell signalling pathways which lead to apoptosis (Jaffrezou *et al.*, 1996; Liu *et al.*, 1998; Levade & Jaffrezou, 1999). Lin and colleagues have shown that ceramide can induce cell death via activation of caspase 9 and caspase 3 (Lin *et al.*, 2004). This pathway involves a decrease in the membrane potential of mitochondria and release of the normally sequestered cytochrome *c* from mitochondria into the cell cytosol (Lin *et al.*, 2004). Evidence indicative of a role for caspase 8 upstream of ceramide-induced mitochondrial changes has been described and caspase 8 can activate

sphingomyelinase, thereby leading to the generation of ceramide (Heinrich *et al.*, 2004).

Ceramide can have roles in proliferation and development at low concentrations. Metabolism of ceramide by glycosylation or acylation can convert it to less harmful forms. Catabolism of ceramide to sphingosine and subsequent phosphorylation can result in the generation of sphingosine-1-phosphate, which has anti-apoptotic roles (Farooqui *et al.*, 2007).

The objectives of this study were:

(i) To assess age-related changes in activities of the enzymes caspase 8, sphingomyelinase and caspase 3

(ii) To investigate whether there was evidence of parallel age-related changes in the activities of these enzymes in cortical and hippocampal tissue

(iii) To confirm the age-related impairment in LTP and assess whether changes in enzyme activity in hippocampal tissue could be correlated with modulation of LTP

(iv) To investigate if there is a link between sphingomyelinase and caspase-3 using *in vitro* methods

3.2 Methods

Young (2-3 month old) and aged (22-24 month old) rats were anaesthesised as described in Section 2.1.3 and LTP was recorded as previously described (see Section 2.3 for experimental details). Rats were sacrificed by decapitation and the hippocampus and cortex were dissected free. Activities of the initiator caspase, caspase 8 and the executioner caspase, caspase 3 were assessed in hippocampal and cortical tissue (see Sections 2.9.2 and 2.9.3). Portions of cortex and hippocampus were separated into cytosolic and membrane fractions (see Section 2.4.2) and sphingomyelinase activity was assessed in each of these (see Section 2.9.1).

Primary cortical neurons were cultured (see Section 2.5.3) and stimulated with LPS (100ng/ml; see Section 2.5.6) in order to induce an inflammatory response in these cells and activities of caspases and sphingomyelinase were investigated. Caspase 3 activity was assessed in cortical neurons treated with LPS, in the presence and absence of a selective inhibitor of sphingomyelinase (GW4869; 20μM; Sigma, UK).

In a separate *in vivo* experiment, young and aged rats were anaesthesised and administered either sterile 0.9% w/v saline or lipopolysaccharide (LPS; 100±g/kg in sterile 0.9% w/v saline) by ip injection (see Section 2.1.3) and after 3h were sacrificed and cortical tissue dissected. Activity of caspase 3 was assessed.

Chapter 3 Results

3.3 Results

3.3.1 Age-related changes in activities of caspase 8, sphingomyelinase and caspase 3

Activities of caspase 8 and 3 were examined in the cortex and hippocampus of young and aged rats. The data show that there was a significant increase in caspase 8 activity in cortical tissue prepared from aged, compared with young, animals (401.43 pmol/mg/min ± 6.17 vs. 301.20 pmol/mg/min ± 0.50; ***$p<0.001$; students *t* test; $n\geq5$; Figure 3.1A). In parallel, there was an age-related increase in caspase 3 activity (317.80 pmol/mg/min ± 5.88 vs. 285.88 pmol/mg/min ± 3.21; **$p<0.01$; students *t* test; $n\geq5$; Figure 3.1B). Sphingomyelinase activity was assessed in cytosolic and membrane fractions prepared from cortical tissue and the data provide evidence of a significant age-related increase in sphingomyelinase activity in membrane fractions (0.10 fluorescence units/mg ± 0.02 vs. 0.04 fluorescence units/mg ± 0.01; *$p<0.05$; students *t* test; $n\geq5$; Figure 3.2A), but no significant change in sphingomyelinase activity was observed in cortical cytosolic fractions (0.24 fluorescence units/mg ± 0.02 vs. 0.20 fluorescence units/mg ± 0.01; $n\geq5$; Figure 3.2B).

Reflecting the data obtained in cortical tissue, caspase 8 activity was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (350.14 pmol/mg/min ± 6.51 vs. 253.56 pmol/mg/min ± 6.22; ***$p<0.001$; students *t* test; $n\geq5$; Figure 3.3A) and, concomitantly, there was an age-related increase in caspase 3 activity in the hippocampus (316.64 pmol/mg/min ± 6.35 vs. 278.29 pmol/mg/min ± 2.74; ***$p<0.001$; students *t* test; $n\geq5$; Figure 3.3B). The data show that there was a significant increase in sphingomyelinase activity in membrane preparations obtained from the hippocampi of aged, compared with young, rats (0.047 fluorescence units/mg ± 0.003 vs. 0.027 fluorescence units/mg ± 0.005; **$p<0.01$; students *t* test; $n\geq6$; Figure 3.4A). A similar change was observed in cytosolic fractions (0.015 fluorescence units/mg ± 0.001 vs. 0.006 fluorescence units/mg ± 0.001; ***$p<0.001$; students *t* test; $n\geq6$; Figure 3.4B).

3.3.2 Age-related impairment in LTP

Figure 3.5C and D shows that LTP was significantly reduced in aged, compared with young rats. The mean percentage changes in EPSP slope in the last 5min of recording of the experiment were 130.99% ± 0.60 and 121.09% ± 0.52 in young and aged rats respectively. These values represent a significant decrease in aged compared with young animals (***$p<0.001$; students *t* test; $n\geq12$; Figure 3.5D).

3.3.3 Effect of LPS on activities of sphingomyelinase and caspase

Administration of LPS, a component of the cell wall of Gram-negative bacteria, is known to induce inflammation in the brain because it consistently increases the concentration of IL-1β (Lonergan *et al.*, 2004). Here, the effect of LPS on activities of caspase and sphingomyelinase in primary cultured cortical neurons was assessed. The data demonstrate that LPS induced a significant increase in sphingomyelinase activity (0.050 fluorescence units/mg ± 0.003 vs. 0.037 fluorescence units/mg ± 0.004 (controls); *$p<0.05$; students *t* test; n=6; Figure 3.6B). The data also show that LPS significantly increased caspase 3 activity (355.06 pmol/mg/min ± 31.42 vs. 172.37 pmol/mg/min ± 4.15; ***$p<0.001$; students *t* test; n=12; Figure 3.7B).

In order to investigate the relationship between sphingomyelinase and caspase-3, the effect of LPS on caspase-3 activity was assessed in the presence of GW4869, a specific selective inhibitor of sphingomyelinase. While LPS stimulated an increase in caspase-3 activity (355.06 pmol/mg/min ± 31.42 vs. 172.37 pmol/mg/min ± 4.15; ***$p<0.001$; ANOVA; n=12; Figure 3.8B), the presence of the inhibitor of sphingomyelinase abrogated the LPS-induced effect (137.40 pmol/mg/min ± 3.00 vs. 355.06 pmol/mg/min ± 31.42; $^{+++}p<0.001$; ANOVA; $n\geq6$; Figure 3.8B). This finding implicates sphingomyelinase as having a crucial role in the LPS-induced activation of caspase-3.

Caspase 3 activity was assessed in the cortex of young and aged rats which had received either an intraperitoneal injection of saline or LPS. There was an age-related increase in caspase 3 activity (156.29 pmol/mg/min ± 1.47 vs. 104.18 pmol/mg/min ± 0.51; $^{+++}p<0.001$; ANOVA; n≥5; Figure 3.9B). LPS induced an increase in caspase 3 activity in both young (131.05 pmol/mg/min ± 1.33 vs. 104.18 pmol/mg/min ± 0.51; ***p<0.001; ANOVA; n≥5; Figure 3.9B) and aged animals (173.21 pmol/mg/min ± 1.25 vs. 156.29 pmol/mg/min ± 1.47; $^{###}p<0.001$; ANOVA; n≥5; Figure 3.9B) and this increase was statistically significantly greater in aged LPS-treated, compared with young LPS-treated rats (173.2 pmol/mg/min ± 1.253 vs. 131.1 pmol/mg/min ± 1.334; $^{\$\$\$}p<0.001$; ANOVA; n≥5; Figure 3.9B). This tissue was kindly obtained from Dr. Aileen Lynch.

Chapter 3 Figures

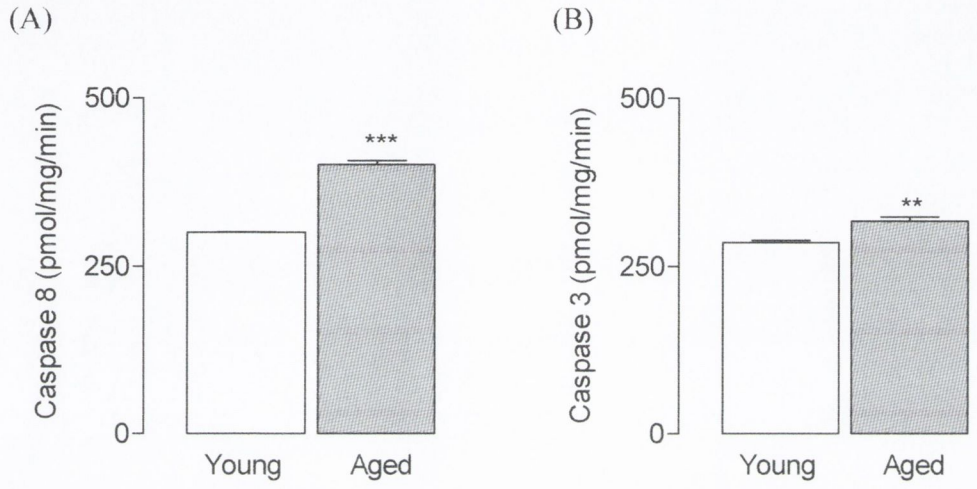


Figure 3.1 Activities of caspase 8 and caspase 3 are significantly increased in the cortex of aged rats

Mean activities of caspase 8 (A) and caspase 3 (B) were significantly increased in cortical homogenate prepared from aged, compared with young, rats (***$p<0.001$ (A) and **$p<0.01$ (B); students *t* test for independent means). Values are expressed as pmol/min, corrected for protein concentration, and are means ± SEM ($n\geq5$).

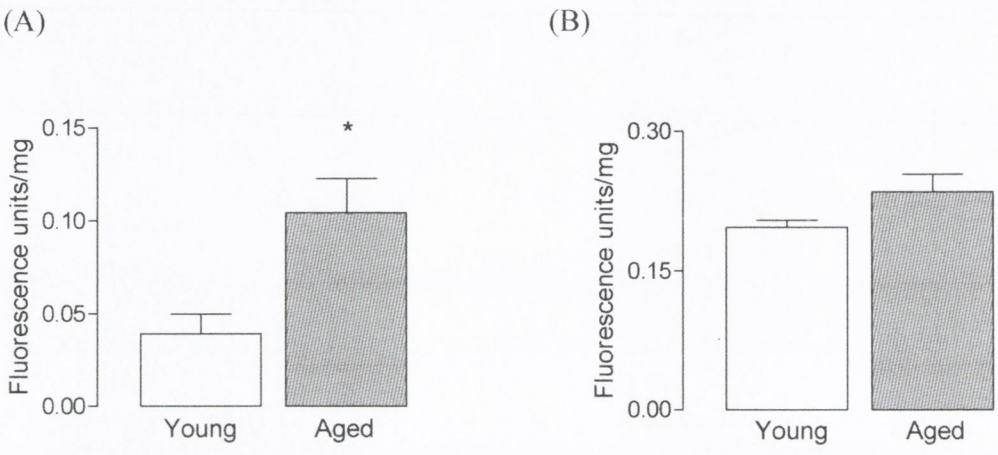


Figure 3.2 Sphingomyelinase is increased in a membrane preparation obtained from cortical tissue of aged rats

Sphingomyelinase activity was significantly increased in membrane (A) fractions prepared from cortical tissue of aged, compared with young, rats (*$p<0.05$ (A); students *t* test for independent means). Sphingomyelinase activity was not significantly altered with age in cytosolic fractions (B). Values are expressed as fluorescence units, corrected for protein concentration, and are means ± SEM ($n\geq5$).

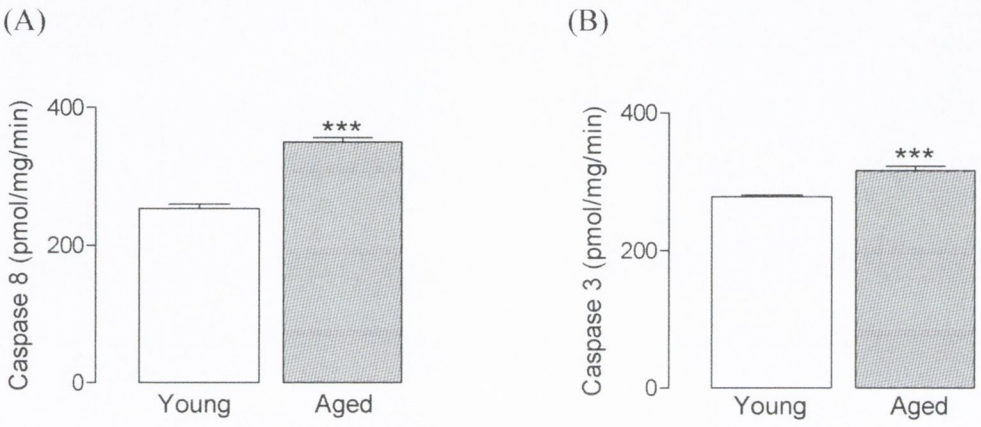


Figure 3.3 Activities of caspase 8 and caspase 3 are significantly increased in the hippocampus of aged rats

Mean activities of caspase 8 (A) and caspase 3 (B) were significantly increased in hippocampal homogenate prepared from aged, compared with young, rats (***$p<0.001$; students *t* test for independent means). Values are expressed as pmol/min, corrected for protein concentration, and are means ± SEM ($n\geq5$).

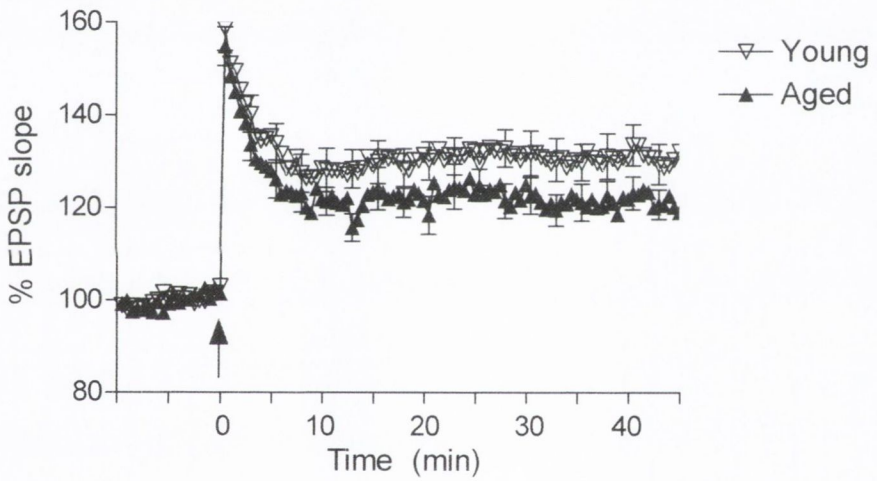
Figure 3.4 Membrane and cytosolic activities of sphingomyelinase are increased in hippocampal tissue prepared from aged rats

Sphingomyelinase activity was significantly increased in membrane (A) and cytosolic (B) fractions prepared from hippocampal tissue of aged, compared with young, rats (**$p<0.01$ (A) and ***$p<0.001$ (B); students *t* test for independent means). Values are expressed as fluorescence units, corrected for protein concentration, and are means ± SEM ($n\geq6$).

(A)

(B)

(C)


160
140
120
100
80
% EPSP slope
0
10
20
30
40
Time (min)
Young
Aged

(D)

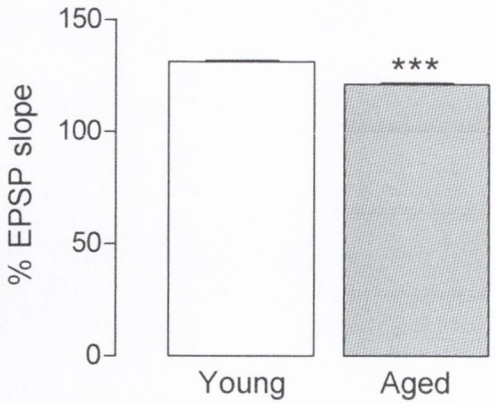
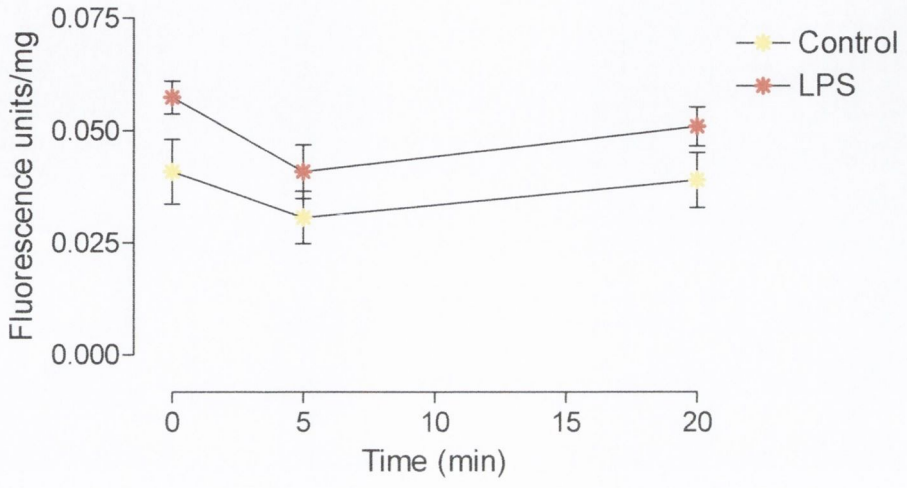

150
100
50
0
% EPSP slope
***
Young
Aged

Figure 3.5 Age is associated with reduced LTP in dentate gyrus

The mean slope of the population EPSP, evoked by test stimuli delivered at 30 sec intervals, before and after tetanic stimulation is shown. Tetanic stimulation (arrow; 3 trains of stimuli at 250Hz for 200ms; C) induced an immediate and sustained increase in EPSP slope. Mean percentage change in population EPSP slope was significantly decreased in the last 5min of recording post tetanus, in aged, compared with young, rats (***$p<0.001$; students *t* test for independent means; D). Sample EPSP slopes are shown for each group, showing trace recordings pre- and post-HFS (A; young, B; aged). Data are expressed as percentage change in EPSP slope and are means ± SEM ($n\geq12$).

(A)



(B)

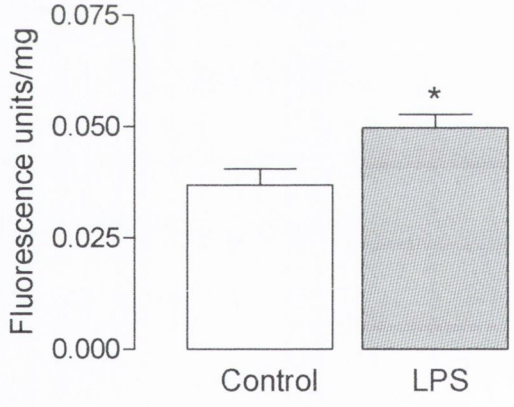
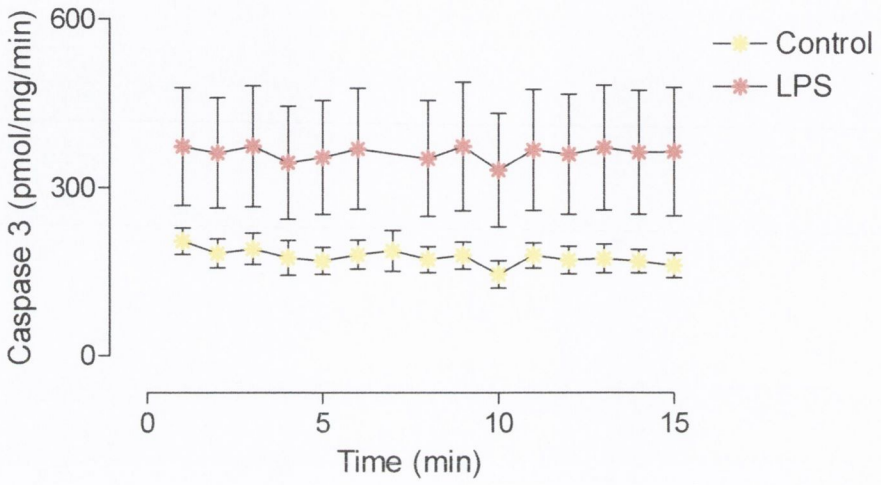


Figure 3.6 LPS increases sphingomyelinase activity in cultured cortical neurons

Incubation of neurons in the presence of LPS (100ng/ml) significantly increased sphingomyelinase activity over the 20 minute recording period (A). Mean activity of sphingomyelinase over the 20 minute recording period was significantly increased in LPS-treated cells (B; *$p<0.05$; students *t* test for independent means). Values are expressed as fluorescence units, corrected for protein concentration, and are means ± SEM (n=6).

(A)



(B)

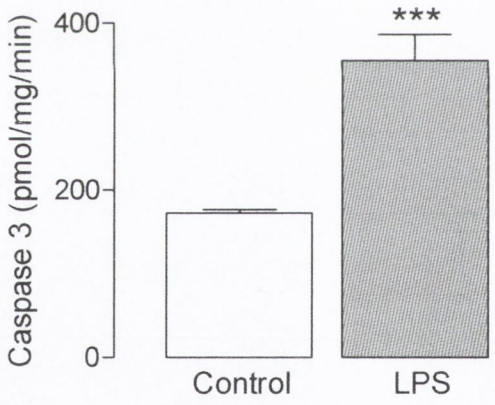


Figure 3.7 LPS induces an increase in caspase 3 activity in cortical neurons

LPS (100ng/ml) significantly increased caspase 3 activity in cortical neurons over the 15 minute recording period (A). Mean activity of caspase 3 was significantly increased in LPS-treated cells (***$p<0.001$; students *t* test for independent means). Values are expressed as pmol/min, corrected for protein concentration, and are means ± SEM (n=12).

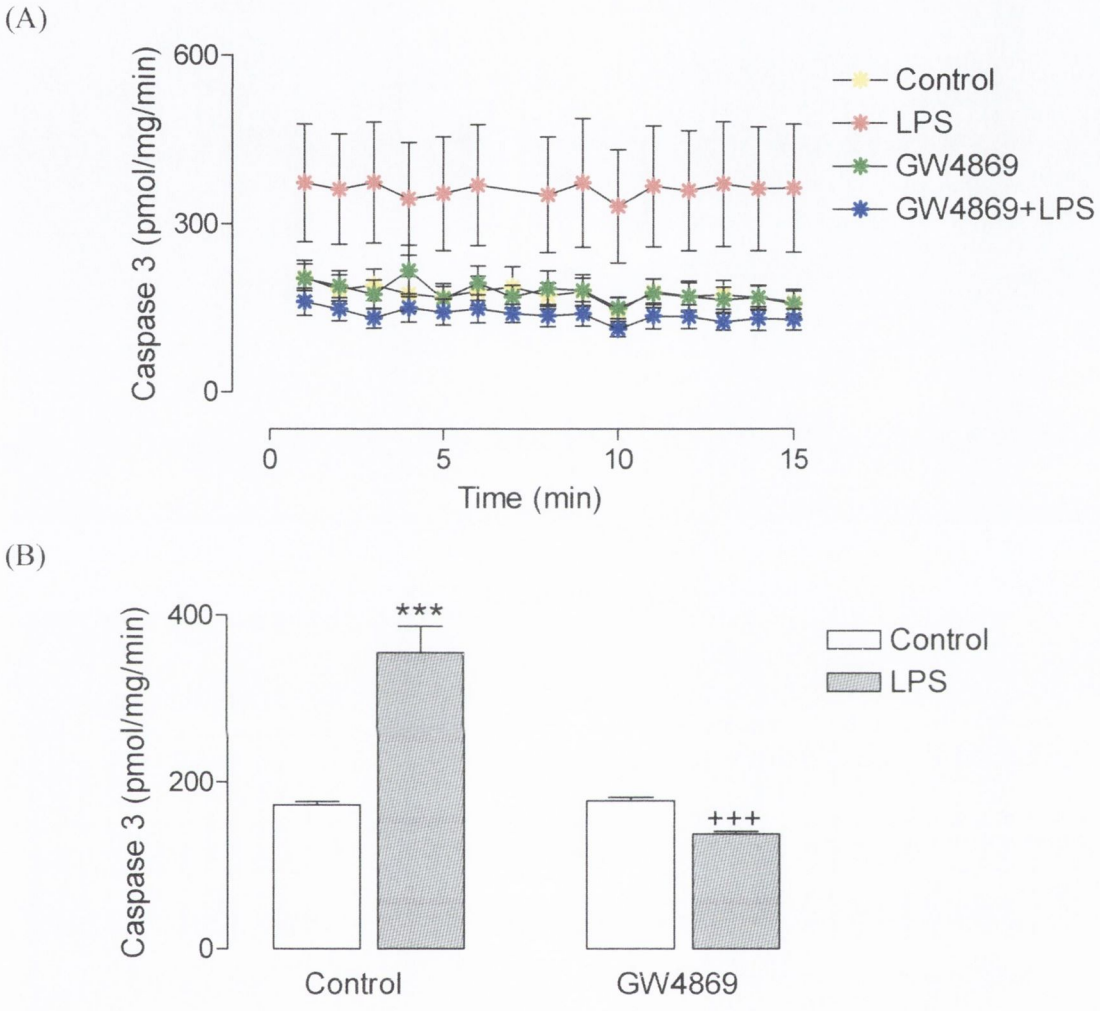


Figure 3.8 Sphingomyelinase inhibition blocks the LPS-induced increase in caspase 3 *in vitro*

LPS (100ng/ml) significantly increased caspase-3 activity in cortical neurons over the 15 minute recording period (A). Mean caspase-3 activity was significantly increased in LPS-treated cells (***$p<0.001$; ANOVA). The LPS-induced increase in caspase 3 activity was attenuated by pre-incubation of cells, 1 hour prior to LPS, in the presence of the sphingomyelinase inhibitor, GW4869 (20μM; $^{+++}p<0.001$; ANOVA). Values are expressed as pmol/min, corrected for protein concentration, and are means ± SEM ($n\geq6$).

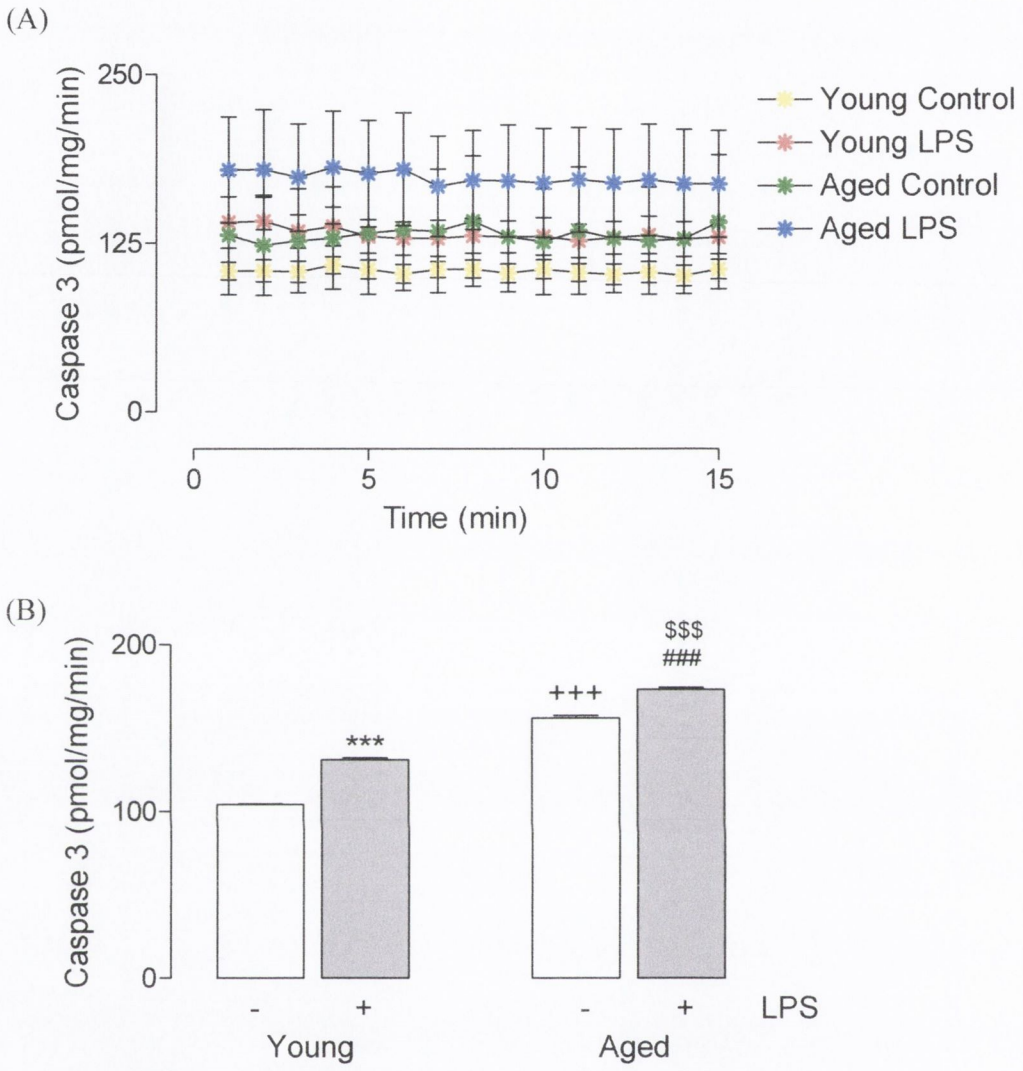


Figure 3.9 LPS induces an increase in caspase 3 activity in the cortex of young and aged rats

Caspase 3 activity was assessed in cortical homogenate over the 15 minute recording period (A). Mean caspase 3 activity was significantly increased in control-treated aged, compared with young, rats (B; $^{+++}p<0.001$; ANOVA). Caspase 3 activity was significantly increased in young rats which received an i.p. LPS injection (100µg/kg) compared with control-treated animals (***$p<0.001$; ANOVA) and in aged LPS-treated compared with control-treated rats ($^{###}p<0.001$; ANOVA). Mean caspase 3 activity was also significantly increased in aged LPS-treated, compared with young LPS-treated, animals ($^{\$\$\$}p<0.001$; ANOVA). Values are expressed as pmol/min, corrected for protein concentration, and are means ± SEM ($n\geq5$).

Chapter 3 Discussion

3.4 Discussion

The objective of this study was to investigate whether there was evidence of similar age-related changes in activities of caspase 8, sphingomyelinase and caspase 3 in cortical and hippocampal tissue and to discern whether changes in the hippocampus could be linked with a change in LTP. The data show that activity of each of these enzymes was increased in cortical and hippocampal tissue prepared from aged, compared with young, rats. These age-related changes in hippocampus were coupled with deficits in LTP.

3.4.1 Age- and LPS-related changes in activities of caspase 8, sphingomyelinase and caspase 3

This age-related increase in caspase 3 activity concurs with previous evidence (Martin *et al.*, 2002a), in which an age-related increase in caspase 3 activity was observed in cortical tissue. Caspase 8 has been reported to be increased in numerous neurodegenerative diseases, for example Alzheimer's disease (Rohn *et al.*, 2001; Yew *et al.*, 2004) or subsequent to injury or insult to the brain (Keane *et al.*, 2001); however, a search of the literature failed to uncover previous evidence of an age-related increase. The coupled increases in caspases are in agreement with the fact that the executioner caspase 3 is a substrate for the initiator caspase 8 (Stennicke *et al.*, 1998). The data provide evidence of an age-induced deterioration of cell function.

LPS has been shown to increase caspase 3 activity both *in vivo* (Lynch *et al.*, 2004; Choi *et al.*, 2007) and *in vitro* (Lee *et al.*, 2004). The data from this current study corroborates these observations as both intraperitoneal injection of LPS *in vivo* and treatment of cortical neurons *in vitro* increased activity of caspase 3. It is interesting that the LPS-associated increase in activity of caspase 3 in cortical tissue was further increased in tissue prepared from aged rats and this is consistent with data from the Johnson group which has shown that aged mice are more susceptible to the effects of LPS than young animals (Gaykema *et al.*, 2007; Huang *et al.*, 2007;

Godbout *et al.*, 2008). Additionally, the Johnson group have shown that peripheral activation of the immune system by LPS leads to deficits in Morris water maze performance in aged mice more so than young, in agreement with earlier studies on cognitive impairments associated with infection (Squire *et al.*, 1992), and have associated this deficit in memory with increased pro-inflammatory cytokine expression in the dentate gyrus and CA regions of the hippocampus (Chen *et al.*, 2008). Immunohistochemical analysis has shown that LPS injected into the striatum can induce caspase 3 activation and this has been shown to be activated within neurons by double staining with NeuN (Choi *et al.*, 2007).

The data presented indicate that caspase 8 activity was significantly increased in tissue prepared from aged, compared with young, rats. Activation of caspase 8 occurs following its recruitment to activated death receptors including TNFα, TRAIL and Fas (Ashkenazi & Dixit, 1998). Caspase 8 may also be upregulated in response to accumulation of reactive oxygen species and there is evidence of a build up of reactive oxygen species in the brain of aged rats (O'Donnell *et al.*, 2000). Significantly, increased production of the pro-inflammatory cytokine IL-1β, which is a feature of the aged brain (Murray & Lynch, 1998b; Martin *et al.*, 2002a), has been associated with enhanced reactive oxygen species generation (Vereker *et al.*, 2001) and IL-1β has been reported to lead to increased activation of sphingomyelinase and subsequent generation of ceramide (Kolesnick & Golde, 1994; Nalivaeva *et al.*, 2000; Davis *et al.*, 2006).

Sphingomyelinase activity in membrane and cytosolic fractions prepared from cortical and hippocampal tissue of aged rats was analysed and was shown to be increased in both fractions relative to values in tissue prepared from young animals. In a study by Crivello and colleagues (2005), membrane- and cytosolic-associated neutral sphingomyelinase activity was evaluated in brain striatum, hippocampus and frontal cortex. They reported greater sphingomyelinase activity in membrane fractions of all brain regions assessed, compared with cytosolic fractions (Crivello *et al.*, 2005). Sphingomyelinase activity in neuroblastoma and liver cells of the rat has

also been shown to be higher in membrane than cytosol (Hostetler & Yazaki, 1979; Spence *et al.*, 1979; Spence *et al.*, 1982; Das *et al.*, 1984). Hippocampal and cortical membrane-associated sphingomyelinase were increased with age (Crivello *et al.*, 2005). The age-related change observed by Crivello and colleagues was greater in the hippocampal tissue than cortical tissue (Crivello *et al.*, 2005). Crivello *et al.*, report that the membrane-associated increase in sphingomyelinase in hippocampus, though not cortex, was coupled with a decrease in cytosolic sphingomyelinase (Crivello *et al.*, 2005). In this study, sphingomyelinase activity was increased with age in the hippocampal membrane fraction. The age-related increase in sphingomyelinase in membrane fractions is in agreement with a study in which membrane sphingomyelinase activity was increased in cerebral cortex in senescence-accelerated mice (Kim *et al.*, 1997). Because of the consequential increase in ceramide, the age-related increase in sphingomyelinase is one change in the brain membrane lipid environment that may be contributing to increased brain vulnerability and sensitivity to inflammation and stress or insult.

In an effort to explore the relationship between sphingomyelinase and caspase-3, *in vitro* experiments were carried out. The data showed that LPS significantly induced increases in caspase-3 and sphingomyelinase activity in cultured cortical neurons. The effect of LPS on caspase-3 was assessed in the presence of GW4869, a specific selective inhibitor of sphingomyelinase. While LPS induced an increase in caspase-3, the presence of the inhibitor of sphingomyelinase abrogated the LPS-induced change in caspase-3. This finding implicates sphingomyelinase as having a crucial signalling role in the LPS-induced activation of caspase-3 and is in agreement with previous findings that activation of the sphingomyelin-sphingomyelinase signalling cascade can induce apoptosis via alteration of mitochondrial function and induction of caspase activation (France-Lanord *et al.*, 1997; Garcia-Ruiz *et al.*, 1997). This finding also suggests that ceramide may be the trigger for the increase in caspase 3 activity.

It has been suggested that caspase 8 has the ability to act on membrane-associated sphingomyelinase (Smyth *et al.*, 1996). This pathway involves the activation of membrane-associated sphingomyelinase in endosomes or lysosomes leading to production of ceramide. This study provides evidence of parallel age–related increases in caspase 8 and caspase 3. There is evidence of apoptosis induced by TNFα and the protein kinase inhibitor, staurosporine, in oligodendrocytes that is dependent upon sequential activation of caspase 8, neutral sphingomyelinase and caspase 3 (Testai *et al.*, 2004). The data presented here, which reveals parallel age-related changes in these enzymes is in agreement with this paper, although it did not examine whether the changes occurred sequentially. Interestingly, induction of apoptosis with an anti-Fas antibody has been shown to be dependent upon sphingomyelinase activation brought about by caspase 8 activity (Sawada *et al.*, 2002) and this provides further evidence of linked changes in these enzymes and a possible causal relationship between them.

3.4.2 Age-related impairment in LTP

Importantly, the age-related increase in enzyme activity in hippocampus was coupled with a reduced ability of rats to sustain LTP. In the case of caspase 3 activity, this is in accordance with the previous finding that there is an inverse correlation between activity of caspase 3 and LTP (Lynch & Lynch, 2002) but there is no evidence to date linking an increase in activation of caspase 8 and sphingomyelinase with a deficit in LTP.

The present findings identify a fundamental relationship between activation of sphingomyelinase and caspase 3 and reveal that the age-associated decrease in LTP is coupled with upregulation of enzyme activity. It is possible that the increases in sphingomyelinase, caspase 8 and caspase 3 are consequences of increased reactive oxygen species production and the pro-inflammatory cytokine IL-1β, both of which are increased in the hippocampus of aged rats and both of which negatively impact on synaptic plasticity and contribute to the reduced ability of aged and LPS-treated rats

to sustain LTP (Vereker *et al.*, 2000a; Kelly *et al.*, 2003). Moreover, LPS has been shown to increase IL-1β which can increase reactive oxygen species (Vereker *et al.*, 2000b) and many proinflammatory mediators, including inducible nitric oxide synthase, a marker of nitric oxide production, the marker of lipid peroxidation 4-hydroxynonenal and 8-OHdG, a marker of oxidative DNA damage (Choi *et al.*, 2007) and these changes have been linked with a reduction in neuronal viability.

Chapter 4 Introduction and methods

4

4.1 Introduction

Omega-3 PUFA are known to have a wide range of beneficial effects and recent evidence indicates that they also possess neuroprotective effects. The most well-known of the n-3 PUFA is DHA (c22:6n-3). DHA is made from the parent fatty acid α-linolenic acid, which is desaturated and elongated in a series of steps to form DHA. Two of the steps in this pathway involve the formation of EPA (c20:5n-3) and its metabolite DPA (c22:5n-3). Anti-inflammatory and protective effects of EPA have been investigated in several studies and its effects are further investigated here. A review of the literature reveals very little about the n-3 PUFA DPA and its actions. There is evidence of effects induced by the n-6 PUFA DPA (c22:5n-6), which has been shown to accumulate in neuronal membranes in the case of n-3 PUFA deficiency (Kim *et al.*, 2003). Evidence indicates that increased n-6 DPA can have negative effects, not protecting neurons from apoptosis (Kim *et al.*, 2003) and n-6 DPA substitution for DHA has been shown to lead to a loss in spatial task performance (Lim *et al.*, 2005b). Here, it is proposed that DPA (c22:5n-3) may have similar actions to EPA and DHA, as these are such closely related molecules and its effects are investigated in this study in *in vitro* experiments. EPA has been shown to have neuroprotective effects in *in vivo* studies, for example, it attenuates the LPS-related changes in synaptic function in the hippocampus (Lonergan *et al.*, 2004). Specifically, EPA was shown to inhibit the LPS-induced increase in IL-1β and this was associated with restoration of LTP. It was also shown to attenuate the age-related increase in microglial activation and IL-1β and to attenuate the age-related impairment in LTP. These data suggest that EPA may exert actions on glia and neurons and this study was designed to investigate this, using cultured cells.

Although there has been a particular focus on investigating the role of microglia in neuroinflammatory changes, recent studies have highlighted the

importance of astrocytes in the regulation of inflammation in the central nervous system. These are the most abundant cell of the brain. Astrocytes express TLRs (Bowman *et al.*, 2003) and when in an activated state can, like microglia, release pro-inflammatory mediators such as IL-6 and IL-1β (Lieberman *et al.*, 1989) that act in the inflammatory response.

The evidence pointing to a neuroprotective effect of EPA includes the finding that EPA attenuated the increase in caspase 3 in cortical homogenate prepared from aged rats. Several upstream events contribute to caspase 3 activation and it has been shown that it is a consequence of increased caspase 8. Here, the effects of EPA and DPA on caspase 3 were assessed in cultured neurons and the possibility that sphingomyelinase played a role in caspase 3 activation was investigated.

The objectives of this study were:

(i) To investigate whether EPA and DPA could modulate the activities of sphingomyelinase and caspase 3 in cultured cortical neurons

(ii) To examine whether EPA and DPA could modulate LPS-induced changes in cytokine secretion from glia

4.2 Methods

Primary cortical neurons (see Section 2.5.3), cortical mixed glia (see Section 2.5.4) and cortical astrocytes (see Section 2.5.5) were prepared from neonatal rats. Cells were pre-treated with EPA (50μM) and DPA (50μM; see Section 2.5.6) and after 24h, cells were stimulated with LPS (100ng/ml; see Section 2.5.6) in order to induce an inflammatory response. Cells were harvested in lysis buffer (see Section 2.5.8) and activities of caspase 3 and sphingomyelinase were assessed (see Sections 2.9.2 and 2.9.1). Concentrations of the cytokines IL-1β, IL-6 and TNFα were assessed in samples of supernatant by ELISA (see Section 2.6.4).

In one experiment neurons were pre-treated with EPA (50μM) or DPA (50μM; see Section 2.5.6) and after 24h were incubated in the presence of ceramide (100μM; see Section 2.5.6). Cell viability was assessed (see Section 2.5.7).

Chapter 4 Results

4.3 Results

4.3.1 Effect of EPA and DPA on neurons

Sphingomyelinase activity was assessed in cultured cortical neurons incubated with LPS, in the presence and absence of EPA and DPA. The data reveal that LPS induced a significant increase in sphingomyelinase compared with controls (0.050 fluorescence units/mg ± 0.003 vs. 0.037 fluorescence units/mg ± 0.004; *$p<0.05$; ANOVA; n=6; Figure 4.1B). The LPS-induced increase in sphingomyelinase activity in neurons pre-treated with DPA (0.030 fluorescence units/mg ± 0.003) and EPA (0.032 fluorescence units/mg ± 0.003) was significantly decreased compared with LPS-treated neurons (0.050 fluorescence units/mg ± 0.003; $^{+++}p<0.001$ and $^{++}p<0.01$ respectively; ANOVA; n=6; Figure 4.1B). Treatment with DPA (0.031 fluorescence units/mg ± 0.004) and EPA (0.032 fluorescence units/mg ± 0.005) alone had no significant effect on sphingomyelinase activity (controls; 0.037 fluorescence units/mg ± 0.004; n=6; Figure 4.1B).

The data show that LPS induced a significant increase in caspase 3 activity compared to controls (355.06 pmol/mg/min ± 31.42 vs. 172.37 pmol/mg/min ± 4.15; ***$p<0.001$; ANOVA; n≥6; Figure 4.2B). This LPS-induced effect was attenuated by incubating cells in the presence of EPA (203.34 pmol/mg/min ± 4.73 vs. 355.06 pmol/mg/min ± 31.42; $^{+++}p<0.001$; ANOVA; n≥6; Figure 4.2B) or DPA (198.41 pmol/mg/min ± 10.47 vs. 355.06 pmol/mg/min ± 31.42; $^{+++}p<0.001$; ANOVA; n≥6; Figure 4.2B).

As EPA and DPA demonstrated an ability to affect the LPS-induced change in sphingomyelinase activity, which leads to increased generation of the pro-apoptotic

second messenger ceramide. The effect of ceramide on cell viability was investigated, and the modulatory effects of EPA and DPA on ceramide-induced changes were assessed. Cell viability was significantly decreased in ceramide-treated neurons compared to control-treated cells. Data are expressed as a percentage of the control which is given as 100%; this was significantly decreased to 69.96% ± 3.60 in ceramide-treated cells (***$p<0.001$; ANOVA; n=6; Figure 4.3). The ceramide-induced decrease in cell viability was not significantly affected by treating cells with DPA (69.96% ± 3.60 vs. 71.11% ± 2.79; n=6; Figure 4.3) but was significantly attenuated by EPA (69.96% ± 3.60 vs. 83.84% ± 3.94; ($^{+}p<0.05$; ANOVA; n=6; Figure 4.3).

4.3.2 Effect of EPA and DPA on mixed glia

LPS induced a significant increase in IL-1β concentration in supernatant prepared from cortical mixed glial cells compared with supernatant obtained from control-treated cells (347.76 pg/ml ± 18.52 vs. 9.16 pg/ml ± 2.85;***$p<0.001$; ANOVA; n≥5; Figure 4.4). This LPS-induced increase in IL-1β levels was attenuated in LPS-treated cells which were pre-treated with DPA (303.69 pg/ml ± 20.14 vs. 347.76 pg/ml ± 18.52; $^{+}p<0.05$; ANOVA; n≥5; Figure 4.4) and EPA (291.80 pg/ml ± 21.67 vs. 347.76 pg/ml ± 18.52; $^{+}p<0.05$; ANOVA; n≥5; Figure 4.4).

In parallel with the LPS-induced change in IL-1β, mean IL-6 concentration was significantly increased in supernatant prepared from cultured cortical mixed glia incubated in the presence of LPS, compared with controls (2,514.85 pg/ml ± 287.33 vs. 0.00 pg/ml ± 0.00; ***$p<0.001$; ANOVA; n=6; Figure 4.5). However, in contrast to the changes in IL-1β, this effect was not significantly altered by pre-treatment of cells with EPA (2885.09 pg/ml ± 338.81 vs. 2,514.85 pg/ml ± 287.33; n=6; Figure 4.5). LPS also induced an increase in TNFα concentration in supernatant prepared from cortical mixed glia (450.26 pg/ml ± 49.36 vs. 0.00 pg/ml ± 0.00; ***$p<0.001$; ANOVA; n=6; Figure 4.6) and, like IL-6, this LPS effect was not attenuated by pre-

treatment of these cells with EPA (540.84 pg/ml ± 89.37 vs. 450.26 pg/ml ± 49.36; n=6; Figure 4.6).

In mixed glia, LPS induced an increase in sphingomyelinase activity compared with control-treated cells (0.020 fluorescence units/ml ± 0.001 vs. 0.013 fluorescence units/ml ± 0.002; *$p<0.05$; ANOVA; $n\geq5$; Figure 4.7B). This LPS-induced increase was attenuated by pre-treatment with EPA (0.013 fluorescence units/ml ± 0.002 vs. 0.020 fluorescence units/ml ± 0.001; $^{+}p<0.05$; ANOVA; $n\geq5$; Figure 4.7B).

4.3.3 Effect of EPA and DPA on astrocytes

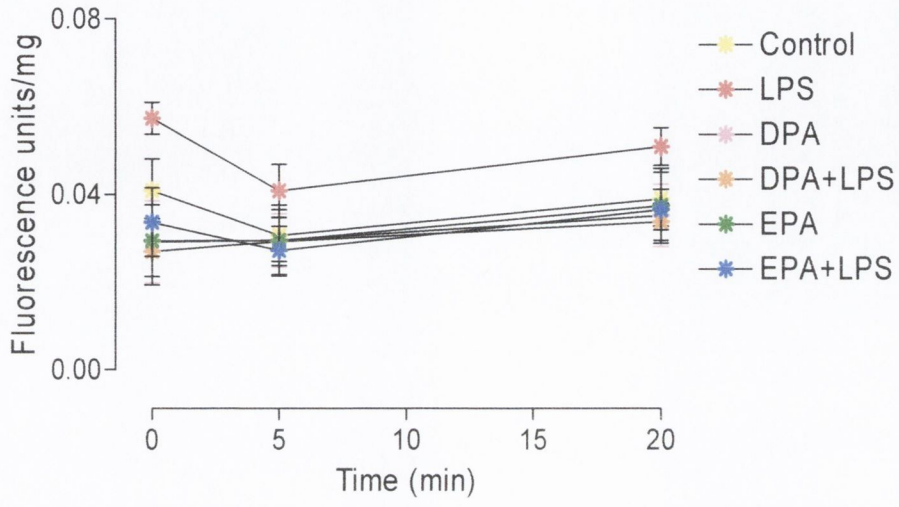
IL-1β concentration was assessed in supernatant prepared from cortical astrocytes treated with LPS in the presence or absence of DPA or EPA. LPS induced a significant increase in IL-1β concentration, compared with controls (46.11 pg/ml ± 13.33 vs. 0.00 pg/ml ± 0.00; ***$p<0.001$; ANOVA; n=6; Figure 4.8). This increase in IL-1β was significantly attenuated by pre-treatment with DPA (9.72 pg/ml ± 2.95 vs. 46.11 pg/ml ± 13.33; $^{+++}p<0.001$; ANOVA; n=6; Figure 4.8) and with EPA (0.63 pg/ml ± 0.58 vs. 46.11 pg/ml ± 13.33; $^{+++}p<0.001$; ANOVA; n=6; Figure 4.8).

Analysis of IL-6 in supernatant obtained from cortical astrocytes revealed an LPS-induced increase in IL-6, compared with controls (23.32 pg/ml ± 5.32 vs 2.16 pg/ml ± 2.16; **$p<0.01$; students *t* test; n=6; Figure 4.9). This LPS-induced increase in IL-6 was not evident in LPS-treated cells in the presence of DPA or EPA so that IL-6 concentration in supernatant from these cells was not significantly different from control-treated supernatants (DPA+LPS 9.22 pg/ml ± 5.56 vs. EPA+LPS 7.30 pg/ml ± 5.38 vs. control 2.16 pg/ml ± 2.16; n=6; Figure 4.9). Similarly, LPS induced an increase in TNFα concentration in supernatant prepared from astrocytes, compared with control-treated cells (1,037.72 pg/ml ± 26.70 vs. 102.23 pg/ml ± 55.49; ***$p<0.001$; ANOVA; n=6; Figure 4.10). This increase was attenuated in supernatants prepared from cells which were pre-treated with DPA (689.18 pg/ml ±

95.16 vs. 1,037.72 pg/ml ± 26.70; $^{++}p<0.01$; ANOVA; n=6 Figure 4.10) and EPA (578.32 pg/ml ± 119.22 vs. 1,037.72 pg/ml ± 26.70; $^{+++}p<0.001$; ANOVA; n=6; Figure 4.10).

Chapter 4 Figures

(A)



(B)

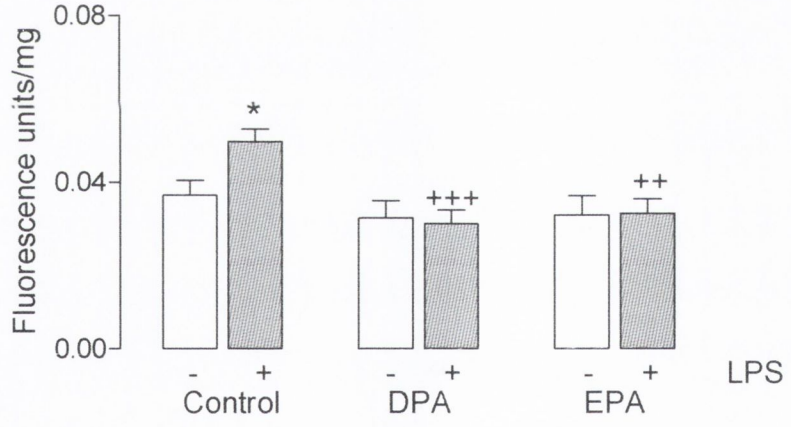
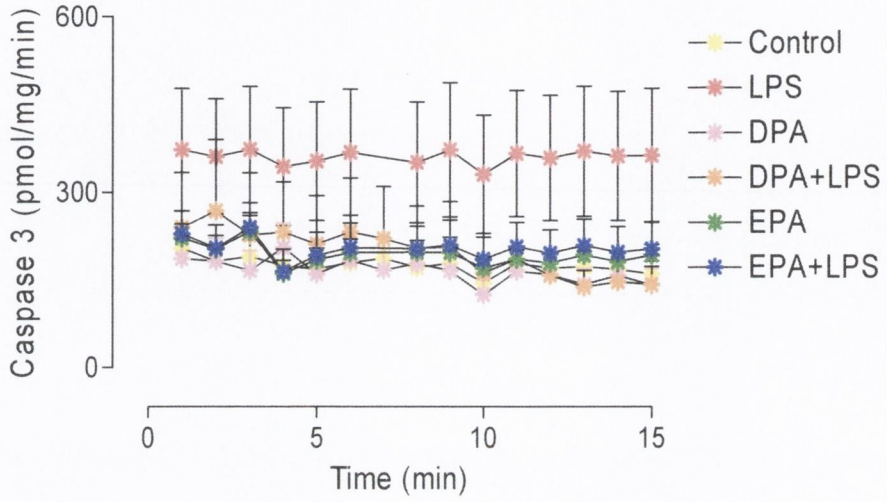


Figure 4.1 EPA and DPA attenuate the LPS-induced increase in sphingomyelinase activity in cultured cortical neurons

Sphingomyelinase activity was assessed following addition of substrate. Mean sphingomyelinase activity in neurons incubated in the presence of LPS was significantly increased compared with controls (B; *$p<0.05$; ANOVA) at 0, 5, 20 minutes. There was a significant difference in sphingomyelinase activity in cortical neurons which were incubated in the presence of LPS + DPA (50μM) and LPS + EPA (50μM) compared with cells incubated in the presence of LPS alone (B; $^{+++}p<0.001$, $^{++}p<0.01$; ANOVA). Values are expressed as fluorescence units, corrected for protein concentration, and are means ± SEM n=6).

(A)



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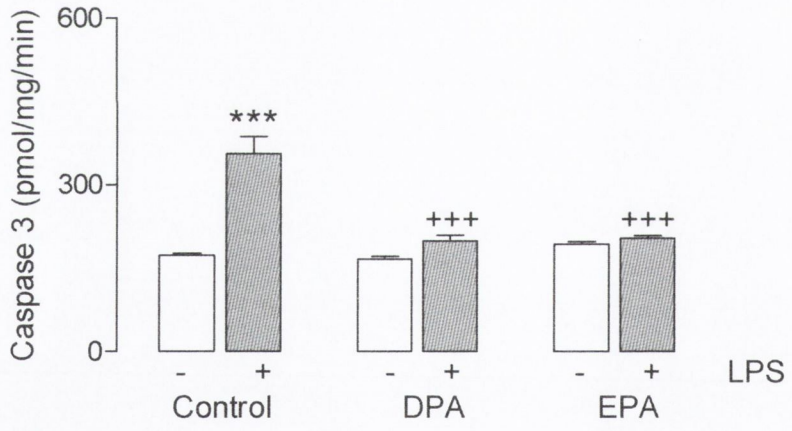


Figure 4.2 EPA and DPA attenuate the LPS-induced increase in caspase 3 activity in cortical neurons

Caspase 3 activity was assessed following addition of substrate. LPS significantly increased caspase 3 activity in cortical neurons over a 15 minute recording period (A). Mean caspase 3 activity was significantly increased in cells which were incubated with LPS (***$p<0.001$; ANOVA) compared with controls. This LPS-induced increase was attenuated by EPA and DPA (50μM; $^{+++}p<0.001$; ANOVA). Values are expressed as pmol/min corrected for protein concentration and are means ± SEM ($n\geq6$).

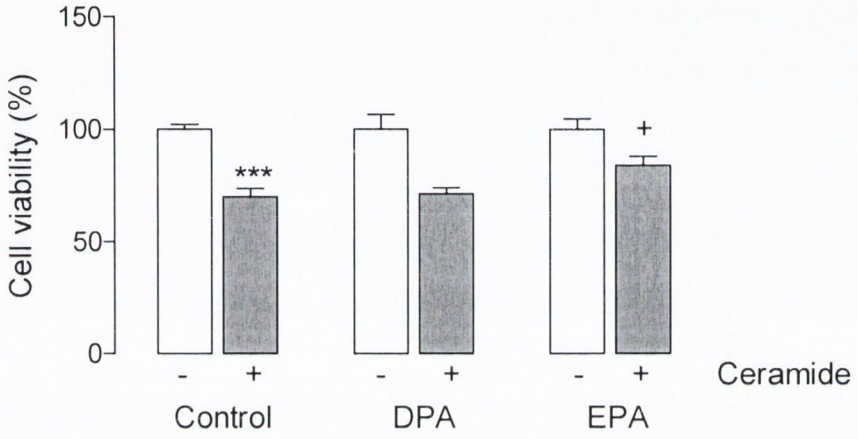


Figure 4.3 EPA attenuates the ceramide-induced decrease in cell viability

Cell viability was assessed in cultured cortical neurons which were incubated in the presence or absence of ceramide (100μM) and which were pre-incubated in the presence or absence of DPA (50μM) or EPA (50μM). Cell viability was significantly decreased in cells incubated with ceramide (***$p<0.001$; ANOVA) compared with controls. The ceramide-induced decrease was attenuated by EPA ($^{+}p<0.05$; ANOVA) but not affected by DPA. Values are expressed as percentage change in cell viability compared to control and are means ± SEM (n=6).

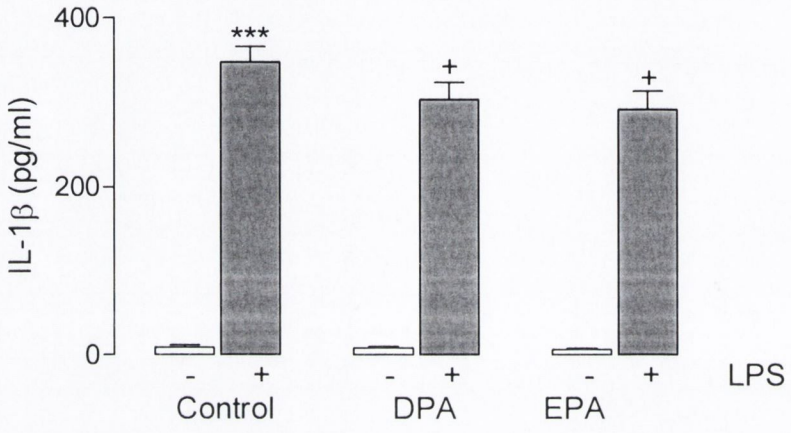


Figure 4.4 EPA and DPA attenuate the LPS-induced increase in IL-1β concentration in supernatant prepared from cultured cortical glia

LPS (100ng/ml) increased IL-1β concentration in supernatant obtained from cortical mixed glia (***$p<0.001$; ANOVA). Incubation in the presence of DPA or EPA significantly attenuated the LPS-induced change ($^{+}p<0.05$; ANOVA). Values are expressed as pg IL-1β/ml and are means ± SEM ($n\geq5$).

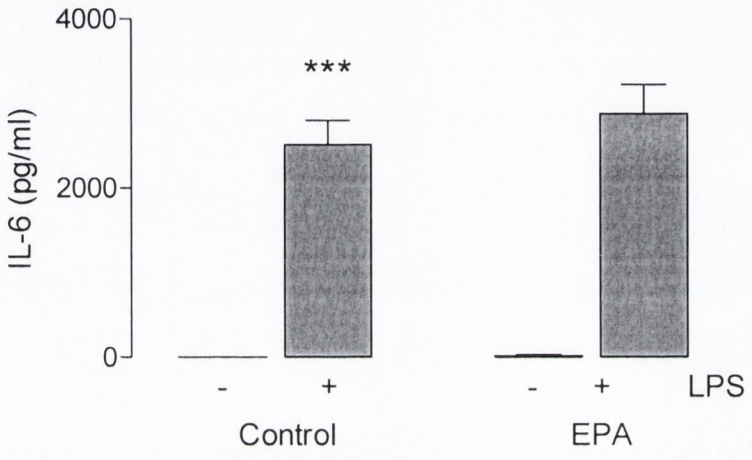


Figure 4.5 LPS induces an increase in IL-6 concentration in supernatant prepared from cultured cortical glia

LPS (100ng/ml) increased IL-6 concentration in supernatant obtained from cortical mixed glia (***$p<0.001$; ANOVA). Incubation in the presence of EPA did not significantly attenuate the LPS-induced change (ANOVA). Values are expressed as pg IL-6/ml and are means ± SEM (n=6).

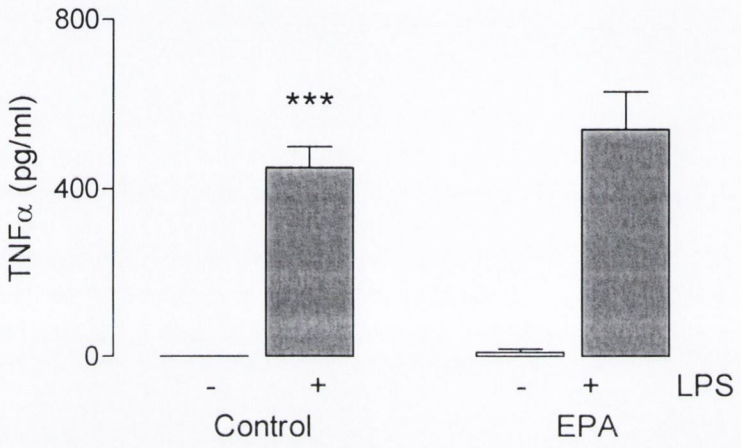
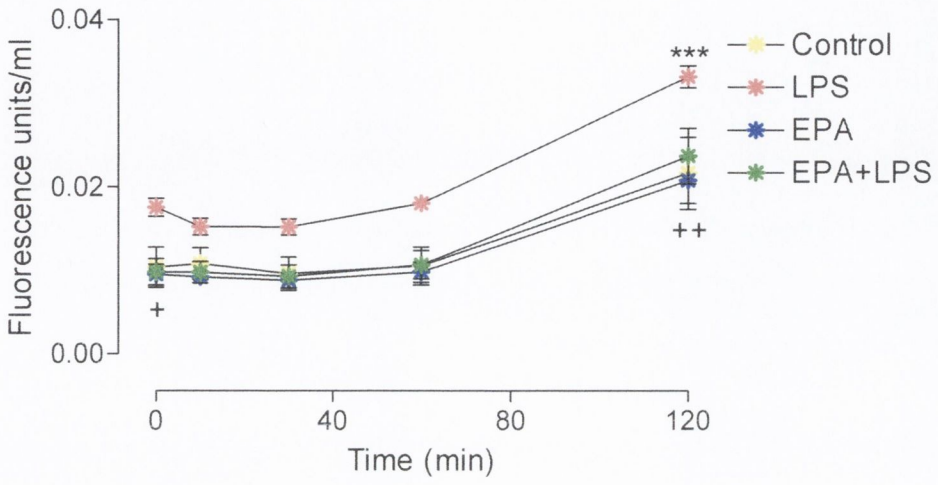


Figure 4.6 LPS induces an increase in TNFα concentration in supernatant prepared from cultured cortical glia

LPS (100ng/ml) increased TNFα concentration in supernatant obtained from cortical mixed glia (***$p<0.001$; ANOVA). Incubation in the presence of EPA did not significantly attenuate the LPS-induced change (ANOVA). Values are expressed as pg TNFα/ml and are means ± SEM (n=6).

(A)



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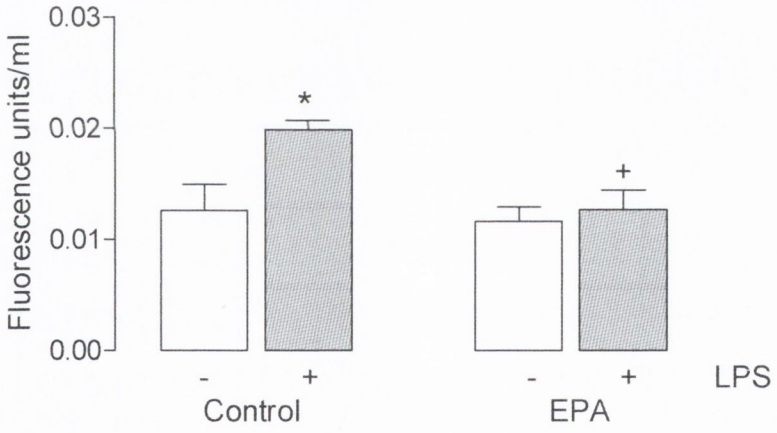


Figure 4.7 EPA attenuates the LPS-induced increase in sphingomyelinase activity in cultured cortical glia

Sphingomyelinase activity was measured over 120min after incubation of the reaction. Sphingomyelinase activity was increased in cortical glia which were incubated in the presence of LPS (100ng/ml) compared with control (A; ***$p<0.001$; 120min; 2 way ANOVA). Sphingomyelinase activity was significantly reduced in LPS-treated cortical glia which were pre-treated with EPA (50μM) compared with cells treated with LPS alone ($^{+}p<0.05$, 0min; $^{++}p<0.01$, 120min; 2 way ANOVA). Figure B shows the mean of all values of sphingomyelinase activity for each experimental group (B). Values are expressed as Fluorescence units per ml in samples corrected for protein and are means ± SEM ($n\geq5$).

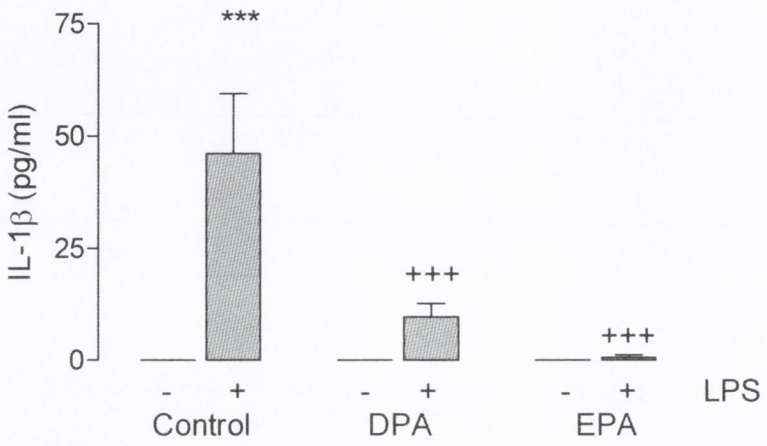


Figure 4.8 EPA and DPA attenuate the LPS-induced increase in IL-1β concentration in supernatant prepared from cultured cortical astrocytes

LPS (100ng/ml) increased IL-1β concentration in supernatant obtained from cortical astrocytes (***$p<0.001$; ANOVA). Incubation in the presence of DPA or EPA significantly attenuated the LPS-induced change ($^{+++}p<0.001$; ANOVA). Values are expressed as pg IL-1β/ml and are means ± SEM (n=6).

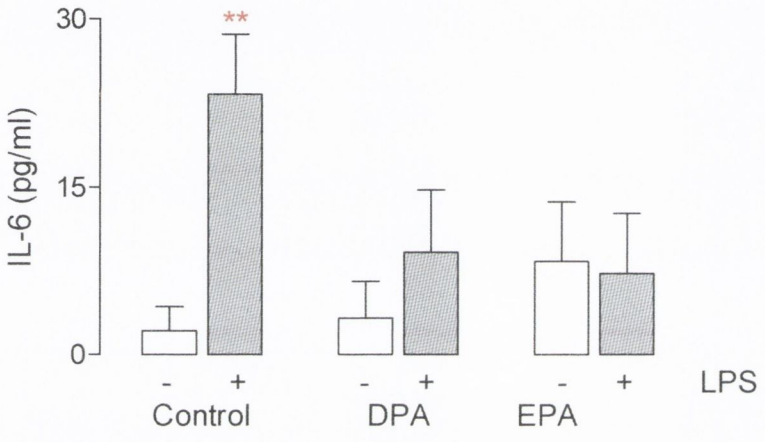


Figure 4.9 EPA and DPA attenuate the LPS-induced increase in IL-6 concentration in supernatant prepared from cultured cortical astrocytes

LPS (100ng/ml) increased IL-6 concentration in supernatant obtained from cortical astrocytes (**$p<0.01$; students *t* test). The LPS-induced increase in IL-6 was not evident in LPS-treated cells in the presence of DPA or EPA so that IL-6 concentration in supernatant from these cells was not significantly different from control-treated supernatants. Values are expressed as pg IL-6/ml and are means ± SEM (n=6).

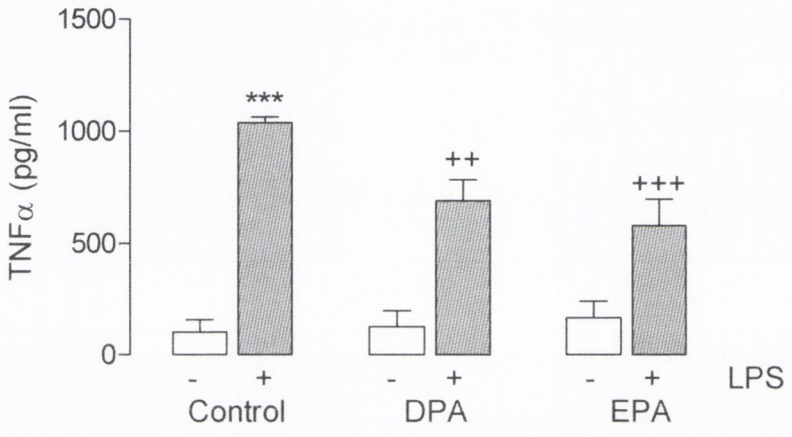


Figure 4.10 EPA and DPA attenuate the LPS-induced increase in TNFα concentration in supernatant prepared from cultured cortical astrocytes

LPS (100ng/ml) increased TNFα concentration in supernatant obtained from cortical astrocytes (***$p<0.001$; ANOVA). Incubation in the presence of DPA or EPA significantly attenuated the LPS-induced change ($^{++}p<0.01$ and $^{+++}p<0.001$ respectively; ANOVA). Values are expressed as pg TNFα/ml and are means ± SEM (n=6).

Chapter 4 Discussion

4.4 Discussion

The objective of this study was to evaluate the potential modulatory effect of DPA and EPA on LPS-induced changes *in vitro*. Evidence is presented which indicates a neuroprotective role for EPA and its metabolite DPA; both attenuate the LPS-induced increases in sphingomyelinase and caspase 3 in neurons. The data also reveal an anti-inflammatory effect of EPA and DPA which were shown to attenuate the LPS-induced increase in the release of the pro-inflammatory cytokine, IL-1β, from glia.

4.4.1 Effect of EPA and DPA on neurons

LPS induced a significant increase in neutral sphingomyelinase activity in neuronal cells, which is consistent with the finding that incubation of C6 rat glioma cells with LPS increases intracellular ceramide, together with a rise in the expression of inducible nitric oxide synthase (iNOS). These increases in ceramide and iNOS were shown to be induced through the action of neutral sphingomyelinase, as when neutral sphingomyelinase was blocked with the inhibitor 3-O-methylsphingomyelin, the changes were not evident (Won *et al.*, 2004). In contrast, inhibition of acid sphingomyelinase and inhibition of *de novo* synthesis of ceramide did not affect iNOS expression. The data presented here concur with these (Won *et al.*, 2004). Interestingly, LPS has been shown to induce membrane-associated sphingomyelinase activity in macrophages (Amtmann *et al.*, 2003), with a consequential production of ceramide (MacKichan & DeFranco, 1999). *In vivo* experiments have also revealed that LPS can increase sphingomyelinase. Thus, intraperitoneal injection of mice with LPS has been shown to increase sphingomyelinase activity 2 to 2.5 fold in serum (Wong *et al.*, 2000).

The data from this study establish that EPA and DPA abrogate the LPS-induced increase in sphingomyelinase activity in neurons, which is consistent with a neuroprotective effect. This apparently contrasts with the findings of Kishida and

colleagues, who reported no effect of DHA on ceramide-induced apoptosis, but they showed that DHA attenuated sphingosine-associated apoptosis (Kishida *et al.*, 1998). They proposed that a 24h incubation period with DHA was necessary for this protective effect, since 6h incubation with DHA failed to have an effect suggesting that DHA may need to be incorporated into membrane to exert its effect. Similar findings were observed in neuro 2A cells (Kim *et al.*, 2000). Interestingly, EPA did not affect sphingosine-induced apoptosis in HL60 cells (Kishida *et al.*, 1998).

The actions of EPA and DPA on LPS-induced sphingomyelinase reported here in this study may be indicative of protection as sphingomyelinase acts on sphingomyelin and results in the generation of ceramide. The present data show that ceramide induced a significant decrease in cell viability and this decrease was attenuated only by EPA, but not DPA. The detrimental effects of ceramide on cell viability have been shown previously, for example, incubation of primary cortical neuron cultures with C2-ceramide induces apoptosis, which has been associated with activation of p38 and c-Jun N-terminal kinase (JNK) phosphorylation coupled with caspase 3 activation (Willaime *et al.*, 2001). This is in agreement with several other studies; for example, Toman and colleagues have shown that C2-ceramide and exogenous sphingomyelinase induce apoptosis in rat cerebellar granule cells and cortical neurons in a time-and a dose-dependent manner (Toman *et al.*, 2002).

Similarly, short-chain ceramide analogs cause caspase 3 activation and poly ADP ribose polymerase cleavage and in this way induce apoptosis (Smyth *et al.*, 1996). The mechanism by which ceramide induces apoptosis has been extensively studied and it has been shown that ceramide functions upstream of Bcl-2 and caspase 3 in the cell death cascade (Dbaibo *et al.*, 1997) and downstream of p53 in apoptosis induced by γ-irradiation (Dbaibo *et al.*, 1998). Ceramide increases cathepsin D (Heinrich *et al.*, 2004), which acts on Bid, linking in with the pathway described earlier involving the direct action of caspase 8 on Bid. Caspase 8 has been shown to act on membrane-associated sphingomyelinase, in endosomes for example, and in this way lead to the generation of ceramide (Monney *et al.*, 1998).

Although both EPA and DPA attenuated the LPS-induced increases in activity of sphingomyelinase, the evidence shows that only EPA significantly attenuated the effect of ceramide on cell viability. Several studies have revealed a neuroprotective effect of EPA (Lynch *et al.*, 2003; Kavanagh *et al.*, 2004; Lonergan *et al.*, 2004) and its ability to prevent apoptosis in renal tubule cells has also been reported in a study in which DHA failed to exert a similar effect (Sasaki & Takita, 2006).

Neutral sphingomyelinase activity has been linked with caspase 3 activation and, in a recent study, has been identified as a crucial enzyme in the pathway of ceramide generation and apoptosis induced by heat stress in zebrafish embryonic cultured cells (Yabu *et al.*, 2008). Similarly, inhibition of sphingomyelinase resulted in an attenuation of serum/glucose deprivation-induced increases in ceramide generation, JNK activation and caspase 3 activity (Soeda *et al.*, 2004) and reduced cerebral infarct volume in mice following middle cerebral artery occlusion (Soeda *et al.*, 2004).

Here, the LPS-induced increase in sphingomyelinase activity was paralleled by an increase in caspase 3 activity and, in both cases; EPA and DPA attenuated the LPS-associated changes. These data are consistent with the findings which indicate that sphingomyelinase plays a role in modulating caspase 3. In a previous study, the relationship between sphingomyelinase and caspase 3 has been elegantly made. Incubation of primary cultured mouse neurons with agents to induce lysosomal and endosomal membrane destabilization resulted in an increase in sphingomyelin hydrolysis and accumulation of ceramide within endosomes and lysosomes which correlated with activation of pro-caspase 8, pro-caspase 9 and caspase 3, and subsequent cell death (Ditaranto-Desimone *et al.*, 2003).

The present data suggest that the protective role of EPA and DPA may derive from their ability to attenuate the LPS-induced increase in sphingomyelinase, and, in this way, modulate caspase 3 activity. *In vitro* evidence using a sphingomyelinase inhibitor provides evidence to support this (see Chapter 3).

Interestingly, in a recent study by Leroy *et al.*, 2008, rat neonatal cardiomyocytes were incubated in the presence of EPA and then apoptosis was induced after two days by exposure of the cells to palmitate, this time in the absence of EPA from the media. They report that incubation with EPA resulted in significant membrane enrichment with n-3 PUFAs, most notably in DPA. Which the authors suggested was responsible for the EPA-associated attenuation of palmitate-induced increases in caspase 3 activation, Bax translocation to the mitochondrial membrane and consequent release of cytochrome *c* (Leroy *et al.*, 2008).

4.4.2 Effect of EPA and DPA on glia

As the principal cell of the central nervous system involved in the innate immune response, microglia have been shown to release pro-inflammatory mediators such as the cytokines IL-1β (Giulian *et al.*, 1986), IL-6 (Frei *et al.*, 1989), TNFα (Sawada *et al.*, 1989), reactive oxygen species and nitric oxide in response to amyloid-β protein and interferon gamma (Tanaka *et al.*, 1994; Meda *et al.*, 1995).

However, astrocytes also participate in the immune response and there is evidence showing that astrocytes release TNFα (Sawada *et al.*, 1989), while production of the cytokines IL-1β and IL-6 has been detected in astrocytes following LPS stimulation (Lieberman et al., 1989). The present data indicate that treatment of astrocytes resulted in increased IL-1β, TNFα and IL-6. Significantly greater concentrations of IL-1β and IL-6, but not TNFα, were released from mixed glia compared with astrocytes; this probably reflects the presence of microglia (approximately 30%) in the mixed cultures. LPS is the best known activator of TLR4. TLRs are important for the identification of infectious agents and endogenous inflammatory signals which indicate to the body that there is cell damage and trigger responses. TLRs are widely expressed in the central nervous system; it has emerged that astrocytes, microglia and oligodendrocytes express TLRs, hallmark pattern-recognition receptors of the innate immune system (Bsibsi *et al.*, 2002; Bowman *et al.*, 2003; Lehnardt *et al.*, 2003; Olson & Miller, 2004; Carpentier *et al.*, 2005; Jack *et*

al., 2005; Walter *et al.*, 2007) involved in innate immunity. Additionally, it has come to light that neurons express TLRs (Lafon *et al.*, 2006; Ma *et al.*, 2006; Kigerl *et al.*, 2007; Rolls *et al.*, 2007), in particular TLR4 has been shown to be expressed in cerebral cortical neurons (Tang *et al.*, 2007).

Cultured human astrocytes have been shown to express TLR2, TLR3 (Bsibsi *et al.*, 2002) and the presence of TLR4 on cultured murine astrocytes has been observed by immunofluorescence (Bowman *et al.*, 2003). LPS-responsive cells, including peripheral blood leukocytes, monocytes, macrophages and microglia, become activated upon the interaction of LPS with circulating LPS-binding protein and CD14, which is a glycosylphosphatidlyinositol-linked cell surface glycoprotein essential for the LPS response (Schletter *et al.*, 1995; Ulevitch & Tobias, 1995; Chow *et al.*, 1999). The active form of TLR4 causes an increase in NFκB and its regulated genes for IL-1 and IL-6 (Medzhitov & Janeway, 2002).

The present data suggest that LPS interacts with TLR4 to release these pro-inflammatory cytokines and this is consistent with previous reports which demonstrated that incubation of mixed glial cultures or co-cultures of astrocytes and microglia with LPS increased release of these cytokines (Kong *et al.*, 1997; Wierinckx *et al.*, 2005).

The present data indicate that incubation of cells with EPA or DPA attenuated the LPS-induced changes but the effect was more robust in astrocytes than in mixed glial cultures. EPA has been shown to exert similar effects previously. EPA can significantly attenuate LPS-induced increases in nitric oxide, PGE_2, IL-1β, IL-6 and TNFα in BV2 microglia, as well as cyclooxygenase-2, iNOS, activation of NFκB, extracellular signal-regulated kinase, p38 and JNK (Moon *et al.*, 2007). However, in another study, while DHA attenuated LPS-induced IL-1β mRNA expression in spleen leukocytes, EPA was ineffective (Watanabe *et al.*, 2000). Anti-inflammatory effects of EPA have also been identified *in vivo*; thus treatment of rats with EPA has been shown to reduce the age-related increase in hippocampal concentration of IL-1β

(Martin *et al.*, 2002a; Martin *et al.*, 2002b), while EPA and DHA both decreased serum concentrations of pro-inflammatory cytokines in LPS-treated dogs (LeBlanc *et al.*, 2008). One of the most significant and novel findings in this study is that DPA also possesses anti-inflammatory effects; these are reflected by its ability to mimic the effect of EPA in reducing IL-1β production from mixed glia and astrocytes. To my knowledge, similar effects of DPA have not been described previously.

In contrast to the modulatory effects of EPA on the LPS-induced increase in IL-1β from mixed glia, there was no evidence of a similar effect on production of IL-6. This suggests that the mechanisms by which cytokine production and release are controlled are cytokine-specific. The finding that LPS-triggered TNFα release from astrocytes is attenuated by EPA and DPA, whereas release from mixed glia is not, provides evidence that mechanisms underlying modulation of cytokine production and release is also cell-specific. These findings, which were obtained from studying primary cortical cultures contrast with data obtained from human THP-1 monocyte-derived macrophages, where the LPS-induced increases in IL-1β, IL-6 and TNFα mRNA and protein were all attenuated by pre-incubation of cells in the presence of EPA or DHA (Weldon *et al.*, 2007).

The data show that LPS increased sphingomyelinase activity in mixed glia as it did in neurons and that EPA exerted a similar modulatory effect in both cell types. Interestingly, sphingomyelinase activity was higher in neurons than in mixed glia and this is largely consistent with the findings of Kilkus and colleagues (2008), who reported that neurons have relatively high sphingomyelinase activity compared with glia, specifically oligodendrocytes (Kilkus *et al.*, 2008). It has been shown that sphingomyelinase activity is coupled with NFκB activation (Pahan *et al.*, 1998) and that its activation can lead to production of inflammatory cytokines like IL-6 (Fiebich *et al.*, 1995). The possibility exists therefore that the LPS-induced increase in sphingomyelinase activity in mixed glia described here is a factor which contributes to cytokine production.

It has been reported that, through inducing IL-1β and TNFα, LPS-treated astrocytes can have harmful effects on neurons and can even induce apoptosis (Chao *et al.*, 1996; Mizuno *et al.*, 2005). A study by Suzumura and colleagues has reported that microglia and astrocytes stimulated by LPS can have a toxic effect on neurons; the evidence showed that the combination of IL-1β, IL-6 and TNFα induced significant neuronal death, but none alone were capable of doing so (Suzumura *et al.*, 2006). TNFα has also been shown to induce apoptosis in neurons (Chao *et al.*, 1995).

In vivo, activated astrocytes, not unlike microglia, can produce and release molecules to target nearby cells and propagate the inflammatory response, including signals for leukocyte recruitment. The activation of astrocytes by LPS has been shown to stimulate these cells to release a variety of soluble inflammatory mediators such as IL-6 and these mediators influence and regulate neuroinflammation (Farina *et al.*, 2007).

Vedin and colleagues have shown that a 6 month supplementation of DHA or EPA to Alzheimer's disease patients affects cytokine release from peripheral blood mononuclear cells. Plasma concentrations of DHA and EPA were significantly increased following the 6 month intervention, compared with placebo-treated patients. Peripheral blood mononuclear cells obtained from patients which received n-3 PUFA exhibited significantly lower levels of IL-6 and IL-1β secreted following overnight incubation of the cells with LPS (Vedin *et al.*, 2008). In that study, reductions in IL-1β and IL-6 were parallel and correlated with each other (Vedin *et al.*, 2008).

The evidence indicates that LPS reduces neuronal viability and this is shown by increased activities of caspase and sphingomyelinase. EPA and DPA have the ability to modulate LPS-induced changes in sphingomyelinase and caspase activity and therefore exert a neuroprotective effect. These fatty acids also exhibit potentially protective anti-inflammatory effects as revealed by their ability to modulate LPS-

induced increases in the pro-inflammatory mediators IL-1β, IL-6 and TNFα in glial cells.

Chapter 5 Introduction and methods

5

5.1 Introduction

Evidence of beneficial, anti-inflammatory and neuroprotective effects of n-3 PUFA has emerged in recent years and the data presented in chapter 4 have demonstrated anti-inflammatory and protective effects of EPA and DPA *in vitro* in mixed glial cells, astrocytes and neuronal cell cultures in response to insult triggered by LPS and ceramide. Inflammatory stress in ageing can be characterised by alterations in immune function and physiological response to stress, changes in pro- and anti-inflammatory cytokine levels, chemokines and activation of microglial cells. Generation of ceramide via the activation of sphingomyelinase has been shown to modulate responses not only to LPS but other stresses including IL-1β and TNFα (Nikolova-Karakashian *et al.*, 2008). In contrast, dietary supplementation with EPA has been shown to abrogate the age-related deficit in LTP, and attenuate age-associated increases in IL-1β and IFNγ in the hippocampus (Lynch *et al.*, 2007). EPA has anti-inflammatory properties including the attenuation of age-related changes in markers of microglial activation. There is evidence that oral administration of EPA can attenuate age-related increases in MHCII, CD40 protein (Lynch *et al.*, 2007). Another important way in which EPA, and DPA, may control the activation of microglial cells, is by affecting neuronal expression of CD200 ligand or the chemokine fractalkine, which have been shown to interact with their receptors which are present on microglia and consequently maintain microglia in a quiescent state.

The mechanisms underlying the neuroprotective and anti-inflammatory effects of EPA require further investigation and this study sought to investigate these, and the possible anti-inflammatory and protective effects of DPA. In this study, the effects of EPA and DPA on age-related changes in the Morris water maze, LTP and concomitant cellular changes in signalling in the cortex and hippocampus, and changes in activation of microglia were examined.

5.2 Methods

Rats aged between 2 and 3 months and between 22 and 24 months of age were treated for 8 weeks with 200mg/rat/day EPA or 200mg/rat/day DPA (Amarin Neuroscience, UK; see Section 2.1.2) or received normal laboratory chow enriched with monounsaturated fatty acids to ensure isocaloric intake in the treatment groups. Rats were tested for their performance in the Morris water maze and their ability to maintain LTP at the end of the 8 week period in order to assess the effect of oral administration of EPA and DPA (see Section 2.2 and Section 2.3).

Hippocampal and cortical tissue was taken and prepared (see Section 2.4) for investigation of markers of microglial activation by mRNA analysis (see Section 2.8), chemokine production (see Section 2.6), activity of sphingomyelinase (see Section 2.9.1), activities of caspases (see Sections 2.9.2 and 2.9.3) and PPARγ (see Section 2.9.4). DNA was isolated from hippocampal and cortical tissue, digested and assessed for 8-OHdG, a marker of oxidative stress (see Section 2.7).

Chapter 5 Results

5.3 Results

5.3.1 Ageing, n-3 PUFA and learning and memory

Figure 5.1 shows that LTP was significantly reduced in aged, compared with young rats. The mean percentage changes in EPSP slope in the last 5 min of recording of the experiment were 130.99% ± 0.60 and 121.09% ± 0.52 in young and aged rats respectively. These values represent a significant decrease in aged compared with young animals ($p<0.001$; ANOVA; $n\geq4$; Figure 5.1G and H). Mean percentage change in population EPSP slope was significantly increased, in the last 5 min of recording post tetanus, in EPA-treated young, compared with control-treated young, rats (144.20% ± 0.69 vs. 130.99% ± 0.60; $p<0.001$; ANOVA; $n\geq4$; Figure 5.1G and H) and was significantly increased in DPA- and EPA-treated aged, compared with control-treated aged, rats (149.32% ± 0.37 and 132.06% ± 0.43 vs. 121.09% ± 0.52 respectively; $p<0.001$; ANOVA; $n\geq4$; Figure 5.1G and H).

Figure 5.2 shows performance of rats in the Morris water maze task in terms of escape latencies, analysed by 2-way ANOVA with repeated measures. Figure 5.2 shows performance of rats in the Morris water maze task in terms of escape latencies. There was a significant age-related impairment in escape latency (**$p<0.01$, DF 1, $F=73.823$; ANOVA; $n\geq6$; Figure 5.2B). There was a significant treatment-related improvement in escape latency, analysis of DPA- and EPA-treated rats revealed significantly shorter escape latency compared with control-treated rats (control compared with DPA: **$p<0.01$, control compared with EPA: *$p<0.05$; ANOVA; $n\geq6$; Figure 5.2B). There was no significant difference between DPA- and EPA-treated groups (ANOVA; $n\geq6$; Figure 5.2B). There was a significant difference between control-treated young, compared with control-treated aged, rats (***$p<0.001$; ANOVA; $n\geq6$; Figure 5.2B). There was a significant difference between DPA-treated young, compared with DPA-treated aged, rats (***$p<0.001$; ANOVA; $n\geq6$; Figure 5.2B). There was no significant difference between EPA-treated young, compared with EPA-treated aged, rats (ANOVA; $n\geq6$; Figure 5.2B). A

comparison of aged EPA-treated rats with young control-treated rats showed no significant difference between these groups on trial day 5 and there was a significant attenuation of the age-related change in aged EPA-treated rats, compared with aged controls ($^{+++}p<0.001$; ANOVA; n≥6; Figure 5.2B). Animals which received EPA showed no significant age-related impairment in escape latency on day 5 (ANOVA; n≥6; Figure 5.2B).

The total distance swam by animals was investigated and the data reveal that aged, control-treated rats swam a significantly greater total distance than young control-treated rats (**$p<0.01$, DF 1, $F=49.701$; ANOVA; n≥6; Figure 5.3B). There was a significant treatment-related improvement in distance swam ($^{++}p<0.01$, DF 2, $F=4.838$; ANOVA; n≥6; Figure 5.3B). Analysis of DPA-treated rats revealed a significant difference compared with control-treated rats ($^{++}p<0.01$, ANOVA; n≥6; Figure 5.3B). There was no significant difference between EPA-treated and control-treated rats (ANOVA; n≥6; Figure 5.3B), nor between DPA- and EPA-treated groups (ANOVA; n≥6; Figure 5.3B).

5.3.2 Ageing, n-3 PUFA and cell signalling

Activities of caspase 8 and 3 were examined in the cortex of rats in all treatment groups. The data are expressed as a percentage of the control which is given as 100% and show that caspase 8 activity was significantly increased to 131.45% ± 0.72 in cortical tissue prepared from aged, compared with young, animals (***$p<0.001$; ANOVA; n≥6; Figure 5.4A). The age-associated increase in caspase 8 activity was significantly lower in animals which were given DPA (131.45% ± 0.72 vs. 124.53% ± 0.52; $^{+++}p<0.001$; ANOVA n≥6; Figure 5.4A) or EPA (131.45% ± 0.72 vs. 111.60% ± 0.64; $^{+++}p<0.001$; ANOVA n≥6; Figure 5.4A).

In parallel, there was an age-related increase in caspase 3 activity to 143.26% ± 0.73 in cortical tissue prepared from control-treated rats. The age-associated increase in activity of caspase 3 was significantly reduced in animals which were

given DPA (143.26% ± 0.73 vs. 122.65% ± 0.62; $^{+++}p<0.001$; ANOVA n≥6; Figure 5.4B) or EPA (143.26% ± 0.73 vs. 107.91% ± 0.71; $^{+++}p<0.001$; ANOVA n≥6; Figure 5.4B).

Sphingomyelinase activity was assessed in cytosolic and membrane fractions prepared from cortical tissue and the data provide evidence of a significant age-related increase in sphingomyelinase activity in membrane fractions (0.073 fluorescence units/mg ± 0.013 vs. 0.040 fluorescence units/mg ± 0.005; *$p<0.05$; ANOVA; n≥6; Figure 5.5A). There was no significant age-related change in sphingomyelinase activity in cortical membrane fractions prepared from animals which received DPA (0.046 fluorescence units/mg ± 0.003 vs. 0.041 fluorescence units/mg ± 0.001; n≥6; Figure 5.5A) or EPA (0.044 fluorescence units/mg ± 0.002 vs. 0.042 fluorescence units/mg ± 0.003; n≥6; Figure 5.5A). This sphingomyelinase assay and fractionation of this cortical tissue was carried out by Dr. Anne-Marie Miller.

There was no significant age-associated change in sphingomyelinase activity in cortical cytosolic fractions prepared from control-treated animals (0.024 fluorescence units/mg ± 0.001 vs. 0.0024 fluorescence units/mg ± 0.002; n≥6; Figure 5.5B), or DPA-treated (0.023 fluorescence units/mg ± 0.001 vs. 0.025 fluorescence units/mg ± 0.003; n≥6; Figure 5.5B), or EPA-treated rats (0.026 fluorescence units/mg ± 0.003 vs. 0.028 fluorescence units/mg ± 0.003; n≥6; Figure 5.5B).

Sphingomyelinase activity was significantly increased in membrane fractions prepared from hippocampal tissue of aged, compared with young, rats (0.114 fluorescence units/mg ± 0.002 vs. 0.106 fluorescence units/mg ± 0.003; *$p<0.05$; students *t* test; n≥6; Figure 5.6). There was a significant decrease observed in sphingomyelinase activity in young rats which received DPA (0.089 fluorescence units/mg ± 0.005 vs. 0.106 fluorescence units/mg ± 0.003; **$p<0.01$; ANOVA; n≥6; Figure 5.6) or EPA (0.088 fluorescence units/mg ± 0.004 vs. 0.106 fluorescence units/mg ± 0.003; n≥6; Figure 5.6), compared with control-treated young rats.

Furthermore, there was a significant decrease in sphingomyelinase activity in aged animals which received DPA (0.097 fluorescence units/mg ± 0.002 vs. 0.114 fluorescence units/mg ± 0.002; ($^{+++}$p<0.001; ANOVA; n≥6; Figure 5.6) and EPA (0.088 fluorescence units/mg ± 0.002 vs. 0.114 fluorescence units/mg ± 0.002; $^{+++}$p<0.00; ANOVA; n≥6; Figure 5.6), compared with control-treated aged rats.

5.3.3 Ageing, n-3 PUFA and microglial activation

There was a significant increase in CD68 mRNA expression, a marker of microglial activation which is also thought to be indicative of phagocytosis, in cortex prepared from aged, compared with young, animals (0.26 mRNA ± 0.04 vs. 0.12 mRNA ± 0.01; **p<0.01; ANOVA; n≥6; Figure 5.7). This age-related increase in CD68 mRNA expression persisted in animals which received DPA (0.23 mRNA ± 0.02 vs. 0.11 mRNA ± 0.01; **p<0.01; ANOVA; n≥6; Figure 5.7) or EPA (0.27 mRNA ± 0.02 vs. 0.12 mRNA ± 0.01; ***p<0.001; ANOVA; n≥6; Figure 5.7).

MHCII mRNA expression was not significantly altered in cortical tissue prepared from aged, compared with young, rats (0.65 mRNA ± 0.05 vs. 0.76 mRNA ± 0.07; ANOVA; n≥6; Figure 5.8) but it was significantly decreased in cortical tissue prepared from aged rats which received DPA (0.55 mRNA ± 0.02 vs. 0.68 mRNA ± 0.04; *p<0.05; students *t* test; n=6; Figure 5.8) or EPA (0.56 mRNA ± 0.02 vs. 0.69 mRNA ± 0.04; **p<0.01; students *t* test; n=6; Figure 5.8), compared with young DPA- and EPA-treated rats.

The data reveal a significant age-associated increase in cortical CD11b mRNA (0.23 mRNA ± 0.05 vs. 0.10 mRNA ± 0.01; *p<0.05; ANOVA; n≥6; Figure 5.9). This age-related increase was also evident in rats which received DPA (0.20 mRNA ± 0.02 vs. 0.09 mRNA ± 0.01; *p<0.05; ANOVA; n≥6; Figure 5.9) but the difference did not reach statistical significance in the cortex of aged rats which received EPA (0.19 mRNA ± 0.02 vs. 0.11 mRNA ± 0.02; ANOVA; n≥6; Figure 5.9).

There was no significant effect of age (1.82 mRNA ± 1.33 vs. 0.68 mRNA ± 0.15; ANOVA; n=6; Figure 5.10) or treatment (1.34 mRNA ± 0.35 vs. 1.04 mRNA ± 0.31; ANOVA; n=6; DPA; 1.91 mRNA ± 0.86 vs. 1.23 mRNA ± 0.19; ANOVA; n=6; EPA; Figure 5.10) on IL-1β mRNA expression in the cortex.

There was a significant increase in CD68 mRNA expression in hippocampal tissue prepared from aged, compared with young, animals (5.27 mRNA ± 0.64 vs. 1.35 mRNA ± 0.08; ***$p<0.001$; ANOVA; $n\geq6$; Figure 5.11). This age-related increase in CD68 mRNA expression persisted in animals which received DPA (5.07 mRNA ± 0.50 vs. 1.44 mRNA ± 0.14; ***$p<0.001$; ANOVA; $n\geq6$; Figure 5.11) or EPA (5.26 mRNA ± 0.21 vs. 1.65 mRNA ± 0.18; ***$p<0.001$; ANOVA; $n\geq6$; Figure 5.11).

MHCII mRNA was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (2.40 mRNA ± 0.42 vs. 1.26 mRNA ± 0.19; **$p<0.01$; ANOVA; $n\geq6$; Figure 5.12). This age-related increase in MHCII mRNA was significantly attenuated in aged rats which received DPA (1.14 mRNA ± 0.05 vs. 2.40 mRNA ± 0.42; $^{+}p<0.05$; ANOVA; $n\geq6$; Figure 5.12) or EPA (1.17 mRNA ± 0.05 vs. 2.40 mRNA ± 0.42; $^{+}p<0.05$; ANOVA; $n\geq6$; Figure 5.12).

The data reveal an age-related increase in CD11b mRNA expression in the hippocampus (3.38 mRNA ± 0.36 vs. 0.87 mRNA ± 0.04; ***$p<0.001$; ANOVA; $n\geq6$; Figure 5.13). This age-associated increase in CD11b mRNA expression was not affected by oral administration of DPA (3.50 mRNA ± 0.41 vs. 0.78 mRNA ± 0.10; ***$p<0.001$; ANOVA; $n\geq6$; Figure 5.13) or EPA (3.38 mRNA ± 0.13 vs. 1.06 mRNA ± 0.15; ***$p<0.001$; ANOVA; $n\geq6$; Figure 5.13).

There was a significant increase in IL-1β mRNA expression in the hippocampus of aged, compared with young, rats (10.29 mRNA ± 3.70 vs. 2.44 mRNA ± 0.64; *$p<0.05$; students *t* test; $n\geq6$; Figure 5.14). This age-related increase in IL-1β mRNA was not observed in aged rats which received EPA (6.59 mRNA ±

1.15 vs. 3.04 mRNA ± 0.44; ANOVA; n≥6; Figure 5.14) but was evident in tissue prepared from aged DPA-treated rats (35.21 mRNA ± 6.28 vs. 10.87 mRNA ± 3.56; ***p<0.001; ANOVA; n≥6; Figure 5.14).

5.3.4 Ageing, n-3 PUFA and oxidative stress

8-OHdG was measured in samples of DNA prepared from cortex and the data revealed no significant age-related change in this marker of oxidative stress (0.030 μg/pg ± 0.010 vs. 0.011 μg/pg ± 0.002; ANOVA; n≥6; Figure 5.15). However, although not statistically significant, the value in tissue prepared from aged rats was double that in tissue prepared from young rats (0.030 μg/pg ± 0.010 vs. 0.011 μg/pg ± 0.002). The age-related increase was attenuated, though not significantly, by DPA (0.016 μg/pg ± 0.006 vs. 0.030 μg/pg ± 0.010; ANOVA; n≥6; Figure 5.15) and EPA (0.009 μg/pg ± 0.004 vs. 0.030 μg/pg ± 0.010; ANOVA; n≥6; Figure 5.15).

In hippocampal samples, there was no significant age-related change in 8-OHdG (0.12 μg/pg ± 0.06 vs. 0.06 μg/pg ± 0.01; ANOVA; n≥6; Figure 5.16) and treatment with DPA (0.09 μg/pg ± 0.02 vs. 0.12 μg/pg ± 0.06; ANOVA; n≥6; Figure 5.16) or EPA (0.05 μg/pg ± 0.01 vs. 0.12 μg/pg ± 0.06; ANOVA; n≥6; Figure 5.16) exerted no significant effect.

5.3.5 Ageing, n-3 PUFA and PPARγ

The data reveal that PPARγ DNA binding activity was significantly decreased in nuclear fractions prepared from cortex of aged, compared with young, rats (0.13 arbitrary units ± 0.02 vs. 0.18 arbitrary units ± 0.01; *p<0.05; students *t* test; n=6; Figure 5.17). There was a significant increase in PPARγ DNA binding activity in aged rats which were given DPA, compared with age-matched controls (0.21 arbitrary units ± 0.03 vs. 0.13 arbitrary units ± 0.02; $^{\#}$p<0.05; students *t* test; n=6; Figure 5.17). There was no age-related change in PPARγ DNA binding activity in nuclear fractions prepared from the hippocampus of control-treated rats (0.10

arbitrary units ± 0.01 vs. 0.11 arbitrary units ± 0.01; ANOVA; n=6; Figure 5.18), DPA-treated rats (0.10 arbitrary units ± 0.01 vs. 0.09 arbitrary units ± 0.00; ANOVA; n=6; Figure 5.18), or EPA-treated rats (0.09 arbitrary units ± 0.00 vs. 0.11 arbitrary units ± 0.01; ANOVA; n=6; Figure 5.18).

Expression of the proxy marker of PPARγ activation, CD36 mRNA expression, was investigated. The data revealed no significant age-related change in CD36 mRNA expression in cortical tissue prepared from control animals (2.05 mRNA ± 0.54 vs. 1.93 mRNA ± 0.37; ANOVA; n=6; Figure 5.19). However, there was a significant increase in CD36 mRNA expression in tissue prepared from aged rats which received EPA, compared with young EPA-treated animals (3.98 mRNA ± 0.63 vs. 1.78 mRNA ± 0.15; *$p<0.05$; ANOVA; n=6; Figure 5.19) but no difference in tissue prepared from DPA-treated rats (3.06 mRNA ± 0.45 vs. 2.58 mRNA ± 0.57; ANOVA; n=6; Figure 5.19). Analysis of hippocampal tissue showed a significant age-related increase in CD36 mRNA expression (2.62 mRNA ± 0.46 vs. 0.97 mRNA ± 0.12; *$p<0.05$; ANOVA; n=6; Figure 5.20). Additionally, there was a significant increase in CD36 mRNA in tissue prepared from young animals which received EPA, compared with young controls (1.43 mRNA ± 0.09 vs. 0.97 mRNA ± 0.12; *$p<0.05$; students *t* test; n=6; Figure 5.20).

CD200L mRNA expression was significantly increased in cortical tissue prepared from control-treated aged, compared with young, rats (1.49 mRNA ± 0.06 vs. 1.01 mRNA ± 0.03; **$p<0.01$; ANOVA; n=6; Figure 5.21) and was not significantly altered with age in the cortex of animals which received DPA (1.33 mRNA ± 0.07 vs. 1.15 mRNA ± 0.05; ANOVA; n≥6; Figure 5.21) or EPA (1.28 mRNA ± 0.09 vs. 1.23 mRNA ± 0.09; ANOVA; n=6; Figure 5.21).

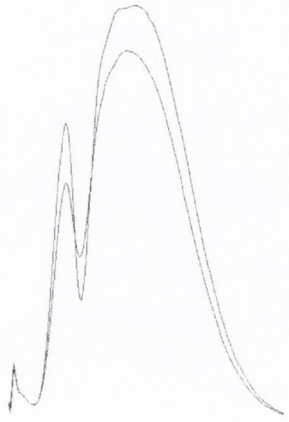
No age-related or treatment-related change in CD200L mRNA was observed in the hippocampus (1.27 mRNA ± 0.17 vs. 1.20 mRNA ± 0.09; ANOVA; n=6; Figure 5.22). DPA (1.14 mRNA ± 0.11 vs. 1.09 mRNA ± 0.09; ANOVA; n=6; Figure

5.22) and EPA (1.08 mRNA ± 0.06 vs. 1.16 mRNA ± 0.04; ANOVA; n=6; Figure 5.22) had no significant effect on CD200L mRNA expression.

Fractalkine was significantly decreased in membrane fractions prepared from cortical tissue obtained from aged, compared with young. rats (8,055.24 pg/mg ± 567.85 vs. 10,367.51 pg/mg ± 448.85; **$p<0.01$; ANOVA; n=6; Figure 5.23). There was no significant age-related difference in fractalkine concentration in the cortex of animals which received DPA (9,426.09 pg/mg ± 379.01 vs. 9,332.86 pg/mg ± 244.75; ANOVA; n≥6; Figure 5.23) or EPA (9,709.52 pg/mg ± 338.83 vs. 8,933.69 pg/mg ± 557.58; ANOVA; n=6; Figure 5.23). In hippocampal membrane fractions, there was no significant effect of age on fractalkine concentration (12,682.22 pg/mg ± 2,001.02 vs. 11,968.70 pg/mg ± 1,906.31; ANOVA; n=6; Figure 5.24). Age, DPA (14,500.51 pg/mg ± 588.95 vs. 14,494.06 pg/mg ± 1,157.30; ANOVA; n=6; Figure 5.24) and EPA (12,391.87 pg/mg ± 882.13 vs. 12,127.97 pg/mg ± 853.74; ANOVA; n=6; Figure 5.24) had no effect upon fractalkine in the hippocampus.

Chapter 5 Figures

(A)



(B)

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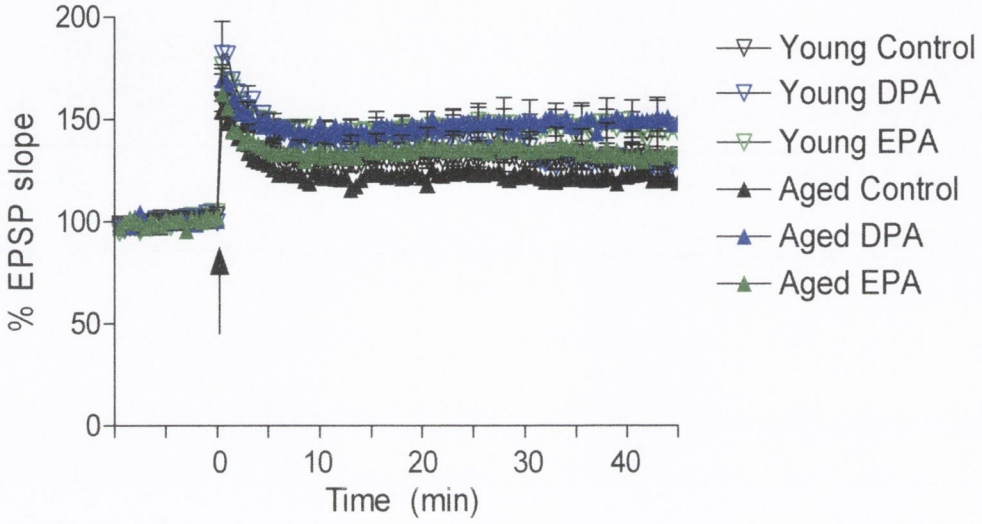
(G)


200
150
100
50
0
% EPSP slope
0
10
20
30
40
Time (min)
Young Control
Young DPA
Young EPA
Aged Control
Aged DPA
Aged EPA

(H)

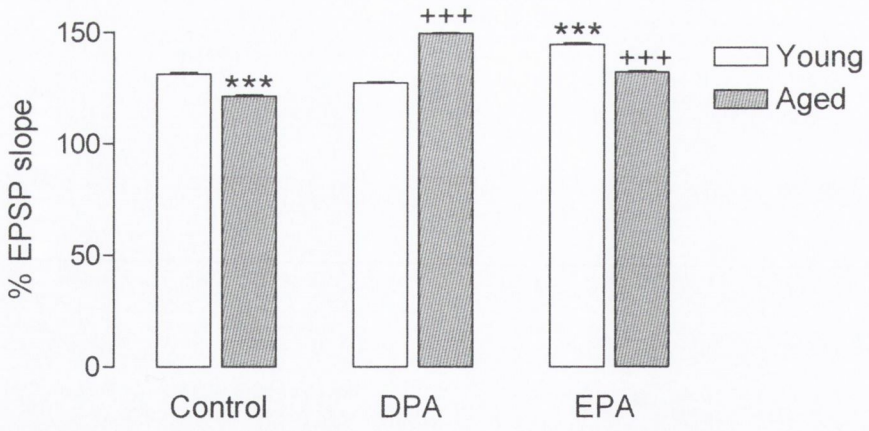
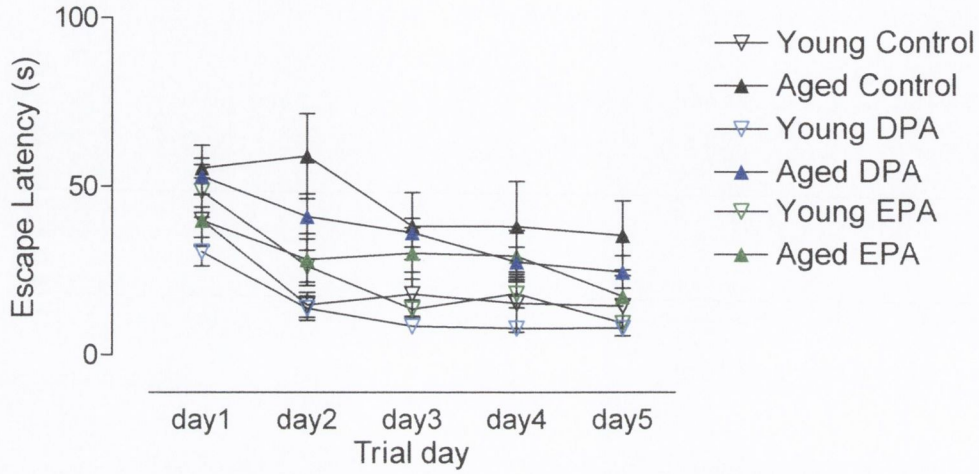

150
100
50
0
% EPSP slope
***
+++
***
+++
Control
DPA
EPA
Young
Aged

Figure 5.1 The age-associated reduction in LTP in dentate gyrus is attenuated by DPA and EPA

The mean slope of the population EPSP evoked by test stimuli delivered at 30 sec intervals, before and after tetanic stimulation is shown. Tetanic stimulation (G; arrow; 3 trains of stimuli at 250Hz for 200ms) induced an immediate and sustained increase in mean population EPSP slope. Mean percentage change in population EPSP slope was significantly decreased in the last 5 min of recording post tetanus, in control-treated aged, compared with control-treated young, rats (H; ***$p<0.001$; ANOVA). It was significantly increased in EPA-treated young, compared with control-treated young, rats (H; ***$p<0.001$; ANOVA). In addition, it was significantly increased in EPA- and DPA-treated aged, compared with control-treated aged, rats (H; $^{+++}p<0.001$; ANOVA). Data are expressed as percentage change in EPSP slope and are means ± SEM ($n\geq4$). Sample EPSP slopes are shown for each treatment group, showing trace recordings pre- and post-HFS (A; young control, B; aged control, C; young DPA-treated, D; aged DPA-treated, E; young EPA-treated and F; aged EPA-treated).

(A)



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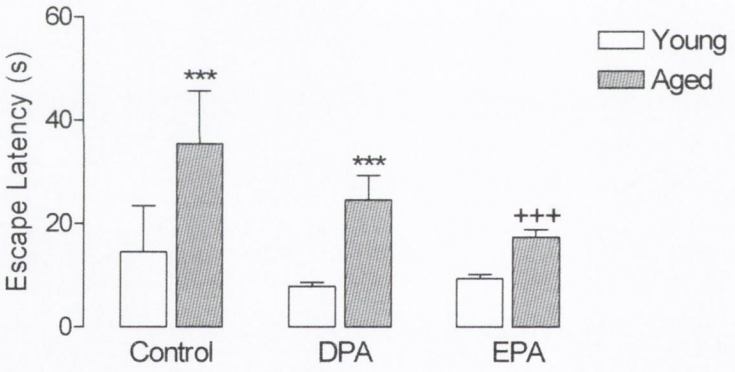
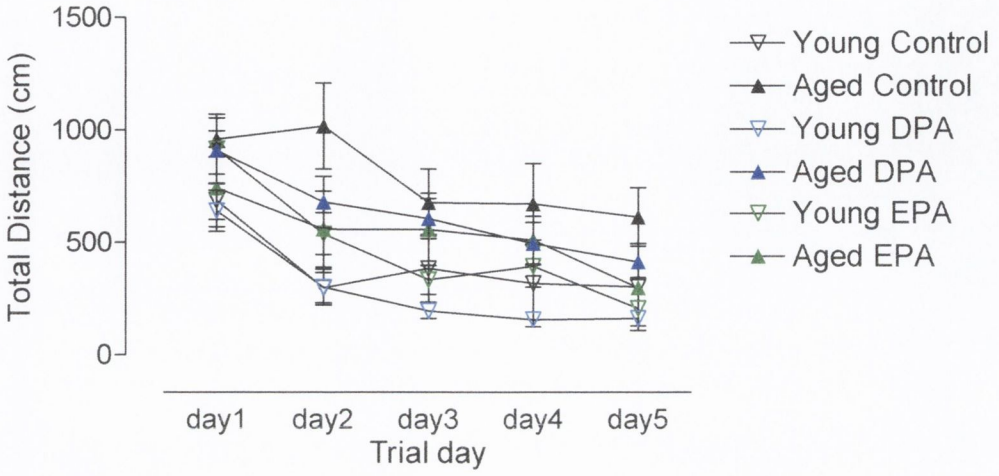


Figure 5.2 Age affects escape latency in 5 -day Morris water maze task

The mean latency to find the hidden platform in the Morris water maze was significantly greater in aged, compared with young, rats on day 5 (B; ***$p<0.001$; ANOVA). Oral administration of EPA significantly attenuated the age-related impairment in escape latency (B; $^{+++}p<0.001$; ANOVA). EPA and DPA had no significant effect on young animals, compared with controls. There was a significant difference between DPA-treated young, compared with DPA-treated aged, rats (B; ***$p<0.001$; ANOVA). Values are expressed as seconds and are means ± SEM ($n\geq6$).

(A)



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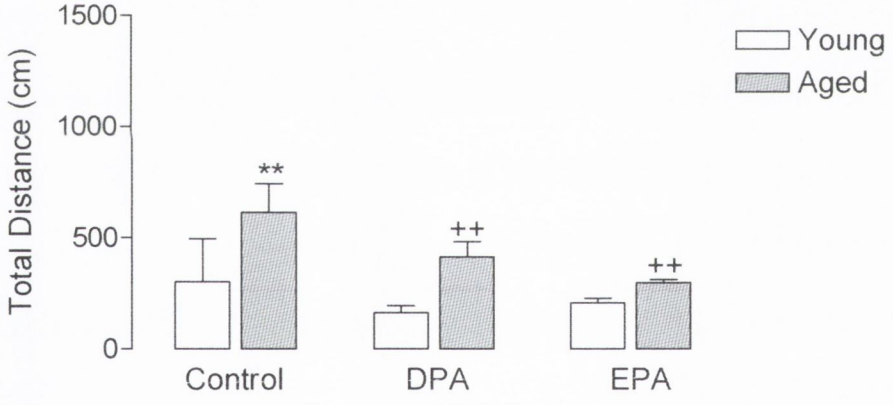


Figure 5.3 Age affects total distance swam to escape platform in 5 -day Morris water maze task

The average total distance (cm) swam to the hidden platform in the Morris water maze was significantly greater in aged, compared with young, rats on day 5 (B; **$p<0.01$; ANOVA). There was a significant treatment-related improvement in distance swam (B; $^{++}p<0.01$, ANOVA). Analysis of aged DPA- and EPA-treated rats revealed a significant difference compared with control-treated aged rats (B; $^{++}p<0.01$, ANOVA). Values are expressed as cm and are means ± SEM ($n\geq6$).

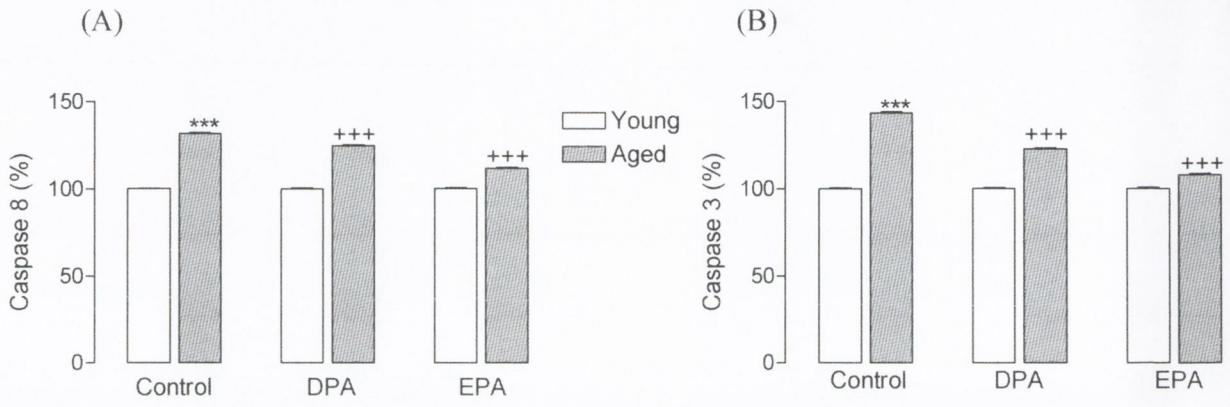
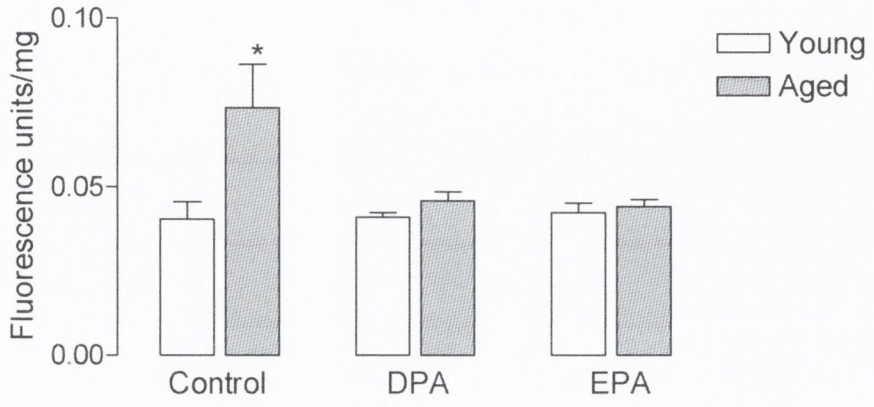


Figure 5.4 Activities of caspase 8 and caspase 3 are significantly increased in the cortex of aged rats and reduced with DPA and EPA

Mean activities of caspase 8 (A) and caspase 3 (B) were significantly increased in cortical homogenate prepared from aged, compared with young, rats (***$p<0.001$; ANOVA) and this increase was significantly reduced in aged DPA- and EPA-treated rats ($^{+++}p<0.001$; EPA- or DPA-treated versus control-treated aged rats; ANOVA). Values are expressed as pmol/min corrected for protein concentration and are means ± SEM ($n\geq6$).

(A)



(B)

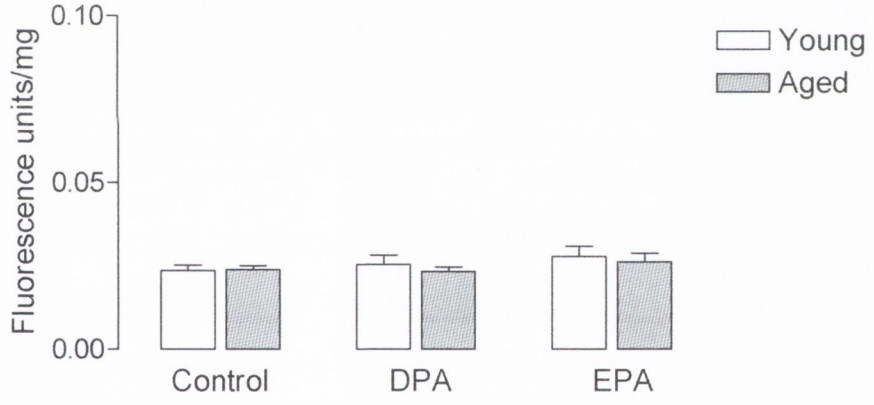


Figure 5.5 Sphingomyelinase is increased in a membrane preparation obtained from cortical tissue of aged rats

Sphingomyelinase activity was significantly increased in membrane (A) fractions prepared from cortical tissue of aged, compared with young, rats (A; *$p<0.05$; ANOVA). Treatment with EPA or DPA attenuated this age-related change, though not statistically significantly. Sphingomyelinase activity was not significantly altered with age in cytosolic fractions (B; ANOVA). Values are expressed as fluorescence units, corrected for protein concentration, and are means ± SEM ($n\geq6$).

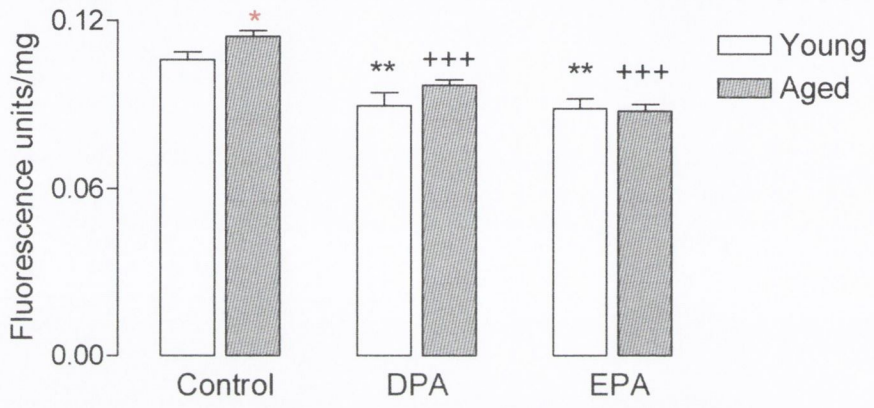


Figure 5.6 DPA and EPA attenuate the age-related increase in membrane sphingomyelinase activity in the hippocampus

Sphingomyelinase activity was significantly increased in membrane fractions prepared from hippocampal tissue of aged compared with young rats (*$p<0.05$; students *t* test). There was a significant decrease observed in sphingomyelinase activity in tissue prepared from young rats which received oral administration of DPA and EPA (**$p<0.01$; ANOVA) compared with control-treated young rats. There was a significant decrease in sphingomyelinase activity in aged animals which received DPA and EPA compared with control-treated aged rats ($^{+++}p<0.001$, ANOVA). Values are expressed as fluorescence units corrected for protein concentration and are means ± SEM ($n\geq6$).

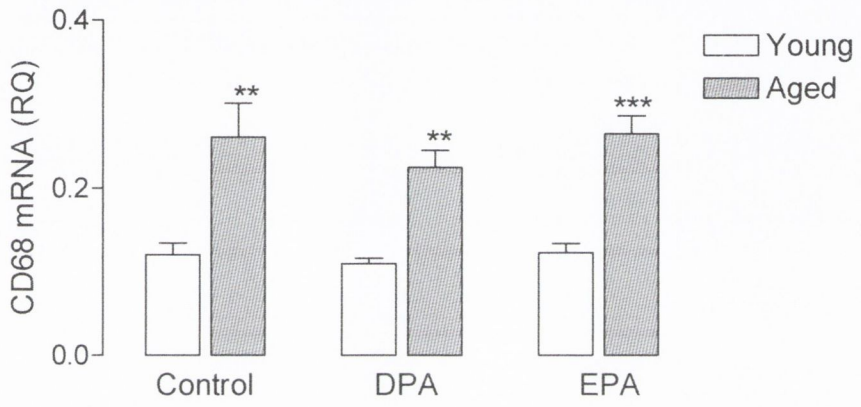


Figure 5.7 Age increases CD68 mRNA expression in the cortex

CD68 mRNA was significantly increased in cortex prepared from aged, compared with young, rats irrespective of treatment (**$p<0.01$ and ***$p<0.001$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

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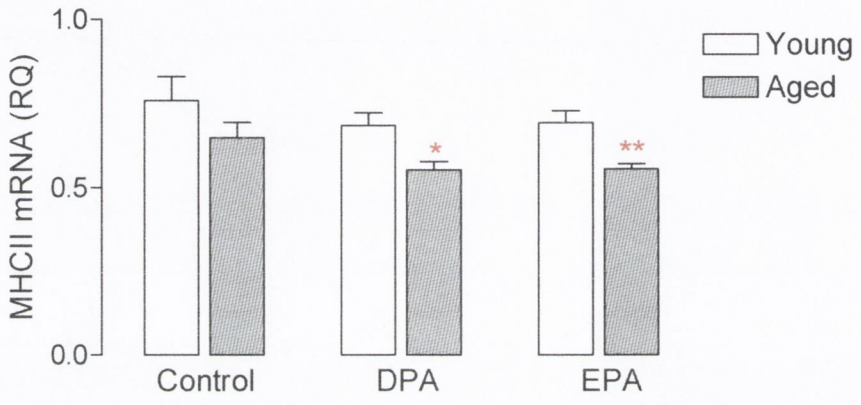

Figure 5.8 Cortical MHCII mRNA expression was decreased by EPA and DPA

MHCII mRNA was similar in cortical tissue prepared from young and aged rats control-treated rats but was significantly decreased in cortical tissue prepared from aged rats which received DPA or EPA, compared with young DPA- and EPA-treated rats (*$p<0.05$ and **$p<0.01$ respectively; students *t* test). Values are expressed as RQ and are means ± SEM (n=6).

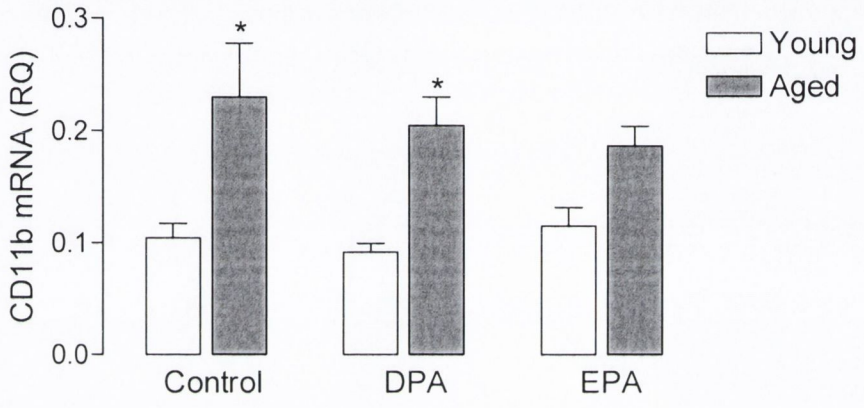

Figure 5.9 Age increases CD11b mRNA expression in the cortex

CD11b mRNA was significantly increased in cortex prepared from aged, compared with young, rats (*$p<0.05$; ANOVA) and was significantly increased in aged rats which received oral administration of DPA (*$p<0.05$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

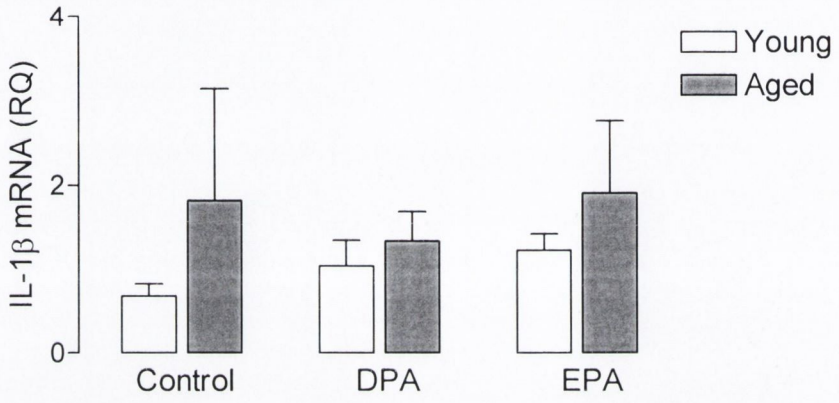

Figure 5.10 Cortical IL-1β mRNA expression unaffected by age

IL-1β mRNA was similar in cortical tissue prepared from young and aged rats which received control, DPA and EPA oral administration.Values are expressed as RQ and are means ± SEM (n=6).

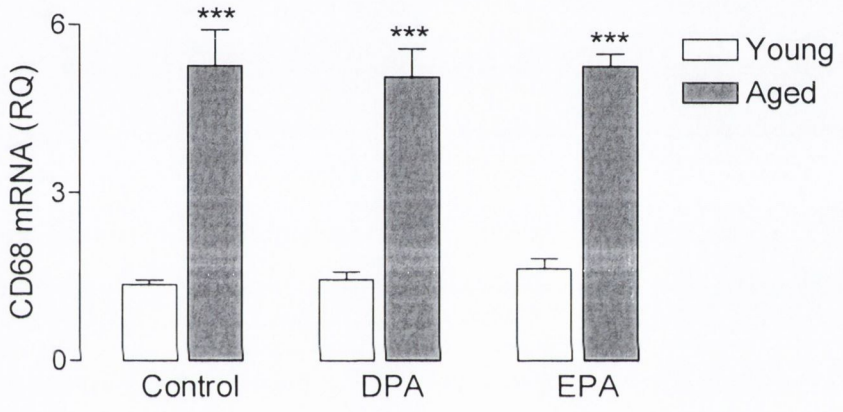

Figure 5.11 Age increases CD68 mRNA expression in the hippocampus

CD68 mRNA was significantly increased in hippocampus prepared from aged, compared with young, rats (***p<0.001; ANOVA) and was significantly increased in rats which received oral administration of DPA and EPA (***p<0.001; ANOVA). Values are expressed as RQ and are means ± SEM (n≥6).

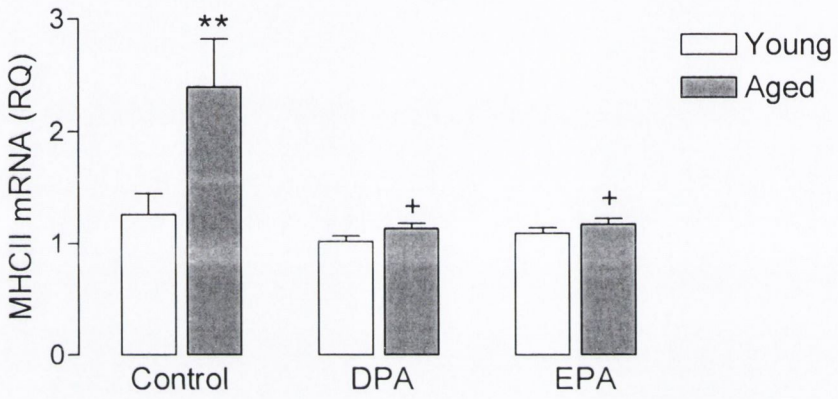

Figure 5.12 EPA and DPA attenuate the age-related increase in MHCII mRNA in hippocampus

MHCII mRNA was significantly increased in aged compared with young rats which received oral administration of control diet (**$p<0.01$; ANOVA). This age-related increase in MHCII mRNA was significantly attenuated in aged rats which received DPA and EPA ($^{+}p<0.05$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

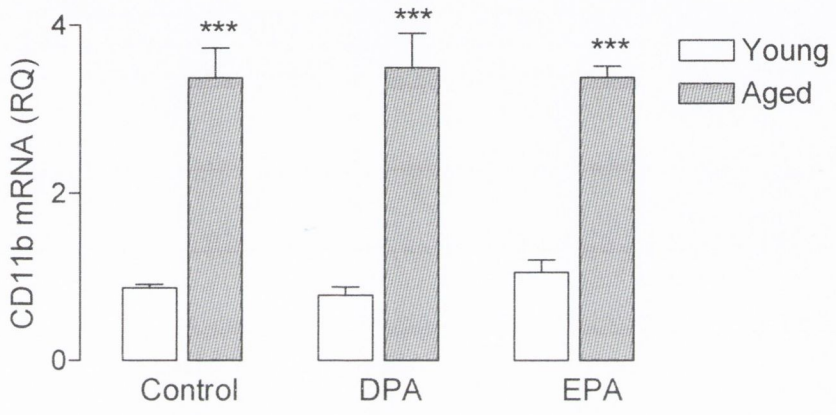

Figure 5.13 Age increases CD11b mRNA expression in the hippocampus

CD11b mRNA was significantly increased in hippocampal tissue prepared from aged, compared with young, rats (***$p<0.001$; ANOVA) and was significantly increased in rats which received oral administration of DPA and EPA (***$p<0.001$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

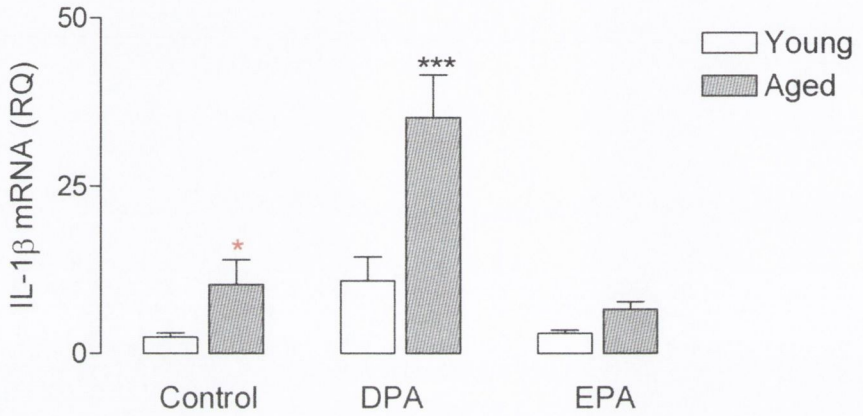

Figure 5.14 EPA attenuates the age-related increase in IL-1β mRNA in hippocampus

IL-1β mRNA was significantly increased in aged, compared with young, rats which received oral administration of control diet (*$p<0.05$; students *t* test). This significant age-related change was not evident in tissue prepared from aged rats which received EPA but was significantly increased in aged DPA-treated rats (***$p<0.001$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

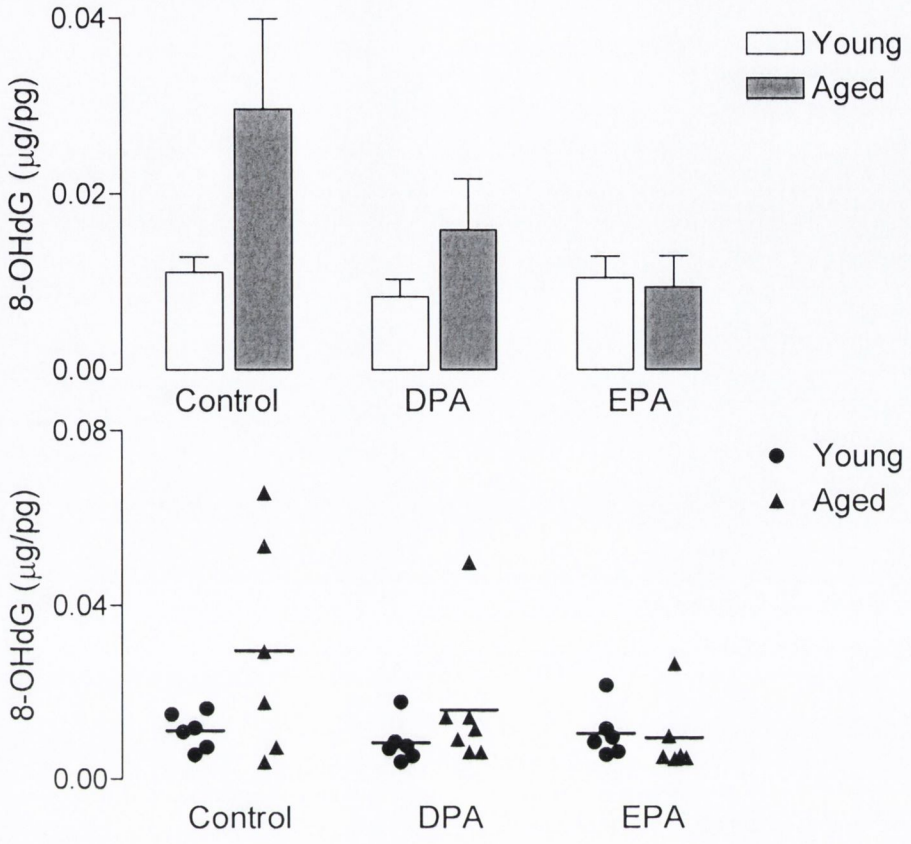

Figure 5.15 8-hydroxy-2'-deoxyguanosine was unaffected by age, DPA and EPA in cortical samples

Values for 8-hydroxy-2'-deoxyguanosine were similar in preparations of DNA obtained from cortical tissue prepared from young and aged rats which received control, DPA and EPA oral administration. Values are expressed as µg/pg and are means ± SEM (n≥6).

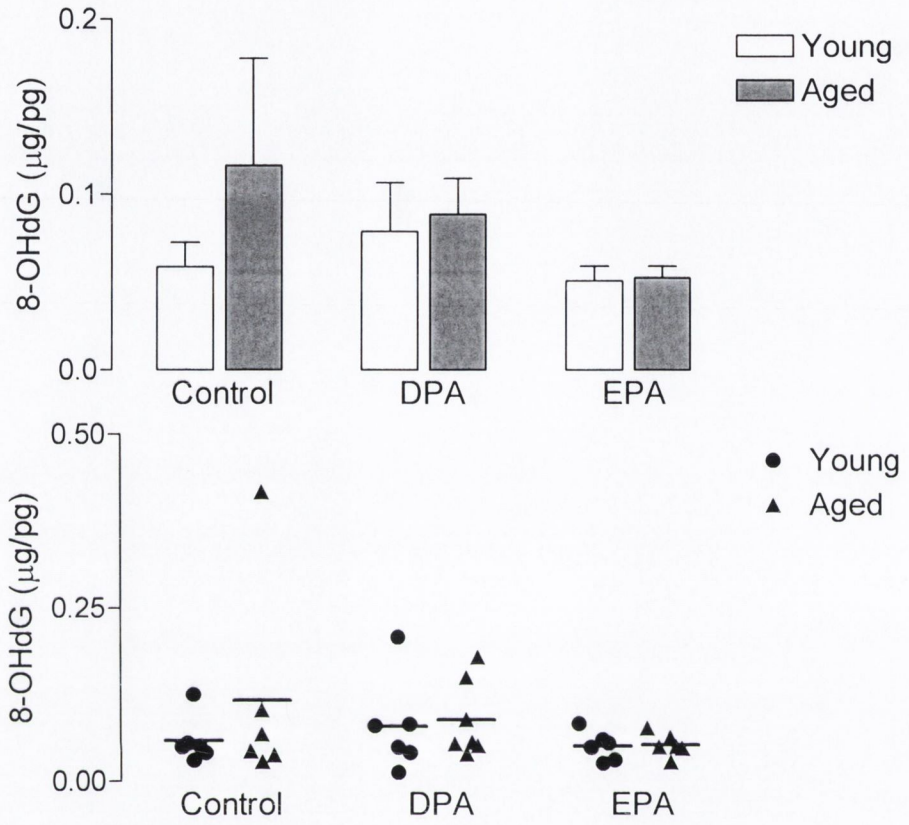

Figure 5.16 8-hydroxy-2'-deoxyguanosine was unaffected by age, DPA and EPA in hippocampal samples

Values for 8-hydroxy-2'-deoxyguanosine was similar in preparations of DNA obtained from hippocampal tissue prepared from young and aged rats which received control, DPA and EPA oral administration. Values are expressed as µg/pg and are means ± SEM (n≥6).

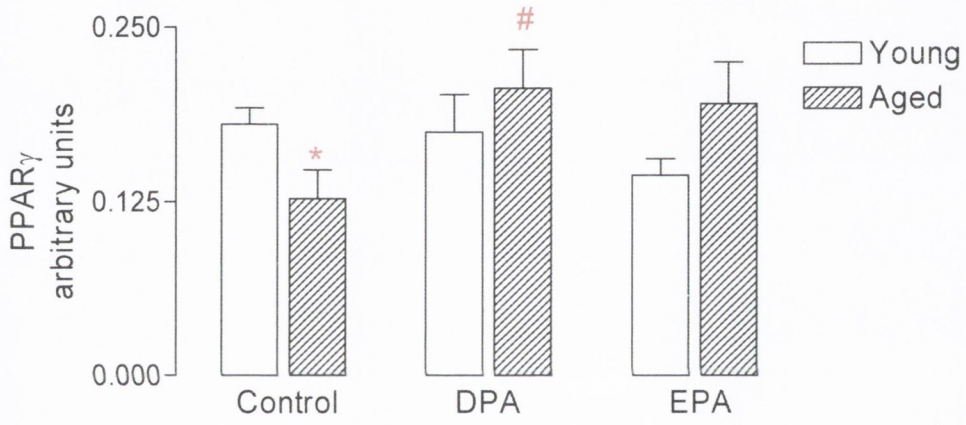

Figure 5.17 Cortical PPARγ DNA binding activity is decreased with age

PPARγ activity was assessed in isolated nuclear fractions of cortical tissue. PPARγ activity was significantly decreased in aged, compared with young, control-treated rats (*$p<0.05$; students *t* test). There was a significant increase in PPARγ in aged rats which received DPA compared with age-matched control-treated rats ($^{\#}p<0.05$; students *t* test). Values are expressed as means ± SEM ($n\geq6$).

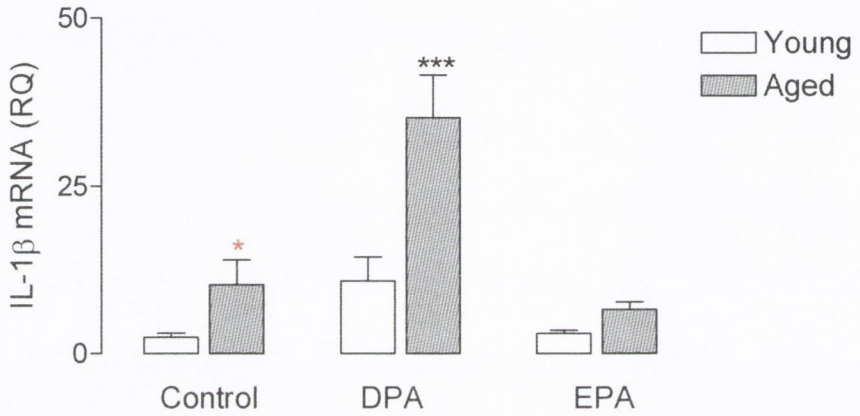

Figure 5.14 EPA attenuates the age-related increase in IL-1β mRNA in hippocampus

IL-1β mRNA was significantly increased in aged, compared with young, rats which received oral administration of control diet (*$p<0.05$; students *t* test). This significant age-related change was not evident in tissue prepared from aged rats which received EPA but was significantly increased in aged DPA-treated rats (***$p<0.001$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

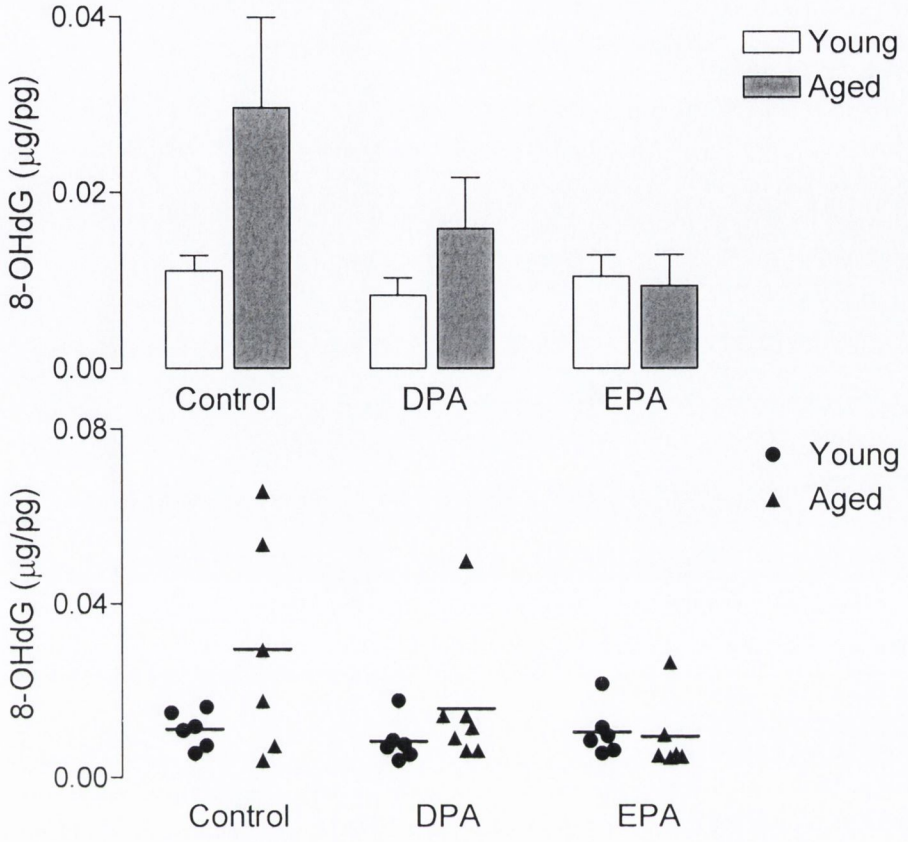

Figure 5.15 8-hydroxy-2'-deoxyguanosine was unaffected by age, DPA and EPA in cortical samples

Values for 8-hydroxy-2'-deoxyguanosine were similar in preparations of DNA obtained from cortical tissue prepared from young and aged rats which received control, DPA and EPA oral administration. Values are expressed as μg/pg and are means ± SEM (n≥6).

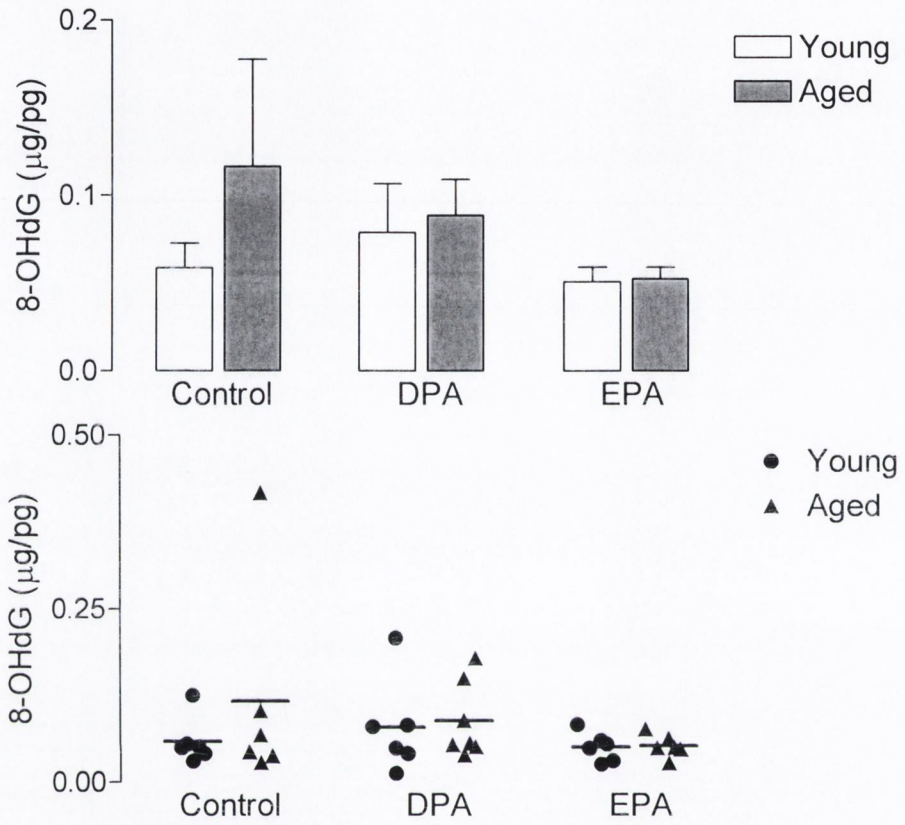

Figure 5.16 8-hydroxy-2'-deoxyguanosine was unaffected by age, DPA and EPA in hippocampal samples

Values for 8-hydroxy-2'-deoxyguanosine was similar in preparations of DNA obtained from hippocampal tissue prepared from young and aged rats which received control, DPA and EPA oral administration. Values are expressed as µg/pg and are means ± SEM (n≥6).

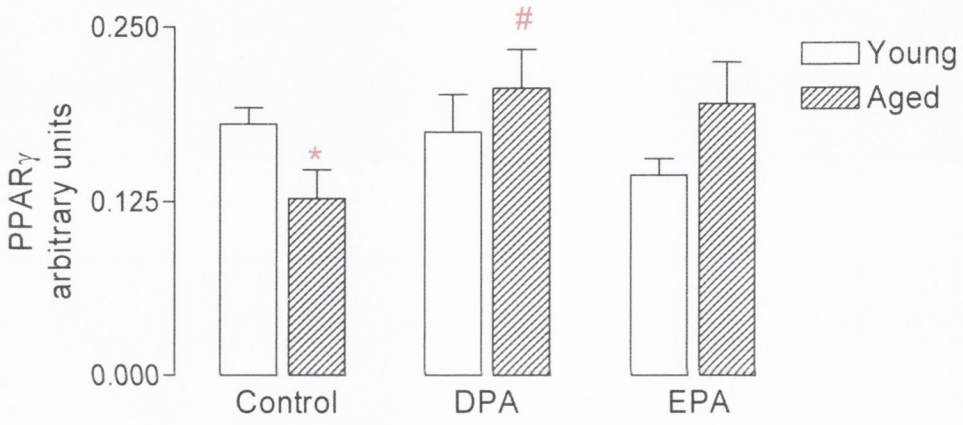

Figure 5.17 Cortical PPARγ DNA binding activity is decreased with age

PPARγ activity was assessed in isolated nuclear fractions of cortical tissue. PPARγ activity was significantly decreased in aged, compared with young, control-treated rats (*$p<0.05$; students *t* test). There was a significant increase in PPARγ in aged rats which received DPA compared with age-matched control-treated rats ($^{\#}p<0.05$; students *t* test). Values are expressed as means ± SEM ($n\geq6$).

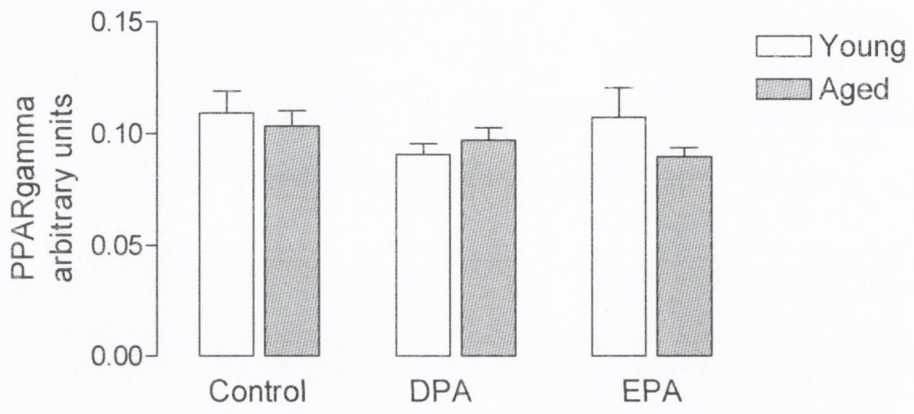

Figure 5.18 Hippocampal PPARγ DNA binding activity is unaffected by age, DPA and EPA

PPARγ activity was assessed in isolated nuclear fractions of hippocampal tissue. PPARγ activity was not significantly altered in in any of the experimental groups. Values are expressed as means ± SEM ($n \geq 6$).

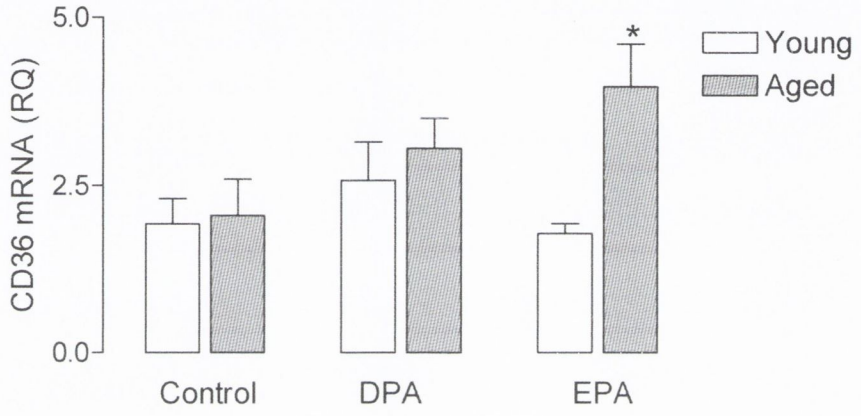

Figure 5.19 CD36 mRNA is increased in cortex of aged rats treated with EPA

CD36 mRNA was unchanged in cortical tissue prepared from young and aged control-treated rats. There was a significant increase in CD36 mRNA in aged rats which received EPA, compared with young EPA-treated rats (*$p<0.05$; ANOVA). Values are expressed as RQ and are means ± SEM ($n\geq6$).

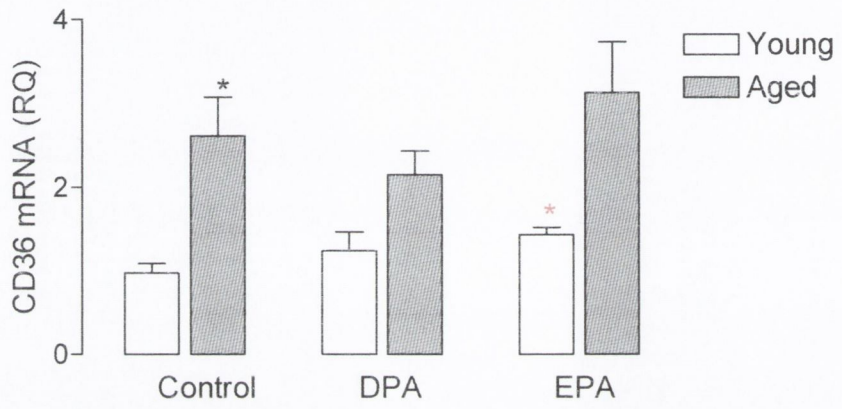

Figure 5.20 CD36 mRNA is increased with age and EPA in hippocampus of aged rats

CD36 mRNA was significantly increased in hippocampal tissue prepared from control-treated aged, compared with young, rats (*$p<0.05$; ANOVA). There was a significant increase in CD36 mRNA in young EPA-treated rats compared with age-matched control-treated rats (*$p<0.05$; students *t* test). Values are expressed as RQ and are means ± SEM ($n\geq6$).

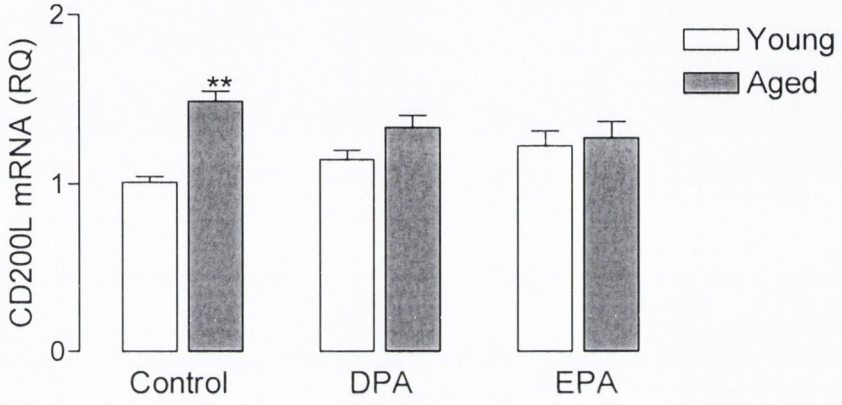

Figure 5.21 Cortical CD200L mRNA expression is increased with age, and unaffected by DPA and EPA

CD200L mRNA was significantly increased in cortical tissue prepared from control-treated aged, compared with young, rats (**$p<0.01$; ANOVA) and was not significantly altered by DPA and EPA oral administration. Values are expressed as RQ and are means ± SEM ($n\geq6$).

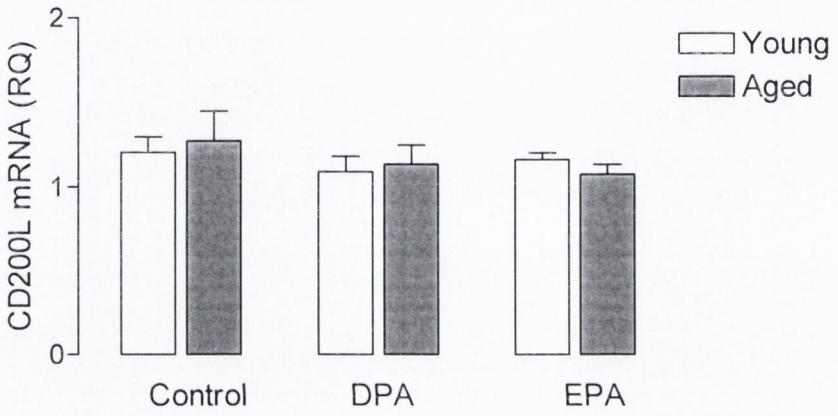

Figure 5.22 Hippocampal CD200L mRNA expression is unaffected by age, DPA and EPA

CD200L mRNA expression was not significantly altered in any of the experimental groups. Values are expressed as means ± SEM. Values are expressed as RQ and are means ± SEM (n≥6).

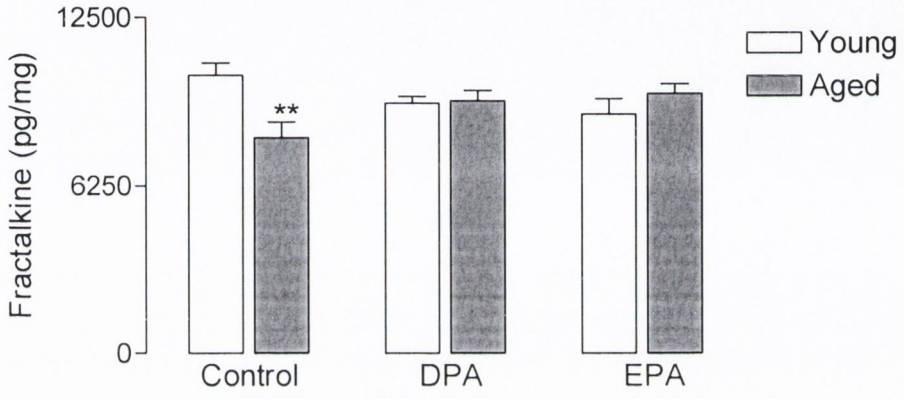

Figure 5.23 Fractalkine is decreased with age, and unaffected by DPA and EPA in the cortex

Fractalkine was assessed by ELISA on membrane fractions prepared from cortical tissue. Fractalkine was significantly decreased in control-treated aged, compared with young, rats (**$p<0.01$; ANOVA) and was not significantly altered by DPA and EPA oral administration. Values are expressed as pg/mg and are means ± SEM ($n\geq6$).

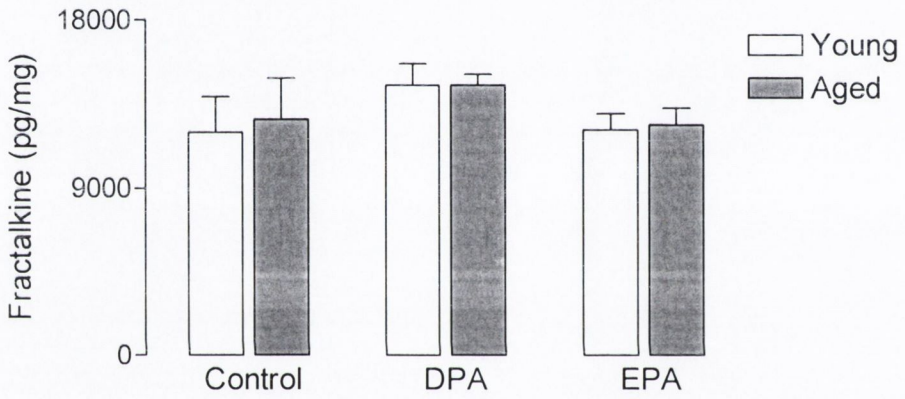

Figure 5.24 Fractalkine is unaffected by age, DPA and EPA in the hippocampus

Fractalkine was assessed by ELISA on membrane fractions prepared from hippocampal tissue. Fractalkine was not significantly altered in any of the experimental groups. Values are expressed as pg/mg and are means ± SEM ($n \geq 6$).

Chapter 5 Discussion

5.4 Discussion

The objectives of this study were to investigate the effects of administration of DPA and EPA on the modulation of age-related changes in the rat. One of the main aims was to investigate whether any impairment in spatial learning could be correlated with a change in the ability of rats to sustain LTP and with changes in neurochemical markers in the brain.

5.4.1 Ageing, n-3 PUFA and learning and memory

This study provides evidence for an age-related impairment in water maze performance, in terms of latencies to find the platform and average distance swam to escape platform. The data reveal significant improvements in performance in the aged groups which received DPA and EPA, in terms of distance swum to the escape platform and latencies to find the platform, compared with aged control animals. Importantly, these findings correlate closely with the findings described here also, showing that the age-related impairment in the ability of rats to sustain LTP was rescued by treatment with the PUFAs.

In humans, an age-related decline in learning and memory has been recognised (Davis *et al.*, 2003) and in rodents, ageing is associated with deterioration of function in the brain and spatial learning tasks, such as the Morris water maze, are commonly used to assess hippocampal-dependent memory. Impairment in cognitive function (Barnes, 1979, , 1988; Barnes *et al.*, 1990; Forster *et al.*, 1996), along with reduced ability to maintain LTP (Landfield *et al.*, 1978; Barnes, 1979; De Toledo-Morrell & Morrell, 1985; Lynch & Voss, 1994; McGahon *et al.*, 1997; Murray & Lynch, 1998b, 1998a; O'Donnell *et al.*, 2000; Martin *et al.*, 2002b), has been associated with age. In parallel with the poorer performance in the Morris water maze, there was an age-related decrease in LTP, while the restorative effects of DPA and EPA in Morris water maze performance was also reflected by a restoration of LTP. These changes in synaptic plasticity are thought to utilise cellular and molecular

events which bring about information storage (Elgersma & Silva, 1999; Luscher *et al.*, 2000; Martin *et al.*, 2000).

A number of studies have reported that n-3 fatty acids, in particular DHA, are decreased with age (McGahon *et al.*, 1999a; Barcelo-Coblijn *et al.*, 2003) and fatty acid analysis was undertaken by Dr Anna Nicolaou (University of Bradford) in cortical tissue prepared from rats in each of the treatment groups in this study. Her data revealed that there was an age-related decrease in n-3 PUFAs in general, and DHA specifically (Nicolaou, personal communication) coupling with the age-related decrease in behaviour and also LTP. Others have also associated an age-related decrease in DHA with poorer performance in the water maze test (Barcelo-Coblijn *et al.*, 2003) and decreased LTP in the dentate gyrus (McGahon *et al.*, 1999a). In contrast with the evidence relating to the age-related decrease in DHA, there is conflicting evidence relating to arachidonic acid, with no change described by some investigators (Barcelo-Coblijn *et al.*, 2003) and an age-related decrease described by others (Gaiti, 1989; Terracina *et al.*, 1992; Lynch & Voss, 1994; McGahon *et al.*, 1997). In human studies, a loss of DHA has been shown in the brains of Alzheimer's disease sufferers and this has been correlated with impairment in learning and memory (Soderberg *et al.*, 1991), but there is evidence of age-related impairment in memory, together with loss of DHA, in the non-Alzheimer's diseased brain also (Delion *et al.*, 1996; Delion *et al.*, 1997; Favreliere *et al.*, 2003).

One important finding of this study is that the aged rats which received DPA and EPA performed in the Morris water maze in a manner which was similar to young rats. To my knowledge, this is the first demonstration of such an effect of these fatty acids, although others have found similar effects with DHA. One the one hand, decreased DHA has been associated with poorer cognitive function and supplementation with DHA improves function. In one study, young and aged rats were given either a control diet or a diet enriched with 11% DHA for a period of 4 weeks. Whereas DHA was decreased by about 15% in control-treated aged rats, aged rats which received additional DHA had levels which were comparable to young rats

(Barcelo-Coblijn *et al.*, 2003). Although in this study, a direct correlation between performance in the water maze task was not evident, others have reported that DHA (in comparison with n-6 PUFAs) improves performance in the Morris water maze (Lim *et al.*, 2005a; Lim *et al.*, 2005b). Similarly, chronic administration of DHA (300mg/kg/day for 10 weeks) to essential fatty acid deficient young rats can improve DHA levels in brain and also improves memory (Gamoh *et al.*, 1999). DHA has been shown to protect against impairment associated with Alzheimer's disease (Hashimoto *et al.*, 2002) and the evidence indicates that a DHA supplemented diet can improve LTP in the rat dentate gyrus (McGahon *et al.*, 1999a). EPA has previously been shown, in our laboratory, to attenuate the age-related impairment in LTP (Lynch *et al.*, 2007).

Moriguchi and colleagues investigated the effect of decreased concentration of DHA on spatial learning in the Morris water maze over the course of 3 generations of animals fed on a diet deficient in n-3 PUFA. They reported that n-3 PUFA was decreased by over 80% in the second and third generation rats, that there was a compensatory increase in n-6 PUFA in the brain and that behavioural deficits were evident in the n-3 deficient rats (Moriguchi *et al.*, 2000). A further study revealed that by replenishing n-3 PUFA performance in the Morris water maze could be improved (Moriguchi & Salem, 2003), although in another study, the effects of supplementation of fish oil to the diet of young, mature and aged mice was investigated and no improvement in Morris water maze performance was observed (Carrie *et al.*, 2000).

One of the potential clinical applications of n-3 PUFA is in the treatment of attention deficit hyperactivity disorder. In one study, designed to investigate the possible efficacy of n-3 PUFAs, spontaneously hypertensive rats, an animal model of attention deficit hyperactivity disorder, were assessed in the water maze. Compared with their progenitor strain (Wistar-Kyoto), spontaneously hypertensive rats were shown to be more impulsive, demonstrated increased exploratory behaviour, and showed evidence of hyperactivity and slower adjustment to a novel environment. They have also been shown to over-react to stress (Hendley, 2000) and to perform

poorly in the water maze task (Gattu *et al.*, 1997a; Gattu *et al.*, 1997b). Although these animals have lower n-3 PUFA levels than Wistar-Kyoto rats (Mills *et al.*, 1990), dietary supplementation with DHA and arachidonic acid failed to alter phospholipid DHA concentration and no effect on performance in the water maze was observed (Clements *et al.*, 2003).

5.4.2 Ageing, n-3 PUFA and oxidative stress

One theory suggests that the cause of the age-associated decline in cognitive function is due to an accumulation of reactive oxygen species and associated damage to neurons. In support of this, the age-related decline in cognitive function has been linked with oxidative stress in the mammalian brain (Forster *et al.*, 1996; Fukui *et al.*, 2001; Butterfield *et al.*, 2006a; Butterfield *et al.*, 2006b), and specifically there is evidence of hippocampal oxidative stress in rodents in which spatial learning is impaired (Nicolle *et al.*, 2001). Similarly deficits in LTP have been linked with oxidative stress in hippocampus (O'Donnell & Lynch, 1998) and these changes were attenuated in animals which received a diet containing antioxidant vitamin E (Murray & Lynch, 1998a). Consistent with these findings, H_2O_2 has been shown to inhibit LTP in CA1 *in vitro* (Pellmar *et al.*, 1991) and in the dentate gyrus *in vivo* (Vereker *et al.*, 2001).

Among the consequences of oxidative stress is oxidative damage to RNA, DNA, proteins and lipids which can stimulate stress-activated signalling pathways leading to deterioration of cell function (Linseman, 2008). In this study, the DNA adduct 8-OHdG, which is the oxidised form of deoxyguanosine (dG) and therefore a marker of oxidative DNA damage, was analysed (Kasai *et al.*, 1986) and the evidence indicated that there was an age-related increase in 8-OHdG in the hippocampus and cortex, which was attenuated in tissue prepared from rats treated with DPA or EPA. In agreement with the findings of this study, Balu *et al.* (2006) reported age-related increases in 8-OHdG, analysed by HPLC, in the cortex, striatum, hippocampus and spinal cord (Balu *et al.*, 2006); this is significant because the striatum and

hippocampus are particularly vulnerable to oxidative stress due to the high oxygen consumption rate in these brain regions (Floyd & Carney, 1991). Others have also reported age-related increases in 8-OHdG and oxidative stress in rodent brain (Sohal & Dubey, 1994; Sohal *et al.*, 1994; Kaneko *et al.*, 1997) as well as pituitary gland (Kondo *et al.*, 2001), kidney, heart, liver and skeletal muscle (Sohal *et al.*, 1994; Kaneko *et al.*, 1997).

A review of the literature reveals few studies which directly assessed the effects of fatty acids on 8-OHdG. One study investigated the effect of DHA and EPA supplementation, from week twenty of pregnancy up until delivery, on urinary 8-OHdG; no effect of EPA or DPA was observed (Shoji *et al.*, 2006). In a second study, dietary supplementation of rats for 12 weeks revealed an increase 8-OHdG in bone marrow of aged animals which received DHA (Umegaki *et al.*, 2001).

5.4.3 Ageing, n-3 PUFA and cell signalling

The ways in which PUFA modulate the inflammatory response and mediate their neuroprotective actions is still under investigation. The mechanisms for these positive effects may be linked with the ability of n-3 PUFAs to control and alter concentrations of inflammatory mediators, change cell-cell communication or modulate signal transduction pathways (Meydani *et al.*, 1991; von Schacky, 1996; Jolly *et al.*, 2001). Here the effects of EPA and DPA were examined for evidence of their effects in the hippocampus and cortex of aged rats and the evidence reveals a protective effect of both fatty acids. This is consistent with previous data which indicated that EPA attenuated the decrease in LTP associated with amyloid-β (Lynch *et al.*, 2007), LPS treatment (Kavanagh *et al.*, 2004; Lonergan *et al.*, 2004) and which occurs in aged rats (Lynch *et al.*, 2007) and rats exposed to γ-irradiation (Lonergan *et al.*, 2002).

Previous evidence has indicated that the deficit in LTP in these experimental conditions is associated with increased hippocampal concentration of IL-1β, and that,

in parallel with its ability to restore LTP in aged rats, EPA also attenuated the age-related increase in IL-1β (Lynch *et al.*, 2007). Here the data show that EPA attenuated the age-related increase in IL-1β mRNA in parallel with its ability to improve performance in the Morris water maze, and its ability to restore LTP. Dietary n-3 PUFAs have been reported to reduce synthesis of TNFα and IL-1β in healthy male volunteers (Caughey *et al.*, 1996) and several studies have reported similar anti-inflammatory effects in different experimental conditions. For example, previous studies have shown that EPA can prevent IL-1β production in human monocytes *in vitro* (Baldie *et al.*, 1993), in rodent macrophages (Yaqoob & Calder, 1995; Wallace *et al.*, 2000) and in mice after ip administration of LPS (Sadeghi *et al.*, 1999). The effect of dietary supplementation with DHA, in the absence of EPA, was investigated on the inflammatory response in the challenge phase of the contact hypersensitivity reaction in the ears of mice sensitized with 2,4-dinitro-1-fluorobenzene. It was observed that DHA acted to decrease IL-1β, IFNγ, IL-2 and IL-6 mRNA expression in this *in vivo* contact hypersensitivity model in mice (Tomobe *et al.*, 2000). Interestingly, in this study, while EPA attenuated the age-related increase in IL-1β mRNA, DPA markedly increased it in hippocampus of both young and aged rats. This unexpected finding is at variance with previous studies from this laboratory which have, to this point, demonstrated an inverse correlation between hippocampal concentration of IL-1β and the ability of rats to sustain LTP and this warrants further investigation.

Ageing is a multifaceted process with many factors contributing to the deficit in synaptic plasticity and cognitive function. These factors include modulation of signalling cascades. Here analysis of cortical tissue identified an age-related increase in activities of caspase 8 and caspase 3 and sphingomyelinase, while *in vitro* analysis suggested that the change in caspase 3 was dependent on increased sphingomyelinase activity. The evidence indicated that the age-related change in activities of caspases were reduced in EPA- and DPA-treated animals. Dietary supplementation with EPA has previously been shown to protect endothelial cells from anoikis, a type of apoptosis induced by the detachment of cells from the surrounding extracellular

matrix, through the inhibition of caspase 8 (Suzuki *et al.*, 2003). The action of EPA was dependent on upregulation of the cellular FLICE (Fas-associating protein with death domain-like interleukin-1-converting enzyme)-inhibitory protein (cFLIP), which is an endogenous inhibitor of caspase 8 (Suzuki *et al.*, 2003). EPA also inhibited the TNF-mediated increase in caspase 8 activity in murine differentiating skeletal muscle cells (Magee *et al.*, 2008).

Membrane sphingomyelinase activity was increased significantly in the cortex and hippocampus of aged animals and this increase was not present in DPA- or EPA-treated aged animals. Interestingly, in the hippocampus, DPA and EPA reduced sphingomyelinase activity in young animals, compared with age-matched controls.

Several factors have been shown to increase sphingomyelinase activity including reactive oxygen species and inflammatory cytokines. For example, TNFα triggers sphingomyelin degradation and ceramide generation *in vivo* and *in vitro* (Malagarie-Cazenave *et al.*, 2002), while IL-1β has been reported to increase ceramide accumulation in epithelial cells (Homaidan *et al.*, 2002), increase neutral sphingomyelinase in a thymoma cell line (Hofmeister *et al.*, 1997) and increase acidic sphingomyelinase in Schwann cells (Carlson & Hart, 1996). H_2O_2 has also been shown to increase activity of neutral sphingomyelinase and increase production of ceramide, and subsequently to induce apoptosis, in human airway epithelial cells (Goldkorn *et al.*, 1998; Chan & Goldkorn, 2000; Lavrentiadou *et al.*, 2001; Ravid *et al.*, 2003; Levy *et al.*, 2006). In this study the trigger leading to sphingomyelinase activity has not been identified although the findings indicate an age-related increase in oxidative stress, suggesting that increased production of reactive oxygen species occurs and this may be the trigger. These findings are consistent with previous observations which indicated that production of reactive oxygen species is markedly increased in brains of aged, compared with young, rats (O'Donnell *et al.*, 2000). Consistently, it has been shown recently that the rate of H_2O_2 production, in synaptosomes prepared from young and aged rats, is greater in aged, compared with young, animals (Kilbride *et al.*, 2008).

Several studies have reported that reactive oxygen species also activates caspase 3. H_2O_2, in combination with nitric oxide, has been shown to activate p38 MAPK and caspase 3 in cortical neurons and this was associated with a decrease in cell viability, and an increase in DNA fragmentation (Wang *et al.*, 2003). Similarly, oxidative stress induced by 6-hydroxydopamine, triggers activation of caspase 8, caspase 9, truncation of Bid and activation of caspase 3 in cortical neurons (Han *et al.*, 2003; Choi *et al.*, 2004). Several factors can contribute to activation of caspase 3 including upstream activation of caspase 8 and caspase 9 (Hengartner, 2000), and production of ceramide (Mizushima *et al.*, 1996; Medzhitov & Janeway, 2002), although it has been shown that ceramide does not directly act on caspase 3 (Heinrich *et al.*, 1999). One pathway has been described in which TNF activates caspase 8, which in turn acts on endosomal acidic sphingomyelinase, increasing production of ceramide (Heinrich *et al.*, 2004). Ceramide has been shown to act on cathepsin D, co-localised with Bid in HeLa cells, and consequently, induce cleavage of Bid by cathepsin D, as shown *in vitro*. The importance of cathepsin D is highlighted in this sequence of events as Bid has been shown not to be activated in cathepsin-deficient fibroblasts (Heinrich *et al.*, 2004). The authors report a robust decrease in TNF-induced stimulation of caspase 3 in cathepsin D deficient fibroblasts derived from cathepsin $D^{-/-}$ mice and acidic sphingomyelinase-deficient lymphocytes (Heinrich *et al.*, 2004).

In agreement with this study, is another report from 2001, in which Cutler and Mattson proposed that interaction of TNF with the p55 receptor led to recruitment of a novel protein, FAN, which couples to neutral sphingomyelinase, leading to production of ceramide. Ceramide is known to affect mitochondrial membrane permeability ultimately leading to the release of cytochrome *c* which activates caspase 3 (Cutler & Mattson, 2001). The parallel increases in caspase 8, caspase 3 and sphingomyelinase which are described in this current study suggest that this pathway may be activated, with reactive oxygen species as the possible trigger, although the age-related increase in IL-1β may also act as a trigger.

5.4.4 Ageing, n-3 PUFA and microglial activation

MHCII mRNA was increased significantly in the hippocampus of aged control animals and this increase was attenuated by EPA and DPA. This finding is in agreement with previous studies indicating that MHCII mRNA expression is increased in the hippocampus with age (Lynch *et al.*, 2007), in parallel with evidence of an increase in staining for MHCII (Moore *et al.*, 2007) and with many studies which have reported changes in microglia in the aged brain (Vaughan & Peters, 1974; Peters *et al.*, 1991; Perry *et al.*, 1993; DiPatre & Gelman, 1997; Sheffield & Berman, 1998; Sheng *et al.*, 1998; Sloane *et al.*, 1999; Kullberg *et al.*, 2001; Peters & Sethares, 2002). Treatment of rats with DPA or EPA completely abrogated the age-related increase in MHCII mRNA; this finding is in agreement with other reports (Lynch *et al.*, 2007) and mirrors the effects observed *in vitro* in which the fatty acids reversed the LPS-induced increase in microglial activation (Chapter 4). In contrast to the findings in the hippocampus, there was no evidence of an age-related increase in MHCII mRNA in cortex, although it is noteworthy that EPA and DPA significantly decreased MHCII mRNA expression in cortex of aged animals. The important finding is that DPA, which has not been examined for its anti-inflammatory effects previously, is capable of dampening activity of microglia and therefore mimics the effect of EPA.

In parallel with the age-related increase in MHCII mRNA, the data show that expression of two other markers of microglial activation, CD11b and CD68, were also increased in the hippocampus and cortex of aged rats. Increased CD11b expression has been correlated with the degree of microglial activation and with morphological changes in various neuroinflammatory diseases (Bellinger *et al.*, 1993; Gonzalez-Scarano & Baltuch, 1999; Rock *et al.*, 2004), while expression of CD68 which is a proposed indicator of phagocytosis (Ramprasad *et al.*, 1996; de Beer *et al.*, 2003), surrounds amyloid plaques in Alzheimer's disease (Kobayashi *et al.*, 1998) and in animal models of Alzheimer's Disease (Bornemann *et al.*, 2001; Sasaki *et al.*, 2002). Increased expression of CD68 has also been reported in the corpus callosum

and striatum, but not hippocampus, of aged mice (Wong *et al.*, 2005), and its expression has been shown to be increased in response to LPS in a mouse model of Alzheimer's disease (Herber *et al.*, 2007).

However neither DPA nor EPA exerted any effect on expression of either CD11b or CD68 in young or aged rats. Interestingly no significant effect of EPA and DHA was observed on the proportion of human peripheral blood mononuclear cells expressing CD11b (Yaqoob *et al.*, 2000). This lack of effect of fatty acids on CD11b expression may have little impact on function since, unlike MHCII, CD11b appears not to play a major role in mediating cell-cell interactions, instead it has been used in a number of studies as merely a marker of microglial cells, rather than activated microglial cells (Sedgwick *et al.*, 1991; Nair & Bonneau, 2006; Henry *et al.*, 2008). Given that expression of CD68 is considered to be a marker of phagocytosis (Ramprasad *et al.*, 1996; de Beer *et al.*, 2003), the finding that DPA and EPA did not affect CD68 may be indicative of the fact that they did not decrease phagocytic activity in brains of aged animals. Although not shown in this thesis, data from this laboratory has established that phagocytic activity, as assessed by uptake of fluorescent particles using flow cytometry, is markedly increased in microglia obtained from aged, compared with young, rats. It might be argued that the phagocytic ability to microglia in the aged brain is desirable since cell turnover, which will generate cell debris, has been described (Napoli & Neumann, 2008) and that the lack of effect of EPA and DPA on the age-related change in CD68 is appropriate.

Several factors contribute to the activation state of microglia including the concentration of inflammatory cytokines and the interaction between microglia and other cells (Lyons *et al.*, 2007). A number of mechanisms of cell-cell interaction have been described. CD200 which is expressed on neurons and endothelial cells, interacts with its receptor CD200R which is expressed on microglia (Barclay *et al.*, 2002) leading to a signalling cascade which helps to maintain microglia in a quiescent state (Wright *et al.*, 2003). Previous evidence has indicated that CD200 is decreased in the

hippocampus of aged rats (Lyons *et al.*, 2007). However analysis of CD200 mRNA expression in this study revealed an age-related increase in cortex and no change in hippocampus. This suggests a lack of correlation between mRNA and protein but confirmation of this requires further study. Neither EPA nor DPA exerted any significant effect on CD200 mRNA expression and it must be concluded that their ability to decrease microglial activation is unlikely to rely on modulation of CD200.

The chemokine fractalkine, CX_3CL1, which is expressed on neurons (Harrison *et al.*, 1998), by binding with its specific receptor, CX_3CR1, which is expressed by microglial cells (Harrison *et al.*, 1998) has been shown play a role in modulating the activation state of microglia (Cardona *et al.*, 2006). Here it was considered that one factor which might contribute to the age-related microglial activation might be a decrease in fractalkine, while EPA and DPA may exert their modulatory effects by upregulating fractalkine expression. However neither age nor treatment significantly affected fractalkine expression in this study.

5.4.5 Ageing, n-3 PUFA and PPARγ

PUFAs have been demonstrated to be natural ligands for PPARγ (Keller *et al.*, 1993; Forman *et al.*, 1997; Kliewer *et al.*, 1997; Krey *et al.*, 1997) and the evidence has indicated that EPA is capable of upregulating expression of PPARγ mRNA in freshly isolated human adipocytes (Chambrier *et al.*, 2002). In this study, PPARγ binding activity in nuclear fractions prepared from cortical tissue was significantly decreased with age and both DPA and EPA increased binding in tissue prepared from aged rats, although the difference reached statistical significance only in the case of DPA. The fatty acid-induced change may contribute to the neuroprotective effect which has been observed since activation of PPARγ can decrease reactive species production (Reilly *et al.*, 2001; Von Knethen & Brune, 2001; Dehmer *et al.*, 2004). It is known that PPARγ agonists can attenuate macrophage activation (Nagy *et al.*, 1998; Tontonoz *et al.*, 1998; Ricote *et al.*, 1999) and can negatively regulate LPS and IFNγ target genes (Welch *et al.*, 2003), while the PPAR agonist rosiglitazone (as well

as EPA (Minogue *et al.*, 2007)) attenuates the age-related increase in IL-1β (Loane *et al.*, 2007). Interestingly this effect of rosiglitazone was accompanied by an attenuation of the age-related decrease in LTP (Loane *et al.*, 2007).

PPARγ expression has been reported to be decreased in the hippocampus (Minogue *et al.*, 2007) and in adipose tissue with age (Hotta *et al.*, 1999; Kirkland *et al.*, 2002), while an age-related reduction in PPARγ activity was observed in myoblasts (Taylor-Jones *et al.*, 2002). However in this study, in contrast with the effect on cortical tissue, PPARγ activity was unchanged in hippocampus with age or treatment, although EPA treatment increased CD36 mRNA, expression of which is dependent upon activation of PPARγ (Hodgkinson & Ye, 2003). It is clear that further work is required to unravel the effects of age and fatty acid treatment on PPARγ activity since the lack of effect of age on PPARγ binding in hippocampus is accompanied by an age-related increase in CD36 mRNA. It would be helpful to assess PPARγ expression, as well as activation, and perhaps to use more sensitive assays to investigate PPARγ binding, for example gel shift, while additional readouts might include lipoprotein lipase expression, which is another PPARγ-induced gene product.

The prostanoid 15-deoxy-Δ12,14 PGJ_2 is a potent activator of PPARγ also, but it is not thought to be present in sufficient quantities to be the main natural ligand (Forman *et al.*, 1995; Kliewer *et al.*, 1995). However, *in vitro* studies have revealed that it inhibits LPS-induced activation of microglial cells (Petrova *et al.*, 1999; Bernardo *et al.*, 2000; Combs *et al.*, 2000; Combs *et al.*, 2001; Kim *et al.*, 2002) and protects neurons against LPS- and IFNγ-induced apoptosis *in vivo* and *in vitro* (Heneka *et al.*, 2000). In this context, it is interesting that the PGJ_2 precursor, PGD_2, was markedly increased in cortical tissue prepared from aged rats which received EPA and DPA (Nicolaou, personal communication). This effect may contribute to the neuroprotective effects of the fatty acids described here.

Concluding remarks

The mechanism by which EPA and DPA attenuate the age-related decrease in performance of rats in the Morris water maze is unclear but the data presented here suggest that they may exert their primary effect on microglia, reducing their activity and therefore release of potentially neurotoxic inflammatory cytokines and reactive oxygen species, or they may act directly on neurons attenuating the age-related upregulation of the caspase cascade.

Both EPA and DPA attenuate the age-related increase in MHCII mRNA which suggests that they act to decrease microglial activation. This is consistent with the observation that both fatty acids also block the age-related increase in reactive oxygen species production, as assessed by 8-OHdG. However while EPA attenuates the age-related increase in IL-1β mRNA affirming its anti-inflammatory action described previously in brain (Lynch *et al.*, 2003; Kavanagh *et al.*, 2004; Lonergan *et al.*, 2004; Lynch *et al.*, 2007; Das, 2008) and other tissues (Hayashi *et al.*, 1999; Babcock *et al.*, 2000), DPA does not share this property with EPA.

Although somewhat inconsistent, some evidence presented here suggests that EPA and DPA modulate PPARγ activity; this may contribute to the effect of the fatty acids on microglial activation since PPARγ agonists can attenuate macrophage activation (Nagy *et al.*, 1998; Tontonoz *et al.*, 1998; Ricote *et al.*, 1999), which share many similarities with microglia. Since neither EPA nor DPA affected either CD200 or fractalkine, it must be concluded that interaction between microglia and other cells, does not rely on engagement of CD200 or fractalkine receptor.

The data also show that EPA and DPA block the age-related increases in activities of caspase 8, sphingomyelinase and caspase 3; the *in vitro* evidence (Chapter 4), together with evidence from immunohistochemical staining of activated caspase 3 (Belinda Grehan, unpublished) suggested that the effect was on neurons rather than glia. An important aspect of the action of EPA may be its conversion, by

elongation, to DPA, as recent evidence shows that EPA treatment of rat neonatal cardiomyocytes resulted in significant membrane enrichment of DPA and protection against palmitate-induced increase in caspase 3 activity (Leroy *et al.*, 2008).

The conclusion of this study is that EPA and DPA exert effects on both microglia and neurons and that both fatty acids possess anti-oxidant, anti-inflammatory and neuroprotective effects.

Chapter 6 General discussion

6 General Discussion

The primary aim of this thesis was to establish whether the n-3 fatty acids EPA and its metabolite, DPA, might modulate the age-related changes in the hippocampus which contribute to the impairment in LTP, and to use *in vitro* analysis to gain an understanding of the mechanisms involved in the proposed neuroprotective effects of these fatty acids. Among the most novel data, is the finding that DPA, which has not been examined in any detail previously, is as effective as EPA in restoring LTP in aged rats and is, like EPA, capable of reducing the age-related deficit in performance in the Morris water maze. The data show that DPA and EPA reverse the age-related increases in activities of caspases 8 and 3, as well as the increase in sphingomyelinase, and *in vitro* analyses suggest that increased sphingomyelinase activity is a key change which drives the increase in activity of caspase 3.

This study set out to assess the effects of age on neuronal activation, and to seek to understand the mechanisms by which changes occur by using an *in vitro* model of stress, i.e. exposure of cortical neurons to LPS. The data showed that both age and LPS affect caspase 3 activity. Thus, there was an age-related increase in caspase 3, indicative of cell stress, in the cortex and a parallel change was observed with LPS in cultured cortical neurons. Previous findings have demonstrated that both age and LPS treatment have been associated with impairment in LTP in the dentate gyrus in urethane-anaesthesised rats (Lonergan *et al.*, 2004; Barry *et al.*, 2005; Hennigan *et al.*, 2007; Lynch *et al.*, 2007), and the *in vivo* data from this study confirm the age-related change. This study is the first to report neuroprotective effects of the EPA metabolite, DPA. Presented here is evidence of a neuroprotective role for EPA and DPA *in vivo*; both fatty acids attenuated the age-related increases in activities of sphingomyelinase, caspase 8 and 3 and the accompanying age-related impairment in LTP. *In vitro* evidence of a neuroprotective role of these PUFAs includes the demonstration that both DPA and EPA abrogate the LPS-induced increases in activities of sphingomyelinase and caspase 3 in cortical neurons.

Significantly, the LPS-induced activation of caspase 3 was blocked by sphingomyelinase inhibition, and mimicked by ceramide, the product of sphingomyelin hydrolysis. Therefore, it can be concluded that upstream activation of sphingomyelinase is one factor which contributes to the LPS-induced increase in caspase 3 activity.

As shown in Figure 6.1, sphingomyelin hydrolysis by sphingomyelinase to produce ceramide is the first step in a cascade of reactions, the products of which have differential effects on cell survival/ cell death. Whereas ceramide has been shown to lead to the demise of the cell, the action of ceramidase on ceramide leads to the generation of sphingosine-1-phosphate, which exerts protective effects. Therefore, it must be recognised that it is the balance between enzymes that is important, and, in this study, only one component of the signalling pathway was measured. The data show that DPA and EPA attenuate the age-related and LPS-induced increases in sphingomyelinase *in vivo* and *in vitro*, respectively. However, the protective effects of both fatty acids may also be due to their ability to increase sequential activity of ceramidase and sphingosine kinase, leading to the generation of sphingosine-1-phosphate (see Figure 6.1 and Figure 6.3).

Sphingoine-1-phosphate exerts multiple effects on cells, including promotion of proliferation, survival, regulation of cell motility and the organisation of cytoskeletal proteins. These effects are mediated via secretion of sphingosine-1-phosphate from the cell through the plasma membrane and binding of sphingosine-1-phosphate with sphingosine-1-phosphate receptors at the cell surface in either a paracrine or an autocrine action. Sphingosine-1-phosphate can also act through its second messenger actions intracellularly (Payne *et al.*, 2002; Okada *et al.*, 2008). Sphingosine-1-phosphate is known to have anti-apoptotic, anti-inflammatory effects and can act on sphingosine-1-phosphate receptors (Rivera *et al.*, 2008). Wong and colleagues have demonstrated that sphingosine-1-phosphate can prevent human embryonic stem cell apoptosis by activation of the MAPK ERK (Wong *et al.*, 2007). Wenderfer and colleagues report increased survival and reduced renal injury in MRL-

lpr mice treated with a novel sphingosine-1-phosphate receptor agonist (Wenderfer *et al.*, 2008). Thus ceramide and sphingosine-1-phosphate are critical factors in sphingolipid signalling, as ceramide modulates cell stress responses in regulation of apoptosis (Obeid *et al.*, 1993) and cell senescence (Venable *et al.*, 1995), whereas sphingosine-1-phosphate regulates cell survival, migration and inflammation (Hla, 2004).

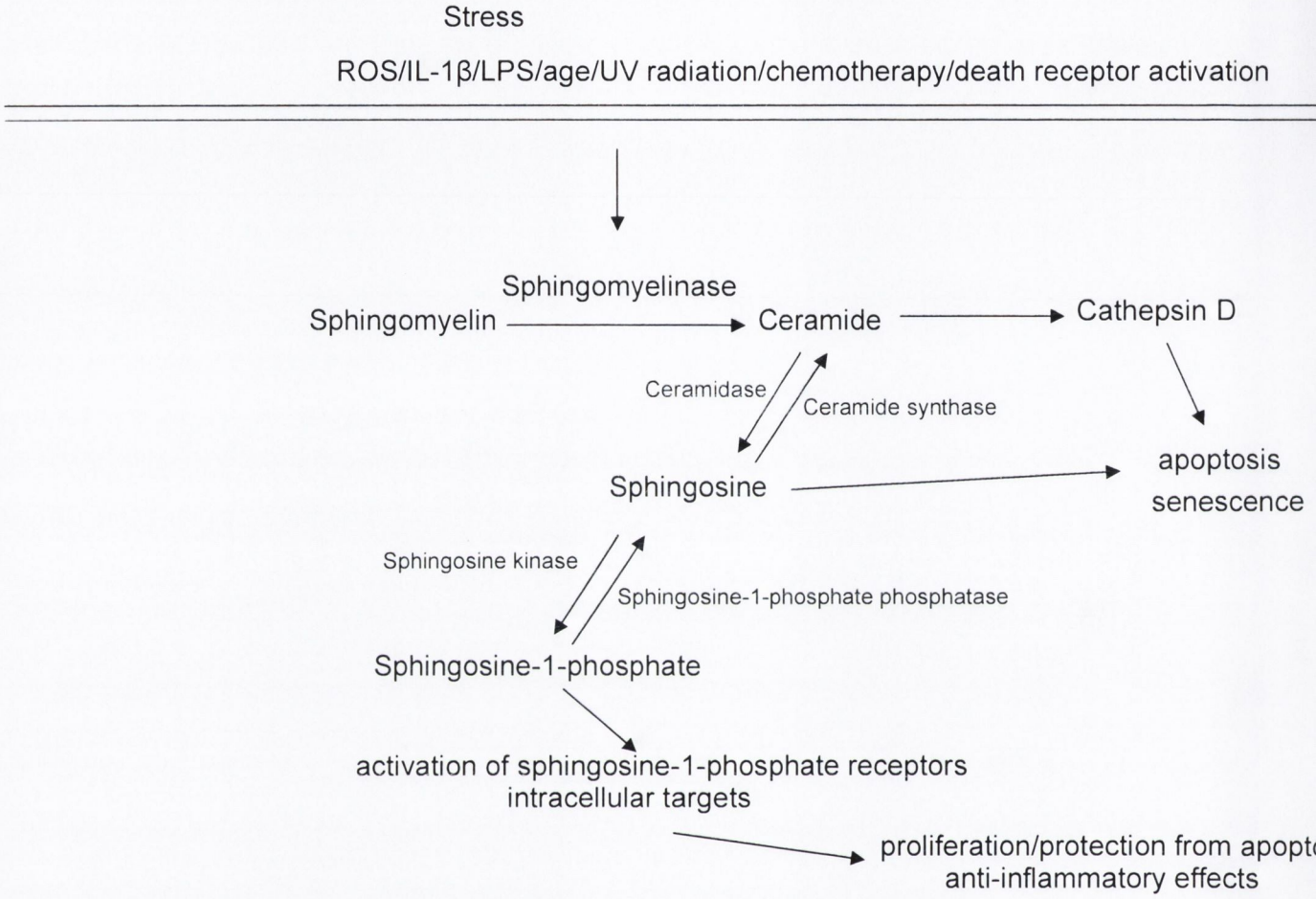

Figure 6.1 Sphingolipid signalling

Adapted from Hannun and Obeid, 2008 and Okada *et al.*, 2008 (Hannun & Obeid, 2008; Okada *et al.*, 2008).

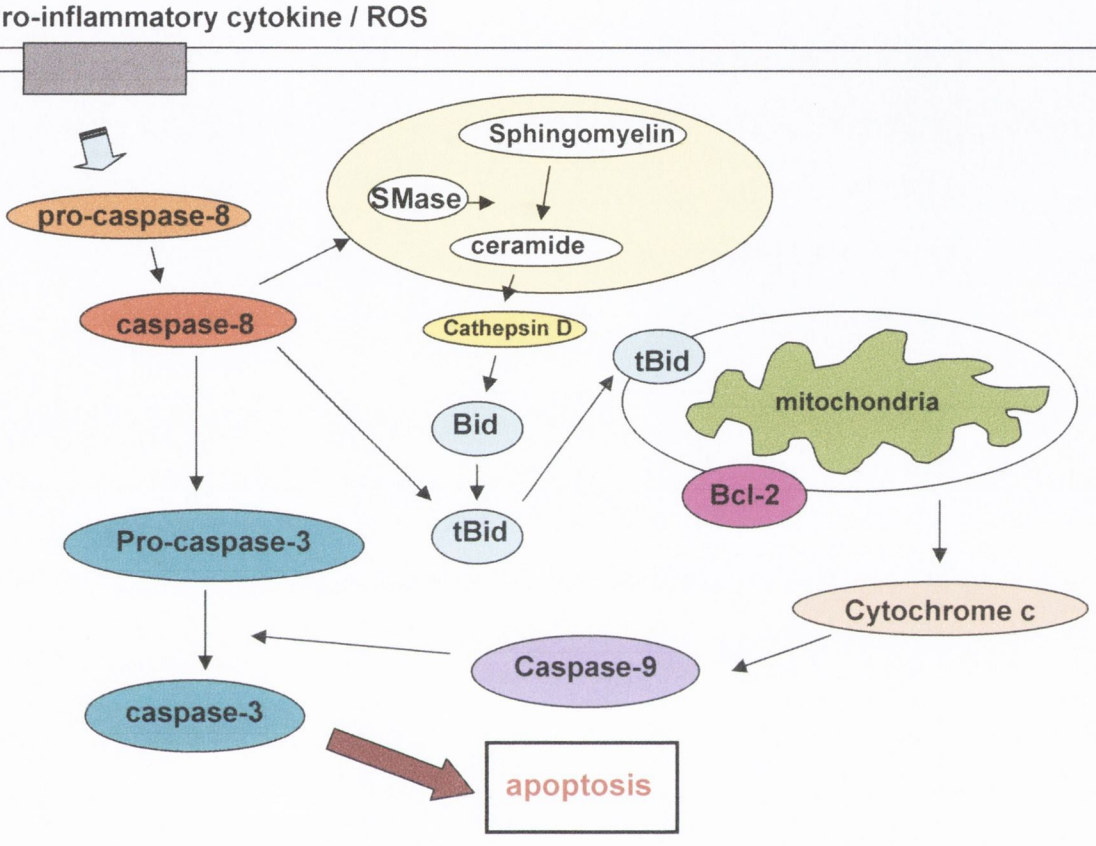

Figure 6.2 Molecular cascade in the activation of caspase 8 and caspase 3

Adapted from Heinrich *et al.*, 2004 (Heinrich *et al.*, 2004).

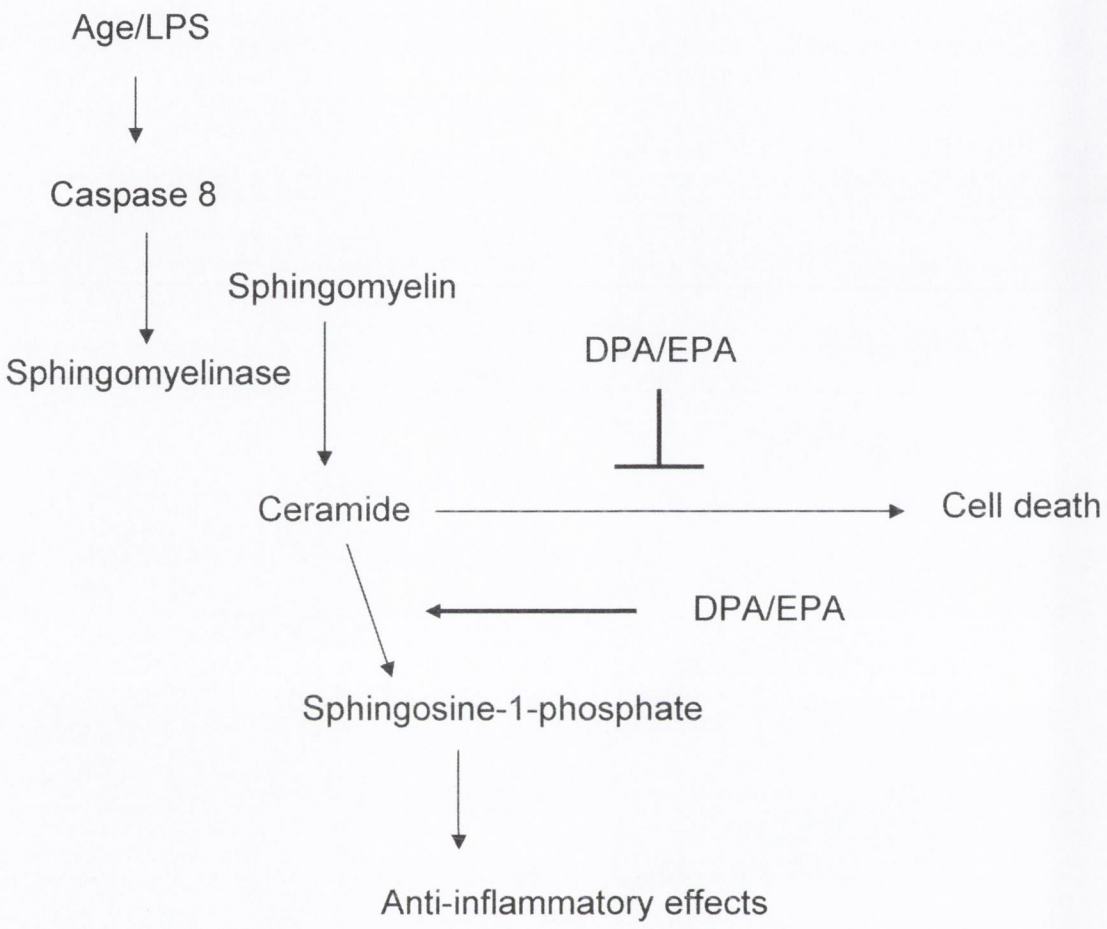

Figure 6.3 Proposed action of EPA and DPA on the sphingomyelin signalling pathway

Recent evidence has shown that sphingomyelinase can be activated by reactive oxygen species (Castillo *et al.*, 2007). Heinrich and colleagues proposed that TNFα activated sphingomyelinase which lead to cathepsin D acting on Bid which resulted in caspase 9 and caspase 3 activation, as shown in HeLa cells (Heinrich *et al.*, 2004).

The initiating event which is responsible for the protective actions of DPA and EPA is still to be elucidated. One possibility is that the fatty acids are incorporated into cell membranes and that they exert their effects by modulating, perhaps, membrane fluidity. It is also possible that EPA and DPA act by binding to specific receptors. There are a number of recently-described G-protein coupled receptors (GPR) on which PUFAs like EPA might act. GPR40-43 were first identified and described as orphan receptors. GPR40 is known to be 30% homologous to GPR41 and GPR43 (Briscoe *et al.*, 2003), with short chain fatty acids capable of activating GPR41 and GPR43, but not GPR40 (Brown *et al.*, 2003). GPR40 is known to be expressed in the brain and pancreas (Briscoe *et al.*, 2003) and has been shown to be expressed in the adult primate central nervous system, in particular the hippocampus (Ma *et al.*, 2007). Recent evidence points to the ability of medium and long chain fatty acids, with carbon chain lengths of greater than six, to activate this receptor (Briscoe *et al.*, 2003). The finding that GPR40 is expressed on neuronal cells could therefore be important, as it could be through this receptor that DHA signalling occurs. It is interesting that astrocytes have been reported to release DHA (Moore, 1993; Kim & Edsall, 1999), and it has been suggested that its release is in sufficient quantities for it to act as an extracellular signalling molecule. Further work is necessary to establish whether the neuroprotective effects of DPA and /or EPA require interaction with one or more of these receptors.

Another possible candidate receptor for EPA and DPA is the G-protein coupled receptor for the chemoattractant protein Chemerin, ChemR23. ChemR23 is known to be activated by resolvin E1, which is a bio-active product of EPA. Resolvin E1 is a member of a novel family of aspirin-triggered bioactive lipids, called the

resolvins (resolution-phase interaction products), which are synthesized at a stage in the resolution phase of acute inflammation *in vivo* (Arita *et al.*, 2005). These molecules have been implicated in the mechanisms of the protective actions of omega-3 PUFAs (Serhan *et al.*, 2002) and the evidence suggests that resolvin E1, which activates inflammation-resolution signalling pathways (Serhan *et al.*, 2000; Arita *et al.*, 2005) and increases macrophage phagocytic activity (Schwab *et al.*, 2007), plays a role in mediating the anti-inflammatory effect of n-3 PUFAs. I carried out preliminary *in vitro* experiments to assess whether GPR40 or ChemR23 were expressed on astrocytes, microglia and/or neuronal cells and the data showed that GPR40 was expressed on neurons, but not on astrocytes or microglia. In an effort to examine whether EPA and/or DPA might modulate LPS-induced effects by interacting with GPR40 or ChemR23, the effect of blocking of GPR40 or ChemR23 with antibodies was assessed but these experiments were inconclusive. Further work is necessary to identify the appropriate culture conditions to systematically examine this question and it must be acknowledged that identification of a fatty acid receptor which mediated the neuroprotective effects of EPA and/or DPA could provide potential for therapeutics. Not only could this be important for modulation of neuronal function, but also there is evidence to suggest that regulation of pancreatic islets could be modulated by this receptor (Briscoe *et al.*, 2003).

The data presented here show, for the first time, that DPA can modulate activation of microglia. The data show that one marker of microglial activation, MHCII mRNA, was significantly increased in the hippocampus of aged rats, confirming previously reported findings (Loane *et al.*, 2007; Moore *et al.*, 2007). This age-related increase in MHCII mRNA was significantly attenuated by both DPA and EPA and, since cell surface expression of MHCII enables interaction with T cells, it must be concluded that DPA and EPA have the potential to indirectly affect T cells. However, expression of CD11b, which was also increased in the hippocampus and cortex of the aged brain, was not attenuated by DPA or EPA. Although this cell surface marker also increases in activated microglia, it may be that CD11b does not function to interact with other cells. Interestingly, in this laboratory, none of the anti-

inflammatory or neuroprotective agents which have been shown to modulate the age-related increase in expression of MHCII mRNA, like atorvastatin or rosiglitazone, successfully attenuated the increase in CD11b expression.

CD68 is a marker of microglial activation and has significant homology with a family of lysosomal membrane proteins which appear to play a role in phagocytosis (Ramprasad *et al.*, 1996; de Beer *et al.*, 2003). The evidence presented here indicates that CD68 mRNA is upregulated in hippocampal cortical tissue prepared from aged rats, but this age-related increase was not attenuated by DPA or EPA. This finding may indicate that phagocytic activity is increased with age, perhaps in response to cell turnover; the evidence suggests that attenuation of phagocytosis by EPA and/or DPA may not be beneficial. Indeed, the neuroprotective effects of the fatty acids may, in part, be due to the maintenance of phagocytic activity. In separate experiments in this laboratory, analysis of phagocytic activity by investigating uptake of fluorescent particles has been shown to be increased in microglia prepared from aged, compared with young, rats (O'Reilly *et al.*, unpublished). It would be important to use this technique to directly assess the effects of the fatty acids on phagocytosis in CD11b-positive cells prepared from young and aged rats using flow cytometry.

Activated microglial cells produce reactive oxygen species as well as inflammatory factors, such as TNFα, nitric oxide and superoxide (Wang *et al.*, 2005). In this study, 8-OHdG, a marker of DNA oxidative damage (Kasai *et al.*, 1986), was examined as evidence of oxidative change in the hippocampus and cortex of aged and young rats. There was an age-related increase in 8-OHdG which was not evident in aged animals which had received DPA or EPA. This is important as it reveals a neuroprotective effect of DPA and EPA in inhibiting oxidative stress, which can be detrimental to cells. It is possible that the primary effect of DPA and /or EPA is to inhibit microglial activation and that one of the consequences of this may be a reduction in release of reactive oxygen species, which may contribute to the neuroprotective effect. Therefore future work should include an investigation of the effect of oxidative stress on neurons, in which the modulatory effect of DPA and

EPA on H_2O_2-induced changes in caspase 8, caspase 3 and sphingomyelinase activities in neurons would be examined. In addition to reactive oxygen species, pro-inflammatory mediators are also produced by activated microglial cells, and this study shows evidence of increases in TNFα, IL-6 and IL-1β induced by LPS from astrocytes with attenuation of release of each of these pro-inflammatory cytokines by DPA and EPA, revealing anti-inflammatory properties of EPA and DPA.

It must be considered that the anti-inflammatory effects of DPA and /or EPA may be a consequence of a change in lipid metabolism which generates active metabolites, for example, prostaglandins, which could activate microglial cells. To address this, cortical tissue obtained from rats in this study were assessed for prostaglandins by Mir and Nicolaou. The unpublished data revealed a significant increase in cortical concentration of PGD_2 in animals which received DPA and EPA and this is significant because there is a great deal of evidence indicating that PGD_2 exerts anti-inflammatory effects (Sandig *et al.*, 2007), while it is a precursor of the endogenous PPARγ agonist PGJ_2 (Scher & Pillinger, 2005), which has also been shown to possess anti-inflammatory properties (data not shown here).

These anti-inflammatory and anti-oxidant effects of DPA and EPA have consequence for synaptic function and the evidence from this study links the neuroprotective effects of both fatty acids with restoration of neuronal function in aged animals as measured by performance in the Morris water maze and by the ability of rats to sustain LTP. Importantly, the data reveal that DPA is as effective or almost as effective as EPA in the analysis undertaken here and therefore provide the first evidence of such effects for this EPA metabolite.

Future experiments

The findings of this study have identified several mechanisms by which DPA and/or EPA may exert their neuroprotective effects but several questions must be addressed in future experiments to provide clarity. For example, it is not known whether the effects of the PUFAs are a consequence of modulating the lipid composition of the membrane, or of interacting with a membrane-associated receptor. While *in vitro* analysis has indicated that the fatty acids are capable of exerting effects on both neurons and glia, preliminary evidence, which is not presented here, suggested that GPR40 was expressed on neurons, but not on astrocytes or microglia. This should be further examined and expression of other fatty acid receptors on neurons and glia, particularly ChemR23, should be investigated. One of the very interesting findings of this study is that both DPA and EPA attenuate the age-related increase in MHCII mRNA, but do not affect the increase in CD68 expression. One interpretation of this result is that there may be a number of states of microglial activation which include expression of cell surface markers (and subsequent interaction with other cells) and phagocytic activity. I would be important to directly assess the effects of the fatty acids on phagocytosis in CD11b-positive cells prepared from young and aged rats using flow cytometry and to couple this with analysis, by flow cytometry, of other cell surface markers, such as intercellular adhesion molecule (ICAM), CD80 and CD86.

The evidence suggests that DPA and EPA decrease reactive oxygen species production in the hippocampus and cortex of aged rats and it has been speculated that this is a result of their ability to modulate microglial activation. Direct evidence for this could be obtained by analysis of the effects of PUFAs in pure microglial cultures. Similarly, it is speculated that the increase in reactive oxygen species production described here exerts detrimental effects on neurons and contributes to the deterioration in cell function leading to depression in LTP. The effect of reactive oxygen species should be examined on cultured neurons by assessing the changes (for example, in activities of caspases and sphingomyelinase) induced by H_2O_2.

It is unclear whether the effects of the fatty acids are a consequence of modulating lipid metabolism, for example, triggering a change in prostaglandin metabolism or sphingomyelin metabolism. Analysis of their effects on PGJ_2 expression would be interesting and could provide a mechanism by which DPA and /or EPA triggers PPARγ activation. It would be particularly interesting to assess whether DPA and EPA affect activity of ceramidase and sphingosine kinase, increasing production of sphingosine-1-phosphate, which has been shown to interact with sphingosine-1-phosphate receptors, exerting neuroprotective effects. Finally, it is not known if oral administration of DPA or EPA could lead to the production of resolvin E1, or another member of this family of molecules, and, if so, how the protective effects of this molecule might confer neuroprotection.

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VIII References

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IX Appendix I Mean data

Table 1.A The effect of age in the cortex. Values are expressed as means ± SEM.

	Young	Aged	Units
caspase 8	301.20 ± 0.50	401.43 ± 6.17	pmol/mg/min
caspase 3	285.88 ± 3.21	317.80 ± 5.88	pmol/mg/min
membrane sphingomyelinase	0.04 ± 0.01	0.10 ± 0.02	fluorescence units/mg
cytosolic sphingomyelinase	0.20 ± 0.01	0.24 ± 0.02	fluorescence units/mg

Table 1.B The effect of age in the hippocampus. Values are expressed as means ± SEM.

	Young	Aged	Units
caspase 8	253.56 ± 6.22	350.14 ± 6.51	pmol/mg/min
caspase 3	278.29 ± 2.74	316.64 ± 6.35	pmol/mg/min
membrane sphingomyelinase	0.027 ± 0.005	0.047 ± 0.003	fluorescence units/mg
cytosolic sphingomyelinase	0.006 ± 0.001	0.015 ± 0.001	fluorescence units/mg

Table 1.C The effect of age on LTP in the last 5 min of recording post tetanic stimulation. Values are expressed as means ± SEM.

Young	Aged	Units
130.99 ± 0.60	121.09 ± 0.52	EPSP slope (%)

Table 1.D The effect of LPS in cultured cortical neurons. Values are expressed as means ± SEM.

	Young	Aged	Units
sphingomyelinase	0.037 ± 0.004	0.050 ± 0.003	fluorescence units/mg
caspase 3	172.37 ± 4.15	355.06 ± 31.42	pmol/mg/min

Table 1.E The effect of sphingomyelinase inhibition on the LPS-induced increase in caspase 3 in neurons. Values are expressed as means ± SEM.

	Control	LPS	GW	GW+LPS	Units
caspase 3	172.37 ± 4.15	355.06 ± 31.42	177.22 ± 4.50	137.40 ± 3.00	pmol/mg/min

Table 1.F The effect of LPS in the cortex of young and aged rats. Values are expressed as means ± SEM.

	Young Control	Young LPS	Aged Control	Aged LPS	Units
caspase 3	104.18 ± 0.51	131.05 ± 1.33	156.29 ± 1.42	173.21 ± 1.25	pmol/mg/min

Table 2.A The effect of DPA and EPA on LPS-induced changes in neurons. Values are expressed as means ± SEM.

	Control	LPS	DPA	DPA+ LPS	EPA	EPA+LPS	Units
sphingomyelinase	0.037 ± 0.004	0.050 ± 0.003	0.031 ± 0.004	0.030 ± 0.003	0.032 ± 0.005	0.032 ± 0.003	fluorescence units/mg
caspase 3	172.37 ± 4.15	355.06 ± 31.42	165.64 ± 5.19	198.41 ± 10.47	192.58 ± 4.99	203.34 ± 4.73	pmol/mg/min

Table 2.B The effect of DPA and EPA on the ceramide-induced reduction in cell viability. Values are expressed as means ± SEM.

	Control	Ceramide	DPA	DPA +ceramide	EPA	EPA +ceramide	Units
cell viability	100.00 ± 2.135	69.96 ± 3.60	100.00 ± 6.61	71.11 ± 2.79	100.00 ± 4.49	83.84 ± 3.94	cell viability (%)

Table 2.C The effect of DPA and EPA on LPS-induced changes in cultured cortical mixed glia. Values are expressed as means ± SEM.

	Control	LPS	DPA	DPA +LPS	EPA	EPA +LPS	Units
IL-1β	9.16 ± 2.85	347.76 ± 18.52	8.27 ± 1.78	303.70 ± 20.14	6.22 ± 1.68	291.80 ± 21.67	pg/ml

Table 2.D The effect of EPA on LPS-induced changes in cultured cortical mixed glia. Values are expressed as means ± SEM.

	Control	LPS	EPA	EPA+LPS	Units
IL-6	0.00 ± 0.00	2514.85 ± 287.33	15.55 ± 15.55	2885.09 ± 338.81	pg/ml
TNFα	0.00 ± 0.00	450.39 ± 49.36	9.13 ± 9.13	540.84 ± 89.37	pg/ml
sphingomyelinase	0.013 ± 0.002	0.020 ± 0.001	0.012 ± 0.001	0.013 ± 0.002	fluorescence units/ml

Table 2.E The effect of DPA and EPA on LPS-induced changes in cultured cortical astrocytes. Values are expressed as means ± SEM.

	Control	LPS	DPA	DPA+LPS	EPA	EPA+LPS	Units
IL-1β	0.00 ± 0.00	46.11 ± 13.33	0.00 ± 0.00	9.72 ± 2.95	0.00 ± 0.00	0.63 ± 0.58	pg/ml
IL-6	2.16 ± 2.16	23.32 ± 5.32	3.30 ± 3.30	9.22 ± 5.56	8.37 ± 5.35	7.30 ± 5.38	pg/ml
TNFα	102.23 ± 55.49	1,037.72 ± 26.70	126.38 ± 71.41	689.18 ± 95.16	165.59 ± 75.83	578.32 ± 119.22	pg/ml

Table 3.A The effect of DPA and EPA on age-related changes in learning and memory and LTP. The effect of DPA and EPA on the age-related impairment in LTP in the last 5 min of recording post tetanic stimulation (compared with the 10 min period immediately preceding tetanus). The effect of DPA and EPA on age-related impairments in performance in the Morris water maze (MWM). Values are expressed as means ± SEM.

	Young Control	Aged Control	Young DPA	Aged DPA	Young EPA	Aged EPA	Units
LTP	130.99 ± 0.60	121.09 ± 0.52	126.99 ± 0.43	149.32 ± 0.37	144.20 ± 0.69	132.06 ± 0.43	EPSP slope (%)
MWM escape latency, Day 5	14.50 ± 8.94	35.46 ± 10.21	7.89 ± 0.78	24.57 ± 4.79	9.37 ± 0.80	17.36 ± 1.50	s
MWM distance, Day 5	301.39 ± 193.26	613.21 ± 130.89	161.19 ± 32.07	413.38 ± 69.70	206.04 ± 21.56	297.46 ± 13.78	cm

Table 3.B The effect of DPA and EPA on age-related changes in the cortex. Values are expressed as means ± SEM.

	Young Control	Aged Control	Young DPA	Aged DPA	Young EPA	Aged EPA	Units
caspase 8	100.00 ± 0.15	131.45 ± 0.72	100.00 ± 0.53	124.53 ± 0.52	100.00 ± 0.64	111.60 ± 0.64	%
caspase 3	100.00 ± 0.43	143.36 ± 0.73	100.00 ± 0.58	122.65 ± 0.62	100.00 ± 0.82	107.91 ± 0.71	%
membrane sphingomyelinase	0.040 ± 0.005	0.073 ± 0.013	0.041 ± 0.001	0.046 ± 0.003	0.042 ± 0.003	0.044 ± 0.002	fluorescenc units/mg
cytosolic sphingomyelinase	0.024 ± 0.002	0.024 ± 0.001	0.025 ± 0.003	0.023 ± 0.001	0.028 ± 0.003	0.026 ± 0.003	fluorescenc units/mg
CD68 mRNA	0.12 ± 0.01	0.26 ± 0.04	0.11 ± 0.01	0.23 ± 0.02	0.12 ± 0.01	0.27 ± 0.02	RQ
MHCII mRNA	0.76 ± 0.07	0.65 ± 0.05	0.68 ± 0.04	0.55 ± 0.02	0.69 ± 0.04	0.56 ± 0.02	RQ
CD11b mRNA	0.10 ± 0.01	0.23 ± 0.05	0.09 ± 0.01	0.20 ± 0.02	0.11 ± 0.02	0.19 ± 0.02	RQ
IL-1β mRNA	0.68 ± 0.15	1.82 ± 1.33	1.04 ± 0.31	1.34 ± 0.35	1.23 ± 0.19	1.91 ± 0.86	RQ
8-OHdG	0.011 ± 0.002	0.030 ± 0.010	0.008 ± 0.002	0.016 ± 0.006	0.011 ± 0.002	0.009 ± 0.004	µg/pg
PPARγ	0.18 ± 0.01	0.13 ± 0.02	0.17 ± 0.03	0.21 ± 0.03	0.14 ± 0.01	0.20 ± 0.03	arbitrary units
CD36 mRNA	1.93 ± 0.37	2.05 ± 0.54	2.58 ± 0.57	3.06 ± 0.45	1.78 ± 0.15	3.98 ± 0.63	RQ
CD200L mRNA	1.01 ± 0.03	1.49 ± 0.06	1.15 ± 0.05	1.33 ± 0.07	1.23 ± 0.09	1.28 ± 0.09	RQ
fractalkine	10,367.51 ± 448.85	8,055.24 ± 567.85	9,332.86 ± 244.75	9,426.09 ± 379.01	8,933.69 ± 557.58	9,709.52 ± 338.83	pg/mg

Table 3.C The effect of DPA and EPA on age-related changes in the hippocampus. Values are expressed as means ± SEM.

	Young Control	Aged Control	Young DPA	Aged DPA	Young EPA	Aged EPA	Units
membrane sphingomyelinase	0.106 ± 0.003	0.114 ± 0.002	0.089 ± 0.005	0.097 ± 0.002	0.088 ± 0.004	0.088 ± 0.002	fluorescence units/mg
CD68 mRNA	1.35 ± 0.08	5.27 ± 0.64	1.44 ± 0.14	5.07 ± 0.50	1.65 ± 0.18	5.26 ± 0.21	RQ
MHCII mRNA	1.26 ± 0.19	2.40 ± 0.42	1.02 ± 0.05	1.14 ± 0.05	1.09 ± 0.05	1.17 ± 0.05	RQ
CD11b mRNA	0.87 ± 0.04	3.38 ± 0.36	0.78 ± 0.10	3.50 ± 0.41	1.06 ± 0.15	3.38 ± 0.13	RQ
IL-1β mRNA	2.44 ± 0.64	10.29 ± 3.70	10.87 ± 3.56	35.21 ± 6.28	3.04 ± 0.44	6.59 ± 1.15	RQ
8-OHdG	0.06 ± 0.01	0.12 ± 0.06	0.08 ± 0.03	0.09 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	µg/pg
PPARγ	0.11 ± 0.01	0.10 ± 0.01	0.09 ± 0.00	0.10 ± 0.01	0.11 ± 0.01	0.09 ± 0.00	arbitrary units
CD36 mRNA	0.97 ± 0.12	2.62 ± 0.46	1.24 ± 0.22	2.15 ± 0.28	1.43 ± 0.09	3.13 ± 0.61	RQ
CD200L mRNA	1.20 ± 0.09	1.27 ± 0.17	1.09 ± 0.09	1.14 ± 0.11	1.16 ± 0.04	1.08 ± 0.06	RQ
fractalkine	11,968.70 ± 1,906.31	12,682.22 ± 2,001.02	14,494.06 ± 1,157.30	14,500.51 ± 588.95	12,127.97 ± 855.74	12,391.87 ± 882.13	pg/mg

X Appendix II Materials

Ara-c	Sigma
Autoclave tape	Sigma
BCA protein assay kit	Pierce
BioRad dye reagent concentrate	BioRad
Bovine serum albumin	Sigma
B27 supplement	Gibco
Calcium chloride	Lennox
Caspase 3 activity assay	Biomol
Caspase 8 activity assay	Biomol
cDNA kit	Applied Biosystems
Ceramide	Sigma
Chloroform	Sigma
Constant isolation unit	Isoflex
Cytosine arabinofuranoside	Sigma
Dimethyl sulphoxide	Sigma
Dithiothreitol	Sigma
DNase	Sigma
DNase-RNase free water	Sigma
Docosapentaenoic acid	Amarin Neuroscience
Dulbecco's modified eagle media	Gibco
EDTA	Sigma
Electrodes	Clark Electromedical
ELISA substrate solution	R&D Systems
Ethanol	Sigma
Ethyl eicosapentaenoic acid	Amarin Neuroscience
Falcon tubes	Sarstedt
Foetal bovine serum	Sigma
Fractalkine ELISA DuoSet	R&D Systems
Glass coverslips	Chance Propper

Glucose	Lennox
Glutamax	Gibco
Glycerol	Sigma
GW4869	Sigma
HEPES	Sigma
Horse serum (heat-inactivated)	Gibco
Hydrochloric acid	Lennox
IL-1β ELISA DuoSet	R&D Systems
IL-6 ELISA kit	BD Biosciences
Immuno plates	Nunc
Laminar flow hood	AGB Scientific
Lipopolysaccharide	Alexis
LTP software	Spike 2, version 5
Macrophage colony stimulating factor	R&D Systems
Magnesium chloride	Sigma
Magnesium sulphate	Sigma
M-CSF	R&D Systems
MTS proliferation assay	Promega
NanoDrop spectrophotometer	NanoDrop Technologies Inc
Neurobasal media	Gibco
Normal goat serum	Vector
Normal laboratory chow	Red Mills
NucleoSpin® RNA extraction kit	Macherey-Nagel
Penicillin	Gibco
Plate reader	Labsystems Multiwell RC
Platform shaker	Stuart Scientific
Poly-L-lysine	Sigma
Polytron	Kinematica
Potassium chloride	Sigma

Potassium hydrogen phosphate	Sigma
PPARγ assay kit	Cayman Chemical
Pre-amplifier	World Precision Instruments
Protease inhibitor cocktail	Sigma
ProteoExtract® Subcellular Preteome Extraction Kit	Calbiochem
Scalpel	Schwann-Mann
Sphingomyelinase activity assay kit	Molecular Probes, Invitrogen
Sodium carbonate	Sigma
Sodium chloride	Sigma
Sodium hydrogen carbonate	Lennox
Sodium hydroxide	Lennox
Sodium phosphate	Sigma
Statistical analysis software	GraphPad Prism
Stereotaxic frame	ASI Instruments
Sterile mesh cell strainer	BD Biosciences
Sterile phosphate-buffered saline (10X)	Sigma
Streptomycin	Gibco
Sulphuric acid	Lennox
Taqman universal PCR master mix	Applied Biosystems
Thermocycler	MJ Research, Biosciences
Tissue chopper	McIlwain
TMB	R&D Systems
TNFα ELISA kit	BD Biosciences
Tris-HCl	Sigma
Trypsin	Sigma
Trypsin inhibitor	Sigma
Tween-20	Sigma
Urethane	Sigma

8-OHdG ELISA kit	Gentaur
96-well plates	Sarstedt

XI Appendix III Addresses

AGB Scientific	AGB Scientific Ltd. Slaney Close Dublin Industrial Estate Dublin 11 Ireland
Alexis	Axxora (UK) Ltd. P.O. Box 6757 Bingham Nottingham NG13 8LS UK
Amarin Neuroscience	Amarin Neuroscience Ltd Oxford, England OX4 4GA UK
Applied Biosystems	Applied Biosystems Dormstadt Germany
ASI Instruments	ASI Instruments, Inc. 12900 East Ten Mile Road Warren, MI 48089 USA

BD Biosciences	BD Biosciences The Danby Building Edmund Halley Road Oxford Science Park OX4 4DQ Oxford UK
Biomol	Biomol International, L.P. Palatine House Matford Court Exeter EX2 8NL UK
BioRad	Bio-Rad Laboratories 1000 Alfred Nobel Drive Hercules, CA 94547 USA
Biosource	Biosource International 542 Flynn Road Camarillo CA 93012 USA
Cayman Chemical Company	Cayman Chemical Company 1180 East Ellsworth Road Ann Arbor, Michigan 48108 USA

Chance Propper	Chance Propper Ltd Uraniumweg 23 3812 RJ Amersfoort The Netherlands
GE Healthcare	GE Healthcare Life Sciences The Grove Centre White Lion Road Amersham Bucks HP7 9LL UK
Gentaur	Gentaur Av. de l' Armée 68 B-1040 Brussels Belgium
Gibco	Gibco Ltd 3 Fountain Drive Inchinnan Drive Paisley PA4 RF Scotland
GraphPad Software	GraphPad Software, Inc. 11452 El Camino Real, #215 San Diego CA92130 USA

Invitrogen	Invitrogen Ltd 3 Fountain Drive Inchinnan Drive Paisley PA4 RF Scotland
Kinematica	Kinematica AG Luzernerstrasse 147a CH-6014 Littau-Lucerne Switzerland
Labsytems	MTX Lab Systems, Inc. 8456 Tyco Road, Building D Vienna Virginia 22182 USA
Lennox	Lennox Laboratory Supplies Ltd. John F. Kennedy Drive Naas Road Dublin 12 Ireland
Macherey-Nagel KG	Macherey-Nagel GmbH & Co. Postfach 10 13 52 D-52313 Düren Neumann Neander Str. 6-8 D-52355 Düren

Merck Biosciences	Merck Biosciences Ltd Boulevard Industrial Park Padge Road Beeston Nottingham NG9 2JR UK
Molecular Probes	Molecular Probes Europe B.V. Poort Gebouw Rijnsburgerweg 10 2333 AA Leiden The Netherlands
NanoDrop Technologies	NanoDrop Technologies Inc. NanoDrop products 3411 Silverside Rd Bancroft Building Wilmington, DE 19810 USA
Nunc	Nunc 75 Panorama Creek Drive Rochester, NY 14625-2385 USA

Pierce	Pierce Biotechnology Inc 3747 N. Meridian Road P.O. Box 117 Rockford IL 61105 USA
Promega	Promega UK Ltd Delta House Southampton Science Park Southampton Hampshire SO16 7NS UK
R & D Systems	R & D Systems 614 McKinley Place NE Minneapolis MN 55413 USA
Red Mills	Red Mills Goresbridge County Kilkenny Ireland
Sarstedt	Sarstedt Ltd Sinnottstown Lane Drinagh Wexford Ireland

Sigma	Sigma-Aldrich Company Ltd Fancy Road Poole Dorset BH12 4QH UK
Stuart Scientific	Stuart Scientific Beacon Road Stone Staffordshire ST15 0SA UK
Vector	Vector Laboratories, Ltd., 3 Accent Park Bakewell Road Orton Southgate Peterborough PE2 6XS England
World Precision Instruments	World Precision Instruments Ltd. Astonbury Farm Business Centre Aston Stevenage. SG2 7EG. UK.

XII Appendix IV Solutions

Krebs solution containing $CaCl_2$, pH 7.4

136mM NaCl

2.54mM KCl

1.18mM KH_2PO_4

1.18mM $MgSO_4$

16mM $NaHCO_3$

10mM glucose

2mM $CaCl_2$

Phosphate buffered saline, pH 7.3

137mM NaCl

2.7mM KCl

8.1mM Na_2HPO_4

1.5mM KH_2PO_4

Lysis buffer, pH 7.4

10mM Tris-HCl

50mM NaCl

10mM $Na_4P_2O_7.10H_2O$

50mM NaF

with 1% IGEPAL

and 1mM Na_3VO_4

1mM PMSF

and protease inhibitor cocktail (Sigma, UK)

Harvesting buffer, pH 7.4

50mM HEPES

1mM EDTA

XIII Appendix V Publications

1. Kelly L., Lynch A., Woods O., Noonan J., Lynch M. (2007) The neuroprotective effect of eicosapentaenoic acid is coupled with its ability to downregulate sphingomyelinase activity. *British Neuroscience Association Abstracts* (65.07).

   British Neuroscience Association 19th National Meeting, 1st – 4th April, 2007, Harrogate, England.

2. Kelly L., Della Chiesa A., Cowley T., O'Dwyer L., Kerskens C., Lynch M. (2007) Characterisation of effects of ageing in the rat brain. *1st School of Medicine Postgraduate Research Day Abstracts* (SMM4).

   1st School of Medicine Postgraduate Research Day, 7th September, 2007, Institute of Molecular Medicine & Trinity Centre for Health Sciences, St. James's Hospital, Dublin, Ireland.

3. Kelly L.C., Lynch M.A. (2008) The age-related deficit in long-term potentiation in the rat hippocampus is associated with increases in activity of sphingomyelinase and caspases. *International Neuroimmunology Symposium Abstracts* (39) and published in the *Journal of Neuroimmunology,* 197 (2008) 159–176.

   International Neuroimmunology Symposium, 14th of March, 2008, The Conway Institute, University College Dublin, Ireland.

4. Kelly L.C., Cowley T.R., Lynch M.A. (2008) Age and LPS induce increases in activity of caspases and sphingomyelinase in the brain: neuroprotective

effects of the omega-3 fatty acids eicosapentaenoic acid and docosapentaenoic acid. *FENS Abstracts,* vol.4, 015.13, 2008.

6th Forum of European Neuroscience, 12th – 16th July, 2008, Geneva, Switzerland.