Molecular Mechanisms and Nutritional Factors Influencing Post-stroke Affective Disorders

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Abstract
The overall aim for this thesis was to study the biological mechanisms underlying stroke related affective symptomatology, specifically ischemia related depressive disorder, anxiety and hyperactive delirium. The pathogenesis of these conditions is currently unknown. This thesis explored the hypothesis that ischemia associated degeneration of cellular networks is closely linked to stroke associated behavioural and affective disorders. Working from this premise, this thesis additionally aimed to study the relationship between nutritional factors, namely polyunsaturated fatty acids and homocysteine, which have been reported to be capable of mediating cellular degeneration, and are implicated in aetiology of affective symptomatology. In chapter 2 the mechanisms leading to cellular degeneration under conditions of inflammatory insult, such as ischemic stroke have been explored and this chapter has been published. The mediating effects of polyunsaturated fatty acids on these mechanisms have been highlighted in chapter 3; this chapter has also been published.

The first experimental study using a rodent model of ischemia has been submitted for publication and presented in chapter 5. This chapter reports the influence of middle cerebral artery occlusion on a number of behavioural outcomes, designed to model human clinical affective disorders. These are anxiety and depressive-like behaviours and hyperactive locomotion. The influence of dietary supplementation with polyunsaturated fatty acids, on these behaviours has additionally been reported.

The second experimental study is presented in chapter 6. This study investigated the influence of middle cerebral artery occlusion on cellular degeneration, and cellular proliferation, in brain regions known to be involved in the aetiology of ischemia associated behavioural and affective conditions. These regions of investigation are the thalamic region and the hippocampus. The influence of polyunsaturated fatty acid supplementation, on both cell death/proliferation, and the correlations between cell death and behavioural outcomes was also investigated.

The final empirical chapter of this thesis is a clinical study that has been published and is presented in chapter 7. This chapter investigated whether homocysteine, a nutritional factor that has cellular degenerative properties, at elevated levels, was associated with depressive mood in a post-stroke clinical population.
The overall findings of the present thesis suggest that ischemia induced cell death is associated with the presentation of depressive, anxious and hyperactive symptomatology, and that nutritional factors, that influence the processes of cell death, may also influence the presentation of these behavioural outcomes. However, a possible detrimental effect of nutritional supplementation, on risk of bleeding, was identified and requires further investigation.

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Completing this thesis has been quite a journey. I have messed up a great deal, learnt a lot, gotten some things right, and at the end of it have come out more passionate about science than I could have expected. The many people who have contributed to the completion of this thesis have made this happy outcome possible. Certainly this has been a work of collaboration.

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To my family, thank you for your belief in me. Thank you for the love and laughter. Thank you for making the world away from thesis a bright place. I am indescribably fortunate to belong to you. My adoration is limitless.


“I am only a child playing on the beach, while vast oceans of truth lie undiscovered before me.”

Sir Isaac Newton.
Authors Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma, except where due reference is made in the text of the examinable outcome; to the best of my knowledge, it contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, discloses the relative contributions of the respective workers or authors has been made.

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Signature:

Date: August 2013
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
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<tr>
<td>BDNF</td>
<td>Brain-Derived Neurotrophic Factor</td>
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<tr>
<td>CA1</td>
<td>Cornu Ammonis 1 region of the hippocampus</td>
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<td>Ca²⁺</td>
<td>Calcium</td>
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<td>Ca²⁺ permeable acid sensing ion channel-1</td>
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<td>CMS</td>
<td>Chronic Mild Stress</td>
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<td>Central Nervous System</td>
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<td>Cyclooxygenase</td>
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<tr>
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<td>Cyclooxygenase-2</td>
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<tr>
<td>CRH</td>
<td>Corticotrophin-Releasing Hormone</td>
</tr>
<tr>
<td>CT</td>
<td>Computer Tomography (scan)</td>
</tr>
<tr>
<td>DSM-III-R</td>
<td>Diagnostic and Statistical Manual of Mental Disorders</td>
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<td>FAME</td>
<td>Fatty Acid Methyl Esters</td>
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<td>GLA</td>
<td>Gamma-linolenic Acid</td>
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<tr>
<td>GluR2 subunit</td>
<td>Subunit Glutamate Receptor 2</td>
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<td>Hypothalamic–Pituitary–Adrenal Axis</td>
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<td>Natural Killer (Cell)</td>
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<td>NF-κB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells</td>
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<td>eNOS</td>
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Chapter 1 Introduction

Fifteen million people experience ischemic stroke, the result of interrupted blood supply to the brain, each year making stroke a leading cause of death and disability worldwide [1]. In Australia, stroke is the second leading cause of death [2]. Prognostic outcome after stroke is effected by many factors, one of which is the development of post-stroke behavioural and affective disorders, including anxiety [3, 4], clinical depression [5] and hyperactive delirium (although hyperactive delirium is not a clinical problem of the magnitude that anxiety or depression are) [6]. These post-stroke behavioural and affective complications are associated with poorer recovery and rehabilitation, reduced quality of life, and lowered functional ability [7]. The biological mechanisms resulting in post-stroke affective and behavioural disorders are unclear. The pathogenesis of affective disorders in non-stroke affected populations is also not known. Much evidence indicates however that stroke induced cellular degeneration, and proliferation, which is an increase in cell number through the process of cellular division or mitosis, in key brain regions may be a contributing factor [8].

Ischemia immediately induces necrotic cell death in the core of the ischemic area [9]. Necrosis is a form of cell injury that results in the premature death of cells [10]. In addition, ischemia disrupts normal cellular osmotic homeostasis in the penumbra, which is the area surrounding the ischemic event [9], inducing both necrotic and apoptotic, or naturally occurring cell death [11]. Infarct size is determined by the extent of cell death [12], 50% of which can result from apoptosis in the penumbra [13]. Additionally, research suggests new immature cells migrate to the site of ischemic damage from the subventricular zone (SVZ) [14-16], and that new cells can be generated directly in the infarct tissue (an area more traditionally believed to be populated only by inflammatory cells) [17-21].

In non-stroke animal models, cellular degeneration and reduced cellular proliferation appear to be key factors in the presentation of depressive and anxious symptomatology [8, 22-24]. Increased cell death in hippocampus of the limbic region (a brain region involved in the regulation of mood and emotion) is a widely acknowledged to be characteristic of major depressive and anxiety disorders, in both clinical population and animal models [8, 22, 24]. Conventional anti-depressants reduce cell death in the
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hippocampus in animal models [25, 26]. Cellular damage in the hippocampus is similarly associated with post-stroke hyperactive locomotion, in the rat model [27-29]. After cerebral ischemia, animal models demonstrate that hippocampus cells are particularly vulnerable to cellular degeneration [30, 31] and that cellular proliferation occurs in the hippocampus [32].

Animal models demonstrate that neural pathways to cortical areas involved in motor and sensory functions project through the thalamic region [33, 34] highlighting the key role the thalamus plays in the information processes necessary for motor control [33-37]. Accordingly, cellular damage in the thalamus is also associated with an increased risk of post-stroke hyperactive delirium, in human stroke survivors [6, 38]. Animal models demonstrate that ischemic stroke results in secondary cellular damage in the thalamic region of the ipsilesional hemisphere. This occurs as late as nine months post-surgery in rat [11, 39-41], and may reflect retrograde degeneration of cortical-thalamic projections [42, 43].

The process of cellular degeneration and more specifically apoptosis, is controlled by a range of cell signals, which may be either extracellular or intracellular. Intracellular or intrinsic signals are initiated in response to stress and result in cell death [9]. These signals, which result in apoptosis are associated with and appear to be regulated by cytokines. Cytokines are small cell signaling protein molecules associated with the immune system and not usually expressed in healthy brain. Cytokines, which can be either pro-inflammatory or anti-inflammatory, play a key role in the innate immune response [44]. Accordingly, pathological conditions, such as ischemic stroke, induce the rapid synthesis of a range of inflammatory cytokines, to aid in removal of dead tissue and which result in brain inflammation [45], and the later expression of anti-inflammatory cytokines [46], which likely reflects the brain’s attempt to re-establish homeostasis [47]. Pro-inflammatory cytokines also induce depression associated cellular apoptosis [48]. This hypothesis is systematically reviewed in chapter 2 of this thesis.

After ischemic stroke, apoptotic cells in the penumbra remain viable for a period of time and so could potentially be rescued [13]. Therefore previous research has focused on identifying potential targets for neuroprotective therapies [13]. We similarly aim to

3
explore the potential therapeutic effects of a nutrition based neuroprotective therapy, with the aim of achieving a better outcome for survivors of stroke.

Omega 3 derived polyunsaturated fatty acids, are an alternative or compliment to conventional medication, for the treatment of hyperactivity [49] depressive [50] and anxiety symptoms [51], in human non-stroke affected clinical populations. Polyunsaturated fatty acids, which are highly bioactive compounds, are commonly derived from dietary fish oil and are essential and necessary for every cell membrane. Polyunsaturated fatty acids protect against cellular degeneration, when administered in optimal doses [52] and have anti-inflammatory effects [50].

Homocysteine is an amino acid that has been shown to contribute to cellular apoptosis, as it results in the production of oxidants, which are toxic to cells [53, 54]. A high expression of homocysteine is associated with a 70% increased risk of clinical depression, in elderly individuals [55], while folate (folate vitamin deficiency results in elevated homocysteine) is decreased in blood samples of depressed individuals [56]. Low folate is associated with a worse than expected response to conventional anti-depressant medications, while higher folate levels are associated with better response [57, 58]. Supplementation with folate has been shown to have antidepressant effects and in some cases, to be as effective as conventional antidepressants [55, 59, 60].

Given that an exceptionally high number of stroke survivors experience affective disorders and that these affective disorders are likely to be mediated, at least in part, by the processes of cellular degeneration, the aim of the present thesis is to explore the mechanisms of cellular degeneration post-stroke, and how these relate to the presentation of behavioural and affective outcomes. Therefore, we aimed to first review the mechanisms leading to cellular degeneration under conditions of ischemic stroke, through a systemic review that is presented in chapter 2. We then aimed to systemically review the hypothesis that polyunsaturated fatty acids, like cytokines, may influence the processes of cellular degeneration, via regulation of intrinsic apoptosis inducing factors, including excitotoxicity and free radicals, and present these findings in chapter 3. A series of experimental studies, presented in chapters 5, 6 and 7, aimed to study the influence of middle cerebral artery occlusion, on cellular degeneration, and cellular proliferation in brain regions believed to be involved in the aetiology of ischemia associated behavioural and affective conditions, the thalamic region and the
hippocampus. We aimed to study correlations between cell death/proliferation, and behaviours that are believed to represent anxious, depressive-like and hyperactive locomotion in these animals. It was aimed to investigate the effects of polyunsaturated fatty acid supplementation, on the presentation of these behaviours, argued to represent anxious, depressive-like and hyperactive locomotion disorders. Finally, we aimed to study the association between homocysteine and persistent depressive mood, at a year and half after stroke onset, in a clinical population, and present these findings in chapter 7.

We found that dietary polyunsaturated fatty acid supplementation influences stroke associated cell proliferation and degeneration in the thalamic region of the contralesional hemisphere in the rodent model. Cellular degeneration and proliferation correlated with hyperactive locomotion and anxiety-like behaviours. Dietary supplementation with polyunsaturated fatty acids, was associated with less stroke associated hyperactive locomotion and anxiety-like behaviours. However, supplementation with polyunsaturated fatty acids is also associated with an increased risk of hemorrhagic bleeding, during reperfusion, in stroke-operated rats. In a clinical population, homocysteine expression is associated with a depression diagnosis post-stroke, and a high cellular expression of homocysteine is a significant predictor of depressive symptoms, after accounting for age and gender. The overall findings of the present thesis are consistent with the argument that nutritional factors that are capable of influencing the mechanism of cell death, post-stroke, are important in understanding the presentation of affective and behavioural disorders, and their prevention.
Chapter 2: Inflammation and Depression: Why Post-Stroke Depression May
be the Norm and Not the Exception

2.1 Abstract

Ischemic stroke often precedes the appearance of clinical depression. Post-stroke depression in turn influences the prognostic outcome. In the interest of advancing our understanding of the biological mechanisms underlying the development of post-stroke depression, this systematic review explores the immunological processes driving the development of inflammation-related cell death in mood-related brain regions. Particular attention has been paid to cytokine driven intrinsic apoptosis factors, including intracellular calcium, glutamate excitotoxicity and free radicals that appear in the brain following ischemic damage and whose presence significantly increases the likelihood of clinically defined depression.

Key Words: Ischemia, Depression, Inflammation, Cytokine, Apoptosis
2.2 Introduction
Ischemic stroke is the result of interrupted blood supply to the brain. Worldwide, 15 million people suffer strokes each year [1]. In Australia alone, 40,000-48,000 strokes occur annually [61]. The direct cost per person over the first year post-stroke is approximately $25,000 [62]. Given the high prevalence of stroke in Western societies and associated costs for the community, it is important to understand the mechanisms that may impact on outcome, in order to achieve the best possible prognostic outcomes.

In this review we address the co-relationships between inflammation and depression and stroke on the PubMed and Google databases. While there are at least 2270 recent papers found dealing with the relation between inflammation and depression, and even more (4300) addressing stroke and depression, there are only 91 studies linking all three areas. Papers have been reviewed in terms of design, sample size and apparent reliability of data collection. The abstracts of these papers have then been exposed to a Pathway Studio (Ariadne Genomics) quantitative reanalysis of degree of overlap of supporting conclusions. Although multiple references exist in many areas we have restricted our choice to first mention of a new point, we additionally have restricted references to those most recent, where references overlapped.

2.3 Post-Stroke Depression
Prognostic outcome after stroke is dependent on a number of factors. One important factor impacting on functional recovery is the development of post-stroke depression. Pooled estimates of clinical populations indicate that approximately 33% of stroke survivors experience clinical depression [63] compared to 13% of population controls [5, 64]. Yet, this is likely to be a conservative estimate, given that: most studies excluded patients with communication difficulties [65] thus underestimating high risk groups; very few studies report on the cumulative incidence of depression; and important depressive symptoms may be experienced in stroke survivors with sub threshold scores on depression measures [65]. Post-stroke depression is associated with poorer recovery and rehabilitation, reduced quality of life, and lowered functional ability [7]. Therefore, recognition of the causal factors contributing to post-stroke depression is important.

The pathological profiles of ischemia and major depression show a number of similarities. Of particular interest is that both ischemia and depression are associated
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with an increased inflammatory state and greater rates of central nervous system (CNS) cell death. In individuals diagnosed with major depressive disorders, inflammation mediates both depressive symptoms and cell death, and treatment methods normalize both inflammatory markers and cell death [25, 26]. Such observations suggest that major depressive disorder is associated with the abnormal physiological and immunological responses, and a resultant increase in inflammatory markers. Ischemic cell death similarly elicits the inflammatory mechanisms, which precede post-stroke associated depressive symptoms, and later long-lasting clinical depression.

2.4 Depression and Inflammation

A recent review identifies that inflammation is closely related to depressive disorder [66]. Individuals with depression have higher circulating levels of pro-inflammatory cytokine Interferon-γ (INF-γ) [66]. A number of investigative clinical studies has found individuals with depression to have higher circulating levels of pro-inflammatory cytokines, Interleukin-1beta (IL-1β) [67] and Tumour necrosis factor-alpha (TNF-α) [68] and Interleukin-6 (IL-6) [69]. Additionally, unpublished data [70] shows that individuals with clinical depression exhibit decreased anti-inflammatory Interleukin-10 (IL-10) [70]. Inflammatory Interferon-α (INF-α) [71] induces depressive symptoms in humans, while symptoms terminate with cessation of INF-α therapy [72] and are abrogated by antidepressant treatment [73].

Evidence indicates that antidepressants may reduce depressive symptoms by mediating mood modulating inflammatory markers [25]. Antidepressants reduce pro-inflammatory IL-1 and Interleukin-2 (IL-2) and increase anti-inflammatory IL-10 in animal models. Heightened plasma concentrations of inflammatory cytokines and acute phase proteins, prior to anti-depressant treatment, are associated a with a non-response in clinical populations [66].

2.5 Ischemia and Inflammation

The brain normally has few detectable cytokines. Acute ischemia induces an immediate innate immune response and the rapid synthesis of a range of inflammatory cytokines, which result in brain inflammation [45]. Animals studies show that ischemia induced inflammation occurs within minutes and the inflammation contributes to ischemic damage [74]. Early increases are seen in the expression of pro-inflammatory cytokines,
IL-1, TNF-α, Tumour necrosis factor –β (TNF-β), IFN-α and IL-6, and later increases are seen in anti-inflammatory IL-10 [46].

2.5.1 Interleukin-1 (IL-1)

Members of the IL-1 family of pro-inflammatory cytokines have long been associated with the modulation of depression [66]. However, more recently, animal studies have shown that IL-1β [67] increases within the first few hours of cerebral ischemia, particularly focal ischemia [75]. It is observed in the hippocampus and striatum [76], regions previously identified as contributing to the etiology of depressive disorder [77]. IL-1β administration after experimentally induced ischemia increases infarct size and edema formation [78]. The messenger ribonucleic acid (mRNA) expression of the two well recognized IL-1 cell surface receptors, IL-1 receptor type I (IL-1RI) and IL-1 receptor type II (IL-1RII), are also elevated. Increases in IL-1RII are seen from six hours and increases in Interleukin IL-1RI are seen from five days [79], suggesting that ischemic insult results in an acute pro-inflammatory cascade in an attempt to re-establish physiological homeostasis. In clinical populations increased intrathecal production of IL-1β is present two days after stroke in humans [80].

2.5.2 Tumour Necrosis Factor-alpha (TNF-α)

Animal studies reveal that depression related pro-inflammatory cytokine, TNF-α [68] is a polypeptide that increases in response to ischemic conditions [75]. TNF-α mRNA is present within an hour of partial acute middle cerebral artery occlusion (MCAo) [81]. It remains elevated for five days, peaking at six-12 hours. Pre MCAo treatment with TNF-α exacerbates infarct size, while inhibiting TNF-α reduces infarct size [82]. Animal models reveal that TNF-α is elevated in depression associated hippocampus and striatum [76]. Similar results are found in clinical populations [77].

2.5.3 Interleukin-6 (IL-6)

IL-6 has pro and anti-inflammatory properties [83]. Depression associated IL-6 [69] is increased from about three to 24 hours after global ischemia, with a peak at 12 hours in MCAo animal models [84]. It is present in brain regions including the mood modulating hippocampus [77] and the striatum and also in the cortex [76].
2.5.4 Interleukin-10 (IL-10)

Experimental animal research indicates that the early increases in ischemia associated pro-inflammatory cytokines TNF-α and IL-1β later lead to synthesis of anti-inflammatory cytokines such as IL-10. Delayed induction of anti-inflammatory cytokines is interpreted as an attempt by the brain to re-establish homeostasis [47]. IL-10 is neuroprotective in ischemic conditions. It improves neurological outcome [85] and reduces depression associated neuronal cell death [86]. Not surprisingly from a viewpoint of systemic function, where pro-inflammatory cytokine expression is increased acutely, increased expression of IL-10 mRNA [70] is not present in ischemic conditions [87] until six hours post insult [88], with peaks at days two and six [89]. However understanding of the duration of these changes in cytokine production is limited as most experimental studies in animals have only been assessed in the acute phase. Additionally, the contribution of cytokine induced changes in limbic regions and the development of long lasting depressive disorder has not been reported in either humans or other animal species.

2.6 Ischemia and Cell Death

Ischemia induced cytokines play an important regulatory role in programmed (apoptotic) [9] and necrotic cell death [90]. Necrosis is a form of non programmed cell death induced by external factors such as trauma. Permanent focal ischemia and MCAo induce necrotic neuronal cell death in the core of the ischemic area within minutes [9]. In addition, transient ischemia disrupts normal cellular osmotic homeostasis in the penumbra [9], inducing both necrotic and apoptotic (naturally occurring) cell death [11]. The extent of cell death determines infarct size [12]. About 50% of infarct volume can be attributed to induced apoptotic cell death in the penumbra [13].

Apoptotic cell death is a normal, naturally occurring cellular process. Increased cell death in limbic regions responsible for the regulation of mood and emotion is a widely acknowledged characteristic of major depressive disorder. Apoptosis protease activating genes are identified as a predisposing factor for depression [91]. Clinical studies show that patients with depressive symptoms show increased markers for apoptosis in peripheral blood samples [92]. Animals studies reveal that inflammation and apoptosis are spatiotemporally comparable [93] and take place in a similar time frame [9]. Global
ischemia induces rapid cell death in vulnerable brain regions, including the depression related Cornu Ammonis 1 (CA1) region of the hippocampus [94].

Unlike necrotic cell death, apoptotic neurons often remain viable for a period of time so could potentially be rescued. Thus, investigations into the mechanisms that mediate ischemic cell death are being followed to elucidate potential targets for neuroprotective therapies [13]. Much research has focused on changes in glutamate, calcium (Ca$^{2+}$) homeostasis and the synthesis of free radicals [9]. However, not surprisingly, all of these factors play a critical role in normal brain function, meaning such manipulation may result in unexpected or deleterious side effects. Mood mediating cytokines on the other hand are not expressed in the brain under normal conditions, but are an important factor in the pathogenesis of neurotoxic and neurodegenerative disorders such as ischemia, so are potentially more useful therapeutic targets [74]. Pro-inflammatory cytokines induce depression associated cellular apoptosis [48] in mood related limbic brain regions [95], by influencing intrinsic apoptosis factors including intracellular Ca$^{2+}$, glutamate excitotoxicity and free radicals [9]. Thus the influence of inflammatory cytokine expression on the primary factors mediating ischemic and depression related cell death is a critical area of investigation. Figure 2.1 displays changes in cytokine expression and factors contributing to cell death, following ischemic insult.
Figure 2.1. Ischemic Induction of Pro-Inflammatory and Anti-Inflammatory Factors that Mediate Depression Related Apoptotic Cell Death in Limbic Regions.

Red boxes indicate factors that contribute to apoptosis. Blue boxes indicate factors that reduce apoptosis. Red in the left region indicates an early increased expression; red in the right region indicates a late increased expression. Blue in the right region indicates later increased expression. Solid arrows indicate direct induction resulting from ischemic insult. Dashed arrows represent downstream inductions resulting from ischemic insult. Ca$^{2+}$ - Calcium, IL-1 – Interleukin 1, IL-6 – Interleukin 6, IL-10 – Interleukin 10, INF-γ – Interferon-gamma, K$^+$ - Potassium, Na$^+$ - Sodium, NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells, NMDA - N-methyl-D-aspartic acid, NO – Nitric Oxide, iNOS - Inducible Nitric Oxide, TNF-α – Tumour Necrosis Factor-alpha.
2.7 Antidepressants and Cell Death

Antidepressants both reduce inflammatory activity [66] and suppress apoptotic processes [26]. They inhibit the expression of IL-1, IL-6, TNF-α and INF-γ [66]. Animal studies demonstrate that antidepressants inhibit stress induced apoptosis in the mood related hippocampus, temporal cortex and dentate gyrus [26]. In vitro studies suggest this effect is achieved via blocking transition of the mitochondrial permeability pore, which is an indicator of mitochondrial compromise [96].

Apoptosis is often initiated via destruction of the mitochondria and activation of caspase which induce programmed cell death [9]. Caspase activation is mediated by cytochrome c release from the mitochondrial permeability pore [97], which forms in response to increased intracellular Ca^{2+} [98]. Cytochrome c release is mediated by apoptotic Bcl-2 proteins. Therefore, the ratio between the pro apoptotic (Bid, Bax, Bad, Bag) and anti apoptotic Bcl-2 proteins (Bcl-2, Bcl-XL), is considered an index for apoptosis vulnerability [9]. Over expression of the anti-apoptotic Bcl-2 protein, Bcl-XL reduces infarct volume [99], while blockage of pro apoptotic Bad is similarity neuroprotective [100]. Knockout Bcl-2 mice show greater injury in response to focal cerebral ischemia [101].

2.8 Primary Intracellular Mediators of Depression Related Cell Death

2.8.1 Excitotoxicity and Depression Related Apoptosis

Excitotoxicity is the first intrinsic apoptosis factor for consideration. Excitotoxicity is a necrotic form of neuronal cell death induced by unexpected disruptions in energy processes. Lack of oxygen induces neuronal and glial depolarization and the opening of voltage gated Ca^{2+} channels. The opening of these channels induces the release of excitotoxic amino acids, particularly glutamate, into the extracellular compartment. Oxygen deprivation additionally disrupts potassium (K+) and sodium (Na+) gradients [102] responsible for the uptake of glutamate from the extracellular space.

Accumulated extracellular glutamate leads to excessive activation of the glutamate receptors, N-methyl-D-aspartate (NMDA) [103] and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) [104]. As activated NMDA receptors are permeable to Ca^{2+}, glutamate induced cell death is likely mediated by NMDA receptor induced abnormal amounts of intracellular Ca^{2+} influx [105]. The relative resistance of the
AMPA receptor to excitatory stimulation supports this argument. The AMPA receptor is generally impermeable to Ca\(^{2+}\) due to the subunit glutamate receptor 2 (GluR2 subunit). While less than three minutes of continuous NMDA receptor stimulation triggers neuronal death [106], one study using dissociated murine cortical cultures found that more than 60 minutes of AMPA receptor stimulation is required to elicit the same effect [107]. On the other hand, some cortex and striatum neurons that normally express Ca\(^{2+}\) permeable AMPA receptors show cell death after only 10 minutes of such stimulation [108]. Finally, another single study has identified that AMPA receptors that are vulnerable to hypoxic and ischemic insult in the CA1 area of the hippocampus show reduced expression of the GluR2 subunit 24 hours after insult [109].

Hypoxia and ischemia induce excitotoxic cell death in the core of the infarct area [107]. Glutamate released as a result of necrotic cell death in the ischemic core can diffuse into the penumbra also, leading to further depolarisation of neuronal and glial cells. Attempted repolarisation in these already metabolically stressed cells can contribute to further cell death and increase lesion volume [110]. Experimentally, ischemia induced excitotoxic cell death can be inhibited by blocking NMDA receptors in animals [111]. Similar techniques in humans have been less effective, most probably as antagonists were administered too long after the ischemic insult, when efficacy is reduced, or because pharmacological blockage of glutamate may disrupt a number of other brain functions [112].

Recent review reveals that individuals with major depressive disorder also show increased glutamate expression in both brain and peripheral samples and altered NMDA receptor function [113]. The degree of glutamate elevation correlates with depressive symptom severity, and reducing NMDA activity has antidepressant effects in both humans and rodent models [113]. An earlier single study consistently revealed that depressed individuals show greater neurodegenerative intracellular Ca\(^{2+}\) influx in response to glutamate [114] while another shows that conventional antidepressant treatment effectively reduces glutamate expression [115].

Mood modulating inflammatory cytokines play a pivotal role in ischemic excitotoxic cell death [116]. Pro-inflammatory IL-1 and TNF-\(\alpha\) reduce the expression and activity of glutamate transporters [117], therefore disrupting glutamate metabolism and uptake and contributing to further extracellular glutamate accumulation [118]. IL-6 enhances
NMDD receptor function and so increases NMDA intracellular Ca\(^{2+}\) influx [119]. Anti-inflammatory IL-10, which is reduced among depressed individuals [70], protects against glutamate-mediated cell death [86], by inhibiting factors which contribute to cell death, including TNF-\(\alpha\), IL-1\(\beta\) [120] and Caspase-3 synthesis [121]. IL-10 also inhibits deoxyribonucleic acid (DNA) binding of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-k\(\beta\)) [122], a protein that induces cell death [122] in hippocampal regions following global ischemia, as examined at 24 and 72 hours [123]. NF-k\(\beta\) is also shown to contribute to cell death in response to glutamate [124], via the induction of caspase [125]. Individuals with depressive disorder show a higher expression of NF-k\(\beta\), compared to individuals without depression, and the increase in [126] expression correlates with symptom severity in response to psychosocial stress [126]. NF-k\(\beta\) activity is induced by inflammation and inhibited by mood modulating anti-depressant treatments [127]. Such observations support the argument that depression following stroke is likely and should not be unexpected.

2.8.2 Free Radicals and Depression-Related Apoptosis

Free radicals are the second intrinsic apoptosis factor of concern. In addition to inducing necrotic cell degeneration, ischemic induced excitotoxicity results in oxidative stress in the surviving cells [128]. Elevated intracellular Ca\(^{2+}\) induces free radical production [129]. Under normal conditions free radicals are cleared by antioxidants including glutathione and superoxide dismutase [130]. During ischemic conditions, increased cell death and excitotoxicity lead to an overproduction of free radicals and disruption of the anti-oxidative processes responsible for their clearance [131]. This results in increased free radicals, such as nitric oxide (NO), plus its isoforms, neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthesis (iNOS) [89]. These free radicals induce further mitochondria damage [132]. Neurodegenerative NO is also implicated in the etiology of depressive disorder [133]. Interestingly, antidepressants also inhibit NO production resulting from pro-inflammatory cytokines IL-1 and TNF-\(\alpha\), and prevent corresponding mitochondrial damage in human tissue cultures [134].

2.8.3 Neuronal Nitric Oxide Synthase (nNOS) and Depression Related Apoptosis

In vitro studies indicate that the Ca\(^{2+}\) induced nNOS is neurodegenerative as it increases glutamate excitotoxicity [135]. nNOS freely diffuses across neuronal cell membranes and stimulates NMDA receptors resulting in the death of neighbouring neurons not
containing nNOS [136]. Rodent studies reveal that inhibiting nNOS protects against ischemic induced excitotoxicity one hour after common carotid artery thrombosis [137] and reduces infarct size after forebrain ischemia [138]. Knockout nNOS mice show reduced lesion volume in response to ischemia [139]. Inhibiting the production of nNOS in the hippocampus, via administration of an nNOS inhibitor, eliminates the development of depressive behaviours [140].

2.8.4 Inducible Nitric Oxide Synthase (iNOS) and Depression Related Apoptosis

Neurodegenerative iNOS is Ca$^{2+}$ independent and produces more NO than nNOS in rat astrocytes [89]. Histochemistry shows that ischemia induced iNOS in the striatum is present from three hours following MCAo in rat and peaks between 24 and 72 hours [141]. Even with reperfusion following ischemic stroke, expression of iNOS remains elevated for several days in rat glial cells and neutrophils [142].

iNOS is also implicated in the aetiology of major depressive disorder [133]. Inhibiting the production of iNOS in the hippocampal region prevents the development of depressive behaviours in rodent models [133], while anti-depressant treatment reduces iNOS expression [127]. In corneal endothelium, up regulated expression of iNOS is induced by mood modulating cytokines, TNF-α, IL-1, IL-2 and INF γ and is argued to mediate cytokine induced cell death [48]. Appropriately, iNOS induces apoptotic cell death [143]. This can be prevented by conditions of high oxygen and NMDA receptor antagonists [144]. Inhibition of iNOS both delays and eliminates cytokine-induced apoptosis [48] and reduces necrotic cell death [144].

Nitric oxide has also been shown to react with reperfusion-produced superoxide to produce peroxynitrite [145]. This free radical is converted to the hydroxyl radical and is implicated in ischemic and reperfusion toxicity [146]. Exposure to hydrogen peroxide induces depression related cell death in astrocytes and the induction of Caspase-3 [54]. This can be inhibited by caspase [147] and Bcl-2 inhibitors [148]. Thus, suppressing iNOS increase reduces infarct volume [149] and makes it a potential post-stroke therapy.

2.8.5 Metabolic Acidosis and Depression Related Apoptosis

Acidosis is the third apoptosis mediating factor to be considered. Depletion of adenosine triphosphate ATP following an ischemic event, results in a switch from
Mechanisms of Stroke Affective Disorders

aerobic to anaerobic metabolism [150]. This metabolic shift generates lactic acid and hydrogen (H+) [151] and subsequently, hydrochloric and lactic acidosis [152], which further induces a drop in cellular pH. Each form of metabolic acidosis has differing effects on brain injury and mood related [8] cell death [153, 154].

Hydrochloric acidosis has pro-inflammatory properties. It increases NF-κB DNA binding [153] and TNF-α synthesis [155]. It contributes to depression related cell death, iNOS synthesis and NO production [153]. By contrast, lactic acid acidosis is neuroprotective [154]. It is present in ischemic brains from 20 seconds post insult [152] and inhibits various aspects of the immune response, including TNF synthesis and release [156], NF-κB activation [157] and DNA binding [156]. Lactic acid also decreases NO, mood associated IL-6 [69], and increases the ratio of pro-inflammatory IL-6 to anti-inflammatory IL-10, in a dose-dependent manner [157].

2.8.6 Acidosis and Calcium and Depression Related Apoptosis

The differing effects of lactic and hydrochloric acidosis on depression associated immune activity and cell death may be due to their differing effects on extracellular Ca²⁺ concentrations, cell osmoregulation and maintenance of Na/K+ energy exchanges. A decrease in brain pH levels induces a corresponding fall in extracellular Ca²⁺ and a subsequent intracellular Ca²⁺ influx [158]. Lactic acidosis causes a much smaller decrease in Ca²⁺ than hydrochloric acidosis [159]. Further, hydrogen serves as the ligand for the recently identified Ca²⁺ permeable acid sensing ion channel -1 (ASIC1) [160] The ASIC1 channel is highly expressed in the brain and stimulated by conditions of ischemia associated acidosis. Limiting ASIC activity reduces ischemic damage by approximately 60%, even in the presence of a glutamate antagonist [112], suggesting that ASIC1 channel mediated increases in intracellular Ca²⁺ contributes to ischemic and depression associated neurodegeneration.
2.9 Future Direction and Conclusions

Inflammation is an integral outcome of ischemic stroke. The incidence of post-stroke depression is also high in the months following ischemic stroke. Thus given the mediating role of cytokines in ischemic cell death, and the association between apoptosis in limbic regions and depressive disorder, it is highly likely that ischemic inflammation is contributing to the development of post-stroke depression. Recent research has investigated the role of inflammation in conditions of myocardial infarction, another inflammatory pathological condition associated with increased depression [161] and pro-inflammatory TNF-α and IL-1β expression [162]. In the rat model of myocardial infarction, depressive behaviours correspond with increased apoptosis in limbic regions, including the hypothalamus, hippocampus, amygdala and pre-frontal cortex, as indicated by increased Caspase-3 and apoptosis DNA fragmentation [163]. Additionally, anti-inflammatory antidepressant treatment ameliorates both depressive behaviours and apoptosis [163]. Systematic investigation of the association between these candidate inflammatory markers and depression, following human ischemic stroke, may lead to development of clinically useful biomarkers to predict post-stroke depression. It is the recommendation that all post-stroke patients should be screened for clinical depression, and treatment made readily available where required. In conclusion we would argue that the psychoimmunological evidence presented here, from both animal and human research, demands that all stroke patients should be routinely monitored for clinical depression and that normal antidepressant treatment be made readily available where required.
Chapter 3: What You Eat is What You Are - A Role For Polyunsaturated Fatty Acids in Neuroinflammation Induced Depression?

3.1 Abstract
As essential polyunsaturated fatty acids (PUFAs) influence both inflammatory and depressive disorders, nutrition related treatment methods deserve great research interest. However, currently biological mechanisms underlying the depression modulating effects of the PUFA Omega-3 (ω-3) and Omega-6 (ω-6) derived eicosanoids (central nervous system messengers) are not fully established. Depression related naturally occurring cell death (apoptosis) is thought to be mediated by excitotoxicity and free radicals that appear in the brain immediately following any inflammatory or ischemic damage, and increases the likelihood of clinically defined depression. This review explores the hypothesis that the interaction between ω-6 and ω-3 derived eicosanoids plays a central role in control over apoptosis linked with inflammation and inflammation-driven depression, via regulation of apoptosis inducing factors including excitotoxicity and free radicals.

Key Words: Apoptosis; Cytokine; Ischemia; Myocardial Infarction; Polyunsaturated-Fatty-Acid; Depression
3.2 Introduction - Inflammation and Depression

Inflammatory disorders such as myocardial infarction are associated with unusually high levels of depressive disorder [164]. Clinical studies show that 20% of individuals develop major depressive disorder within 18 months of myocardial infarction, while 65% experience depressive episodes [165]. Pooled estimates reveal that at least one third of stroke survivors also experience clinical depression [5, 63] compared to 13% of the control population [5]. To date, the reasons why inflammatory conditions are associated with an increased risk for clinical depression have not been clearly established [5, 164], though a large literature suggests that the neuroinflammation itself is associated with, and possibly induces the gene and immune responses that drive most depressive conditions [164]. Certainly depressed individuals show increased pro-inflammatory cytokine expression [166] while the direct administration of cytokines can induce depression like behaviours in healthy animal models [166] and clinical populations [72]. This has led to a focus of research attention on the role of cytokines, signalling molecules involved in immune regulation in depression [164].

Our current working hypothesis is that sustained inflammation may be driving the development of depressive disorder by mediating cellular apoptosis, the classical form of programmed cell death. Apoptosis is known to be higher among depressed individuals than in normal age matched controls and considered a predisposing factor for clinical depression [48, 91, 92]. Apoptotic cell death is directly induced by lesion driven gene and immune changes including pro-inflammatory cytokines such as Tumour Necrosis Factor-alpha (TNF-α), Interleukin-1 (IL-1), Interleukin-2 (IL-2) and Interferon-gamma (INF-γ) [48].

Omega-3 (ω-3) derived PUFAs have been argued to inhibit inflammatory activity [167] and depression associated apoptosis [52]. The purpose of this review is to explore the biochemical mechanisms underlying the anti-apoptotic effects of immune [168] and depression [169] modulating ω-3 PUFAs, and how these anti-apoptotic effects may contribute to their antidepressant effects. We searched PubMed and Google databases for studies investigating the co-relationships between polyunsaturated fatty acids and depression. There are at least 272 papers dealing with the relation between polyunsaturated fatty acids and depression, and even more (n = 686) addressing inflammation and polyunsaturated fatty acids. Although multiple references exist in
many areas we have restricted our choice to first mention of new data or new interpretation of older data. Further, where references overlap, we have restricted references to the most recent.

### 3.3 Polyunsaturated Fatty Acids (PUFAs)

PUFAs are dietary lipids found in cell membranes, most abundantly in the central nervous system [170]. Structurally, PUFAs are those essential fatty acids containing two or more double bonds, or carbon atoms capable of bonding to two or more hydrogen atoms. The number of carbon atoms determines the length of the fatty acid chain. Short-chain essential fatty acids, containing 18 or less carbons, serve as a substrate for the more biologically active longer chain fatty acids, which contain 20 or more carbon atoms [171]. The most commonly researched fatty acid types are the $n$−3 and $n$−6 groups commonly referred to as $\omega$-3 and Omega 6 ($\omega$-6). The terminology $\omega$-3 or $\omega$-6 refers to the position along the chain at which the first double bond occurs from the terminal methyl CH$_3$ end ($n$), being either the third or sixth carbon-carbon bond, respectively. $\omega$-3 fatty acids are a family of unsaturated fatty acids that contain a final carbon–carbon double bond in the third bond from the methyl end of the fatty acid, Nutritionally important $n$−3 fatty acids include $\alpha$-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), all of which are polyunsaturated [172]. The human body cannot synthesize $\omega$-3 fatty acids de novo, but it can form long chain 20-carbon unsaturated $\omega$-3 fatty acids and 22-carbon unsaturated $\omega$-3 fatty acids from the short chain eighteen-carbon $\omega$-3 fatty acid ALA. [172]

Omega−6 fatty acids are a family of unsaturated fatty acids containing a final carbon–carbon double bond in sixth position from the methyl end of the fatty acid. Competitive interactions with the $\omega$-3 fatty acids affect the relative storage, mobilization, conversion and action of the $\omega$-3 and $\omega$-6 eicosanoid precursors. Functionally, $\omega$-3 and $\omega$-6 fatty acids are incorporated into cell membranes to form the structural components of the lipid bilayer, where they serve as substrates for lipid mediators. $\omega$-3 and $\omega$-6 fatty acids have a wide range of effects on biochemical and physiological functions [171, 172]. Much recent literature indicates that $\omega$-3 fatty acid derived signalling molecules also have potent anti-inflammatory effects [167].

The central nervous system (CNS) signalling messengers derived from 20 carbon essential fatty acids (either $\omega$-3 or $\omega$-6) are referred to as eicosanoids and are known to
play a key role in many important biochemical functions including depression associated inflammatory processes [173]. The networks that depend upon eicosanoids are among the most complex in the human body. The biological effects of the ω-6 fatty acids are largely mediated by their conversion to ω-6 eicosanoids that bind to diverse receptors found in every tissue of the body [172]. The amounts and balance of these fats in an individual’s diet will affect the body's eicosanoid-controlled functions [167].

There are four families of eicosanoids. These are the prostaglandins, leukotrienes, prostacyclins and thromboxanes, each of which is represented by two or three separate series. Of particular interest to the present review are the prostaglandins.

PUFAs cannot be synthesized by vertebrates and must be obtained from diet [174]. Therefore, the cellular concentrations of ω-3 and ω-6, and their relative derived eicosanoids are determined by their relative dietary intake [175]. PUFA ω-6 linoleic acid (LA) (18 carbons with 2 double bonds) is present in foods such as soy, corn, safflower and sunflower oils [172] and as illustrated in Figure 3.1, LA is converted to γ-Linolenic acid (18 carbons with 3 double bonds), and then to dihomo-γ-linolenic acid (20 carbons with 3 double bonds), and then finally to the ω-6 arachidonic acid (AA) which contains 20 carbons with 4 double bonds. The longer chain ω-3 α-linolenic acid (ALA) (18 carbons with 3 double bonds) is found in leafy green vegetables, flaxseed, canola oils and marine fish. ALA is converted into stearidonic acid (18 carbons with 4 double bonds) and then into eicosapentaenoic acid (EPA) (20 carbons with 5 double bonds) and docosahexaenoic acid (DHA) (22 carbons and 6 double bonds).
Omega-3 derived factors produce the longest and most unsaturated fatty acid, docosahexaenoic acid (DHA).
3.4 ω-3 and ω-6 Derived Prostaglandins

Competitive interaction exists between ω-6 AA and ω-3 derived EPA for their shared enzymatic pathways, with the eicosanoids released by ω-3 being anti-inflammatory comparative to those released by ω-6 [174]. Therefore, the relative amount of ω-6 and ω-3 derived prostaglandins released in response to depression associated inflammatory stimuli that initiate inflammatory driven and depression related cell death, is determined by the fatty acid composition of the cell membrane phospholipids [174]. Accordingly, increasing ω-3 in cell membranes reduces the release of ω-6 AA derived prostaglandins and thus inflammatory activity [176]. Prostaglandins are synthesized via the COX pathway and accordingly initiation of this pathway is necessary for a normal immune response [177].

3.4.1 ω-6 Derived Prostaglandins

Inflammatory prostaglandin E series (PGE) appears to have a direct role in depression associated inflammatory apoptotic processes. Inflammatory prostaglandin series E² (PGE²) results in inflammatory related sickness behaviours and is argued to be the main central mediator for cytokine induced fever, hypothalamic–pituitary–adrenal axis (HPA) activation [178] of adrenocorticotropic hormone (ACTH) and corticosterone [179]. Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria, which is often used to induce an acute immune response [180]. Both IL-1 and LPS increase PGE² receptor subtype 4 (EP₄) expression [181] within corticotrophin-releasing hormone (CRH) neurons of the paraventricular nucleus (PVN) of the hypothalamus [182], a brain region involved in the aetiology of depressive disorder [183]. Converse to the ω-3 derived prostaglandin series E³ (PGE³), ω-6 derived PGE² is synthesized by the inflammatory and depression associated AA [167]. At doses above 40µg ω-6 related AA [184] and gamma-linolenic acid (GLA) contribute to cell death [185]. In cell cultures, sustained induction [186] of PGE² induces apoptosis in resting human T cells [187]. Low doses of PGE² induce TNF-α production [188]. Inflammatory interleukin-1beta (IL-1β) induces PGE² [167] while TNF-α induces AA [189].

In rat, six weeks of supplementation with 0.5% of AA similarly increases corticosterone release and the basal inflammatory response [190]. Both inflammatory AA and PGE² directly induce an acute inflammatory response and depression related sickness.
behaviours including anorexia, disrupted sleep, reduced activity and attention deficits in rats [190]. Intracerebroventricular injection of agonists of the PGE\(^2\) receptor subtype 3 (EP\(^3\)) [181] induce fever and cortisol release in pigs [191]. In rats, microinjection of PGE\(^2\) receptor agonists result in hyperalgesia, which is increased sensitivity to pain [192].

Modified EP\(^3\) knockout mice fail to show an immune response when subjected to IL-1\(\beta\) or PGE\(^2\) [193]. In rats, inhibiting PGE\(^2\) abolishes the anorexic induced effects of IL-1 [194]. Corticosterone secretion [179], PGE\(^2\) mediated fever [195] and IL-1 are suppressed by COX inhibitors [196], as the COX pathway is necessary for a normal immune response [180]. This effect is dose dependent and appears in the hypothalamus and medulla [196], brain regions implicated in the aetiology of clinical depression [183]. On the other hand disrupting the activity of IL-1 similarly inhibits IL-1\(\beta\) induced synthesis of PGE [197] and fever mediated by cyclooxygenase-2 (COX-2), a COX isozyme [198]. Appropriately IL-1\(\beta\) knockout mice do not show COX-2 induction [177].

In response to conditions of inflammation, associated with depression such as hypoxia, the expression of COX-2 is rapidly elevated [199]. Specifically, TNF-\(\alpha\) initiates the transcriptional gene encoding COX-2 [200] while IL-1\(\beta\) increases COX-2 mRNA by ten-fold and COX-2 transcription by eight-fold [201]. Increased PGE\(^2\) [202], inflammatory AA [203] and phospholipase A2 (PLA2), an enzyme that releases AA [70], are similarly elevated in individuals with depressive disorder [204]. The olfactory bullectomized rat model of depression shows increased PGE\(^2\) in serum and brain, which is normalized by six weeks of 1\% EPA supplementation [205]. \textit{In vitro}, apoptosis limiting [26] antidepressants (lithium, valproate and carbamazepine) comparably decrease basal expression of PGE\(^2\), AA turnover, COX [206] and caspase expression [207]. Anti-depressants, Fluoxetine (0.3 microg/ml, 1 microg/ml, and 3 microg/ml) and amitriptyline (1 microg/ml and 3 microg/ml), similarly result in a time and dose dependent reduction in PGE\(^2\) induced by the presence of depression associated [67] [68] interleukin 1 \textit{alpha} (IL-1\(\alpha\)) and TNF-\(\alpha\) [134] suggesting that these factors are involved in the aetiology of depressive disorder and have potential in relation to its therapeutic treatment.
3.4.2 \(\omega-3\) Derived Prostaglandins

In a small study of nine healthy volunteers, \(\omega-3\) administration for six weeks was shown to inhibit blood mononuclear cell proliferation by 70% [208] and suppress synthesis of depression associated pro-inflammatory cytokines, such as TNF-\(\alpha\) and IL-1, with levels returning to baseline after cessation of supplementation [209]. Four weeks of dietary \(\omega-3\) rich flaxseed oil similarly suppresses depression associated inflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) by 30% [210], while dietary supplementation of \(\omega-9\) rich olive oil (largely oleic acid) inhibits phagocyte activity and IL-1 production in mice [211]. Physiologically, four months supplementation with 6 grams a day of EPA and DHA inhibits the expression of the molecule that constitutes the IL-2 receptor, CD25, in clinical populations [212]. Dietary supplementation for six and half months with 10% fish oil, which is rich in \(\omega-3\) inhibits messenger ribonucleic acid (mRNA) production of inflammatory cytokines, IL-1, Interleukin-6 (IL-6) and TNF-\(\alpha\) in mice [213]. Recent review highlights that conventional antidepressant treatments similarly inhibit the expression of IL-1, IL-6, TNF-\(\alpha\) and INF-\(\gamma\) [164]. In healthy males, decreasing \(\omega-3\) intake augments natural killer (NK) cell activity. NK cells are cytotoxic lymphocytes, signalled by pro-inflammatory cytokines and involved in the innate immune response [214]. Low EPA is associated with increased interferon-alpha (INF-\(\alpha\)) induced sickness behaviour in chronic hepatic patients [174]. In a sample of 27 university students, low serum levels of \(\omega-3\) and a higher \(\omega-6:3\) ratio corresponds with greater inflammatory TNF-\(\alpha\) and INF-\(\alpha\) production in response to psychological stress [215]. Finally, dietary supplementation with 0.5% of \(\omega-3\) increases the secretion of the anti-inflammatory cytokine Interleukin-10 (IL-10) in rats [190] and inhibits the IL-1\(\beta\) driven suppression of IL-10 [216]. The anti-inflammatory IL-10 has similarly been identified as influencing depressive symptomatology, as increasing IL-10 expression appears to contribute to the efficiency of conventional antidepressant treatments [217]. Similar to \(\omega-3\), administration with conventional anti-depressants, amitriptyline and desipramine (10 mg/kg) increase IL-10 secretion in mice [25]. Clomipramine and sertraline also increase IL-10 secretion in response to inflammatory LPS, in human blood culture samples [217]. Interestingly, recent review reveals that a decreased ratio of anti-inflammatory \(\omega-3\) derived eicosanoids compared to \(\omega-6\) derived eicosanoids increases the risk of inflammatory conditions [218]. Neuroprotectin D1 (ND1) is a docosanoid derived from DHA. In an inflammatory mouse stroke model ND1 inhibits IL-1\(\beta\) driven COX-2,
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which is an isozyme of COX and involved in inflammatory responses [219]. In inflammatory conditions such as stroke, ND1 has a neuroprotective influence by inhibiting IL-1β driven nuclear factor kappa-β (NF-κβ), which induces depression related cell death in rodent models [219].

Omega-3 likely mediates these anti-inflammatory effects via its production of PGE³, which inhibits ω-6 derived PGE² and has anti-inflammatory properties. In vitro, PGE³ potently inhibits inflammatory T cell mitogen-induced lymphocyte proliferation [220] while supplementation with six grams a day of dietary DHA, for a period of 90 days, lowers ω-6 derived inflammatory PGE² production by 60-75%, in response to LPS in healthy men [221]. Fish oil inhibits inflammatory PGE² induced by LPS in cell cultures [222] while dietary supplementation with 0.5% EPA inhibits PGE² and corticosterone production in rats [190]. Six weeks supplementation with daily doses of 3.2 g of eicosapentaenoic acid and 2.2 g of docosahexaenoic acid reduces AA by 37% in neutrophils and 39% in monocytes of healthy volunteers [223]. Thus ω-3 supplementation results in a reduction in inflammatory related eicosanoids.

Accordingly, individuals diagnosed with inflammatory related depression show a higher ratio of ω-6 relative to ω-3 [224] and individuals diagnosed with major depression, have a higher ω-6: ω-3 ratio and lower overall ω-3 than those diagnosed with minor depression [225]. As a percentage of total fatty acid, ω-3 derived DHA, as well as the ratio of ω-6 compared to anti-inflammatory ω-3 are reliable predictors of suicide attempts [226]. In a sample of 15 depressed patients, the fatty acid composition of phospholipid in cell membranes from red blood cells show an overall reduction in ω-3 among depressed patients [227]. These findings are confirmed by post mortem studies which showed reduced DHA in the orbitofrontal cortex of 15 depressed individuals [228]. Deficiency in ω-3 derived DHA is associated with a dysfunction in the transmission of serotonin, norepinephrine and dopamine. Improving ω-3 consumption increases 5-hydroxy-indoleacetic acid, a metabolite and indicator of serotonin turnover [229]. Six weeks of dietary supplementation with 0.5% of anti-inflammatory ω-3 derived EPA inhibits stress and anxiety behaviours in animal models [190]. Eight weeks of supplementation with 1% EPA inhibits IL-1β related memory impairments, corticosterone release [70]. Six weeks of supplementation with 17% menhaden oil, rich
in ω-3 fatty acids, similarly inhibits LPS induced lethargy, anorexia, fever and loss in body weight [222].

Decreased consumption of anti-inflammatory ω-3 ALA by most first world countries is argued to contribute to rising rates of inflammatory associated clinical depression in Western cultures [230]. Cross national studies reveal that national consumption levels of fish, high in anti-inflammatory ω-3 derived DHA and EPA, negatively correlates with the prevalence of mood disorders [169]. Consumption of anti-inflammatory ω-3 is associated with greater grey matter volume of brain regions involved in the regulation of mood [8, 231]. This includes the amygdala, hippocampus and anterior cingulate cortex, [231]. These limbic brain regions are critically involved in the aetiology of depressive disorder [8]. As ω-3 consumption is associated with increased grey matter volume in the circuitry supporting emotional regulation it potentially contributes to the biological aetiology of clinical depression and may aid therapeutic and psychosocial treatments. Like conventional antidepressants, daily capsules of EPA and DHA, for 6 months modulates depression associated inflammatory activity [232] and can be effective in the management of ischemia associated clinical depression [174, 233]. Increased intake of ω-3 PUFAs as a therapy for depression also has the advantage of not inducing the unpleasant associated side of conventional antidepressant treatment such as sleep difficulties and nausea [234]. See Figure 3.2 below depicting the comparable action of antidepressants and PUFA on depression related cytokines and apoptotic activity.
Figure 3.2. Comparable Anti-inflammatory Profiles after Antidepressant and Omega 3 Exposure.

IL-10 – Interleukin 10; eNOS – endothelial nitric oxide synthase; IL-6 – Interleukin 6; IL-1 – Interleukin 1, TNF-α – Tumour Necrosis Factor-alpha; NO – Nitric Oxide; iNOS – Inducible Nitric Oxide Synthase; NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells, PGE2 – Prostaglandin E2.
3.5 PUFAs and Apoptosis

PUFAs are widely acknowledged to influence cell death and are neuroprotective at optimal doses [52]. In cultured cells AA and LA inhibit apoptosis at doses of 10µg [52], while EPA inhibits cell death at doses of 10µg to 40µg [52, 184, 235] DHA reduces apoptosis at doses of 5, 10, 20, 25 and 50µg after 24 hours of incubation [52, 235-237] and at doses of 25µg and 0.5 µM after 48 hours [207, 238]. Human studies also confirm that 2 weeks of DHA (400, 800 mg) supplementation reduces apoptosis in monocytes of healthy individuals [239]. One factor identified to contribute to DHA inhibition of apoptosis is its production of NPD1 [240]. In vitro, NF-κB protein (nuclear factor kappa-light-chain-enhancer of activated B cells) directly induces cellular apoptosis [124], oxidative stress induced cell death, leukocyte infiltration and total infarct volume [240]. Tricyclic antidepressants (10-15 µM) decrease LPS induced NF-κB activity in cell cultures [127]. In individuals with depressive disorder, a higher expression of NF-κB is seen [126]. The degree of expression of NF-κB also corresponds with symptom severity in response to psychosocial stress [126].

Omega-3 derived PUFAs are also likely to protect against inflammation driven depressive disorder via regulation of Caspase-3 activity [238, 241]. Caspase activation, which directly induces apoptotic cell death, [242] is determined by the level of anti apoptotic Bcl-2 proteins. Indeed, apoptosis vulnerability is indicated by the ratio of pro (Bid, Bax, Bad, Bag) to anti apoptotic (Bcl-2, Bcl-XL) Bcl-2 proteins [243]. DHA and NPD1 decrease the pro-apoptotic enzyme Caspase-3, and Bcl-2 pro-apoptotic proteins, Bax, Bad and Bik. In vitro, DHA and NPD1 increase the anti-apoptotic Bcl-2 proteins, Bcl-xl, Bcl-2 and Bfl-1(A1) in depression related hippocampal cells [241]. DHA further suppresses amyloid-beta 42 (Aβ42) peptide release, which is shown to induce the pro apoptotic Bik and Bax [241]. In rodent models, two weeks of DHA administration (0.4 g/kg/day, by gavage) prior to inflammatory stroke induction decreases the appearance of apoptotic neurons in hippocampus brain tissue [244].

In depression related inflammatory conditions, pro-inflammatory cytokines appear to contribute to depression associated cell death, at least in part, by influencing intrinsic apoptotic pathways [9]. As PUFAs influence cytokine activity, it is reasonable to speculate that they also influence these intrinsic apoptotic pathways. Figure 3.3 illustrates the proposed pathway by which ω-3 PUFAs may protect against
inflammatory ischemic induced cell death, by decreasing many of the pro-apoptotic factors that contribute to depression related neurodegeneration.

Figure 3.3. Mirror Image Profile of Ischemic and Omega 3 Induced Modulation of Pro-Inflammatory and Anti-inflammatory Factors That Mediate Depression Related Apoptotic Cell Death in Limbic Regions.

Solid arrows indicate direct induction resulting either after ischemic insult or ω-3 exposure. Dashed arrows represent downstream inductions resulting after ischemic insult and ω-3 exposure. NO – Nitric Oxide; iNOS - Inducible Nitric Oxide Synthase; Ca²⁺ - Calcium; NMDA - N-methyl-D-aspartic acid; IL-6 – Interleukin 6; K⁺ - Potassium, Na⁺ - Sodium; TNF-α – Tumour Necrosis Factor-alpha; IL-1 – Interleukin 1; INF-γ – Interferon-gamma; NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells; IL-10 – Interleukin 10.
3.6 PUFAs and Intracellular Apoptosis Pathways

3.6.1 PUFAs and Excitotoxicity

Inflammatory conditions are coupled with a high occurrence of excitotoxic cell death [9, 144]. Excitotoxicity is a glutamate driven necrotic form of neuronal cell death [245] associated with excessive intracellular calcium (Ca$^{2+}$) influx [246] and the induction of downstream apoptosis [110]. In vitro cell culture studies reveal excitotoxicity associated intracellular Ca$^{2+}$ influx, which results in the release of inflammatory ω-6 AA [247, 248]. Arachidonic acid (AA) acts to potentiate Ca$^{2+}$ sensitive N-methyl-D-aspartic acid (NMDA) receptors. NMDA receptors are permeable to Ca$^{2+}$ and thus AA amplifies glutamate driven and depression associated neurodegenerative intracellular Ca$^{2+}$ influx [249].

Anti-inflammatory ω-3 derived EPA and DHA block sodium (Na$^{+}$) channels in a dose and time dependent manner in neonatal rat ventricular myocytes [250, 251]. This in turn limits the activity of the Na$^{+}$/Ca$^{2+}$ exchanger, which regulates intracellular Ca$^{2+}$ influx. Indeed, in vivo cell culture studies demonstrate that both EPA and DHA increase cellular inactivation in the CA1 region of the hippocampus thus reducing Ca$^{2+}$ influx associated cellular activity [252, 253]. Thus PUFAs are likely to reduce intracellular Ca$^{2+}$ influx associated excitotoxicity [254, 255].

Conversely, anti-inflammatory and anti-depressive ω-3 DHA reduces neural excitotoxicity associated with stimulation of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) glutamate channel in vitro. This has previously been demonstrated in brain tissue taken from the rat Cornu Ammonis area 1 (CA1) region of the hippocampus. The CA1 region has also been identified to contribute to the aetiology of depressive disorder, presumably ω-3 DHA acts here to inhibit excitotoxic cell death [256]. Indeed, in rat hippocampus ω-3 acts to decrease the release of the neurotransmitter acetylcholine that contributes to cellular excitability and thus excitotoxic cell death [257]. Furthermore a depletion of ω-3 DHA in hippocampus amplifies acetylcholine release, and thus likely contributes to excitotoxic cell death [258]. Meanwhile, nerve growth factor (NGF), a factor which contributes to cell survival, is decreased in the hippocampus region of DHA deficient female rats [259] and is similarly identified to be reduced in the hippocampal region of olfactory...
bulbectomized rats, a rodent model of human clinical depression. Again NGF deficiency can be reversed by EPA administration [205].

3.6.2 PUFAs and Oxidants

In addition to regulating excitotoxic cell death, PUFAs play a mediating role in the production of oxidants and anti-oxidants. Neurotoxic free radicals are produced in response to excitotoxicity related elevated intracellular Ca\(^{2+}\) [260] and are a second apoptosis mediating factor associated with depressive disorder [134]. Free radicals such as nitric oxide (NO), hydrogen peroxide, hydroxyl and superoxide all contribute to neuronal death anywhere in the brain at any time and particularly in case of ischemic neuronal death in its association with depression associated neuronal cell death [145]. Accordingly, neurodegenerative NO is implicated in the aetiology of depressive disorder [133] and cell culture studies reveal that conventional antidepressants, Fluoxetine and Amitriptyline inhibit NO release by as much as 70% in response to IL-1 and TNF-\(\alpha\) [134].

In addition to increasing oxidants that contribute to depression associated apoptosis, depression related inflammatory conditions, such as ischemia, can disrupt the anti-oxidative processes responsible for the clearance of oxidants [131]. This disruption further contributes to neuronal death [145]. Depression and anti-inflammatory related EPA and DHA increase the antioxidants, superoxide dismutase, glutathione peroxides [189] and vitamin E, which reduces tumour necrosis factor (TNF) induced apoptosis [235]. Additionally, anti-inflammatory and antidepressant DHA and EPA reduce neurodegenerative superoxide production in human polymorphonuclear leukocytes, presumably via a prostaglandin-dependent pathway, given that a cyclooxygenase (COX) inhibitor reduced \textit{in vitro} effects in leukocytes [261]. Indeed DHA (0.4 g/kg/day, by gavage) administration has been shown to reduce the expression of NO in the hippocampus in rat in response to inflammatory insult [244]. In addition, two weeks of DHA supplementation (0.4 g/kg/day, by gavage) increases hippocampus expression of the anti-oxidant superoxide dismutase, which is responsible for the clearance of neurodegenerative oxidants such as superoxide and thus is considered neuroprotective [244]. These findings strongly suggest that \(\omega-3\) is capable of altering the ratio of oxidants to antioxidants [189] which mediate depression associated apoptosis,
consistent with the effect seen in response to neuroprotective antidepressants, amitriptyline or fluoxetine (50/100µmol/L) in cultured rat pheochromocytoma [262].

3.6.3 PUFAs and Endothelium Nitric Oxide (eNOS)

Ischemic induced [263] endothelium nitric oxide (eNOS) is the only NO synthase enzyme associated with a reduction in inflammatory and depression related neurodegeneration [142]. Clinical studies reveal that EPA and DHA increase eNOS expression [189]. This effect is similarly present after 8 weeks of paroxetine supplementation, which is a conventional antidepressant, in healthy volunteers [264]. In cultured human endothelial cells, ω-3 derived EPA (0.3 mM) rapidly elevates eNOS as well as the ratio of anti-inflammatory EPA to inflammatory AA [265]. These eNOS elevations are dose dependent [266], present from as early as three minutes after EPA exposure, and remain heightened until at least 30 minutes [265]. By increasing neuroprotective eNOS ω-3 likely protects inhibiting depression related apoptosis.

3.6.4 PUFAs and Inducible Nitric Oxide Synthase (iNOS)

Inducible NO synthase (iNOS) is a NO enzyme stimulated by pro-inflammatory and depression associated cytokines, such as TNF-α, IL-1, IL-2 and INF γ [48]. In rat, iNOS is present within a few hours of ischemic insult [267]. In cerebral endothelial cells excised cornea and in transformed and primary corneal endothelial cells it contributes to cytokine induced apoptotic cell death [48, 143]. In ischemic rodent brains, 0.4 g/kg/day gavage feeding of anti-inflammatory ω-3 derived DHA and EPA decrease iNOS and inhibit depression associated hippocampal neuronal death [244]. In macrophage cells, ω-3 derived DHA, EPA and alpha-linolenic acids (ALA) all modulate iNOS expression in a dose dependent fashion [268]. In vitro cell cultures, EPA and DHA decrease iNOS expression when incubated in human dendritic cells for 18 hours at a dose 0.1 µmol/L, [269]. The ω-3 derived DHA and EPA inhibit iNOS mRNA and NF-κβ binding, which as mentioned previously is a protein which induces cell death [124], at doses between 10µg and 20µg in human monocytes [270]. Both DHA and EPA also reduce human monocyte [271] and leukocyte production of the oxidant superoxide [261]. Six weeks of ω-3 rich cod liver oil supplementation decreases superoxide by 64%, which is accompanied by a greater expression of anti-inflammatory EPA and a reduced expression of inflammatory AA in human polymorphonuclear leukocytes samples taken from healthy volunteers, [272]. Conversely, ω-6 derived inflammatory AA increases
iNOS in osteoblastic MC3T3-E1 cells [273]. Conventional anti-depressants such as clomipramine (15µM) and imipramine (10µM) similarly reduce iNOS expression in microglia and astrocyte cultures [127].

It is important to note however that optimal cellular function is dependent on the ratio of ω-3: ω-6 [171, 172] and that ω-3 PUFAs can also be neurodegenerative when in excess, possibly due to oxidation [238]. At doses above 40µg, AA [184, 185, 274] and GLA contribute to cell death [185, 275, 276]. At doses of 120 and 240µg similar results are found using EPA [277]. Greater than 20µg of DHA [277-280] and extended incubation periods of 72 hours increase cell death, [52] while 30 µM of DHA can increase IL-1β induced iNOS expression after 24 hours of incubation, in rat cell cultures [281]. These results are consistent with findings that DHA can increase apoptosis in tumour cells [282] and neonatal monocytes [280]. Thus, low doses of DHA (5 to 10µg) are considered to be most neuroprotective [52].

3.7 Future Directions and Conclusions

Given the regulatory role of cytokines in mediating intrinsic apoptotic pathways and the association between apoptosis and depression, [91, 92] it is not surprising that inflammatory conditions are associated with unusually high levels of clinical depression [5]. Omega-3 PUFAs that have immune modulating properties are also capable of inhibiting cytokine activity suggesting that increasing the consumption of ω-3 comparative to ω-6 shifts the production of eicosanoids away from those with potent inflammatory properties, and toward those with reduced inflammatory properties [167]. This shift has been demonstrated here to influence the intrinsic immune/cell metabolism pathways mediating apoptosis. Thus, it is reasonable to speculate that PUFAs are theoretically capable of reducing cardiovascularly induced neuroinflammation driven neurodegeneration and related depressive disorders. The development of potential treatment methods requires systematic investigation.
Chapter 4 : Methodology

The specific methodological details for each published empirical study are presented in the chapter relating to that study. The present chapter provides a general explanation of the theoretical bases for the decisions behind relevant methodologies, pertaining to the animal based research, and that were unable to be addressed in the relevant empirical chapters. This chapter begins with an outline of the rationale behind the species selection, diet selection, and period and method of dietary supplementation. It follows with a discussion regarding the selection of particular tests employed in the animal stroke model, to study affective and locomotor behaviours.

4.1 Species

Rodents are commonly employed in investigative research because they are classified as lower level sentient beings [283]. While mice are less expensive than rats, they have been shown to have species specific immune-modular responses to polyunsaturated fatty acids, which differ to those seen in humans, making them an inappropriate model for the present research [168]. In both rats and humans [284] female sex hormones, progesterone and estrogen, appear to be neuroprotective against ischemia [285-287], and the particular mechanisms underlying the gender specific differences are not yet well elucidated [288]. Thus to avoid gender related confounds, the research presented in this thesis only used male rats.

4.2 Middle Cerebral Artery Occlusion (MCAo)

The middle cerebral artery occlusion (MCAo) model of ischemic stroke induces an infarct in the striatum, followed by a more delayed cortical infarction or penumbra, involving opening of the blood brain barrier and apoptotic cell death [289, 290]. The pattern of cell death induced by the MCAo model closely models the pattern of cell death resulting from middle cerebral artery ischemia in clinical populations, and thus is often utilized [291]. The delayed progressive cortical cell death observed in the MCAo model provides an ischemic penumbra, which has formed the focus of a number of studies into neuroprotective targets [289]. This model has been shown to be reproducible and the acute neurologic and neuropathological outcomes have been well defined [292].
The MCAo stroke model has additionally been associated with behavioural changes, which the authors have interpreted to reflect depressive-like behaviours (specifically reduced sucrose consumption, argued to indicate desensitisation of the brain reward mechanism, a behaviour argued to reflect anhedonia, which is a symptom of clinical depression), for up to six weeks post-surgery in rodents. Indeed, in these animals the administration of a conventional antidepressant, citalopram, has been observed to increased sucrose consumption in MCAo operated animals, compared to MCAo operated animals not receiving citalopram [293, 294].

4.3 Weight Gain, Food and Water Consumption

In the first five days following surgery, animal weight gain, eating and drinking behaviour in MCAo and sham operated rats was monitored daily across diet conditions, as a measure of animal recovery and sickness behaviour (often defined by anorexia, weight loss, reduced activity in rodents [295]). In the weeks 2-6 post-surgery, and after any acute sickness behaviours were expected to dissipate (previous experience with MCAo stroke modelling in our lab indicates that by two weeks post-surgery, surviving animals consume an amount of food and water expected by a healthy rat, have regained lost body weight and are mobile and active, which is interpreted to indicate a dissipation of the earlier surgery related sickness behaviours) animal weight gain, eating and drinking behaviour were monitored as a more general measure of animal well-being. Blunted weight gain corresponds with increased frequency of grooming behaviour, which is interpreted to reflect anxious like behaviours in the rat [296]. Blunted weight gain is seen in animals who experience the chronic mild stress (CMS) model, which is involves exposing an animal to a series of mild stressors over a number of days or weeks, including flashing lights, restraint or social isolation [296-300]. CMS induced blunted weight gain is associated with an increased expression of neurodegenerative oxidants and a decreased expression of neuroprotective anti-oxidants, in hippocampus region, in the rat [297] and corresponds with a number of behavioural changes that have been argued to reflect anxious/depressive-like symptomatology [296-300].
4.4 Acute Stroke-Related Motor Impairment Measures

Post-surgery behavioural tests of motor impairment are widely used in stroke modelling research, as they provide a means by which confirm the presence of acute physical disability, expected to result from a successful MCAo surgery (i.e. the MCAo surgery has resulted in cortical infarct) [301, 302]. Animals with larger cortical infarcts are expected to show greater impairment. MCAo effected animals are expected to hold the left forelimb between 45 and 90° to the right when lifted from the base of the tail [301], show a curling of the head and forelimbs toward the left paralytic side of the body, when held via their tail above a table [301], show weakened resistance when held behind the shoulders and pushed towards the paralytic left side, circling toward the opposite side of the infarct damage (anti-clockwise direction) [301], decreased general motor ability, consisting walking, grooming, and rearing behaviours. In the present research, we conducted basic motor neurological testing as outlined above, in the first five days, or acute recovery phase post-surgery. This provided an indication of consistency between our neurological outcomes and those observed in previous research. We aimed to confirm that the MCAo and Sham operated animals differed in these outcomes, and to investigate if dietary supplementation influenced acute stroke-induced motor impairments, that are observed after stroke induction, in the rat model. Additionally, these basic motor neurological testing outcomes provided an indication of animal recovery from stroke.

4.5 Behavioural Assessment Longer Term Ischemia Associated Behavioural and Affective Disorders

In the present research, we studied a number of behavioural outcomes interpreted to reflect animal responses to fear or anxiety, and locomotor hyperactivity, within the restraints introduced by stroke modelling. For example, the expected stroke associated acute motor disabilities and acute sickness behaviours (such as anorexia, disrupted sleep, reduced activity, and attention deficits in rats [295]), are likely to mask behaviours believed to reflect depression-like symptoms in animal models. Many of the behaviours studied in the present research (and described below) are commonly used to assess anxiety-like, depressive and hyperactive locomotion behaviours. Previous research indicates that these behaviours are also influenced by factors that induce an acute immune response, such as Lipopolysaccharide (LPS) (the major component of the
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outer membrane of Gram-negative bacteria) [303], and can therefore also be considered indicators of sickness behaviour, which is very closely linked with depressive behaviours [295]. It was important in the present research to ensure that the behaviours observed reflected longer-term depressive, anxiety-like and hyperactive locomotor behaviours, rather than acute surgery induced sickness behaviours. Therefore, these behaviours were not studied until two weeks post-stroke, by which time the animal is not expected to display any obvious sickness behaviours or motor disabilities. Indeed, previous research indicating that rats regain mobility by 10 days post-stroke [304-306].

Previous authors have questioned the validity of using rodents, as ‘models’ of human affective and behavioural disorders, such as anxiety and depression, given that these pathologies are complex, highly variable and largely subjective [307, 308]. While it is largely accepted that a rat does not think like a human, it is argued that the brain mechanisms and behavioural responses of anxious states, such as fear, are so essential for survival, that by necessity, these would have evolved very early in mammals and are highly conserved [308]. As animals appear to be incapable of suicidal thoughts, self-damaging unhelpful thought processes and speech, only their behavioural and physiological responses can be observed [309]. Previous authors have argued that for the purpose of translational research, animal models of human clinical pathologies should be based on identifying critical components of behaviour, that represent a more complex phenomena. In the words of Gottesman, “animal models based on endophenotypes that represent evolutionarily selected and quantifiable traits, are believed to better lend themselves to investigation of psychiatric phenomena, than models based on face-valid diagnostic phenotypes [309]”. In the case of anxiety/depressive-like symptoms, patterns of physiological and behavioural responses to fear, stress or anxiety, which are linked to relevant neural circuits and hormone systems, have been argued to be the most appropriate [309, 310]. Changes in brain plasticity and neurogenesis are also commonly studied in animal models of anxious and depressive-like behaviours [24]. Thus in the present study, both animal behaviours and changes in brain plasticity were studied.

In the animal model, behaviours believed to reflect depressive and anxious like states have traditionally been studied using a number of well recognized ‘despair behaviour tests’ [311]. These include the forced swim and tail suspension test. Previous authors
have argued that increased periods of rodent immobility in these tests reflect clinically relevant indicators of depressive symptoms. Indeed, a wide range of clinically active antidepressant drugs have been shown to decrease the periods of immobility in the forced swim and tail suspension test [311]. However, these measures are inherently stressful to the animal and so may confound the immunological changes expected to result from the MCAo procedure. The present research was interested in ischemia associated inflammatory response per se, and how this related to cellular degeneration, proliferation, and post-stroke affective and behavioural outcomes, and not behaviour testing induced stress. It was therefore deemed important not to introduce any additional confound, such as additional anxiety through the process of behavioural testing. Thus, the present research employed a number of behavioural tests, designed to be as minimally stressful as possible. These are the novel object exploration test, the free exploration test and the spatial displacement recognition test, which are described below. The interpretation of the behaviours observed in these tests is also described below.

Previous authors have observed behaviours argued to reflect anxiety-like and hyperactive locomotion at various points post-stroke, in animal models. For example, two weeks after myocardial infarction, rats show an increased duration of immobility in the forced swim, compared with rats exposed to myocardial infarction and then treated with a conventional anti-depressant, sertraline. The authors interpreted the increased duration of immobility to reflect depressive-like behaviour [312]. Others authors have demonstrated a reduction in sucrose consumption, in rats at six weeks post MCAo, compared to rats exposed to MCAo and treated with a conventional antidepressant, citalopram. The authors interpreted reduced sucrose consumption to reflect a desensitisation of the brain reward mechanism, a behaviour argued to reflect anhedonia, which is a symptom of clinical depression [293]. In order to maximize the detection of ischemia associated behavioural changes, behavioural testing to detect anxious/depressive-like and hyperactive loctomotion, in this project, commenced in the second week post-surgery, and reoccurred at bi-weekly intervals, until the sixth week.
4.5.1 Free Exploration Test

Rodents display locomotive hyperactivity after stroke induction [27, 313-315], which may be considered to be homologous with the hyperactive delirium common among human stroke survivors, and associated with poorer prognostic outcome [6]. Locomotive hyperactivity is also seen in many animal models of clinical depression [316-320].

Rate of locomotion activity in animals is commonly studied using an open field test, in which the total area explored by the animal, within a specific area and finite period of time, is recorded [28]. In rodent models, the MCAo surgery has been observed to be associated with hyperactive locomotor behaviours, and this hyperactive locomotion has commonly been studied using the open-field test [27, 28, 315]. Anxiety-like behaviours are also often studied using this test, by measuring how long the animal spends in the centre, which has been argued to be the anxiety provoking region of the arena, compared to the side walls, which have been argued to be less anxiety provoking [321]. This traditional open-field test requires experimenter handling and forces exploration of the open field, the size and illumination of which induces anxiety in nocturnal rodents, who prefer small dark environments. Accordingly, the open field test is often employed as a measure of an animal’s ability to respond to a stressful event [321].

The free exploration test a derivative of the open field test, designed to eliminate the forced exploration associated with the open-field test [322, 323]. In the free exploration test, the animal is placed inside a familiar hide box and can choose to emerge into the open field or not. If the animal does emerge from the hide box, then its movement around the open field can be studied, including its propensity to spend time in different areas of the open field, such as the anxiety provoking centre of the arena, or against the side walls [321].

In order to avoid experimenter handling and forced exploration of open field, the present project employed the free exploration test, rather than the more commonly used open field test, to study changes in locomotive behaviours and anxiety-like behaviours post MCAo. Animals were scored for the number of times they fully emerged from the hide box and the total time they spent fully emerged from hide box and moving around the arena. This was defined as locomotor hyperactivity. While the above mentioned behaviours are traditionally interpreted to reflect anxiety-like states [324], the MCAo has been widely shown to results in hyperactive locomotion in the rodent [27, 313-315],
and thus we only interpret these to measure hyperactive locomotion, not anxiety-like states. Animals were however also scored for the percentage of time spent in the centre area of the arena, compared to along the sidewall of the arena, which was defined as anxiety-like behaviours.

4.5.2 Spatial Displacement Recognition
Previous authors have suggested that locomotor hyperactivity in rodent models, following MCAo surgery, may result from hippocampus damage, that leads to spatial mapping difficulties [28]. Thus, in the present study, it was considered important to assess deficits in spatial mapping abilities, in stroke-affected rats as compared to sham-operated rats. Spatial mapping abilities in the rodent can be studied using the spatial displacement recognition test [325-330], during which animals are habituated to a testing environment containing a number of objects. Once the animal is familiar with the spatial layout of the environment, the location of one of the object is moved, and the duration of the rats’ interactions with each of the objects is recorded. Previous research demonstrates that rats without spatial mapping deficits spend more time exploring the displaced objects, than they spend exploring non-displaced objects [325-330]. Animals argued to have spatial mapping deficits however, have been observed not to spend more time exploring displaced objects, compared to non-displaced objects [325-330]. In the present study, we assessed deficits in spatial mapping abilities, using the spatial displacement recognition test. The number of object contacts and the duration of these contacts defined object interaction.

4.5.3 Novel Object Exploration
Time spent exploring a novel object while in a familiar environment has been commonly studied to measure approach–avoidance conflict behaviours [328, 330-334]. This is because rats are innately driven to approach or explore new stimuli/environments, but simultaneously are likely to be driven to avoid the unfamiliar, and thus fear inducing stimuli/environments. Avoidance rather than exploration of the novel object has been interpreted to reflect an anxiety-like state, induced by the unfamiliar object, which is pronounced enough to override the innate drive to explore it [334-336].
Previous research has demonstrated that novel object exploration can be inhibited by 14 days of physical stress, including electrical shock, short term food and water deprivation, conditions of cold and heat and tail pinch, in rodents [337]. Rats who spend less time exploring a novel object, have also been shown to have a longer duration of immobility, when in the forced swim test, less exploration of a novel environment in the emergence test (thought to reflect increased avoidance behaviours due to an anxious like state, induced by the potential danger contained in the novel environment) a higher expression of corticosterone (a stress response related hormone) and more defecation (a behaviour argued to reflect an anxious state, in the rodent model) [337]. Decreased novel object exploration has been observed in the Flinders Sensitive Line rat, which is a selectively bred genetic animal model of depression, as compared to Sprague-Dawley rats [338]. In the Flinders Sensitive Line rat, an increase in duration of exploration, of a novel object is observed after treatment using acute electrical stimulation. Acute electrical stimulation is a variation of the clinical anti-depressant treatment, called electroconvulsive treatment (ECT), and is used in rodent models [339]. Previous authors have additionally demonstrated that in the rat model, inhibiting the expression of depression-associated serotonin reduces exploration of novel stimuli [340]. Dopamine and brain-derived neurotrophic factor (BDNF) are similarly both associated with the presentation of depressive and anxious symptomatology. Mice with a genetic knockout of the dopamine D4 receptor [332], as well as mice with a pharmacologically induced reduction of brain-derived neurotrophic factor (BDNF) [341], have been demonstrated to spend less time exploring novel stimuli. Administration of gaseous nitric oxide (which results in central nervous system cell death) [342], and post natal injection of the LPS, are associated with a decrease in novel object exploration, in the rat [303]. In the LPS treated rats, decreased novel object and open field exploration, has been shown to correspond with an increased expression of depression associated cell death, and increased body temperature, which is an indicator of inflammation [303].

In the present study, we used a novel object exploration test to study anxiety related, approach-avoidance behaviour. Approach-avoidance behaviour was defined by the duration of time spent interacting with the novel object, with a longer period of interaction with the object being defined as approach behaviour.
4.6 Diet Supplementation

The fatty acids, Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA) appear to be differentially absorbed, and thus to have differing immune modulating effects, according to the methods of administration [343]. Oral supplementation with omega 3 (ω-3), which best models the method of human consumption, has been demonstrated to increase secretion of the anti-inflammatory cytokine, Interleukin 10 [190], and to reduce immobility time in the forced swim test, which is described above, and which the authors interpreted to reflect a decrease in ‘despair like’ behaviours [344-346]. Oral consumption of ω-3 has previously been reported to correspond with increased brain DHA in the rat [344]. EPA consumption has been shown to reduce Prostaglandin E2 secretion, induced by the pro-inflammatory cytokine Interleukin-1 (IL-1), and to increase anti-inflammatory Interleukin-10 secretion, from whole blood cells [190]. Therefore, oral dietary consumption was deemed to be an appropriate method of ω-3 supplementation for the present research.

Previous research has shown that shorter term fatty acid supplementation, via gavage is associated with reduced apoptosis, nitric oxide generation and increased superoxide dismutase in the hippocampus [244]. Sustained durations of gavage-administered supplementation however, have been reported to increase neuronal cell death [347]. Indeed, optimal cellular function is dependent on the ratio of ω-3: ω-6 [172] and ω-3 PUFAs can also be neurodegenerative when in excess [277-280]. Therefore, in the present research, it was important to select an appropriate dose and duration of ω-3 PUFAs dietary supplementation.

Fatty acids supplementation via oral consumption has previously been reported to require a number of weeks to induce immune modular and mood related behavioural changes. While a reduced inflammatory response to IL-1, has been demonstrated after four weeks of supplementation in rodent models [348], a greater body of research has demonstrated immunosuppressive effects, after weeks of supplementation [190, 349-351]. Behavioural effects have similarly been reported after six weeks of oral administration, as defined by reduced immobility in the forced swim test and tail suspension test [345]. Finally, six weeks of dietary supplementation with ω-3 has been reported to increase hippocampus volume in mice [345]. Therefore, in order to study the potential behavioural, affective and cellular protective benefits of supplementation with
polyunsaturated fatty acids, as a post-stroke intervention, in the present research rats were supplemented with a ω-3 rich diet for a six-week period, post-stroke. Additionally, to avoid excessive animal weight loss within the first week following surgery, animals were acclimated to their respective diets in the week prior to surgery. The ingredients, calculated nutritional parameters, amino acids, vitamins, and minerals, of the experimental fish oil enriched SF09-109 diet and the basal control AIN93G diet, are presented in the Appendices.

4.7 Cellular Markers and Techniques

Post-stroke functional recovery is undoubtedly dependent upon the proliferation profile of, and interaction between, many cell types including astrocytes, microglia, oligodendrocytes, and neurons [15, 352, 353]. In future research it will be valuable to study the proliferation profiles of different cell types post-stroke, and the relationship between these. However, it first must be established that stroke related cellular proliferation in general is associated with the presentation of, and apparent improvement in depressive/anxiety-like and locomotor behaviours post-stroke. Thus, in the present research, we have used Ki-67 as a general marker for cellular proliferation. The Ki-67 protein is established as a reliable marker, present during all active phases of the cell cycle, and commonly employed to detect cellular proliferation [354]. Indeed, previous authors have demonstrated increased Ki-67 expression, in the dentate subgranular zone, as late as seven weeks post MCAo in rodent models [32]. Therefore, in the present research, Ki-67 was deemed an appropriate marker to study cellular genesis in the rat brain, at six weeks post MCAo.

As outlined previously, apoptosis is often initiated via destruction of the mitochondria and activation of Caspase-3, which directly induces programmed cell death [9]. It is also activated in the apoptotic cell by extrinsic death ligand pathway [355]. Accordingly, in rodent models, Caspase-3 is widely employed as a marker of cell death after traumatic brain injury [356] and after MCAo [357]. Caspase activation has been observed in rodent brain tissue from as early as few hours post-stroke, to as late as several weeks post-stroke [31, 357-360], and thus was considered to be an appropriate apoptosis marker to study cellular degeneration, at the six week time point in the present research.
4.8 Western Blot Analysis and Appropriate House Keeping Genes

For the purpose of Western Blot analysis, loading controls are used to confirm that protein loading is the same across each well contained in the gel. To be an appropriate loading control, a gene needs to be constitutively and stably expressed at high levels in the tissue of interest. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is an enzyme that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules, that is expressed in large amounts in rat brain tissue and is required for multiple cell maintenance functions and at all times [361]. Accordingly, GAPDH is one of the most commonly used loading control genes, often employed as a loading control gene for the purpose of Western Blot protein analysis [362-365]. Additionally, an appropriate loading needs to have a different molecular weight than that of the protein of interest, in order to ensure that the bands observed are distinct and the expression levels are quantifiable. As GAPDH has larger molecular weight then Caspase-3, it was deemed an appropriate loading control for the purpose of Western Blot analysis in the present research. It should be noted however that even the most widely used Western Blot housekeeping genes are involved in multiple cell functions and that the expression levels of these genes are affected by many factors [366]. Therefore the validity of even the most commonly used housekeeping genes, as internal standards for measuring gene expression, has been called into question [366]. This is particularly relevant in cases of lesion damage, where cellular energy supplies could be expected to confound.
Chapter 5 Fish Oil Diet Reduces Longer Term Stroke-Related Sickness Behaviours, and Motor Impairment in Rat, But is Also Associated with Acute Reperfusion Related Hemorrhage

5.1 Abstract

Background: Ischemic stroke in humans is associated with both acute and longer-term complications, including motor impairment and increased incidence of affective disorders such as clinical depression. In non-stroke populations, successful management of such affective disorders and symptoms including anxiety, hyperactivity and agitation has been reported following diet supplementation with long chain omega-3 polyunsaturated fatty acids (PUFAs) or fish oils. However, the potential protective effects of omega-3 polyunsaturated fatty acids supplementation on affective behaviours after induced stroke and sham surgery have not been examined previously in animal models.

Method: Thus this study investigated the behavioural effects of dietary PUFA supplementation over a six-week period following either middle cerebral artery occlusion or sham surgery in the hooded Wistar rats.

Results: Most importantly PUFA supplementation was found to be associated with an increased risk of reperfusion related haemorrhage at the time of surgery, whereas in surviving animals, PUFA supplementation was found to moderate sickness behaviours, and acute motor impairment and longer-term locomotor hyperactivity and depression/anxiety-like behaviour, as measured using the number and duration of emergences into an open-field and duration of exploration of a novel object.

General Conclusion: Dietary supplementation with fish oil is also associated with acute reperfusion related hemorrhage and in surviving animals appears to reduce sickness behaviour and depression/anxiety-like behaviour.

Key Words: PUFAs, MCAo, EPA, DHA, Mood, Stroke.
5.2 Introduction

Ischemic stroke affects 15 million people each year and is a leading cause of disability worldwide [367]. Unfortunately recovery after stroke is associated with unusually high incidences of behavioural and affective disorders including anxiety and [3, 4] clinical depression [5] which are commonly co-morbid [368, 369]. In non-stroke affected populations, depressive disorder is associated with hyperactive delirium, (i.e. increased motor activity with agitated behaviour) [6] [370, 371]. Delirium is also common following stroke [6]. Anxiety, depression and delirium all negatively influence recovery after stroke [7]. Depressive disorder is also similarly closely linked to inflammation related ‘sickness behaviours’ [372]. Sickness behaviours are defined as the normal, ubiquitous responses to infection seen in all animal species examined and bear close biological resemblance to clinical depression [372]. Sickness behaviours are characterized by innate immune, endocrine, peripheral and central nervous system behavioural responses, that raise temperature to fight infection or macrophage dead or dying cells, and usually suppress normal activities such as exploring for food and eating and drinking and irrespective of the initiating cause. Thus to achieve the best prognostic outcome for stroke survivors, it is important to design better management regimes for post-stroke affective disorders [5] and sickness behaviours in general.

An alternative therapy for the generic treatment of depression [50] excessive anxiety [51] and hyperactive symptoms [49] are long chain omega-3 polyunsaturated fatty acids, (n-3-LC-PUFA) and in particular eicosapentaenoic acid, (EPA, 20:5n-3) and docosahexaenoic acid, (DHA, 22:6n-3)[49]. However the influence of such n-3-LC-PUFA supplementation on mood and behavioural disorders has not previously been investigated in stroke either in humans or in animals after experimentally induced ischemia via middle cerebral artery occlusion (MCAo). The middle cerebral artery occlusion (MCAo) model of ischemic stroke closely models the pattern of cell death resulting from middle cerebral artery ischemia in clinical populations, and thus is often utilized [291]. The MCAo stroke model has additionally been associated with reduced sucrose consumption, a behaviour argued to reflect anhedonia, which is a symptom of clinical depression, and which is reduced by the administration of a conventional antidepressant, citalopram [293, 294].
n-3-LC-PUFAs are highly bioactive compounds, commonly derived from dietary fish oil, that have anti-inflammatory effects at optimal doses [50]. n-3-LC-PUFAs are not associated with any of the unwanted side effects of conventional antidepressant medications [234], making them an important alternative candidate for the management of behavioural and emotional disturbances irrespective of the cause. Thus in the present study, we aimed to investigate and compare potential protective effects of diet (n-3-LC-PUFA supplementation to the basal diet vs. basal diet) over the 6 weeks after surgery (either Middle Cerebral Artery occlusion [MCAo] or sham surgery) in rats. In particular we aimed to study the effects of diet on surgically induced acute and longer term motor and sickness behaviours, including behaviours commonly interpreted to reflect excessive anxiety or depressive-like behaviours. We hypothesised that following MCAo surgery all animals would show changes in food and water consumption, compared to the sham operated animals. Additionally, we hypothesised that the n3-LC-PUFA diet supplemented animals would show less change in food and water consumption, and body weight post-surgery, compared to the basal diet fed animals. Acute inability to make co-ordinated motor movements have regularly been reported and studied in rats post MCAo compared to sham surgery using a battery of behavioural tests, [301, 302]. Motor impairments decrease in response to neuroprotective agents [373-375]. Thus we hypothesised that the n3-LC-PUFA supplemented animals would also show less acute MCAo related motor impairments during the first five days following surgery, compared to basal diet fed rats. Furthermore as very few studies have examined potential longer-term effects of MCAo surgery on rodent sickness and affective behaviours we have also investigated the extent or type of motor anomalies that might be indicative of sickness behaviours and excessive anxiety and potential depression during recovery from experimental stroke.

To test for longer term sickness behaviours and particularly depression and anxious like states after surgery we chose not to use traditional despair behaviour tests such as the forced swim and tail suspension test [283] on the grounds that these tests are inherently stressful to the animal and likely to augment the immunological changes expected to result from the MCAo procedure [376]. Rather we chose to use the novel object exploration test that measures the conflict between a rat’s mutual drives to both avoid and approach unfamiliar and potentially fear inducing-stimuli/environments, with the greater avoidance thought to reflect anxiety-like behaviour [334-336] [328, 330-334]
and a modified open field test described below that can be conducted while the animal is in a darkened and familiar environment, and thus is minimally fear inducing and more likely to reliably reflect the on-going sickness associated anxiety-like behaviours.

In terms of MCAo surgery, rodents have been reported to display locomotive hyperactivity from the first five minutes to 3 weeks after surgery in tests such as the open field test [27, 313-315] similar to the behaviours seen in models of anxiety and depression on the same test [316-320]. Thus we used the modified free exploration test, [322, 323] which eliminates the forced exploration and related stress normally associated with the open-field test. The free exploration test involves habituating animals to a darkened enclosure that is placed inside the open field and allows access to the larger, illuminated and transparent open field through a small door [322, 323, 332]. Hyperactive locomotion is defined as an increase in the number of emergences from the hide box into the open-field and an increase in the duration of time spend moving around the open field arena, during a prescribed period of time. Given that the MCAo procedure and the associated neurodegenerative damage results in increased locomotor, activity per se [27, 313-315] we suggest that the above-mentioned measures may not reflect anxiety-like behaviours in MCAo operated animals, but instead may only be another measure of locomotion hyperactivity.

Anxiety-like behaviour was defined as avoidance of the centre, considered to be the most anxiety-provoking region of the open field arena. We hypothesised that both the number of emergences from the hide box into the open-field and the duration of time spent moving around the open field arena, would not be positively correlated with the widely accepted anxiety-like behaviour, defined by percentage of emergence time spent in the centre of the open-field arena.

Previous authors have speculated that MCAo related changes in locomotion might arise from an inability to habituate to unfamiliar environments, possibly related to spatial mapping difficulties induced by hippocampus cell death [27, 28, 315, 377]. Therefore, spatial mapping abilities were assessed here using the spatial displacement recognition test, which has previously been employed to study spatial memory deficits post-stroke in the rodent model [28, 326, 327, 330]. This test is based on observations that normally reared healthy rodents spend more time exploring displaced objects, than they do exploring familiar non-displaced objects, while animals with lesions associated with
spatial mapping deficits do not [325-330]. We hypothesised that MCAo operated animals would show more long-term stroke-related anxiety-like and hyperactive locomotor behaviours than sham operated animals (MCAo vs. Sham). Finally, we hypothesised that the MCAo animals supplemented with n-3-LC-PUFA would show less longer-term stroke-related sickness or anxiety-like and locomotor behaviours than basal diet fed animals (n-3-LC-PUFA vs. Basal). Long-term was defined as occurring at 2, 4, or 6 weeks following surgery.
5.3 Methods and Materials

5.3.1 Animals and Design
The experimental design was for two surgery groups, (MCAo and Sham groups) and two diet groups (basal and n-3-LC-PUFA supplementation) \( (n = 11 \text{ n-3-LC-PUFA MCAo}; n = 13 \text{ Basal MCAo}; n = 12 \text{ n-3-LC-PUFA sham}; n = 12 \text{ Basal Sham}) \). Male hooded Wistar rats were obtained from Laboratory Animal Services, The University of Adelaide, Aust. Animals were maintained in a temperature (21°C ± 2) controlled environment on a 12:12 h light-dark cycle (lights on at 07:00 h). Food and water were available \textit{ad libitum} and consumption was measured daily. Animals were housed separately to allow individual monitoring of food and water consumption. Acclimatization or the adjustment period to separate housing occurred in the week prior to surgery. All MCAo and sham surgeries and behavioural testing took place in the lights-on phase. MCAo operated animals weighed between 330 and 360 grams at the time of surgery. A number of rats \( (n = 11) \) in the n-3-LC-PUFA MCAo group died immediately following withdrawal of the suture thread. These animals were replaced. A further number of animals \( (n = 16) \) (n-3-LC-PUFA, \( n = 7 \); Basal, \( n = 9 \)) were culled due to MCAo induced motor impairments and clinical symptoms deemed too large for the animal to survive. These animals were also replaced.

5.3.2 Ethics Statement
Ethics approval was granted by the Austin Health Research Ethics Unit (10/3865) and was conducted in accordance with the Australian Code of Practice for the use of animals for scientific purposes [378].

5.3.3 Dietary Regime
Half the animals were randomly assigned to n-3-LC-PUFA diet condition \( (n = 23 \text{ n-3-LC-PUFA}) \) and the other half randomly assigned to basal diet condition \( (n = 25 \text{ basal diet}) \). To avoid excessive animal weight loss within the first week following surgery, animals were acclimated to their respective diets in the week prior to stroke surgery. Animals were then maintained on respective diets until the time of sacrifice, in the sixth week post-stroke as anti-depressive-like behavioural effects have previously been reported after six weeks of oral supplementation with omega-3 in non surgery effected animals [345]. Animals supplemented with n-3-LC-PUFA were maintained on a fish oil...
enriched standard rodent chow, (Specialty Feeds, Cat No. SF09-109 5% Fat High N3 Modified Rodent Diet; Glen Forest, WA, Aust) that has been demonstrated to reduce expression of pro-inflammatory interleukin-1 and interleukin-6 [379]. Each kg of the 5% Fat High N3 Modified Rodent Diet contained 2700mg of EPA and 11900mg of DHA. Animals fed the basal diet were maintained on a comparable and nutritionally sound standard rodent chow, (Specialty Feeds, Cat No. AIN93G; Glen Forest, WA, Aust). Food consumption, water consumption and animal weight were monitored daily using analogue scales.

5.3.4 Right Middle Cerebral Artery Occlusion (MCAo)

Half the animals were randomly assigned to the MCAo surgery condition \( n = 24 \) MCAo, the other half to the sham surgery condition \( n = 24 \) sham. All surgeries were performed according to the protocol of Longa [380] and its modifications by Spratt [290]. Animals were shaved around incision areas and swabbed with Betadine antiseptic solution (Faulding Pharmaceuticals, VIC, Aust). Isoflurane (Cat No. AHN3640-250ML, Baxter, Old Toongabbie, NSW, Aust.) in 5% oxygen was used to induce anaesthesia that henceforth was maintained with 2% Isoflurane in oxygen via a cone over the nose of the animal during surgery. Oxygen saturation levels, heart rate and temperature were monitored throughout the procedure using iWORX (Cat No. PO2-300D, iWORX, Dover, NH, USA) and body temperature was maintained at 37°C using a locally manufactured rectal temperature regulated heating pad. Atropine (0.2ml 600µg/mL, Pfizer Aust Pty Ltd, NSW, Aust.) was administered intraperitoneally to inhibit bronchial secretions and salivation. Cerebral blood flow was measured using laser doppler flowmetry (LDF). The Laser Doppler (Cat No. IEC-601-1, Moor Instruments, Axminster, Devon, UK) was attached to the scalp, at a point previously thinned using a dental burr. A laboratory-made rubber probe holder was attached to the skull with Loctite instant adhesive (Loctite 406 instant adhesive, Cat No. 265606, Henkel, Sydney, NSW, Aust) to hold the Laser Doppler in place (1 mm caudal and 5 mm lateral to bregma, this area overlies the region of cortex supplied by branches of the middle cerebral artery). To occlude the right middle cerebral artery, an incision was made in the neck, small branching blood vessels were cauterised, the external carotid artery was ligated and an incision was made in the right external carotid artery. A 0.4 mm diameter silicone tipped suture thread was inserted into the incision, passed via the right external carotid and up the internal carotid artery (approximately 18 mm from the carotid
bifurcation) until a drop in cerebral blood flow was detected using the laser Doppler flowmetry. Animals were subjected to 90 min MCAo; incisions were stitched up using 4.0 silk sutures (Cat No. 90352, Dynek Pty Ltd, Port Adelaide, SA, Aust) and animals were removed from anaesthesia during this time. After 90 min, animals were re-anaesthetised, the incisions re-opened and the MCA-occluding suture was retracted from the middle cerebral artery into the external carotid stump. All incisions were again closed using a 4-0 silk suture (Cat No. 90352, Dynek Pty Ltd, Port Adelaide, SA, Aust). Analgesia was provided as paracetamol [Cat No. 569925, Sanofi-Aventis, Macquarie Park NSW, Aust.] that was crushed and dissolved into drinking water at a concentration of 0.15mg of paracetamol per 1 ml of water. To prevent dehydration saline was injected intraperitoneally (i.p.) daily for the first three days following surgery (3 ml Sodium Chloride Solution [0.9%], Cat No. 7647-14-5, Tocris Bioscience, Bristol, United Kingdom) Animals were provided with relevant diet condition pellets, sunflower seeds and soft food (Sustagen® Everyday Nestlé, Notting Hill, VIC, Aust) to encourage eating and weight gain in the week following surgery. Animals were killed six weeks post-MCAo via isoflurane anaesthesia (Cat No. AHN3640-250ML, Baxter, Old Toongabbie, NSW, Aust.) followed by either guillotine or perfusion (see methods below). Sham animals underwent identical procedures with the exclusion of thread insertion into the MCA. Table 5.1 displays animal groups, final successful numbers and behaviour testing timetable.
### Table 5.1. Animal Groups, Numbers, Surgery and behaviour Testing Timetable.

<table>
<thead>
<tr>
<th>Group and Timeline for Behavioural Testing</th>
<th>MCAo n-3-LC-PUFA (n=23)</th>
<th>MCAo Basal (n=21)</th>
<th>Sham n-3-LC-PUFA (n=12)</th>
<th>Sham Basal (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Died due to reperfusion injury (n=11)</td>
<td>Died due to large stroke damage (n=9).</td>
<td>Included in final cohort (n=12)</td>
<td>Died due to large stroke damage (n=7).</td>
<td>Included in final cohort (n=12)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Week Prior to Surgery</th>
<th>Diet/Housing Acclimation</th>
<th>Diet/Housing Acclimation</th>
<th>Diet/Housing Acclimation</th>
<th>Diet/Housing Acclimation</th>
</tr>
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<tbody>
<tr>
<td>Surgery</td>
<td></td>
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|----------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|

<table>
<thead>
<tr>
<th>Four Weeks Post-surgery</th>
<th>Behavioural Testing as Specified in Week 2 Post-Surgery</th>
<th>Behavioural Testing as Specified in Week 2 Post-Surgery</th>
<th>Behavioural Testing as Specified in Week 2 Post-Surgery</th>
<th>Behavioural Testing as Specified in Week 2 Post-Surgery</th>
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<table>
<thead>
<tr>
<th>Sacrifice</th>
<th>Sacrifice</th>
<th>Sacrifice</th>
<th>Sacrifice</th>
</tr>
</thead>
</table>

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats.
5.3.5 *Acute Stroke-Related Motor Impairment Measures*

Motor impairments were assessed daily in the first five days post-surgery using a battery of behavioural tests. The tests included the forelimb flexion test, the torso twisting test, the lateral push test and the circling and motor ability observations. These tests collectively measure the acute inability to make coordinated motor movements that have regularly been reported and studied in rats post MCAo compared to sham surgery [301, 302]. The tests were used to provide an indicator of consistency between our findings and those of previous researchers. Additionally, these motor impairments are seen to be less severe in animals exposed to neuroprotective agents [373-375] and thus we aimed to determine if acute motor disability was decreased after n-3-LC-PUFA supplementation as an alternative treatment agent. Impaired performance on these tests indicates that the MCAo procedure was reliable and as previously reported induced acute paralysis on one side of body. In the forelimb flexion test, MCAo affected rats have been reported to hold the left forelimb between 45 and 90° to the right when lifted from the base of the tail [301]. In the torso twisting test, MCAo affected rats have previously shown a curling of the head and forelimbs toward the left paralytic side of the body, when held via their tail above a table [301]. The lateral push test measures resistance when an animal is held behind the shoulders and pushed either to the left or right sides [301]. MCAo affected rats show weakened resistance when pushed towards the paralytic left side. The circling test involved recording the duration and extent of circling behaviour. Many previous experiments have reported that animals with infarct or lesion damage circle toward the opposite side of the damage and thus animals with infarct in the right hemisphere would be expected to circle in an anti-clockwise direction [301]. Motor ability observations consisted of a general observation of the walking, grooming, and rearing behaviours of all animals after either MCAo or sham-operations and were ranked from 0 to 2, with 0 indicating no disability and 2 indicating severe disability.

5.3.6 *Longer Term Ischemia Associated Behavioural and Affective Disorders*

The battery of behavioural tests designed to model ischemia related affective behaviours, frequently seen in humans were administered and recorded on video in the second, fourth and sixth weeks post-stroke. An experienced rat behaviour observer (NC) who was blinded to the dietary and stroke treatment conditions scored the behaviour
video. Animal behaviours were scored by a second observer (MP), and the inter-rater reliability correlation was statistically significant, $r^2 = .97$.

5.3.7 Free Exploration Test

Animals were habituated to a laboratory constructed black Plexiglass hide box (30 × 15 × 19 cm) for 15 min on the day prior to behavioural testing and re-acclimated (five min) immediately prior to testing. The open field was a transparent Plexiglass arena (1m²) illuminated with ~500 lux of light (Fairway Work light, DCWLT1000, 240 volt). The hide box was positioned inside one wall of the open field such that when the door opened, the animal was allowed access to the open field. Emergence behaviour was recorded for four min using a digital camcorder (Canon, HG20) and was stored as a MPEG-TS video file. Animals were scored for locomotor hyperactivity that was defined as number of times fully emerged from the hide box (i.e. emergence number) and time spent fully emerged from hide box and moving around and exploring the arena over a four minute period (emergence duration). Animals were also scored for anxiety-like behaviour that was defined as the percentage of time spent in the centre area of the arena over a total time of four minutes. The open field and hide box were cleaned with 70% ethanol between each testing session.

5.3.8 Spatial Displacement Recognition

The testing box (40cm x 40cm x 60cm) used for spatial displacement recognition was constructed of 60cm high black walls and had an open top. Animals were acclimated to the testing box for five min on the day prior to testing, at each of the three testing time points. On the day of testing, animals were placed in the testing box and positioned with their nose facing the mid-point of the wall opposite the objects, to prevent an unintentional bias of placing the animal in an orientation favouring a particular object [381]. Interaction with each of the objects was recorded using a digital camcorder (Canon Legria, HG20) and was stored as a MPEG-TS video file. Object contact was defined as when the mouth, nose and/or paw touched the object or when the nose of the rat was within 1 cm surrounding the object. Contact judged as accidental, such as bumping the object as the animal passed or grooming behaviour within 1 cm of the object was not counted as interactive behaviour. The number of object contacts and the duration of these contacts were recorded.
The spatial displacement recognition testing consisted of two phases. The acquisition phase involved two habituation trials (three min each) to two identical objects. These habituation trials were separated by a two-min interval, during which the animal was returned to the home cage. In the spatial displacement recognition phase, the location of one of the two objects was moved and the animal was again placed back in the testing box for a single three min trial. All apparatus were cleaned with 70% ethanol between trials (Cat No. EA043-10L, Chem-Supply, Gillman, SA, Aust).

5.3.9 Novel Object Exploration

The novel object exploration trial took place immediately after the spatial displacement recognition trial, separated only by a two-min interval during which the animal was returned to the home cage. Testing took place in the same box used for the spatial change displacement recognition test, ensuring adequate acclimation of the animal with all aspects of the environment, minus the newly introduced novel object. Rats were placed in the testing box for a single three-min trial. The familiar object was placed in one corner of the box and the novel object was placed in the opposite corner. Object placement and selection were counterbalanced across trials and animals. Contact definition is consistent with that described above for the spatial displacement recognition test. The apparatus was cleaned with 70% ethanol after each trial.

5.3.10 Tissue Collection

Tissue was collected using two methods; the first for whole brain total fatty acid analysis (FFA) \( n = 5 \) n-3-LC-PUFA MCAo; \( n = 6 \) Basal MCAo; \( n = 6 \) n-3-LC-PUFA Sham; \( n = 6 \) Basal Sham) after decapitation. The second was for infarct analysis after reperfusion \( n = 6 \) n-3-LC-PUFA MCAo; \( n = 6 \) Basal MCAo; \( n = 6 \) n-3-LC-PUFA Sham; \( n = 6 \) Basal Sham). For FFA analysis, half the animals were anesthetized via Isoflurane (Cat No. AHN3640-250ML, Baxter, Old Toongabbie, NSW, Aust.) overdose, decapitated via guillotine, the brains removed, snap frozen in liquid nitrogen (Liquid Nitrogen Services Pty Ltd, Melbourne, Vic, Aust) and stored at -80°. For infarct analysis half the animal tissue was preserved using perfusion. Once anesthetized, the rib cage and diaphragm were cut exposing the heart, a perfusion needle placed into the left ventricle of the heart and the right atrium of the heart cut. Saline (90 ml) (1.8%) was infused for five min, followed by paraformaldehyde (PFA) (4%) (270 ml) for fifteen min, using a perfusion pump (Peri-Star Pro 4-channel, high rate pump, Cat No.
PERIPRO-4HS, World Precision Instruments, Hilton, SA, Aust.) (18 ml per minute). The brains were removed and placed directly into PFA (4%) for 24 h, before being changed to 30% sucrose, where they remained until the time of paraffin embedding.

5.3.11 Fatty Acid Analysis

Fatty acid analysis on whole brain tissue was performed to confirm that the n-3-LC-PUFA supplementation resulted in changes in brain phospholipids levels of fatty acids. Whole brain frozen tissue was grounded up and tissue lipids extracted by dichloromethane/methanol (2:1) overnight, as described by Sinclair et al [382]. After addition of dichloromethane/methanol (2:1), samples were filtered. 1ml of 0.9% saline was added to each tube and vortexed for 1 min. Samples were then centrifuged at 1500 g for 10 min to separate the aqueous and organic phases at room temperature. The organic phase containing the lipid was removed and transferred to a new glass tube and evaporated under a stream of nitrogen. The lipid extract was reconstituted in 200 µl of dichloromethane and lipids were then separated by thin layer chromatography (TLC). The lipid extracts were spotted onto silica gel plates (silica gel 60 G, Merck, Germany) and developed in 85:15:2 (v/v) petroleum ether: diethylether : acetic acid in paper-lined tanks. The lipids were visualised with 0.1% (w/v) 2′,7′ – dichloroflurescein indicator in ethanol (Scharlau, Spain). The phospholipid bands from the samples was scraped off into glass screw-capped tubes and were reacted with 5% H2SO4 in 100% methanol for 3 hours at 80 °C to form the fatty acid methyl esters (FAME). After the reaction, the FAME were isolated in 100% petroleum ether and stored in glass vials at -20 °C prior to gas chromatography. Purified FAME were isolated and identified using an Agilent Technologies 7890A GC System (Agilent Technologies; Santa Clara, CA, USA) equipped with an Omegawax 250 capillary column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness, Supelco, Bellefonte, PA, USA), a flame ionization detector (FID), an Agilent Technologies 7693 auto sampler, and a split injection system (split ratio 50: 1). The injection volume was 1µl, the injector and detector temperature were 300 °C and 270 °C, respectively. The temperature program was 50 to 190 at 20 °C min-1, then from 190 to 250 at 4 °C min-1, and held at 250 °C for 8 min. The carrier gas was helium at 1.18 mL min-1, at a constant flow. Each of the fatty acids was identified relative to known external standards (a series of mix and individual standards from Sigma-Aldrich, Inc., St. Louis, MO, USA and from Nu-Chek Prep Inc., Elysian, MN,
USA). The resulting peaks were then corrected by the theoretical relative FID response factors [383] and quantified relative to the internal standard.

5.3.12 Infarct Volume Analysis and Area of Damage

Total infarct volume (mm$^3$) analysis was performed to confirm that the MCAo procedure resulted in expected ischemic tissue damage (see Figure 5.4). For paraffin embedding brains were cut into 2 mm coronal sections using an acrylic rat brain matrix and transferred to plastic cassettes. Coronal slices were placed between two biopsy sponge pads to prevent curling and moving of the tissue with the cassettes. Tissue was processed using a closed linear Tissue Processing System (Cat No. TPC 15, MEDITE GmbH, Wollenweberstr, Burgdorf, Germany) and underwent an overnight protocol whereby it was soaked for 1 h in each solution (4% buffered formalin [40°C], 4% buffered formalin [40°C], 70% ethanol [40°C], 96% ethanol [40°C], 100% ethanol [40°C], 100% xylene [40°C], 100% xylene [40°C], 50% xylene/50% ethanol [40°C], 100% ethanol [40°C], 100% ethanol [40°C], 100% xylene [40°C], 100% xylene [40°C], 100% Paraffin wax [60°C], 100% Paraffin wax [60°C], 100% Paraffin wax). Coronal sections were then embedded in molten paraffin wax using a heated Paraffin Embedding Module (Cat No. Leica EG1150 H, Leica-microsystems, Ernst-Leitz-Straße, Wetzlar, Germany) and cooled to 4 °C using a cold plate for modular tissue embedding (Cat No. Leica EG1150 C, Leica-microsystems, Ernst-Leitz-Straße, Wetzlar, Germany). Once cooled, coronal sections were kept at room temperature until cutting. Paraffin embedded tissue was cut at room temperature (7 µm) using a rotary microtome, (Cat No. Leica 2040, Leica-microsystems, Ernst-Leitz-Straße, Wetzlar, Germany) suspended at 40 °C in a tissue flotation bath (Cat No. TFB45, MEDITE GmbH, Wollenweberstr, Burgdorf, Germany) and attached to silane coated slides (Cat No. CS2460100MK, Mikro-glass, Grale Scientific, Melbourne, Aust.). Tissue slides were dried at 49 °C on a solid state control warming tray (Cat No. WT1, Ratek, Melbourne, Vic, Aust.) and then incubated (Contherm Series Five, Cat No. 105M, Wellington, NZ) at 32° C for at least 24 h before being stained with Haematoxylin (5g Haematoxylin [Cat No. 340374T, VWR International, Pty Ltd., Murarrie, QLD, Aust] 50ml Absolute Ethanol (EtOH) [Cat No. EA043-10L, Chem-Supply, Gillman, SA, Aust], 100g Aluminium potassium sulphate [Cat No. A7167, Sigma, -Aldrich, St. Louis, MO, USA] 1.25g Mercuric oxide [Cat No. 315, Ajax Finechem, Melbourne, VIC, Aust.], 1L dH2O,) and Eosin (10 g Eosin Y [Cat
No. E-4382, Sigma, -Aldrich, St. Louis, MO, USA], 20 g Calcium chloride [Cat No. A608982, Merck KGaA, Darmstadt Germany] 400 ml Absolute EtOH (Cat No. EA043-10L, Chem-Supply, Gillman, SA, Aust) in 600 ml dH2O). This method consisted of de-waxing slides in three consecutive 10 min Histosol washes (Cat No. CP L HISTOSOL 08, HD Scientific, Wetherill Park, NSW, Aust). Slides were then rehydrated washed for five min in each ethanol dilution, (100%, 100%, 90%, 70%, and 50%) (Cat No. EA043-10L, Chem-Supply, Gillman, SA, Aust), rinsed in distilled water (dH2O), dehydrated in a series of two-min ethanol washes (50%, 70%, 90%, 100%, and 100%), followed by two Histosol washes, (three min each). Slides were rehydrated in ethanol washes for two min each, (100%, 100%, 90%, 70%, 50%) rinsed in dH2O and stained with filtered Harris Hematoxylin (three min). Slides were rinsed in dH2O, stained in filtered Eosin Y for one min, washed in dH2O and dehydrated in two-min ethanol washes (50%, 70%, 90%, 100%, 100%). This was followed by two, three-min histosol washes before slides were cover slipped with DPX (Cat No. 1019790500, Merck KGaA, Darmstadt Germany). Stained tissue was examined and using bright field microscopy (Nikon Eclipse 80i, Nikon Instruments Europe) with a Nikon DIGITAL SIGHT DS-U1 camera. Photographs were stored as JPEG Image files. Areas of tissue damage were analysed using Stereo Investigator Version 6 software.

5.3.13 Data Analysis

The present study uses both parametric and not parametric data analysis techniques. Independent sample $t$ Tests were used to compare mean differences in animal weight between groups at time of surgery. Mixed design ANOVAs were conducted to determine differences in infarct volume and total fatty acid levels ($\mu$g) in brain phospholipids. Mixed design ANOVAs (diet condition x surgery condition x time point) were used to assess group differences in weight, food and water consumption (grams) across time points. Mixed design ANOVAs (diet condition x surgery condition x time points) were also conducted to compare differences in spatial change recognition (time) at weeks two, four and six, and differences in stroke behaviours at days zero, one, two, three, four and five post-stroke. Normality of data from behavioural tests was assessed using Q-Q plots and histograms. Outliers were screened for using box plots and no data was deleted, the assumptions of homogeneity was checked using Levene’s Test of Equality of Variance, and sphericity was checked using Mauchly’s Test of Sphericity. A
square root transformation was conducted on spatial change recognition data to achieve a normal distribution.

Data relating the longer-term ischemia associated behavioural and affective disorders (emergence number, emergence duration, percentage of time spent in the centre of the arena and novel object exploration) were not normally distributed and were unable to be transformed to achieve a normal distribution using transformations. Therefore the non-parametric Kruskal-Wallis test was used to identify differences between groups. Unfortunately the Kruskal-Wallis non parametric statistical test is limited in that it can only determine if differences exist between groups, but it cannot determine where the differences exist [384]. Therefore, where possible we used post hoc Mann-Whitney tests to determine where differences between groups occurred. Each new post-hoc group comparison results in an inflated Type 1 Error. Controlling for Type 1 Error requires a correction such as Bonferroni (i.e. dividing the critical level of significance (0.05) by the number of comparisons made [384]. However dividing the critical level of significance by many comparisons also makes Type 2 Error [384] much more likely. Thus in order to control for these errors, prioritization of the most important comparisons was necessary. We determined that comparisons a) differences between surgery conditions and b) differences between diet conditions were the most important/relevant to the current study. Thus, in order to control for Type 1 and Type 2 Error rates, we have not been able to make further comparisons to explore differences between diet groups within each of the different surgery conditions.

Spearman correlations were used to detect correlations between emergence behaviour and infarct damage. Spearman correlations were also conducted between both the number of emergences from the hide box into the open-field and the duration of time spend moving around the open field arena, and the percentage of emergence time spent in the centre of the open-field arena. The accepted p value for all analysis was $p<0.05$. 
5.4 Results

5.4.1 Increased Risk of Reperfusion Related Hemorrhage at the Time of Surgery among n-3 LC-PUFA Supplemented Animals

An unexpected finding of the present research that has not previously been reported was that 39% ($N=11$) of all the n-3-LC-PUFA fed animals that underwent MCAo surgery experienced haemorrhagic bleeding following the reperfusion component of the surgery. Reperfusion consists of withdrawing the silicone tipped thread from the MCA in order to restore blood flow to the ischemic region. This surgical complication was only seen in the n-3-LC-PUFA fed animals and did not occur in any of the basal diet fed rats. Independent sample $t$ tests indicate that the difference in the rate of reperfusion related bleeding between diet groups is significant, $t(17) = 3.29, p < 0.01$. These animals were obviously excluded from the final cohort numbers. Unfortunately, as the death of these n-3-LC-PUFA fed animals was unexpected insufficient time was available to prepare the tissue for further experimental analysis.

5.4.2 Animal Weight from Time of Surgery until Sacrifice

Animal ($N = 48$) mean bodyweight was 337 ($SD = 36.5$) grams at time of surgery. Rats in different diet conditions did not differ in mean bodyweight at time of surgery, $F(1, 47) = 1.14, p = 0.29$ (n-3-LC-PUFA $M = 332$, $SE = 6.7$, Basal $M = 342$, $SE = 6.4$). Between surgery conditions, sham operated rats were slightly lighter than MCAo operated animals, $F(1, 47) = 14.24, p < 0.01$ (MCAo $M = 354$, $SE = 6.6$, Sham $M = 320$, $SE = 6.6$). From weeks one until week six post-surgery, MCAo operated animals did not differ from sham operated animals in mean bodyweight, $F(1, 41) = 0.00, p = 0.99$ (MCAo $M = 345$, $SE = 7.5$; Sham $M = 345$, $SE = 7.0$), nor did the two diet groups differ, $F(1, 41) = 0.24, p = 0.63$ (n-3-LC-PUFA $M = 348$, $SE = 7.4$; Basal $M = 343$, $SE = 7.2$).

5.4.3 Food and Water Consumption from Time of Surgery until Sacrifice

Rats did not differ in food and water consumption at the time of surgery (see Figures 5.1 and 5.2). On average at the time of surgery, sham operated n-3-LC-PUFA diet fed animals were consuming approximately 206 mg of EPA, and 909 mg of DHA per day, per kg of total body weight. On average at the time of surgery MCAo operated n-3-LC-PUFA diet fed rats were consuming 181 mg of EPA and 800 mg of DHA per day, per kg of total body weight. As the basal diet contains no EPA or DHA, basal diet fed
animals in both the MCAo and Sham surgery conditions consumed 0 mg of EPA and DHA daily, per kg of total body weight. All animals increased in bodyweight from week one until week six post-surgery, $F (59,205) = 30.43, p < 0.01$. Food, $F (5,215) = 5.79, p < 0.01$ and water, $F (5,220) = 13.10, p < 0.01$ consumption increased between week one and week six post-surgery. MCAo operated animals continued to consume less food from week one until week six post-surgery, $F (1, 43) = 25.46, p < 0.01$ and drank less water, $F (1, 44) = 4.40, p < 0.05$, than sham operated animals. Over the same period of time, n-3-LC-PUFA supplemented animals consumed more food, $F (1, 43) = 12.47, p < 0.01$, and water, $F (1, 44) = 18.06, p < 0.01$ than basal diet fed rats. During the six weeks post-surgery, sham operated n-3-LC-PUFA diet fed animals consumed an average of 272 mg of EPA and 1200 mg of DHA daily, per kg of total body weight. On average, MCAo operated n-3-LC-PUFA diet fed animals consumed 189 mg of EPA and 833 mg of DHA daily, per kg of total body weight. Again, basal diet fed animals in both the MCAo and Sham surgery conditions consumed 0 mg of EPA and DHA daily, per kg of total body weight. Figure 5.1 shows the mean food consumption in grams between stroke and diet conditions, from week one until week six post-surgery. Figure 5.2 depicts the mean water consumption, in mls, between surgery groups and diet conditions, between week one and week six post-surgery.
Figure 5.1. Mean Food Consumption (shown with Confidence Intervals), Between Stroke and Diet Conditions from Week 1 until Week 6 Post-Surgery.

Note that sham animals always consumed significantly more food than MCAo animals and that in each surgery group the n-3-LC-PUFA diet fed animals consumed more than the basal diet fed animals. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats; Week 1 = Week One post-surgery; Week 2 = Week two post-surgery; Week 3 = Week three post-surgery; Week 4 = Week four post-surgery; Week 5 = Week five post-surgery; Week 6 = Week six post-surgery.
Figure 5.2. Mean Water Consumption (shown with Confidence Intervals), Between Surgery and Diet Conditions from Week 1 until Week 6 Post-Surgery.

Note the large drop in water consumption immediately after surgery and the rapid recovery in drinking behaviour, by week two post-surgery, and by the time that ischemia associated motor impairments were no longer obvious. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats; Week 1 = Week One post-surgery; Week 2 = Week two post-surgery; Week 3 = Week three post-surgery; Week 4 = Week four post-surgery; Week 5 = Week five post-surgery; Week 6 = Week six post-surgery.
5.4.4 Fatty Acid Analysis

Whole brain fatty acid analysis was conducted after euthanasia at six weeks postsurgery. Fatty acid levels are reported as percentage of total FAME. There were no effects of surgery condition on fatty acid expression. ANOVA showed that n-3-LC-PUFA fed animals showed less n-6 in whole brain tissue than animals fed the basal diet, evidenced by less arachidonic acid (AA; 20:4, $n-6$), $F(1, 23) = 8.01, p < 0.05$, linoleic acid (LA; 18:2, $n-6$), $F(1, 23) = 37.41, p < 0.01$ and palmitic acid (16:0) in brain phospholipids, $F(1, 23) = 5.51, p < 0.05$. A trend for higher levels of eicosatrienoic acid (ETA, 22:3, $n-3$), $F(1, 23) = 3.85, p = 0.06$, among n-3-LC-PUFA fed rats than basal fed rats was seen. No significant differences between diet conditions was seen in the expression of docosahexaenoic acid (DHA, 22:6, $n-3$), $F(1, 23) = 0.30, p > 0.05$, or eicosapentaenoic acid (EPA, 20:5, $n-3$), $F(1, 23) = 0.72, p > 0.05$. There were no significant interaction effects of surgery condition and diet condition. Figure 5.3 illustrates difference in the mean fatty acid levels in the brain phospholipids between diet and surgery conditions.
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Figure 5.3. Mean Percentage and Confidence Intervals of Whole Brain Fatty Acid in Fatty Acid Methyl Esters Between Diet and Surgery Conditions.

Note that although no difference between dietary conditions is seen in the expression of Eicosapentaenoic Acid [20:5] or Docosahexaenoic Acid [22:6], the levels of fatty acids (Palmitic Acid [16:0], Arachidonic Acid [20:4], and Linoleic Acid [18.2] were higher in basal diet fed animals, compared to n-3-LC-PUFA fed animals, in whole brain tissue. By comparison, n-3-LC-PUFA fed animals are shown to express more omega-3 derived fatty acids (Eicosatrienoic Acid [22:3]) than basal diet fed animals. No differences are seen in the expression of Fatty Acid in Fatty Acid Methyl Esters between surgery groups. 20:5 n-3 = Eicosapentaenoic Acid; 22:6 n-3 = Docosahexaenoic Acid; 20:4 n-6 = Arachidonic Acid; 18:2 n-6 = Linoleic Acid; 16:0 = Palmitic acid; 22:3 n-3 = Eicosatrienoic Acid. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet condition; n-3-LC-PUFA = n-3 L-C-PUFA enriched diet condition.
5.4.5 Infarct Volume Analysis and Area of Damage

Figure 5.4 illustrates areas of atrophy at six week post-surgery in MCAo affected animals (n = 6 n-3-LC-PUFA MCAo; n = 6 Basal MCAo). MCAo occlusion resulted in damaged tissue in cortical and sub cortical tissue in the right hemisphere. Atrophic areas included the caudate putamen, amygdala, auditory cortex and somatosensory cortex. Damaged, but still existing tissue was seen in the area bordering the infarct area, including the thalamus. Variability was seen in infarct size. Animals with smaller infarcts (n = 5) generally showed atrophy in the area 1 outline, animals with medium infarcts (n = 4) showed atrophy in area 1 and 2. Animals with larger infarcts (n = 3) showed atrophy in area 1, 2 and 3.
Figure 5.4. Outline of Infarct Areas Six weeks After Middle Cerebral Artery Occlusion.

Variability was seen in infarct size. Area 1 outlines the average small infarcts ($n=5$), area 2 outlines medium infarcts ($n=4$), and area 3 outlines the large infarcts ($n=3$). Animals with smaller infarcts generally showed atrophy in the area 1 outline, animals with medium infarcts showed atrophy in area 1 and 2, and animals with larger infarcts showed atrophy in area 1, 2 and 3. 1 = Area of Atrophy in Animals with Smaller Infarcts; 2 = Area of Atrophy Additional to Area A in Animals with Larger Infarcts. A = Motor Areas M1 and M2; B = Somatosensory Cortex; C = Auditory Cortex; D = Caudate Putamen; E = Amygdala Region; F = Ventral Posterolateral Thalamic Nucleus/ Ventral Posteromedial Thalamic Nucleus; G = Lateral Hypothalamic Area. Scale bar = 1mm.

Not surprisingly the brains of MCAo affected animals showed a greater volume ($\text{mm}^3$) of damaged tissue, $F(3, 20) = 21.98, p < 0.01 (M=6.14, SE = 0.92)$ when compared to sham operated animals that did not show any infarct damage ($M=0.02, SE = 0.92$). No effect on infarct volume was found for diet condition under either surgery condition, $F(3, 20) = 0.52, p > 0.05, (\text{n-3-LC-PUFA}, M=3.56, SE = 0.92; \text{basal}, M=2.62, SE = 0.92)$. 

Mechanisms of Stroke Affective Disorders
5.4.6 Acute Stroke-Related Motor Impairment Measures

Tests aimed at measuring acute motor impairments were conducted daily in the first five days following surgery. Sham operated animals consistently scored very low on motor impairment measures at all-time points. Main effects between MCAo and sham surgery conditions were found for all motor impairment measures. Forelimb flexion, $F(1, 36) = 19.32, p < 0.01$, torso twisting, $F(1, 35) = 40.29, p < 0.01$, lateral push, $F(1, 36) = 36.34, p < 0.01$, circling, $F(1, 35) = 52.52, p < 0.01$ and motor ability, $F(1, 36) = 23.04, p < 0.01$. A between group effect of diet on the MCAo surgical group was found for lateral push, $F(1, 36) = 4.34, p < 0.05$. Repeated measures for several behavioural tests showed decreasing motor impairment over time. Animals showed recovery on all measures of motor impairment from day one, until day five post-surgery, suggesting that these tests do not reflect permanent MCAo induced brain damage. Statistically significant improvement was seen in the torso twisting test, $F(3, 118) = 3.23, p < 0.05$, lateral push test, $F(4, 144) = 3.01, p < 0.05$, circling, $F(4, 140) = 4.78, p < 0.01$, and motor ability observations, $F(4, 144) = 5.65, p < 0.01$. Within group interactions were found between torso twisting and stroke condition, $F(3, 118) = 2.70, p < 0.05$, lateral push and stroke condition, $F(4, 144) = 3.01, p < 0.05$ and circling and stroke condition, $F(4, 140) = 4.78, p < 0.01$.

Comparisons of MCAo diet groups showed a within group interaction effect for diet and lateral push, $F(4, 144) = 3.83, p < 0.01$, with n-3-LC-PUFA rats showing a smaller impairment than basal fed rats, particularly on day zero post MCAo surgery (Basal $M=.20$, n-3-LC-PUFA $M=.05$). An interaction effect between surgery and diet condition was found for lateral push, $F(4, 144) = 3.83, p < 0.01$, with sham exposed rats showing minimal impairment, irrespective of diet condition. Between group surgery and diet interactions were also found for motor ability, $F(1, 36) = 5.22, p < 0.05$ and lateral push, $F(1, 36) = 4.34, p < 0.05$. Within the MCAo condition, n-3-LC-PUFA fed rats had better motor ability across all time points (day zero: Basal $M=0.55$, n-3-LC-PUFA $M=0.21$) while within the sham condition, basal fed rats showed less impairment in general motor ability on day zero (Basal $M=.1$; n-3-LC-PUFA $M=.3$).
5.4.7 Longer term Ischemia Associated Behavioural Disorders

5.4.7.1 Free Exploration Test - Locomotor hyperactivity

As outlined in the method, in the free exploration test animals were scored for locomotor hyperactivity, which was defined as emergence number, (number of times animals fully emerged from the hide box) and emergence duration (time spent fully emerged from hide box and moving around and exploring the arena over a four minute period). These measures of locomotor hyperactivity were significantly correlated with each other ($\rho = 0.82, p < 0.00$), indicating that these two outcomes are likely reflecting the same construct. Spearman correlation showed a positive correlation between total infarct volume and emergence number at weeks two ($\rho = 0.47, p < 0.05$) and six, ($\rho = 0.44, p < 0.05$) and a trend at week four, ($\rho = 0.39, p = 0.06$).

Figures 5.5 and 5.6 depict significant group differences in measures of locomotor hyperactivity according to Kruskal-Willis non-parametric analysis. Group differences in the number of full body emergences from the hide box into the open field, were seen at weeks four and six, and group differences were seen in the total time spent emerged and moving in the open field at week four. Post hoc Mann-Whitney tests show that MCAo affected animals made more emergences from the hide box into the open-field than sham affected animals at weeks four, $U=179.5, z = -2.39, p < 0.05$ (MCAo, $mdn=1$, $IQR=2.75$; Sham, $mdn=0$; $IQR=1$), and six, $U=155, z = -2.87, p < 0.01$ (MCAo, $mdn=2$, $IQR=3.5$; Sham, $mdn=0$; $IQR=1.75$). Emergence duration similarly differed between surgery groups at week six, $U=1.72, z = -2.48, p < 0.05$ with MCAo animals ($mdn=145; IQR=177$) spending more seconds emerged than sham ($mdn=8; IQR=116$) affected animals, indicative of more stroke-related locomotor hyperactivity.

A comparison of diet conditions showed that n-3-LC-PUFA animals ($mdn=15; IQR=145$) spent fewer seconds out in the open field area than basal fed rats ($mdn=95; IQR=189$) at week six post-surgery, $U=195, z = -1.99, p < 0.05$, suggesting that n-3-LC-PUFA supplementation reduced locomotor hyperactivity by six weeks post-surgery.
Figure 5.5. Kruskal-Wallis Mean Ranks of Number of Emergences into Open-Field Between Diet and Surgery Conditions.

Visual inspection of 5.5 indicates that sham surgery operated animals were less active than MCAo operated animals and that in each surgery condition, at each time point, the n-3-LC-PUFA animals emerged from the hide box less often, suggesting less hyperactivity. Note that as the Non-parametric Kruskal-Wallis test is unable to specify where differences between groups occur; this information is not specified on the graphs depicting the Kruskal-Wallis Mean Ranks. Significant differences between surgery and diet conditions are presented in text as non-parametric post hoc Mann Whitney results. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats. The Non-parametric Kruskal-Wallis test is unable to specify where differences between groups occur, and therefore this information is not specified in the graphs depicting the Kruskal-Wallis Mean Ranks.
Visual inspection of Figure 5.6 indicates that sham surgery operated animals spent less time moving around in the open-field arena compared to MCAo operated animals. Furthermore, in each surgery condition, n-3-LC-PUFA diet fed animals spent less time moving around the open-field arena than do basal diet fed animals. Note that As the Non-parametric Kruskal-Wallis test is unable to specify where differences between groups occur; this information is not specified on the graphs depicting the Kruskal-Wallis Mean Ranks. Significant differences between surgery and diet conditions are presented in text as non-parametric post hoc Mann Whitney results. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats. The Non-parametric Kruskal-Wallis test is unable to specify where differences between groups occur, and therefore this information is not specified in the graphs depicting the Kruskal-Wallis Mean Ranks.
5.4.7.2 Free Exploration Test - Classically a Test of Anxiety-like Behavior

Animals were scored for anxiety-like behaviour (percentage of total emergence time spent in the centre of the open-field arena) over a total time of four minutes. The above outlined measures of locomotor activity were both found to be negatively correlated with the percentage of total emergence time spent in the centre of the open-field arena, (emergence number $[\rho = -0.35, p < 0.01]$ and duration of time spent moving in the open-field arena $[\rho = -0.45, p < 0.01]$), indicating that they are unlikely to reflect anxiety-like behaviour in operated animals.

A further non parametric Kruskal-Willis test indicated that surgery and diet groups differed in the percentage of total emergence time spent in the centre of the open field arena at four weeks post-surgery, as compared to side wall of the arena, $H (3) = 7.79, p < 0.05$. At six weeks post-surgery, an effect for a difference in the percentage of total emergence time spent in the centre of the open field arena approached significance, $H (3) = 7.25, p = 0.06$, Figure 5.7 depicts these differences. The appropriate post hoc Mann Whitney test indicated that rats supplemented with n-3-LC-PUFA spent significantly more time in the centre of the arena at week four, $U = 16.20, z = -2.72, p < 0.01$ (n-3-LC-PUFA, $mdn=66, IQR=80$; Basal, $mdn=0, IQR=51$), and week six, $U = 161.5, z = -2.65, p < 0.01$ (n-3-LC-PUFA, $mdn=70, IQR=40$; Basal, $mdn=40, IQR=56$), compared to basal diet fed rats. Post hoc tests showed no differences between surgery conditions.
Visual inspection of Figure 5.7 indicates that n-3-LC-PUFA diet fed animals spent more time in the centre of the arena, than did basal diet fed animals. No effects of surgery condition on percentage of time spent in the centre of the arena can be seen. Note that as the Non-parametric Kruskal-Wallis test is unable to specify where differences between groups occur; this information is not specified on the graphs depicting the Kruskal-Wallis Mean Ranks. Significant differences between surgery and diet conditions are presented in text as non-parametric post hoc Mann Whitney results. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats.
5.4.7.3 Spatial Displacement Recognition – Classically Considered a Test for Spatial Memory Deficits

Spatial mapping abilities were assessed using the spatial displacement recognition test. No differences in time spent exploring the displaced object were seen between surgery, $F(1, 44) = 0.14, p = 0.71$, or diet conditions, $F(1, 44) = 0.09, p = 0.77$ at any testing time point post-surgery (data not shown). All animals were found to explore the displaced object equally.

5.4.7.4 Novel Object Exploration – Classically Considered a Test of Approach Avoidance Behaviours

Approach–avoidance conflict was studied using the novel object exploration test. Group differences in mean rank according to Kruskal-Willis non parametric analysis are presented in Figure 5.8. Surgery and diet groups differed in novel object exploration time at four weeks, $H(3) = 9.00, p < 0.01$, and six weeks post-surgery, $H(3) = 12.50, p < 0.01$.

Post hoc comparisons between surgery conditions showed MCAo affected animals explored the novel object less than sham operated animals at week four post-surgery, $U = 175, z = -2.32, p < 0.05$ (MCAo, $mdn = 6$, $IQR = 17$; Sham, $mdn = 17$, $IQR = 42$). Animals did not demonstrate any MACo related motor deficits that might have been responsible for changes in exploration behaviours.

Post hoc comparison of diet conditions showed that the n-3-LC-PUFA supplemented animals in each surgery condition spent more significantly more time exploring the novel object than the basal fed animals at week six, $U = 144, z = -2.95, p < 0.01$ (n-3-LC-PUFA, $mdn = 31$, $IQR = 23$; Basal, $mdn = 14$, $IQR = 15$). At weeks four, an effect for greater exploration of the novel object by n-3-LC-PUFA supplemented animals compared to basal diet fed animals approached significance, $U = 198, z = -1.85, p = 0.06$ (n-3-LC-PUFA, $mdn = 17$, $IQR = 28$; Basal, $mdn = 5$, $IQR = 18$).
Visual inspection of Figure 5.8 indicates that at both time points sham surgery-operated animals supplemented with n-3-LC-PUFA diet spent more time exploring the novel object than sham operated basal diet fed animals. MCAo surgery-operated animals supplemented with n-3-LC-PUFA diet appear to have spent slightly more time exploring the novel object than MCAo operated basal diet fed animals. Note that As the Non-parametric Kruskal-Wallis test is unable to specify where differences between groups occur; this information is not specified on the graphs depicting the Kruskal-Wallis Mean Ranks. Significant differences between surgery and diet conditions are presented in text as non-parametric post hoc Mann Whitney results. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats.
5.5 Discussion

We aimed to investigate potential protective effects of n-3-LC-PUFA supplementation on surgery induced acute and longer term sickness behaviours, acute motor impairment and anxiety-like and locomotor behaviours, when either on a normal, nutritionally sound diet, or n-3-LC-PUFA supplemented diet. To our knowledge, this is the first rodent study to explore the effects of n-3-LC-PUFA supplementation against persistent anxiety-like and locomotor behaviours post-stroke.

Surprisingly, increased reperfusion related haemorrhagic risk in MCAo n-3-LC-PUFA supplemented rats occurred, even after only a short 1-week acclimation period. One interpretation from this data is that although n-3-LC-PUFA supplementation may reduce stroke-related affective and behavioural disorders; it renders ischemic stroke survivors at risk of haemorrhagic transformation. Stroke survivors are already considered particularly high risk for haemorrhagic transformation[385-388] and n-3-LC-PUFAs have anti-thrombotic effects [389] and contribute to reduced platelet coagulation [390]. Therefore, some authors argue that it is prudent to ensure that patients who are classified as high risk for haemorrhage discontinue n-3-LC-PUFA consumption [391]. Others suggest that the empirically supported benefits of n-3-LC-PUFA supplementation simply outweigh the yet unproven potential risks of n-3-LC-PUFA supplementation [389]. We are unable to specify, based on our findings and the conflicting findings of previous research, if the increased risk of haemorrhagic bleeding seen in -3-LC-PUFA MCAO operated fed rats is a clinically relevant finding. Further research is required to clarify the potential risks of n-3-LC-PUFA supplementation on haemorrhagic bleeding in vulnerable populations.

In order to explore whether the increased hemorrhagic risk seen among n-3-LC-PUFA supplemented animals in the present study may be specific to hooded Wistar rats, we sourced data relating to bleeding times, rates of platelet aggregation and responsiveness to dietary EPA/DHA supplementation, in hooded Wistar rats compared to other rat strains. We searched PubMed, Science Info and Google Scholar using the keywords “hooded Wistar” AND “platelet aggregation,” or “bleeding,” AND “fatty acid,” “docosahexaenoic acid,” or “eicosapentaenoic acid,” “fish oil,” “omega 3.” We were unable to identify any relevant articles. We did find research indicating that two weeks of supplementation with fish oil prior to intracerebral haemorrhage is associated with
increased cerebral blood flow in the peri-infarct region and contralateral hemisphere at six hours after surgery, in male Sprague–Dawley rats [392]. Regarding shorter term supplementation, we found one article indicating that a high omega 6 Arachidonic Acid was negatively associated with gastric haemorrhage, and that seven days of fish oil supplementation decreases Arachidonic Acid in liver phosphatidylcholine. Those authors speculated that fish oil may increase gastric bleeding due to modulating the permeability of cell membranes in the gastric mucosa [393] in their inbred rats that were fed 7.5% fish oil diet (our animals were fed only 1.66% total omega 3). Given the limited research, we are unable to specify if hooded Wistar rats differ in bleeding times, rates of platelet aggregation and responsiveness to dietary EPA/DHA supplementation compared to other rat strains.

In surviving animals, from week 1 to 6 post–surgeries, all animals increased in bodyweight, food and water consumption. Prolonged changes and decreases in eating and drinking behaviours are a feature of clinical depression [394] and sickness behaviours in animals or humans [295]. Therefore it is not surprising that sham animals had higher food and water intake than MCAo affected animals from week 1 until week 6 post-surgery. As hypothesised, n-3-LC-PUFA supplemented animals similarly consumed more water than basal fed rats from week one until week six post-surgery. Our unpublished data shows non-surgery affected, normally reared, n-3-LC-PUFA supplemented rats, do not consume more food and water than basal diet fed rats. Therefore, it is unlikely that n-3-LC-PUFA fed animals drink more due to increased salt or dietary change. In the present study increased water consumption among n-3-LC-PUFA supplemented rats after surgery could reflect better recovery from surgery, as animals rapidly replace surgery associated blood volume loss.

Not surprisingly, MCAo affected animals showed acute motor impairment in the five days following surgery; while sham operated animals did not. Symptom severity was significantly reduced from day zero to day 5 post-surgery. MCAo operated animals supplemented with n-3-LC-PUFA showed better motor ability and less left lateral push disability from day zero to day 5 post-surgery, compared to basal fed MCAo animals. Motor impairment scores in the first five days following MCAo did not correlate with infarct size as determined at six weeks post-surgery, indicating that early motor impairments is more likely to reflect short term MCAo related ischemia and associated
oedema rather than longer-term tissue atrophy. Additionally, acute motor deficits were not associated with any obvious histological damage to motor areas M1 and M2 viable at six weeks post-stroke (see Figure 5.1). These findings suggest that the commonly employed motor impairment testing, may not be the most appropriate model to behaviourally assess longer-term MCAo related cellular damage in rodents.

In weeks four and six post-surgery, MCAo affected animals showed increased locomotive behaviours (as defined by the number of emergences into the open-field from the hide box, and the time spent moving in the open-field) compared to sham operated animals, which is consistent with previous research [395]. MCAo exposed animals emerged more often from the hide box into the open field and spent more time moving around in the open field area, than sham-operated rats.

Previous authors have speculated that MCAo related locomotor deficits might arise from an inability to habituate to unfamiliar environments, possibly related to spatial mapping difficulties induced by hippocampus cell death [27, 28, 315, 377]. However, the present research does not indicate ‘habituation’ deficits in ischemia-affected animals, as we found no difference between stroke and sham affected animals in the spatial displacement recognition test at two, four and six weeks, post-surgery. It is possible that ischemia associated cell proliferation in the hippocampus may have already occurred by the time the first spatial displacement recognition test was conducted, at two weeks post-surgery, and corrected any spatial mapping disabilities that may have been previously present. Indeed, in rodents, CA1 neurons are seen to completely repopulate by one-month post infarct [396]. This is associated with improved spatial learning and memory functions, as assessed using a water-maze [29]. Alternatively, Plamondon and Khan, [397] suggest that ischemia affected rodents do not have habituation deficits at all, but rather that the testing periods often employed are not long enough to allow the animal enough time to become familiar with its environment [397].

In the present study extensive tissue damage in the somatosensory cortex following the MCAo surgery (See Figure 5.4) may have contributed to the changes in locomotory behaviours, as pyramidal neurons of the second and third layers of the somatosensory cortex project to the motor cortex [27]. Additionally, previous research has associated amygdala damage with a decreased anxiety responses, that has also been associated
with increased exploratory activity [398]. While extensive damage was seen in the amygdala of MCAo affected animals in the present study, the current results do not suggest that MCAo operated animals showed less anxiety-like behaviours in the free exploration test than sham operated animals, given that there was no difference in the total percentage of time spent by the surgery groups in the central area of the arena of the open-field, over a four minute period. Additionally, we found a negative correlation between both the numbers of emergences from the hide box into the open-field, and the time spent moving around in the open field and the percentage of emergence time spent in the centre of the open-field arena. The percentage of emergence time spent in the centre of the open-field arena is widely interpreted to reflect anxiety-like behaviour, indicating that the change in locomotion is unlikely to result from a change in anxiety-like behaviours in surgery affected animals.

Figure 5.8 suggests that MCAo operated animals explored the novel object less than sham operated animals, irrespective of diet condition. Post hoc comparisons between surgery conditions confirmed this for MCAo vs sham operated at week four post-surgery. These findings are consistent with previous research that indicates that conditions of unpredictable chronic stress stressful interventions, induces sickness behaviour, inhibits novel object exploration [337]. Interestingly, genetically knocking-out the depression associated dopamine D4 receptor in mice has also been shown to be associated with less approach/explore behaviours in the novel object exploration test [332]. Figure 5.8 additionally indicates that animals supplemented with n-3-LC-PUFA explored the novel object for a longer duration than basal diet fed animals indicating more approach/exploration behaviour. Post hoc tests-confirmed that n-3 LC-PUFA supplemented animals spent significantly more time exploring the novel object at six weeks post-surgery than did basal diet fed animals. Consistently, Figure 5.7 suggests that animals supplemented with n-3-LC-PUFA spent a greater percentage of total emergence time in the centre, classically considered the anxiety provoking region of the open-field, as compared to basal diet fed animals, indicating less anxiety-like behaviour. Post hoc tests-confirmed this.

Figure 5.8 further suggests that at six weeks post-surgery, MCAo operated n-3-LC-PUFA fed animals continued to explore the novel object less than sham operated n-3-LC-PUFA fed animals, however as previously outlined, the between diet group with
surgery condition possible effects were unable to be statically tested using post hoc comparisons. In Figure 5.8, no obvious difference in mean ranks can be observed in the exploration time of the novel object between basal diet fed MCAo and sham operated animals. The above may suggest that MCAo surgery does not prevent the beneficial effect of n-3-LC-PUFA supplementation on the exploration behaviour of a novel object. Further beneficial effect of diet condition, irrespective of surgery condition are illustrated in Figure 5.6 and 5.7. Figure 5.6 illustrates that those animals that received n-3-LC-PUFA supplementation showed less total emergence duration time than basal fed animals, six weeks post-surgery. This result suggests that the n-3-LC-PUFA diet reduced hyperactive motor deficits. Figure 5.7 illustrate that n-3-LC-PUFA fed animals spent more time in the centre of the open filed compared to basal diet fed animals. There was no difference between MCAo and sham operated animals in the percentage of emergence time spent in the centre of an open-field arena. These results have been interpreted to indicate reduced locomotor hyperactivity and reduced anxiety-like behaviour in n-3-LC-PUFA fed animals compared to basal diet fed animals. These may be a clinically important findings as they suggests that the positive effect of n-3-LC-PUFAs on affective and depressive like behaviours, such as novel object exploration behaviour, is independent of MCAo. Our findings are consistent with previous research in non surgery operated animals, demonstrating oral administration of omega 3 fatty acids reduce depressive like behaviours in the rodent model (reduced immobility in the forced swim test and tail suspension test) [345] as well as previous research indicating that anti-depressive interventions, such as electroconvulsive treatment, increase novel objects exploration, in the rodent model [339].

A major limitation of the present research is our choice of investigation of concentrations of EPA and DHA in brain tissue following diet supplementation. Unfortunately we did not investigate serum or vascular EPA and DHA expression. Previously, EPA administration post MCAo has been shown to improve local cerebral blood flow and metabolism in the rat when administered for four weeks via gavage [399]. In humans, 12 weeks of dietary supplementation with n-3-LC-PUFA is associated with an improved cerebral blood flow response to cognitive tasks [400] while 4 weeks of EPA dietary supplementation has been shown to be associated with more efficient neural recovery of magnocellular-like visual responses [401]. Serum and vasculature tissue have been shown to be more readily responsive to dietary EPA/DHA
supplementation than brain tissue [402]. Thus it is possible that the behavioural effects of n-3-LC-PUFA dietary supplementation may relate to the likely effects of PUFA supplementation on cerebral blood flow. The present findings also indicate an increased reperfusion related haemorrhagic risk in MCAo n-3-LC-PUFA supplemented rats, after only a short acclimation period. Further research is required to clarify the potential risks of n-3-LC-PUFA supplementation on haemorrhagic bleeding in vulnerable populations. In surviving animals, a positive influence of n-3-LC-PUFA supplementation on both locomotor hyperactivity and anxiety-like behaviours is present.
Chapter 6  Fish Oil Supplementation Associated with Decreased Cellular Degeneration and Increased Cellular Proliferation Six Weeks after Middle Cerebral Artery Occlusion in the Rat

6.1 Abstract

Background: Polyunsaturated fatty acids are both neuroprotective and have anti-depressive effects. The influence of polyunsaturated fatty acid dietary supplementation on cell death and proliferation in adult male hooded Wistar rats are yet to be investigated. The correlation between cell death and proliferation and the presentation of anxiety-like and hyperactive locomotor behaviours previously reported post MCAo in these animals is similarly yet to be studied.

Methods: Male hooded wistar rats were exposed to middle cerebral artery occlusion or sham surgeries. Cell death and proliferation was studied in the dentate gyrus, Cornu Ammonis region of the hippocampus, and the thalamus of the ipsilesional and contralesional hemispheres. The amount of cell death and proliferation was correlated with anxiety-like and hyperactive locomotor behaviours previously reported post MCAo in these animals were also studied. Animals were sacrificed at six weeks post-surgery for molecular or histological analysis. Non-localised protein expression was studied using Western Blot techniques and localised protein expression was studied using immunohistochemistry.

Results: We found that dietary supplementation with n-3-LC-PUFA decreased cell degeneration and increased cell proliferation in the thalamic region of the contralesional hemisphere at six weeks post-stroke. Middle cerebral artery occlusion associated cell degeneration in the hippocampus and thalamus positively correlated with anxiety-like and hyperactive locomotor behaviours.

General Conclusion: Fish Oil Supplementation appears to be to have cellular protective effective weeks after middle cerebral artery occlusion in the rat, which may affect behavioural outcomes.

Key Words: Apoptosis, Polyunsaturated Fatty Acids, Contralesional Hemisphere, Stroke, Anxiety, Depression.
6.2 Introduction

Ischemic stroke is experienced by 15 million people worldwide each year [1]. Stroke is a personal and public health and financial burden, thus it is important to achieve the best possible outcomes. This requires an understanding of the biological mechanisms that influence outcome. In rodent models, ischemia associated central nervous system cell death, and proliferation, influence functional recovery and outcome [29, 314, 403]. Therefore, much research in animal models has focused on attempting to decrease cellular degeneration, and increase cellular proliferation, after infarction [13, 396, 404-407].

In clinical populations, stroke is associated with unusually high incidences of behavioural and affective disorders, including hyperactive delirium [6], anxiety at one year after stroke [3, 4], clinical depression at a year and a half after stroke [5]. In both clinical populations and animal models, cell death in relevant brain regions is involved in the aetiology of these behavioural and affective disorders, or in animal models behaviours believed to reflect these affective and behavioural disorders [6, 8, 27-29, 38, 408, 409]. In clinical populations, the presentation of these affective disorders negatively influence functional recovery, at one year post-stroke [5].

Long chain omega-3 polyunsaturated fatty acids (n-3-LC-PUFA) have been reported to be associated with reduced infarct size, three days after middle cerebral artery occlusion (MCAo) in cats, when diet was supplemented for 24 days prior to surgery [410]. In rodents, three sequential injections of omega-3 alpha-linolenic acid, promoted neural plasticity at 10 days post MCAo surgery [411]. To our knowledge, the influence of n-3-LC-PUFA dietary supplementation, which best models the method of human consumption, on stroke related cellular degeneration and proliferation, is yet to be investigated in either animal models or clinical populations. We have previously shown that six weeks of dietary supplementation with n-3-LC-PUFA is associated with reduced locomotor hyperactivity and anxiety-like behaviour in the rat at six week postsurgery (see chapter 5) (the methods and results of these behavioural tests are outlined briefly in the methods section). We hypothesise that these behavioural changes result in part, from the cellular protective effects of n-3-LC-PUFA supplementation, after stroke or sham surgery. Thus the present study aimed to investigate the influence of n-3-LC-PUFA dietary supplementation, on cell death and proliferation, at six weeks post-
surgery in the male hooded Wistar rat. We additionally aim to study correlations between cell death and proliferation, and the anxiety-like and locomotor behaviours previously seen in these animals (see chapter 5).

Western Blot analysis was performed to confirm the presence of Caspase-3 in whole brain tissue, across all groups of animals. Localised protein was then studied in specific brain regions of interest. In rodent models, MCAo induced cortical infarction produces secondary cellular damage in the thalamic region of the ipsilesional hemisphere, at as late as nine months post-surgery [11, 39-41], possibly due to retrograde degeneration of thalamocortical projections [42, 43]. The thalamus plays a key role in the information processes necessary for motor control [33-37]. Accordingly, hyperactive delirium (increased motor activity with agitated behaviour) after stroke is associated with cellular damage in the thalamus, in clinical populations [6, 38, 409] and cellular damage in the Cornu Ammonis area (CA1) region, in animal models [27-29]. Previous clinical and animal research indicates that after stroke the brain can undergo a spatial remapping or neuroplasticity, whereby contralesional undamaged brain regions are capable of taking over functions of the affected areas to some extent [412-415]. Thus in the present study, cellular proliferation in the thalamic region of the contralesional hemisphere, that may represent functional spatial remapping post MCAo, and cellular proliferation in the thalamic region of the ipsilesional hemisphere, were studied using immunohistochemistry.

Hippocampus cells are considered to be particularly vulnerable to cellular degeneration after cerebral ischemia [30, 31]. The molecular mechanisms underlying this hippocampus vulnerability are not yet well elucidated, and a number of theories currently exist, including an increased sensitivity to over secretion of glucocorticoids, which are a class of steroid hormones that induce apoptosis [416]. A review of these theories is beyond the scope of this study and so will not be discussed, for further reading please see [416-419]. Additionally, the hippocampus is involved in adult neurogenesis, and ischemia induced cellular proliferation has been widely demonstrated to occur in the hippocampus [32]. In the rat model, cell proliferation is seen in the hippocampus for up to two months post-stroke [420]. Finally, a decrease in neurogenesis and an increase in neurodegeneration, in the CA1 and dentate gyrus (DG) of the hippocampus, are widely implicated in the aetiology of depressive and anxiety
related or anxiety-like disorders [8, 408]. Thus in the present study, localised cell death and proliferation in the DG and CA1 of the hippocampus were also studied using immunohistochemistry.

Caspase-3 has been widely employed as a marker of cell death after traumatic brain injury [356], and MCAo [357]. Caspase activation has been observed in rodent brain tissue from as early as a few hours post-stroke, to as late as several weeks post-stroke [31, 357-360]. Caspase-3 binds also to Amyloid Precursor Protein [APP] (an integral membrane protein) and Amyloid Beta (Aβ) (a peptide processed from APP) [40, 41, 421-423], that has been observed in the thalamic region, as late as nine months after stroke in the rodent model [41]. Thus the number of Caspase-3-like immunoreactivity (Caspase-3-ir) labelled cells in thalamic regions and the CA1 and DG regions of the hippocampus, was considered to be an appropriate marker to study ischemia induced cellular degeneration, at six weeks after surgery.

Although identification of the proliferation profiles of different cell types and at different times post-stroke and the relationship between these are important, we have chosen to first establish if n-3-LC-PUFA dietary supplementation influences cell proliferation in general. Thus we examined the relationship between n-3-LC-PUFA supplementation and the number of Ki-67-like immunoreactivity (Ki-67-ir) labelled cells, as a general marker of cellular proliferation [354].

We hypothesised that MCAo surgery compared to sham surgery, would be associated with increased expression of Caspase-3 and Ki-67-ir labelled cells and that dietary supplementation with n-3-LC-PUFA would influence the expression of Caspase-3 and Ki-67-ir labelled cells, in the DG, CA1 region of the hippocampus, and the thalamic region of the ipsilesional and contralesional hemisphere. We further hypothesised that MCAo surgery would similarly be associated with increased whole brain expression of Caspase-3, detected by Western Blot quantification technique, and that dietary supplementation with n-3-LC-PUFA would influence Western Blot expression of whole brain Caspase-3. We also hypothesised that cell death in the thalamus, CA1 region of the hippocampus and DG would be correlated with the presentation of hyperactive locomotor and anxiety-like behaviours, previously observed in these same animals at six weeks post-surgery (see chapter 5).
6.3 Materials and Methods

6.3.1 Experimental Design

Histological Analysis was conducted on the tissue of 48 rats that were randomly allocated to either the basal diet ($n = 25$) or a high n-3-LC-PUFA diet ($n = 23$) and further randomised to either sham or MCAo surgery condition ($n = 11$, n-3-LC-PUFA diet plus MCAo; $n = 13$, basal diet plus MCAo; $n = 12$, n-3-LC-PUFA diet plus sham surgery; $n = 12$, basal diet plus sham surgery). The surgeon conducting the MCAo procedure (MP) was not blinded to animals’ diet condition. As the behavioural tests conducted were new to the laboratory and likely effect size was unknown, a formal power calculation was not performed prior to the commencement of the experiment, instead group sizes were chosen based on previous research investigating similar behavioural outcomes [340].

6.3.2 Animals

Male Hooded Wistar rats (body weight $= 337$, $SD = 36.5$, age 12-14 weeks) were supplied by Laboratory Animal Services, The University of Adelaide and housed in a 12:12 h light-dark cycle (lights on at 07:00 h) at $21 \, ^\circ C \pm 2$ and acclimated to diet and housing during the week prior to surgery. Dietary supplementation continued until the sixth week post-surgery, when the animal was sacrificed for tissue collection. Surgeries were conducted in the light phase. Food and water was available ad libitum. Procedures were approved by the Austin Health Research Ethics Unit (10/3865) and conducted in accordance with the Australian Code of Practice for the use of animals for scientific purposes [378].

6.3.3 Dietary Regime

Supplemented animals were fed a ‘5% Fat High N3 Semi-Pure Modification of AIN93G Rodent Diet’ (Cat No. SF03-076, Speciality Feeds, Glen Forest Western Aust) containing 2700mg of eicosapentaenoic acid and 11900mg of docosahexaenoic acid per 1 kg of food. Basal diet fed animals were maintained on nutritionally comparable standard rodent chow (Cat No. AIN93G, Speciality Feeds Glen Forest Western Aust). To avoid excessive animal weight loss within the first week following surgery, animals were acclimated to their respective diets in the week prior to surgery. Animals were maintained on their respective diets until sacrifice at six weeks post-surgery.
6.3.4 Middle Cerebral Artery Occlusion (MCAo)

The Middle cerebral artery occlusion surgery was conducted in accordance with the method outlined previously (chapter 5).

6.3.5 Behaviour Testing

The battery of behavioural tests was administered and recorded on video in the sixth week post-stroke and has been described previously (chapter 5). To summarise methodologies, hyperactive locomotor behaviours were studied using the free exploration test, where a rat was habituated to a small black hide box and then given access to a larger illuminated transparent open-field, for a period of four minutes. Locomotor hyperactivity was defined as a high number of emergences from the hide box into the open-field arena and the duration of time spent moving around in the open-field arena [27, 28, 315]. Anxiety-like behaviour was defined as the percentage of emergence time spent along the outer wall of the open-field arena, compared to time spent in the centre, which was considered to be the more anxiety provoking area of the arena [321]. Additionally, the total time spent exploring a novel object while in a familiar environment, was recorded and interpreted to indicate approach versus avoidance behaviour. More approach behaviour, or longer time spent exploring the novel object, was interpreted to reflect lower anxiety-like behaviours [328, 331-334].

6.3.6 Cellular Markers

For immunohistochemistry analysis, cell death was defined as the number of Caspase-3-ir labelled cells and cell proliferation was defined as the number of positively stained Ki-67-ir labelled cells. As outlined in the introduction, the brain regions studied using immunohistochemistry were the CA1 region, the granule cell layer of the DG of the hippocampus, and the thalamic region of the ipsilesional and contralesional hemisphere. For Western Blot analysis, cell death expression in whole brain tissue was defined as the total mean protein expression of Caspase-3, normalised to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which is one of the most commonly used loading housekeeping genes and often employed as a loading control gene, for the purpose of Western Blot protein analysis [362, 363]. Additionally, GAPDH has larger molecular weight than Caspase-3, ensuring that the bands observed for each antibody are distinct and that the expression levels are quantifiable.
6.3.7 Tissue Collection

At six weeks post-surgery, animals were killed via one of two methods. The first method of tissue collection was for the purpose of immunohistochemistry and infarct analysis. After anaesthesia using Isoflurane, (Baxter) animals were transcardially perfused using a perfusion pump (Peri-Star Pro 4-channel, high rate pump, World Precision Instruments, Hilton, SA, Aust) with 18 ml of saline (1.8%) per min, for 5 min, followed by 18 ml of paraformaldehyde, (PFA; 4%) per min, for 15 min. Perfused brain tissue was post fixed in PFA (4%) for 24 hours. Brains were then placed in a 30% sucrose solution until paraffin embedding. Brains were cut at 2 mm coronal sections using an acrylic rat brain matrix, transferred to plastic cassettes between two biopsy sponge pads to prevent curling and moving of the tissue and processed using a closed linear tissue processing system (MEDITE GmbH, Wollenweberstr, Burgdorf, Germany; 1 hour each in: 4% buffered formalin [40°C], 4% buffered formalin [40°C], 70% ethanol [40°C], 96% ethanol [40°C], 100% ethanol [40°C], 100% xylene [40°C], 100% xylene [40°C], 50% xylene/50% ethanol [40°C], 100% ethanol [40°C], 100% ethanol [40°C], 100% xylene [40°C], 100% Paraffin wax [60°C], 100% Paraffin wax [60°C], 100% Paraffin wax. Coronal sections were embedded in molten paraffin wax using a heated paraffin embedding module, (Leica-microsystems, Ernst-Leitz-Straße, Wetzlar, Germany) cooled to 4°C using a cold plate for modular tissue embedding (Leica-microsystems, Ernst-Leitz-Straße, Wetzlar, Germany) then kept at room temperature until cutting. Paraffin sections (7 µm) were cut at room temperature using a rotary microtome, (Leica-microsystems, Ernst-Leitz-Straße, Wetzlar, Germany) suspended at 40 °C in a tissue flotation bath (MEDITE GmbH, Wollenweberstr, Burgdorf, Germany) and attached to silane coated slides (Cat No. CS2460100MK, Mikro-glass, Grale Scientific, Melbourne, Aust). Tissue was dried at 49 °C on a solid state control warming tray (Ratek, Melbourne, Vic, Aust.) and incubated (Contherm Series Five, Wellington NZ) at 32°C, for at least 24 h. Paraffin embedded tissue was used for both immunohistochemistry and infarct analysis.

The second method of tissue collection was for the purpose of whole brain Western Blot analysis. After anaesthesia using Isoflurane (Baxter), whole brain was collected via decapitation using a guillotine, placed immediately into liquid nitrogen (Liquid Nitrogen Services Pty Ltd, Melbourne, Vic, Aust) and stored at –80°C until analysis.
6.3.8 Infarct Analysis

The method of infarct analysis has been described previously (chapter 5).

6.3.9 Immunohistochemistry

Slides were incubated at 60°C to remove paraffin wax (Contherm Series Five, Cat No. 105M, Lower Hutt, Wellington NZ) for 15 min followed by histosolve (2 x 20 min) and rehydration using ethanol washes (1 min x 100%, 70% and 50%). Tissue was washed in dH2O (3 x 5 min). For antigen retrieval tissue was soaked in 95 °C 10 mM Sodium citrate buffer (0.294% Tri-sodium citrate [Cat No. 27833.294, Prolabo, Palée, Paris, France] with 0.05% of Tween 20 [Cat No. P7949-500ML, Sigma-Aldrich, St. Louis, MO, USA] distilled water (dH2O) in a water bath (BMUTE, Froilabo-Firlabo Emerainville, France) for 30 min. Tissue was washed in phosphate buffered saline (PBS) (0.1 M) and 0.3% Triton-X (Cat No. T9284, Sigma-Aldrich, St. Louis, MO, USA) (3 x 5 min) blocked for endogenous peroxidises using 0.3% hydrogen peroxide (H2O2; Cat No. 7722-84-1, ChemSupply, Gillman, SA, Aust) in PBS for 60 min, before being washed in PBS (3 x 5 min). Tissue was blocked with 10% normal horse serum (NHS) (Cat No. 26050-088, Invitrogen, Melbourne, Victoria Aust) in PBS, and incubated at 4 °C overnight with the primary antibody (Anti-Ki-67 antibody [SP6] - Monoclonal Proliferation Marker, Cat No. Ab16667, Sapphire Bioscience Pty Ltd, Waterloo, NSW, Aust, 1:1000; or Polyclonal Cleaved Caspase-3 [Asp175] Antibody, Cat No. 9661L, Genesearch Pty Ltd, Arundel Qld, Aust, 1:1000) in 2% NHS + 0.3% Triton X-100 in 0.1M PBS. The next day, tissue was washed in PBS (3 x 5 min), incubated with the secondary antibody (Biotinylated Horse Anti-Rabbit IgG Antibody, cat No. BA-1100, ABACUS ALS, East Brisbane, QLD, Aust) in diluent of 2% NHS, 0.3% Triton X in 0.1M PBS for 60 min. Tissue was again washed in PBS 0.3% Triton-X (3 x 5 min) and incubated with an avidin biotin complex (ABC) solution (2 drops of A + 2 drops of B per 5 ml 0.1M PBS) (VECTASTAIN Elite ABC Kit, Cat No. PK-6100, ABACUS ALS, East Brisbane, QLD, Aust) for 60 min. The ABC reagent was made up 30 min before use. Tissue was washed in PBS and 0.3% Triton-X (3 x 5 min), incubated on an orbital shaker, (Ratek Orbital Mixer, Cat No. EOM5, Melbourne, VIC, Aust) with 1% at 50 mg/ml of 3-3’diaminobenzidine tetrahydrochloride; (DAB) (Cat No. D-8001, Sigma-Aldrich, St. Louis, MO, USA) in a solution containing 44.5% dH2O with 54.5% 0.2 M neutral phosphate buffer (NPB) [24.96g NaH2PO4 in 90.34g

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NaH$_2$PO$_4$ in 4L of dH$_2$O for 20 min. H$_2$O$_2$ (0.01%) (Cat No. 7722-84-1, ChemSupply, Gillman, SA, Aust) in PBS was added to the DAB, and tissue was incubated for a further 15 min, before being washed with PBS and 0.3% Triton-X (3 x 5 min). Tissue was background stained using Hematoxylin and Eosin as previously described (see chapter 5). Primary antibody control sections (omission of the primary antibody only) were run to confirm the specificity of primary antibody binding to the antigen. Secondary antibody control sections (omission of the secondary antibody only) were run to confirm the label was specific to the primary antibody. Results were analysed by an observer blind to experimental treatment condition.

Photomicrographs were taken using a Nikon 90i upright microscope (Coherent Scientific, Hilton, SA, Aust) and a Nikon DS-Fi1 digital camera (Coherent Scientific) and stored as a JPEG 2000 images (1280 x 960 pixels). A standard stereotaxic rat brain atlas [424] was used to identify relevant anatomical regions (See Figure 6.1). Ki-67-ir appeared as an orange/brown precipitate localized to cell nuclei. Caspase-3-ir appeared as an orange/brown precipitate localized to both cell nuclei as well as dense plaque like deposits morphologically consistent with stroke related APP and Aβ staining [40, 41, 421-423] (See Figure 6.14).

Cell number was determined using NIS Elements software (Version 3.2; Coherent Scientific). Cell counting within each photograph was restricted to a predefined area (1064.7 µm x 521.5 µm). Two photographs were taken of the CA1 region and DG of each animal and an average was derived from these two scores. For the thalamic region of the ipsilesional and contralesional hemisphere, photographs were taken of the areas outlined in Figure 6.1, and an average cell number was derived from these. Cell Number was determined using a threshold parameter where only pixels with a given range of colour, identified as representing Caspase-3-ir and Ki-67-ir cells, were included in the analysis. An area restriction of 40 µm$^2$ was set to ensure that dark stained objects; too small to be cell nuclei were not counted in the case of Ki-67-ir cells. In the case of Caspase-3 an area restriction was not set, to ensure that the smaller Caspase-3-ir pigmentation and plaques were detected. Automated counting was significantly correlated with manual counting, $r^2 = .91$. Figure 6.1 outlines the areas from which photographs for immunohistochemistry analysis were taken. Areas include the Mediodorsal Thalamic Nucleus (MDM), the Posterior Thalamic Nuclei Group (Po), the
Ventral Posteromedial Thalamic Nucleus (VPM), the Ventral Posterolateral Thalamic Nucleus (VPL), and the Ventromedial Thalamic Nucleus (VM). Images taken from these regions are analysed together as a single thalamic region outcome, and thus, will collectively be referred to as the thalamic region throughout the results of this paper. Images were also taken from the CA1 and granule cell layer of DG region of the hippocampus.

6.3.10 Western Blot

For sample lysis, tissue was pulverised in liquid nitrogen using mortar and pestle. 1x Laemelli Sample Buffer (63 mM (w/v) Tris-Hydrochloride [Cat No. T-3253, Sigma Aldrich, St. Louis, MO, USA] 10% (v/v) Glycerol, [Cat No. G7757, Sigma Aldrich, St. Louis, MO, USA] 2% (w/v) Sodium Dodecyl Sulfate, [Cat No. 811034, MP Biomedicals Aust, Seven Hills, NSW, Aust] 0.0025% (w/v) Bromophenol Blue [Cat No. B3269, Sigma Aldrich, St. Louis, MO, USA], 7.5% (v/v) 2-mercaptoethanol, [Cat No. 63689, Fluka, Sigma Aldrich, St. Louis, MO, USA]) was heated to 95°C using a heating block (EL-02, Major Science, Saratoga, CA, USA) and added to pulverised tissue samples. Samples were then heated for 5 min at 95°C (EL-02, Major Science, Saratoga, CA, USA), sonicated (2 cycles of 15 secs) using a hand held sonicator (Sonics, Vibra Cell VCX 130, Sonics & Materials Inc., Newtown, CT, USA) and centrifuged at room temperature at 15,000 rpm for 10 min. The pellet was discarded and supernatant stored at –20°C.

Protein concentrations of samples were determined using the RCDC Protein Assay (Bio-Rad Laboratories Pty. Ltd. Gladesville, NSW, Aust). Aliquots of lysates were diluted 10x in dH2O. To precipitate the samples, 500 µl of RC Reagent I (Cat No. 500-0117, Bio-Rad Laboratories Pty. Ltd. Gladesville, NSW, Aust) was added to each sample, incubated for 1 min followed by the addition of 500 µl of reagent II (Bio-Rad Laboratories Pty. Ltd.). Samples were then centrifuged at 15,000 rpm for 5 min, the supernatant discarded, and the tissue pellet was dried on a heating block for 2 hours at 37°C. The pellet was resuspended in a solution containing Reagent A (Cat No. 500-01131, Bio-Rad Laboratories Pty. Ltd. Gladesville, NSW, Aust) and S (Cat No. 500-0115, Bio-Rad Laboratories Pty. Ltd. Gladesville, NSW, Aust) and incubated on a heating block for 3 min at 37°C. (EL-02, Major Science, Saratoga, CA, USA) BSA Standards (0.1 mg/mL – 1.2 mg/mL) were diluted in solution Reagent A and S and
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treated in the same way as sample pellets. After heating, standards and samples were aliquoted in triplicates to a 96 well plate. Reagent B (Cat No. 500-0119, Bio-Rad Laboratories Pty. Ltd. Gladesville, NSW, Aust) was added and left to incubate for 15 minutes at room temperature. Absorbance was measured at a wavelength of 750 nm using a plate reader (Victor V3, PerkinElmer, Waltham, Massachusetts, USA). Concentration of each sample was determined using GraphPad Prism (v.5 for Windows, GraphPad Prism Inc.). Samples were diluted to 1mg/ml in 1x Laemelli Sample Buffer and heated at 95°C for 5 mins (EL-02, Major Science, Saratoga, CA, USA). Samples were loaded into 4-20% precast gels (Cat No. 59511, Lonza, Muenchensteinerstrasse, Basel, Switzerland) in a gel tank (MP 300v Major Sciences, Saratoga, CA, USA) and ran at 150V for approximately 50 minutes in 1 x tris-glycine SDS buffer (Cat No. 50880, Lonza, Muenchensteinerstrasse, Basel, Switzerland) until migration front reached the bottom of the gel. The gel was then removed and transferred to a PVDF membrane using the same gel tank (MP 300v Major Sciences, Saratoga, CA, USA) (110 volts, 1 hour) in 1 x Transfer Buffer (190 mM (w/v) Glycine [Cat No. 6000-43-7 Sigma, Aldrich, St. Louis, MO, USA] 25 mM (w/v) Tris (hydroxymethyl)- aminomethane [Cat No. 161-0716, Bio-Rad, Hercules, CA, USA] 0.005% (w/v) sodium dodecyl sulphate [Cat No. 71725, Sigma, Aldrich, St. Louis, MO, USA], 20% Methanol (v/v) [Cat No. UN1230, Chem-Supply, Gillman, SA, Aust]). The membrane was blocked for one hour in 5% skim milk (w/v) in 1 x TBS-T (8.8% (w/v) NaCl [Cat No. X190, Amresco, Solon, OH, USA], 0.2% (w/v) KCl [Cat No. P9541, Sigma-Aldrich, Inc., St. Louis, MO, USA] 3% (w/v) Tris (hydroxymethyl)-aminomethane [Cat No. 161-0716, Bio-Rad, Hercules, CA, USA] in dH²O), 0.1% (v/v) Tween 20 [Cat No. P1379, Sigma-Aldrich, Inc., St. Louis, MO, USA] in dH²O). The primary antibody (Cleaved Caspase-3 [Asp175 Antibody, Cat No. 9661L, Genesearch Pty Ltd, Arundel Qld, Aust, 1:500 or GAPDH, Cat No. G9545, Sigma-Aldrich, Inc., St. Louis, MO, USA, 1:5000) was added and incubated overnight at 4° with shaking. The day after incubation with the primary antibody, the membrane was washed in five consecutive washes of 1x TBS-T (10 min each) on a shaker before being incubated with the secondary antibody, for 1 hour in 1x TBS-T. After incubation with the secondary antibody that membrane was again washed in five consecutive washes of 1x TBS-T (10 min each) on a shaker. The membrane was then saturated with ECL mix (Cat No. RPN2019V2, GE Healthcare, Waukesha, WI, USA) and developed onto X-ray film.
(100 NIF, FujiFilm, Minato-ku, Tokyo, Japan). The intensity of bands on digital images was analysed using Image Processing and Analysis in Java (ImageJ) Software. A given band intensity was calculated as the sum of the values of the pixels in the image, which is equivalent to the product of area and mean gray value.

6.3.11 Data Analysis
Statistical analysis was performed using PASW (v. 20 for Windows, SPSS.). Outliers were screened using box plots. No cases were removed. Sphericity was checked using Mauchly’s test of sphericity and homogeneity of variance was checked using Levine’s test of equality of error variance. Normality was assessed using Q-Q plots and histograms.

Pearson’s correlation was conducted to explore a) correlation between infarct size and the number of Caspase-3-ir labelled cell in the thalamic region of the ipsilesional hemisphere and contralesional hemisphere, b) correlation between infarct size and the number of Ki-67-ir labelled cell in the thalamic region of the ipsilesional hemisphere and contralesional hemisphere, c) correlations between the number of Caspase-3-ir labelled cells and Ki-67-ir labelled cells.

Analysis of variance (ANOVA; diet condition x surgery condition) was conducted to determine difference between surgery and diet conditions for a) the number of Caspase-3-ir labelled and Ki-67-ir labelled cells in the thalamic region of the ipsilesional hemisphere, b) the number of Caspase-3-ir labelled and Ki-67-ir labelled cells in the thalamic region of the contralesional hemisphere c) the number of Caspase-3-ir labelled and Ki-67-ir labelled cells in the granule cell layer of the DG, d) the number of Caspase-3-ir labelled cells and Ki-67-ir labelled cells in the CA1 region of the hippocampus e) differences in whole brain Western Blot protein expression of Caspase-3 normalised against the density bands for GAPDH.

Within the MCAo diet condition, One-way ANOVA was conducted to determine difference in number of Caspase-3-ir labelled and Ki-67-ir labelled cells between the thalamic regions of the ipsilesional and contralesional hemispheres.

Spearman correlation tests were conducted to detect correlations between the number of Caspase-3-ir and Ki-67-ir labelled cells in brain regions of interest (ipsilesional CA1 region, ipsilesional DG, and ipsilesional thalamic region) and the behaviours of interest,
as previously reported (see chapter 5). We were unable to study correlations between
behavioural outcomes and cell death or proliferation, within surgery and diet groups,
due to small sample sizes. In the present study, behavioural outcomes across diet and
surgery conditions are correlated with the number of Caspase-3-ir or Ki-67-ir labelled
cells, in the above outlined brain regions. That is, the scores for each behaviour of
interest, across both diet and surgery groups, have been collapsed into a single
continuous variable, and correlated with the number of Caspase-3-ir or Ki-67-ir labelled
cells, in the above outlined brain regions. The accepted p value for all analysis was
\( p<0.05 \).
6.4 Results

6.4.1 Infarct Analysis

In MCAo operated animals, right hemisphere infarct was seen in cortical and subcortical tissue, as depicted in Figure 6.1. Variability was seen in infarct size. The area labelled A in Figure 6.1 outlines the infarct region typically seen in animals with smaller infarcts ($n = 5$ [basal diet = 3]). Both the areas labelled A and B outline the infarct region seen in animals with medium infarcts ($n = 4$ [basal diet = 3]). The areas outlined A, B and C outlines the infarct region seen in animals with larger infarcts ($n = 3$ [basal diet = 0]).

Pearson’s correlation showed that the number of Caspase-3-ir cells in the thalamic region of the ipsilesional hemisphere was significantly correlated with infarct size, $r^2 = 0.62, p < 0.01$. The number of Ki-67-ir labelled cells in the thalamic region of the ipsilesional hemisphere similarly correlated with infarct size, $r^2 = 0.69, p < 0.01$. The number of Caspase-3-ir labelled cells in the thalamic region of the ipsilesional hemisphere positively correlated with the number of Ki-67-ir labelled cells, $r^2 = 0.78, p < 0.01$. Infarct size did not correlate with the number of Caspase-3 and Ki-67-ir labelled cells in the thalamic region of the contralesional hemisphere.
Figure 6.1. Line Drawing Showing Infarct Area and Brain Regions Selected for Analysis of Caspase-3-like and Ki-67-like Immunoreactivity, Six Weeks after Middle Cerebral Artery Occlusion.

Shaded areas show the extent of infarct; A = area of atrophy in animals with smaller infarcts; B = area of atrophy additional to area A, in animals with medium infarcts; C = area of atrophy additional to areas A and B, in animals with large infarcts. In both the ipsilesional and contralesional hemispheres, areas MDM, Po, VM, VPL and VPM are analysed together as a single thalamic region outcome, and thus, are collectively be referred to as the thalamic region throughout this paper. Basal, Basal diet fed rats; CA1, field CA1 of the hippocampus; DG, dentate gyrus; MDM, mediodorsal thalamic nucleus; Po, posterior thalamic nuclear group; VM, ventromedial thalamic nucleus; VPL, ventral posterolateral thalamic nucleus; VPM, ventral posteromedial thalamic nucleus.
6.4.2 Immunohistochemistry Between Group Comparisons

6.4.2.1 Caspase-3 and Ki-67 Expression Higher in the Thalamic Region of the Ipsilesional Hemisphere of MCAo Rats Compared to Shams, But Does Not Differ Between Diet Conditions, at Six Weeks Post-Surgery

MCAo operated animals showed a higher expression of Caspase-3-ir labelled cells than sham-operated animals ($F(1, 14) = 37.92$, $p < 0.01$) in the thalamic region of the ipsilesional hemisphere (MCAo $M = 2859$, $SEM = 382$; Sham $M = 148$, $SEM = 28$) (see figure 6.2 and 6.3). No differences were seen between dietary groups. No interaction effects were seen.

MCAo operated animals showed a higher expression of Ki-67-ir labelled cells than sham-operated animals, ($F(1, 14) = 17.68$, $p < 0.01$) in the thalamic region of the ipsilesional hemisphere (MCAo $M = 17.4$, $SEM = 3.7$; Sham $M = 1.2$, $SEM = .19$) (see figure 6.4 and 6.5). No differences were seen between dietary groups. No interaction effects were seen.
Figure 6.2. Mean Number (±SEM) of Caspase-3-ir Cells Between Surgery and Diet Conditions in Ipsilesional Thalamic Region at Six Weeks Post-Surgery.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats; Thalamic Region = Includes the Mediodorsal Thalamic Nucleus (MDM), the Posterior Thalamic Nuclei Group (Po), the Ventral Posteromedial Thalamic Nucleus (VPM), the Ventral Posterolateral Thalamic Nucleus (VPL), and the Ventromedial Thalamic Nucleus (VM).
Figure 6.3. Caspase-3 Immunoreactive Staining in Bregma -3.60 Rostral Section of the Ipsilesional Thalamic Region of Middle Cerebral Artery Occlusion Operated and Sham Operated, n-3-LC-PUFA and Basal Diet Fed Rats, Six Weeks After Surgery.

MCAo operated animals are seen to express more Caspase-3-ir labelled cells than sham operated animals. No effect of diet is seen. Cell counting within each photograph was restricted to a region of interest (1064.7µm x 521.5µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Caspase-3-ir were included in the analysis. A = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a n-3-LC-PUFA diet, MCAo surgery affected rat, scale bar 100µm; B = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a basal diet, MCAo surgery affected rat, scale bar 100µm; C = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a n-3-LC-PUFA diet, sham surgery affected rat, scale bar 100µm; D = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a basal diet, sham surgery affected rat, scale bar 100µm.
Figure 6.4. Mean Number (±SEM) of Ki-67-ir Cells Between Surgery and Diet Conditions in Ipsilesional Thalamic Region at Six Weeks Post-Surgery.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats. Thalamic Region = Includes the Mediodorsal Thalamic Nucleus (MDM), the Posterior Thalamic Nuclei Group (Po), the Ventral Posteromedial Thalamic Nucleus (VPM), the Ventral Posterolateral Thalamic Nucleus (VPL), and the Ventromedial Thalamic Nucleus (VM).
Figure 6.5. Ki-67 Immunoreactive Staining in Rostral Section of the Contralesional Thalamic Region of Middle Cerebral Artery Occlusion Operated and Sham Operated, n-3-LC-PUFA and Basal Diet Fed Rats, Six Weeks After Surgery.

MCAo operated animals are seen to express more Ki-67-ir labelled cells than sham operated animals. No effect of diet is seen. Cell counting within each photograph was restricted to a region of interest (1064.7µm x 521.5µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Ki-67-ir cells were included in the analysis. An area restriction of 40 µm² was set to ensure that dark stained objects; too small to be cell nuclei were not counted in the case of Ki-67-ir cells. A = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a n-3-LC-PUFA diet, MCAo surgery affected rat, scale bar 100µm; B = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a basal diet, MCAo surgery affected rat, scale bar 100µm; C = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a n-3-LC-PUFA diet, sham surgery affected rat, scale bar 100µm; D = Bregma -3.60 10x magnification rostral section of a portion of the ipsilesional thalamic region of a basal diet, sham surgery affected rat, scale bar 100µm.
6.4.2.2 Caspase-3 and Ki-67 Expression Higher in the Thalamic Region of the Contralesional Hemisphere Between Surgery and Diet Conditions at Six Weeks Post-Surgery

ANOVA showed a significant effect of surgery condition on the number of Caspase-3-ir labelled cells \((F(1, 22) = 15.46, p < 0.01)\) in the thalamic region of the contralesional hemisphere, with MCAo operated animals showing more Caspase-3-ir labelled cells (Caspase-3, MCAo \(M = 254, SEM = 31\); Sham \(M = 86, SEM = 28\)). A significant effect of diet condition on the number of Caspase-3-ir labelled cells was also found \((F(1, 22) = 7.64, p < 0.05)\) in the thalamic region of the contralesional hemisphere. Animals supplemented with n-3-LC-PUFA showed fewer Caspase-3-ir labelled cells (n-3-LC-PUFA \(M = 107, SEM = 17\); Basal \(M = 218, SEM = 55\)). No interaction effects were seen, as depicted in Figures 6.6 and 6.7.

No effect of surgery condition was seen on the number of Ki-67-ir labelled cells \((F(1, 22) = 2.25, p = 1.51)\). A significant effect of diet condition on the number of and Ki-67-ir labelled cells was found \((F(1, 22) = 9.72, p < 0.01)\) in the thalamic region of the contralesional hemisphere. Animals supplemented with n-3-LC-PUFA showed more Ki-67-ir labelled cells (Ki-67, n-3-LC-PUFA \(M = 2.6, SEM = 0.29\); Basal \(M = 1.2, SEM = .32\)). No interaction effects were seen, as can be seen in Figure 6.8 and 6.9.
Figure 6.6. Mean Number (±SEM) of Caspase-3-ir Cells Between Surgery and Diet Conditions in Contralesional Thalamic Region at Six Weeks Post-Surgery.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats. Thalamic Region = Includes the Mediodorsal Thalamic Nucleus (MDM), the Posterior Thalamic Nuclei Group (Po), the Ventral Posteromedial Thalamic Nucleus (VPM), the Ventral Posterolateral Thalamic Nucleus (VPL), and the Ventromedial Thalamic Nucleus (VM).
Figure 6.7. Caspase-3 Immunoreactive Staining in Bregma -3.60 Rostral Section of the Contralesional Thalamic Region of Middle Cerebral Artery Occlusion Operated and Sham Operated, n-3-LC-PUFA and Basal Diet Fed Rats, Six Weeks After Surgery.

MCAo operated animals are seen to express more Caspase-3-ir labelled cells than sham operated animals. Animals supplemented with n-3-LC-PUFA are shown to express less Caspase-3-ir labelled cells, than basal diet fed rats, in both surgery conditions. Cell counting within each photograph was restricted to a region of interest (1064.7µm x 521.5µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Caspase-3-ir were included in the analysis. A = Bregma -3.60 10x magnification rostral section of a portion of the contrallesional thalamic region of a n-3-LC-PUFA diet, MCAo surgery affected rat, scale bar 100µm; B = Bregma -3.60 10x magnification rostral section of a portion of the contrallesional thalamic region of a basal diet, MCAo surgery affected rat, scale bar 100µm; C = Bregma -3.60 10x magnification rostral section of a portion of the contrallesional thalamic region of a basal diet, sham surgery affected rat, scale bar 100µm; D = Bregma -3.60 10x magnification rostral section of a portion of the contrallesional thalamic region of a basal diet, sham surgery affected rat, scale bar 100µm.
Figure 6.8. Mean Number (±SEM) of Ki-67-ir Cells Between Surgery and Diet Conditions in Contralesional Thalamic Region at Six Weeks Post-Surgery.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats. Thalamic Region = Includes the Mediodorsal Thalamic Nucleus (MDM), the Posterior Thalamic Nuclei Group (Po), the Ventral Posteromedial Thalamic Nucleus (VPM), the Ventral Posterolateral Thalamic Nucleus (VPL), and the Ventromedial Thalamic Nucleus (VM).
Figure 6.9. Ki-67 Immunoreactive Staining in Bregma -3.60 Rostral Section of the Contralesional Thalamic Region of Middle Cerebral Artery Occlusion Operated and Sham Operated, n-3-LC-PUFA and Basal Diet Fed Rats, Six Weeks After Surgery.

Animals supplemented with n-3-LC-PUFA are shown to expresses more Ki-67-ir labelled cells, than basal diet fed rats, in both surgery conditions. No effect of surgery condition is seen. Cell counting within each photograph was restricted to a region of interest (1064.7µm x 521.5µm) and determined using a threshold parameter, where only pixels with a given range of colour, identified as representing Ki-67-ir cells were included in the analysis. An area restriction of 40 µm² was set to ensure that dark stained objects too small to be cell nuclei were not counted in the case of Ki-67-ir cells. A = Bregma -3.60 10x magnification rostral section of a portion of the contralesional thalamic region of a n-3-LC-PUFA diet, MCAo surgery affected rat, scale bar 100µm; B = Bregma -3.60 10x magnification rostral section of a portion of the contralesional thalamic region of a basal diet, MCAo surgery affected rat, scale bar 100µm; C = Bregma -3.60 10x magnification rostral section of a portion of the contralesional thalamic region of a n-3-LC-PUFA diet, sham surgery affected rat, scale bar 100µm; D = Bregma -3.60 10x magnification rostral section of a portion of the contralesional thalamic region of a basal diet, sham surgery affected rat, scale bar 100µm.
6.4.2.3 Caspase-3 and Ki-67 Expression Did Not Differ in the Dentate Gyrus and CA1 region Between Surgery and Diet Conditions at Six Weeks Post-Surgery

No effects for surgery or diet condition on Caspase-3-ir labelled cells was seen in the DG or CA1 region of the hippocampus, of the ipsilesional hemisphere, at six weeks after surgery, as can be seen in Figure 6.10 and 6.11.

No effects for surgery or diet condition on Ki-67-ir labelled cells were seen in the DG or CA1 region of the hippocampus, of the ipsilesional hemisphere, at six weeks after surgery, as can be seen in Figure 6.12 and 6.13.
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Figure 6.10. Mean Number (±SEM) of Caspase-3-ir Cells Between Surgery and Diet Conditions in CA1 and DG of the Hippocampus at Six Weeks Post-Surgery.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats; CA1 = Cornu Ammonis area of the hippocampus; DG = Dentate Gyrus of the Hippocampus.
Figure 6.11. Caspase 3 Immunoreactive Staining in Rostral Section of the Dentate Gyrus and CA1 Region of Hippocampus of Middle Cerebral Artery Occlusion Operated and Sham Operated, n-3LC-PUFA and Basal Diet Fed Rats, Six Weeks After Surgery.

No effects can be seen for surgery or diet condition on the number of Caspase-3-ir labelled cells in the DG or CA1 region of the hippocampus of the ipsilesional hemisphere. Cell counting within each photograph was restricted to a region of interest (1064.7 µm x 521.5 µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Caspase-3-ir were included in the analysis. A = Dentate Gyrus of a n-3-LC-PUFA diet, MCAo surgery affected rat; B = Dentate Gyrus of a basal diet, MCAo surgery affected rat; C = Dentate Gyrus of a n-3-LC-PUFA diet, sham surgery affected rat; D = Dentate Gyrus of a basal diet, sham surgery affected rat; E = CA1 Region of a n-3-LC-PUFA diet, MCAo surgery affected rat; F = CA1 Region of a basal diet, MCAo surgery affected rat; G = CA1 Region of a n-3-LC-PUFA diet, sham surgery affected rat; H = CA1 Region of a basal diet, sham surgery affected rat; Pictures show bregma -3.60 10x magnification rostral, scale bar 100 µm.
Figure 6.12. Mean Number (±SEM) of Ki-67-ir Cells Between Surgery and Diet Conditions in CA1 and DG of the Hippocampus at Six Weeks Post-Surgery.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats; CA1 = Cornu Ammonis area of the hippocampus; DG = Dentate Gyrus of the Hippocampus.
Figure 6.13. Ki-67 Immunoreactive Staining in Rostral Section of the Dentate Gyrus and CA1 Region of Hippocampus of Middle Cerebral Artery Occlusion Operated and Sham Operated, n-3LC-PUFA and Basal Diet Fed Rats, Six Weeks After Surgery.

No effects can be seen for surgery or diet condition on the number of Ki-67-ir labelled cells in the DG or CA1 region of the hippocampus of the ipsilesional hemisphere. Counting within each photograph was restricted to a region of interest (1064.7 µm x 521.5 µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Ki-67-ir cells were included in the analysis. An area restriction of 40 µm² was set to ensure that dark stained objects too small to be cell nuclei were not counted in the case of Ki-67-ir cells. A = Dentate Gyrus of a n-3-LC-PUFA diet, MCAo surgery affected rat; B = Dentate Gyrus of a basal diet, MCAo surgery affected rat; C = Dentate Gyrus of a n-3-LC-PUFA diet, sham surgery affected rat; D = Dentate Gyrus of a basal diet, sham surgery affected rat; E = CA1 Region of a n-3-LC-PUFA diet, MCAo surgery affected rat; F = CA1 Region of a basal diet, MCAo surgery affected rat; G = CA1 Region of a n-3-LC-PUFA diet, sham surgery affected rat; H = CA1 Region of a basal diet, sham surgery affected rat; Pictures show bregma -3.60 10x magnification rostral, scale bar 100µm.
6.4.3 Immunohistochemistry Within MCAo Surgery Group Comparisons

6.4.3.1 More Caspase-3 and Ki-67-ir labelled Cells in the Ipsilesional Hemisphere, Compared to Contralesional Hemisphere, of MCAo Operated Rats at Six Weeks Post-Surgery

Within group comparisons revealed that MCAo operated animals showed significantly more Caspase-3-ir cells in the thalamic region of the ipsilesional hemisphere, compared to the thalamic region of the contralesional hemisphere, \((F(1, 19) = 45.48, p < 0.01)\) (Caspase-3, Ipsilesional thalamic region \(M = 2738, SEM = 405\); Contralesional thalamic region, \(M = 254, SEM = 55\)). Figure 6.14 shows that Caspase-3-like immunoreactivity (Caspase-3-ir) appeared as an orange/brown precipitate localized to both cell nuclei as well as dense plaque like deposits. This presentation of Caspase-3-ir labelled cells in the present study is morphologically consistent with the presentation of APP and A\(\beta\) that has been previously observed, from as early as one week, to as late as nine months post MCAo, in the rodent model \([40, 41, 421-423, 425]\) (that is small diffuse fragments and large, dense deposits that resemble plaques, and that are most likely harmful for functional recovery \([40, 41, 421-423, 425]\)). No differences in the number of Caspase-3-ir labelled cells were seen between dietary groups.

Similarly, MCAo operated animals showed significantly more Ki-67 in the thalamic region of the ipsilesional, compared to the thalamic region of the contralesional hemisphere, \((F(1, 18) = 19.77, p < 0.01)\) (Ki-67, Ipsilesional thalamic region, \(M = 17, SEM = 3.7\); Contralesional thalamic region, \(M = 1.6, SEM = 0.33\)) (see Figure 6.15). No differences were seen between dietary groups.
Figure 6.14. Caspase-3 Immunoreactive Staining in Bregma -3.60 Rostral Section of the Contralesional and Ipsilesional Thalamic Region of a Middle Cerebral Artery Occlusion Operated Rat, Six Weeks After Surgery.

MCAo operated animals show more Caspase-3-ir cells in the thalamic region of the ipsilesional hemisphere compared to the thalamic region of the contralesional hemisphere. Cell counting within each photograph was restricted to a predefined area (1064.7 µm x 521.5 µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Caspase-3-ir were included in the analysis. A = 2x Bregma -3.60 magnification scale bar 500 µm; B = Bregma -3.60 10x magnification rostral section of a portion of the contralesional thalamic region, scale bar 100 µm; B right hand corner = 40x magnification of a portion of image B, scale bar 25 µm. C = Bregma -3.60 10x magnification photograph of a portion of the ipsilesional thalamic region, scale bar 100 µm; C right hand corner = 40x magnification of a portion of image C, scale bar 25 µm.
Figure 6.15. Ki-67 Immunoreactive Staining in Rostral Section of the Contralesional and Ipsilesional Thalamic Region of a Middle Cerebral Artery Occlusion Operated Rat, Six Weeks After Surgery.

MCAo operated animals show more Ki-67-ir cells in the thalamic region of the ipsilesional hemisphere compared to the thalamic region of the contralesional hemisphere. Cell counting within each photograph was restricted to a predefined area (1064.7µm x 521.5µm) and determined using a threshold parameter where only pixels with a given range of colour, identified as representing Ki-67-ir cells were included in the analysis. An area restriction of 40 µm² was set to ensure that dark stained objects too small to be cell nuclei were not counted in the case of Ki-67-ir cells. A = 2x Bregma -3.60 magnification scale bar 500µm; B = Bregma -3.60 10x magnification rostral section of a portion of the contralesional thalamic region, scale bar 100µm; B right hand corner = 40x magnification of a portion of image B, scale bar 25µm. C = Bregma -3.60 10x magnification photograph of a portion of the ipsilesional thalamic region, scale bar 100µm; C right hand corner = 40x magnification of a portion of image C, scale bar 25µm.
6.4.4 Whole Brain Western Blot Expression of Caspase-3 Does Not Differ Between Surgery or Diet Conditions at Six Weeks Post-Surgery

Western Blot analysis was performed to confirm the presence of Caspase-3 in whole brain tissue, across all groups of animals. Non-localised brain tissue expression of GAPDH did not show a significant difference between surgery conditions, $F(1, 23) = 0.18, p = 0.67$, or between diet conditions, $F(1, 23) = 0.75, p = 0.40$.

Non-localised brain tissue expression of Caspase-3 did not differ between surgery conditions, $F(1, 23) = 1.38, p = 0.25$, nor between diet conditions, $F(1, 23) = 0.56, p = 0.47$, (MCAo n-3-LC-PUFA $M = 0.83, SEM = 0.17$; MCAo Basal $M = 0.82, SEM = 0.15$; Sham n-3-LC-PUFA $M = 0.75, SEM = 0.16$; Sham Basal $M = 0.53, SEM = 0.16$), as can be seen in Figures 6.16 and 6.17.
Figure 6.16. Direct Western Blot Detection of Caspase-3 and GAPDH, from Brain Tissue of MCAo Effected, Sham Operated, n-3-LC-PUFA and Basal Diet Fed Rats, at Six Weeks Post-Surgery.

No effects of surgery of diet condition on whole brain expression, of Caspase-3, according to Western Blot analysis. Samples from eight different animals (two basal diet fed sham operated, two basal diet fed MCAo operated, two n-3-LC-PUFA diet fed sham operated and two n-3-LC-PUFA diet fed MCAo operated animals). Each lane was loaded with 20µg of protein. After separation on SDS-PAGE, the blots were probed with anti-bodies against (Caspase-3 or GAPDH). The selected Caspase-3 antibody (Asp175) detects endogenous levels of the large fragment (17/19kDa) of activated Caspase-3. A clear band was detected at the 17kDa location, however, despite multiple attempts, we were unable to identify a clear band at the 19kDa location in all samples, and thus have excluded the 19kDa band from analysis. This may be as17kDa bands represent activated cleaved Caspase-3, while 19kDa represents intermediate products in the processing of Caspase-3 [426], suggesting that in the present study we observed active Caspase-3 only. For details, see experimental procedures. MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; Basal = Basal diet fed rats; n-3-LC-PUFA = Polyunsaturated fatty acid supplemented rats.
Figure 6.17. Band Intensity (±SEM) of Caspase-3 Protein Expression, Normalised to GAPDH, Between Surgery and Diet Conditions.

Note that no effects of surgery or diet condition on whole brain expression of Caspase-3 according to Western Blot analysis.

MCAo = Middle Cerebral Artery Occlusion surgery condition; Sham = Sham surgery condition; n-3-LC-PUFA = Polyunsaturated enriched Diet Condition; Basal = Basal Diet Condition.
6.4.5 Correlations Between Caspase-3 and Ki-67 Expression and Behavioural Outcomes, at Six Weeks Post-Surgery

The effects for surgery and diet condition on hyperactive locomotor behaviours, in the free exploration test, and novel object exploration have been previously reported (see chapter 5) and thus data is not presented in this paper. However the results are very briefly recounted below to assist with the interpretation of the correlation analysis conducted in the present study. In the free exploration test, MCAo operated animals emerged more from the hide box into the open-field, and spent more time moving around in the open-field, than sham operated animals. Dietary supplementation with n-3-LC-PUFA was associated with a decrease in the number of emergences from the hide box into the open-field, and the duration of time spent moving around the open-field. Rats supplemented with n-3-LC-PUFA spent more time in the centre of the open-field arena. MCAo operated animals explored a novel object less than sham-operated animals, and animals supplemented with n-3-LC-PUFA explored that novel object more than basal diet fed animals.

In the present study, we correlated cellular degeneration and proliferation in the DG, CA1 region of the hippocampus, and in the thalamus of the ipsilesional and contralesional hemispheres, as seen at six weeks post-surgery, with the behavioural outcomes previously reported (i.e. locomotor hyperactivity [number of emergences into open-field and duration of time spent moving around in open-field] and anxiety-like behaviour [percentage of total emergence time spent in the centre of the open-field arena] and novel object exploration at six weeks post-surgery.

Spearman correlations showed a positive correlation between the number of Caspase-3-ir labelled cells in the CA1 region of the hippocampus and the total emergence duration in the open-field ($\rho = 0.44, p < 0.05$). The number of Caspase-3-ir labelled cells in the thalamic region of the ipsilesional hemisphere positively correlated with the number of times that the rat emerged from the hide box, into the open-field, in the free exploration test, at six weeks post-surgery ($\rho =0.48, p < 0.05$). The number of Ki-67-ir labelled cells in the contralesional thalamic region negatively correlated with number of times the rat emerged from the hide box into the open-field arena ($\rho = -0.48, p < 0.05$). A negative correlation between the number of Ki-67-ir labelled cells in the contralesional...
thalamic region and the duration of time spent moving in the open-field arena approached significance ($\rho = -0.42, p > 0.05$). No correlations were seen between cellular degeneration/proliferation in the DG and behavioural outcomes. No correlations were seen between novel object exploration and cellular degeneration and proliferation, in any brain region.
6.5 Discussion

The primary aim of this study was to investigate the influence of n-3-LC-PUFA dietary supplementation, on cell death and proliferation, at six weeks post MCAo, in the male hooded Wistar rat. This study also aimed to study correlations between localised cell death, and the presentation of hyperactive locomotor and anxiety-like behaviours, previously observed and reported in these animals (see chapter 5).

MCAo surgery resulted in variable right hemisphere infarct in cortical and sub cortical tissue. We found that MCAo surgery, as compared to sham surgery condition, was associated with increased cellular degeneration and proliferation in the thalamic region of the ipsilesional hemisphere, which is consistent with previous research [11], and has been speculated to result from retrograde degeneration of thalamocortical projections [42, 43]. Supplementation with n-3-LC-PUFA was associated with increased cellular proliferation, and decreased cell death, as compared to basal diet fed animals, in the contralesional thalamic region of MCAo operated animals. Previous clinical and animal research has shown that the brain can undergo a spatial remapping after ischemic stroke, whereby undamaged brain regions are capable of taking over functions of the affected areas to some extent [413-415]. Increased cellular proliferation post MCAo in n-3-LC-PUFA fed rats may represent a diet assisted functional spatial remapping.

No significant differences in Caspase-3 or Ki-67-ir labelled cells were seen in the hippocampus and CA1 region of the DG of the ipsilesional hemisphere, between surgery and diet conditions. This finding is inconsistent with previous research, demonstrating that cells in the CA1 and DG are particularly vulnerable to ischemia induced cellular degeneration [427]. Alternately, ischemia associated cell degeneration and subsequent proliferation in the hippocampus may have already occurred by six weeks post-surgery, when immunological analysis was conducted. Some previous research suggests that CA1 neurons can completely repopulate by one-month post infarct in rodents [396].

Within the MCAo surgery condition, more localised Caspase-3 and Ki-67-ir cells were seen in the ipsilesional, stroke affected thalamic region, than in the contralesional thalamic region, which is expected and consistent with previous research [428]. The extent of cell death, as measured by Caspase-3-ir labelled cells, was correlated to both infarct size and the number of Ki-67-ir labelled cells. The presentation of Caspase-3
expression, seen in the ipsilesional thalamic region of MCAo operated animals, was morphologically consistent with the presentation of APP and Aβ, previously observed post-stroke in rodent models (that is small diffuse fragments and large, dense deposits that resembled plaques) [425]. The C-terminal cleavage product of APP is co-localised with Caspase-3 at a cellular level [357] and Caspase-3 directly and efficiently cleaves to APP [357, 429, 430]. Previous research demonstrates that one year of dietary supplementation with omega 3 derived docosahexaenoic acid dietary supplementation reduces Aβ deposition in the DG of non MCAo subjected mice [431]. The influence of n-3-LC-PUFA supplementation on the expression of APP and Aβ post-stroke has not been investigated. In the present study the expression of Caspase-3, which directly cleaves to APP, [357, 429, 430] did not differ between dietary conditions, in thalamic region of the ipsilesional hemisphere. However, dual immunofluorescence label, of both Caspase-3 and APP, is required to confirm this circumstantial evidence.

A significant difference was not found between surgery or diet conditions, in relation to whole brain expression of Caspase-3, using Western Blot analysis technique. This is inconsistent with the immunohistochemistry data presented in chapter 6. These inconsistent results may reflect differences in the brain regions studied using the two methods. It is possible that localised differences in the number of Caspase-3 and Ki-67-ir labelled cells, between dietary and surgery conditions, were not large enough to result in significant differences in whole brain tissue. Thus, in future research, it would be more valuable to study localised brain regions, such as the thalamic region, using Western Blot techniques. Alternately, the lack of significant differences between surgery and diet conditions, in Caspase-3 expression, may reflect limitations in commonly used housekeeping genes. Even the most widely used Western Blot housekeeping genes are involved in multiple cell functions and the expression levels of these genes are affected by many factors [366]. The validity of the commonly used housekeeping genes as internal standards for measuring gene expression has been called into question [366]. Indeed, GAPDH has been shown to over express and accumulate in the nucleus during cell death [432, 433]. In the present research, it is possible that the expression levels of GAPDH may be confounded by earlier oxidative stress, resulting from ischemic insult, which may have obscured the experimental results.
We have previously demonstrated that MCAo surgery is associated with behavioural changes, argued to reflect locomotor hyperactivity and anxiety-like behaviours, in the rat. In the free exploration test, MCAo operated animals emerged more times from the hide box into the open-field arena and spent more time moving around the open-field arena, than sham operated rats, suggestive of stroke related hyperactive locomotion. MCAo operated animals also showed less approach behaviour, than sham operated animals, as defined by less time interacting with a novel object, and interpreted to reflect anxiety-like avoidance behaviour. Animals supplemented with n-3-LC-PUFA spent less time moving around in the open-field arena, than basal fed rats, but a higher percentage of emergence time in the centre, anxiety provoking area of the open-field arena. Additionally, n-3-LC-PUFA fed animals spent more time exploring the novel object than basal fed animals, which was interpreted to reflect more approach and less anxiety related avoidance behaviour.

In the present study, we found that cell denegation in the CA1 region of the hippocampus correlates with time spent moving around in the open-field arena, in the free exploration test at six weeks post-surgery. This finding is consistent with previous research, showing that ischemia induced damage to CA1 cells results in increased locomotion in the gerbil, at 2 days following surgery [27, 315]. The number of Caspase-3-ir labelled cells in the thalamic region of the ipsilesional hemisphere positively correlated with the number of times that the rat emerged from the hide box into the open-field, in the free exploration test. Additionally, the number of Ki-67-ir labelled cells in the contralesional thalamic region negatively correlated with number of times the rat emerged from the hide box into the open-field arena. A negative correlation between the number of Ki-67-ir labelled cells in the contralesional thalamic region and the duration of time spent moving in the open-field arena, approached significance. These results may suggests that locomotor hyperactivity post MCAo is associated with both hippocampus and thalamus damage. Indeed, the thalamus plays a key role in the information processes necessary for motor control [33-37] and thalamic damage is associated with post-stroke hyperactive delirium, in clinical populations [6].

An important limitation of the present research is that we were unable to identify the type of degenerating or proliferating cell types using the selected markers, Caspase-3 and Ki-67. Post-stroke functional recovery is undoubtedly dependent upon the
degeneration and proliferation profile of, and interaction between many cell types
including astrocytes, microglia, oligodendrocytes, and neurons [15, 352, 353]. In future
research it will be necessary to study the proliferation profiles of different cell types
post-stroke, and the relationship between these.

In conclusion, this study is the first to demonstrate that n-3-LC-PUFA dietary
supplementation influences MCAo associated cell proliferation and degeneration in the
thalamic region of the contralesional hemisphere. Additionally, this study demonstrated
that cellular degeneration and proliferation at six weeks post-surgery is correlated the
hyperactive locomotor and anxiety-like behaviours previously reported, that is
emergences from the hide box into the open-field arena, time spent moving around the
open-field arena, and percentage of emergence time spent in the centre, anxiety
provoking area of the open-field arena, in the free exploration test, and time spent
interacting with a novel object (see chapter 5).

However, it must be noted that we have also previously identified that n-3-LC-PUFA
supplementation is associated with an increased risk of hemorrhagic bleeding during
reperfusion, in MCAo operated rats (see chapter 5). Therefore, the potential beneficial
effects of n-3-LC-PUFA supplementation in populations that are at risk of hemorrhagic
bleeding should be interpreted with caution, and further research is required to identify
any safety risks of n-3-LC-PUFA supplementation.
Chapter 7: Homocysteine as a Potential Biochemical Marker for Depression in Elderly Stroke Survivors

Chapter 7 is an empirical translational chapter, with the aim to expand on the argument that nutritional factors that influence cell death, have mood modulating effects. This is done in two ways: the first is by studying a clinical population rather than an animal model; the second is by shifting the focus away from PUFAs and towards homocysteine (tHcy), an amino acid associated with the presentation of clinical depression [55]. Like PUFAs, tHcy has been shown to play a role in the mechanisms mediating cell death [56, 434]. A high cellular level of tHcy contributes to depression related apoptosis [56, 434], as it results in the production of hydrogen peroxide and superoxide anion [53], which are toxic to endothelial cells [53]. Exposure to hydrogen peroxide induces cell death and the induction of Caspase-3 [54].

The purpose of chapter 7 is to study the relationship between tHcy expression and the presentation of depressive symptomatology, in a cohort of elderly stroke survivors, a year and a half after stroke. The results of this study are consistent with the argument that stroke related apoptosis contributes to the presentation of depressive symptomatology, as individuals with a higher expression of apoptosis associated tHcy, were found to have more depressive symptomatology, than those without clinically elevated tHcy. These findings suggest that the mood modulating effects of various nutritional factors, such as PUFAs and tHcy, may relate to their influence on depression associated cell degeneration.
7.1 Abstract

Background: Elderly stroke survivors have been reported to be at risk of malnutrition and depression. Vitamin B-related metabolites methylmalonic acid and homocysteine have been implicated in depression. We conducted a study exploring the relationship between homocysteine and post-stroke depression,

Method: Observational cohort study of elderly Swedish patients (n = 149) 1.5 years post-stroke, assessed using Diagnostic and Statistical Manual of Mental Disorders, Montgomery-Åsberg Depression Rating Scale and serum blood levels of methylmalonic acid and homocysteine,

Results: Homocysteine significantly correlated with depressive symptomatology in stroke survivors ($\beta = .18^*$). Individuals with abnormal levels of methylmalonic acid and homocysteine were almost twice more likely to show depressive symptomatology than those with normal levels (depressive symptoms 22%; no depressive symptoms 12%).

Comparison of methylmalonic acid and homocysteine levels with literature data showed fewer stroke survivors had vitamin deficiency than did reference individuals (normal range 66%; elevated 34%).

General Conclusions: Homocysteine is significantly associated with depressive-symptomatology in elderly Swedish stroke survivors.

Key Words: Depression, Ischemia, Nutrition, Neurodegeneration, Geriatric.
7.2 Introduction

Depression is particularly prevalent among stroke survivors, affecting approximately a third of individuals [5]. Post-stroke depression is associated with reduced functional ability and poorer outcome, [7] making the prevention and management of post-stroke depression an important area of research. Vitamin deficiencies are particularly common among stroke survivors and like post-stroke depression, are associated with poor outcome [435].

Vitamin B₁₂ and folate are required dietary vitamin nutrients involved in the biosynthesis of depression-related serotonin, dopamine and norepinephrine [436]. Folic acid and vitamin B₁₂ are also required for the synthesis of methionine, by methionine synthase, from the amino acid homocysteine (tHcy). Methionine cannot be synthesized from tHcy without an adequate supply of folate and vitamin B₁₂, so vitamin deficiency results in an elevated level of tHcy.

Methylmalonyl coenzyme A is the coenzyme A linked form of methylmalonic acid (MMA). Vitamin B₁₂ is required for the biosynthesis of MMA Methylmalonyl-coenzyme A, to succinyl coenzyme A in the citric acid cycle. Therefore, insufficient vitamin B₁₂ levels are associated with increased levels of MMA [437].

High tHcy (≥1.69 mg/L) is associated with a 70 percent increased risk of clinical depression in elderly individuals [55] while folate is decreased in blood samples of depressed individuals [56]. Low folate (less than 200 µg/litre) is associated with a worse than expected response to selective serotonin reuptake inhibitor (SSRI) and tricyclic antidepressants, while higher folate levels are associated with better response [57, 58]. Supplementation with folate has been shown to have antidepressant effects and in some cases, to be as effective as conventional antidepressants [55, 59, 60]. Higher tHcy contributes to depression related naturally occurring cell death, or apoptosis [53, 56, 434].

Thus, as elderly stroke patients are at particular risk of malnourishment [435] and depression [5], and as B vitamin related factors such as folate and Vitamin B₁₂ related tHcy and MMA are implicated in the aetiology of clinical depression, it is particularly important to clarify if these biomarkers are associated with the depressive symptomatology seen in at least one third of elderly stroke patients [5].
We sought to systematically review the role of MMA, tHcy, folate and Vitamin B$_{12}$ and depressive symptomatology in stroke survivors. To do this, we examined the large literature relating to the relationship between B vitamins and clinical depression, and between B vitamins and stroke. No studies were found to directly address the possible role of folate, vitamin B$_{12}$, tHcy or MMA as contributing to depressive symptomatology in a post-stroke sample.

Thus, we aimed to explore the relationship between tHcy and depressive symptomatology among chronic stroke survivors, by determining the percent of individuals diagnosed with clinical depression and vitamin deficiency. We further aimed to determine if elevated tHcy contributed to the observed depressive symptomatology and to clarify the percentage of elderly stroke survivors with abnormal tHcy and MMA, compared to a matched non-stroke affected population. Thus, we compared a population of elderly Swedish stroke survivors to a matched non-stroke population of elderly Swedish individuals [438] to assess nutrition induced risk for deficiency in the depression related metabolites MMA and tHcy, 1.5 year post-stroke.
7.3 Method

7.3.1 Subjects

Participant selection has been described in detail previously [18]. Briefly, participants (n = 149) were drawn from the ‘Gothenburg 70+ Stroke Study’, which consisted of individuals admitted to Sahlgrenska University Hospital Gothenburg, Sweden, between February 1, 1993, and May 17, 1994, aged at least 70 years and presenting with an acute cerebrovascular neurological deficit, as diagnosed by routine investigations by the physician on call and acute computer tomography (CT) scan. Exclusion from the study applied if coma, cerebral tumour, extracerebral or subarachnoid haemorrhage, previous cerebral lesion requiring ongoing care, or a requirement for specialized neurological care were present. Participation was limited also to individuals who received a bed in the stroke unit, presented with symptoms for a time period no greater than 7 days prior to admission and were not residing in a nursing home at the time of admission. There was no control participant data available for this cohort study.

7.3.2 Study Protocol

Stroke patients were contacted by mail one year after stroke and subsequently by phone, to arrange a hospital-based appointment. When unable to attend the hospital-based appointment n=15, 10%), interviews took place in participants’ homes. Informed consent was obtained after having provided verbal and written information to participants or nearest relatives when relevant. Ethics approval was granted by The Ethics Committee for Medical Research, at the University of Gothenburg.

7.3.3 Biomarkers Collection

MMA was analysed from venous blood drawn into gel tubes (filled to at least ¾), turned over at least 10 times and centrifuged at 2300G for 10 minutes. Serum was separated and kept at room temperature before analysis with gas chromatography/mass spectrometry. Samples for tHcy were drawn in Li-heparin gel tubes, (filled to at least ¾) turned over at least five times within 30 minutes and centrifuged at 2300G for 10 minutes, before analysis. Reference values from the Uddevalla Hospital, Uddevalla, Sweden were <0.37 µmol/L for serum MMA, <15 µmol/L for serum tHcy [438].
7.3.4 Diagnostic Criteria

Depression assessments were conducted by a neurologist/psychiatrist who was unaware of the type, size and location of the index stroke at the time of the investigation and throughout the diagnostic procedure. Clinical depression was diagnosed according to DSM-III-R criteria [439] using algorithms based on psychiatric interview and neuropsychiatric examination. Depressive symptomatology was assessed using the Montgomery-Åsberg Depression Rating Scale (MADRS). Adjusted MADRS scores are compiled based on information obtained from ten related criteria, such as suicidal thoughts and pessimistic thoughts. Individuals are given a score for each of these measures that are complied into an overall depressive score. Missing data however on some of these criteria among cases, made MADRS score adjustment implausible in the current study, therefore, raw score data was used as a conservative measure of depressive symptoms. It should be noted however, that the raw score data used in the present study may underestimate the degree of depressive symptomatology among the sample.

Assessment consisted of a semi-structured diagnostic psychiatric interview, neuropsychological tests and a neurological examination. Assessment instruments were the same as those used in the Gerontological and Geriatric Population Studies and the Prospective Population Study on Women and included a semi-structured psychiatric diagnostic interview with the Comprehensive Psychopathological Rating Scale [440]. This form of clinical depression diagnosis was used to categorically classify individuals as either depressed or not depressed, in order to determine the percentage of these individuals with and without elevated MMA and tHcy. For the purpose of multiple regression analysis and descriptive statistics, raw continuous MADRS scores only were used.

7.3.5 Control Reference Data

Control participant reference data were sourced from PubMed and drawn from a population based study of elderly Swedish individuals [438]. This study provided information regarding tHcy and MMA levels in non-stroke elderly Swedish individuals and laboratory cut off points for vitamin deficiency. This control data was compared with the stroke cohort data to assess if elderly stroke survivors were more at risk of
nutritional deficiencies, as indicated by MMA and tHcy 1.5 year post-stroke, than were the non-stroke elderly individuals.

7.3.6 Statistical Methods

Statistical analysis was conducted using the SPSS version 18 package. Hierarchical Multiple Regression was used to determine variance explained by tHcy after accounting for age and gender. Outliers were screened using box plots; linearity and homoscedasticity were checked using scatter plots of standardised residuals. Normality was assessed using a histogram and normal probability plots. Pearson's Correlation was used to determine multi-collinearity between predictors. T tests were used to assess differences in tHcy and MMA between genders. Independent sample t tests were used to determine differences in MMA and tHcy expression between stroke survivors and reference data from non-stroke individuals. The accepted p value was $p<0.05$. 
7.4 Results

7.4.1 Characteristics of the Observational Cohort Study Population

Table 1 illustrates the characteristics of stroke survivors according to gender. The study cohort comprised of 243 patients at baseline (stroke admission). By the time of hospital follow up, at 1.5 years post-stroke (SD 0.4 years), 32% ($n=77$) had passed away and 7% ($n=17$) declined the invitation to participate, leaving 149 individuals. Two thirds, 65% ($n=97$) of individuals were female, and 35% ($n=52$) were male. Patients’ mean age at follow up was 81 years (SD 5.3 years). The mean age of the female survivors was approximately 2 years older than that of the male participants. Female MADRS scores were approximately 3 points higher than male participants. tHcy was comparable between males and females, $t (114) = .308, p = 0.58$. MMA was slightly higher in females than in males, but this effect was just not significant, $t (113) = 3.87, p = 0.052$. Peason’s correlation analysis were conducted between tHcy and MMA and the following factors, serum cholesterol levels, body weight, was the participant a smoker or diagnosed with diabetes. No significant correlations were found.
Table 7.1 Age, Montgomery-Åsberg Depression Rating Scale (MADRS) scores, homocysteine (tHcy) and methylmalonic acid (MMA) in stroke survivors.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean (SE)</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>82 (0.55)</td>
<td>83.08</td>
</tr>
<tr>
<td>MADRS</td>
<td></td>
<td>11.40 (0.90)</td>
<td>9</td>
</tr>
<tr>
<td>tHcy</td>
<td>79</td>
<td>12.24 (0.64)</td>
<td>11</td>
</tr>
<tr>
<td>MMA</td>
<td>78</td>
<td>0.27 (0.01)</td>
<td>0.25</td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td>79.23 (0.64)</td>
<td>78.99</td>
</tr>
<tr>
<td>MADRS</td>
<td></td>
<td>8.23 (0.90)</td>
<td>7</td>
</tr>
<tr>
<td>tHcy</td>
<td>39</td>
<td>12.05 (0.69)</td>
<td>11</td>
</tr>
<tr>
<td>MMA</td>
<td>38</td>
<td>0.24 (0.01)</td>
<td>0.23</td>
</tr>
</tbody>
</table>

>15 µmol/L = cut off point of tHcy. >0.37 µmol/L = cut off point of serum MMA. Higher MADRS scores represent more depressive symptomatology. Means, Medians and SE Including Age, Gender, tHcy Expression and MADRS Scores, Presented by Gender.
7.4.2 Rates of Depression and Vitamin Deficiency among Stroke Survivors

Of the individuals with elevated metabolites, 22% showed depressive symptoms, while 12% showed no depressive symptoms. Of the individuals with no elevated metabolites, only 16% percent showed depressive symptoms, while 37% showed no depressive symptoms. Individuals with elevated tHcy or MMA were almost twice as likely to be classified as clinically depressed, according to Diagnostic and Statistical Manual of Mental Disorders (DSM-III) criteria, than were individuals who did not show elevated tHcy or MMA. As expected, MMA and tHcy were significantly correlated, \( r = 0.17, n = 115, p < 0.05 \).

7.4.3 Multiple Regression

Table 2 shows the results of the Hierarchical Multiple Regression used to determine the predictive roles of age, gender and peripheral blood plasma tHcy expression on raw MADRS scores. Age and gender were entered together into the first block, as previous research with this particular cohort indicated that both contributed to depressive symptoms [5]. Then tHcy was entered into the second block to determine if it contributed to the MADRS scores. Age and gender together explained 3.2% of the variance in depression scores with neither gender nor age predicting depression scores \( F (2, 115) = 1.88, p = 0.16 \). The addition of tHcy to the model increased the amount of variance explained by 3.3%, with tHcy significantly contributing to MADRS scores \( F (1, 114) = 3.99, p < 0.05 \). Table 2 displays the coefficients \( b \) the Standard Error of the coefficients \( SE \ B \) and standardized beta \( \beta \) values of the model.
Table 7.2 Coefficients, Standard Error of the Coefficients and Standardized Beta Values of the Regression Model

<table>
<thead>
<tr>
<th></th>
<th>B</th>
<th>SE B</th>
<th>β</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step 1</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
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<tr>
<td>Constant</td>
<td>12.42</td>
<td>12.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.13</td>
<td>0.15</td>
<td>-0.08</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>-3.08</td>
<td>1.63</td>
<td>-0.18</td>
<td></td>
</tr>
<tr>
<td><strong>Step 2</strong></td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Constant</td>
<td>12.08</td>
<td>12.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.05</td>
<td>0.15</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>-3.13</td>
<td>1.61</td>
<td>-0.18</td>
<td></td>
</tr>
<tr>
<td>tHcy</td>
<td>0.28</td>
<td>0.14</td>
<td>0.18*</td>
<td></td>
</tr>
</tbody>
</table>

Note $R^2 = 0.03$ for Step 1: $\Delta R^2 = 0.06$ for Step 2 ($p < .05$). * is significant at $p < 0.05$
7.4.4 Characteristics of the Study Population as Compared to Literature Sourced Control Data

As no control data was available for the observation cohort study, reference values of tHcy and MMA expression in elderly non stroke affected Swedish individuals was sourced from the literature [438]. One sample $t$ tests were used to show that stroke survivors differed significantly in MMA, $t(147) = -3.528, p < 0.01$ (Stroke = 0.20; Control = 0.16) and tHcy levels, $t(148) = -9.64, p < 0.01$ (Stroke = 9.64; Control = 14.7). Surprisingly, 49% of non-stroke individuals showed elevated tHcy and MMA. Among stroke survivors, 34% of individuals had elevated tHcy or and MMA as compared to the literature sourced reference values [438].
7.5 Discussion

To our knowledge this is the first observational cohort study to examine the relationship between depressive symptoms and tHcy, a sensitive measure of folate and vitamin B\textsubscript{12} status [441], in an at-risk elderly post-stroke population.

Individuals with elevated tHcy or MMA were almost twice more likely to be classified as clinically depressed according to DSM-III criteria than were individuals who did not show elevated tHcy or MMA. The multiple regression showed that peripheral plasma tHcy was significantly associated with MADRS depression scores after having controlled for age and gender. This suggests that tHcy is a predictor of depressive symptomatology in stroke survivors. These findings are consistent with previous research suggesting that in non-stroke individuals, increased folate and vitamin B\textsubscript{12} may decrease depressive symptoms by influencing the production of depression related neurotransmitters [436].

Recently, research has begun to focus also on the role of apoptosis in stroke related depression [50]. Apoptosis is elevated among depressed individuals [92] and considered to be a risk factor for depressive disorder [91]. The metabolite tHcy is toxic to neurons [53]. Thus tHcy may be contributing to depressive symptomatology, in part, via regulation of depression related apoptosis.

In the present study, no gender differences were found in tHcy expression. Females did however have higher MADRS scores than males. This finding is consistent with previous analysis using data from this cohort [5] and the general trend of higher reported levels of subjective depression among females [442].

The present research additionally aimed to explore if stroke survivors were more at risk of vitamin deficiency, as indicated by MMA and tHcy levels, than was a comparable non stroke affected population. Previous research has indicated that age related biological and physiological changes make the elderly particularly vulnerable to vitamin deficiency [443]. Dysphagia and subsequent eating difficulties in up to 80% of patients with acute stroke has been reported to further increase this risk of malnutrition [435]. Stroke survivors have additionally been shown to be particularly at risk of clinical depression [5], thus in the present study it was important to clarify the presence of nutritional deficiencies among stroke survivors in depression associated markers. In this
cohort, Swedish stroke survivors were no more likely to show elevated MMA or tHcy, 1.5 years after stroke, compared to elderly Swedish non-stroke affected individuals. In fact, among stroke survivors, a smaller percentage of individuals were found to be vitamin deficient than in the reference group. However, caution should be exercised when interpreting these results. In the present study, nearly one third of stroke survivors were found to be vitamin deficient as indicated by elevated tHcy and MMA, and thus should still be considered an ‘at risk’ group. This is particularly true as tHcy elevation was associated with depressive symptomatology in the stroke-surviving cohort. Elderly individuals are in general identified as at risk of nutritional deficiencies, due to an extensive variety of factors spanning from psychosocial, social, financial, medical and cognitive [443]. Very high levels of nutritional deficiency among the control group may have overshadowed the observed deficiency among stroke surviving individuals. Indeed, almost half of the elderly Swedes from the reference study had elevated MMA and tHcy [438]. It may also be the case that these particular stroke survivors showed less vitamin deficiency than the non-stroke affected comparison population due to the typically high level of medical care, likely hospitalization and monitoring given post-stroke in Sweden. Finally, the stroke experience may prompt increased self-awareness regarding health and well-being, which in conjunction with high level medical care may have encouraged stroke survivors to consume more nutritionally sound foods.

7.6 Conclusions

The present observational cohort study demonstrates that MMA and tHcy are associated with a depression diagnosis post-stroke, and that high tHcy expression is a significant predictor of depressive symptoms post-stroke after accounting for age and gender. The results are consistent with the argument that nutritional factors post-stroke are important in understanding depressive disorder, and its prevention. This observational cohort data additionally demonstrates that elderly Swedish stroke survivors do not have more B vitamin deficiencies when compared to elderly non stroke affected Swedish individuals. It is important to note however that this data was collected in the nineties and possible changes in nutritional intake since this time among stroke survivors may influence the present day validity of these findings. While we do not anticipate this to be the case, we recommend replicating of the present study to ensure the validity of the results.
Chapter 8: General Discussion

The present thesis aimed to investigate the processes of cellular degeneration after ischemic stroke, as a contributing factor to the high incidence of affective disorders associated with post-stroke recovery. Additionally, this thesis aimed to investigate the influence of polyunsaturated fatty acids, on the processes of cellular degeneration. This thesis aimed to study the influence of middle cerebral artery occlusion, on cellular degeneration, and cellular proliferation in the thalamic region and the hippocampus, which are involved in the aetiology of behavioural and affective conditions. It was also aimed to investigate the effects of polyunsaturated fatty acid supplementation on the apparent anxiety-like, depressive-like and hyperactive locomotion behaviours. It was aimed to study correlations between cell death/proliferation, and the above-mentioned behaviours. Finally, in a clinical study, this thesis aimed to investigate the association between homocysteine and persistent depressive mood, at a year and half after stroke onset. The overall study aims were achieved through two systemic reviews, two experimental rodent model studies, and a single clinical observational cohort study. The findings were consistent with the initial hypotheses of the present body of work, that affective disorders post-stroke are related to cellular apoptosis and cellular genesis. Thus, non-pharmacological nutritional interventions, capable of influencing cellular degeneration, may offer a potential alternative, or supplement to conventional medicine. These findings are particularly valuable to those many people who experience unpleasant side effects associated with conventional antidepressant treatments [234]. However, while the overall findings of the present thesis support the hypothesis that nutritional factors that are associated with less localised cellular degeneration and more cellular proliferation, are effective in the management of stroke related affective disorders, a serious risk of increased haemorrhagic bleeding has been demonstrated, and thus any possible positive effects should be interpreted with extreme caution.

8.1 Important Findings from the Present Investigations

8.1.1 Cellular Degeneration and Behavioural Outcomes Interpreted to Reflect Post-stroke Affective Disorders

Chapter 2 systematically reviewed the relationship between inflammation and cell death post-stroke. Moreover, it demonstrated that ischemia induced cell death may influence
the presentation of stroke related affective disorders, via mediating the intrinsic mechanisms driving cellular degeneration, in brain regions involved in the regulation of mood and emotion. It was then demonstrated that anxiety-like, depressive and hyperactive locomotor behaviours, are seen in the rodent model after experimentally induced stroke, and that these behaviours persist for up to six weeks post-surgery (see chapter 5). It is of particular interest that the finding of stroke related behavioural changes, such as locomotor hyperactivity and anxiety-like behaviours, were as predicted, correlated with the amount of localised cell death, seen in brain regions believed to be of significance, namely the limbic region and hippocampus (see chapter 6). Thus, this body of work has been successful in demonstrating that MCAo associated behavioural changes may be closely linked to the process of cellular degeneration.

These above-mentioned results are clinically significant as they suggest it is possible to mediate mood, and thus prognostic outcome, by reducing cellular degeneration occurring, or possibly the duration of cellular degeneration, after stroke. These findings lend support to those previous authors, who have argued for the development of interventions aimed at favouring cellular proliferation and preservation, post stroke [13, 404].

Although differences were seen in localised cell death using immunohistochemistry, no statistical differences between surgery and dietary groups were seen in whole brain Caspase-3 expression, using Western Blot quantification techniques. It is possible that localised differences in the number of Caspase-3-ir labelled cells, between dietary and surgery conditions, was not large enough to result in significant differences in protein expression, across whole brain tissue. Alternatively, the lack of significant differences between surgery and diet conditions, in Caspase-3 expression according to Western Blot analysis, may reflect limitations in commonly used housekeeping genes. As discussed in chapter 6, GAPDH has been shown to over express and accumulate in the nucleus during cell death [432, 433]. It is possible that the expression levels of GAPDH may have been confounded by earlier oxidative stress, resulting from ischemic insult, and confounded the experimental results.

8.1.3. Nutritional Factors Influencing Post-Stroke Affective Disorders

In chapter 3 the anti-inflammatory effects of polyunsaturated fatty acids (PUFAs) were examined. In particular, the potential cellular protective role of nutritional interventions
was also reviewed, by outlining the biological mechanisms by which PUFAs are capable of mediating the intrinsic mechanisms driving ischemia related cell death, and protecting against affective disorders.

In chapter 5, it was demonstrated that even short term PUFA supplementation is associated with an increased incidence of reperfusion related bleeding risk. In surviving animals, dietary supplementation with anti-inflammatory PUFA was seen to be associated with better recovery, during the first five days post ischemic stroke, and with less longer-term stroke related hyperactive locomotion and anxiety-like behaviours. Again, these behavioural effects were seen up to six weeks post-stroke. The six week time period more accurately represents longer-term ischemic recovery, than protocols that only investigate acute neurological or transient motor deficit (i.e. 24 hours after surgery) [444]. Indeed, a number of previous authors have argued that to improve translational research from animal models to clinical populations, primary outcomes should be longer-term behavioural-functional effects and survival times [445, 446]. Thus in the present thesis, a strength of the experimental studies using a rodent model is that they investigated behavioural and histological outcomes at six weeks post surgery.

This thesis demonstrated for the first time that in MCAo surgery surviving animals, dietary supplementation with PUFA was associated with increased cellular proliferation, in the thalamic region of the contralesional hemisphere, when compared to basal diet fed rats (see chapter 6). The implication of this finding is that nutritional factors can be neuroprotective and may facilitate post ischemic spatial remapping and thus functional outcome after stroke. Collectively, the findings of this thesis suggest that stroke associated affective disorders are likely to be related to ischemia induced cellular degeneration. Additionally, neuroprotective PUFAs appear to be capable of protecting against ischemia related affective disorders, possibly in part, by reducing the related neurodegeneration.

The final empirical study of this body of work (chapter 7), successfully demonstrated that a high cellular level of other nutritional factors, homocysteine and methylmalonic acid, which are known to induce cell death [53, 56] (and which are also associated with increased risk of clinical depression in elderly individuals [55, 434]), are associated with human depressive symptomatology, 1.5 years after stroke. Most importantly this was seen in a clinical, rather than an animal population. The implication of this finding
is that neurodegenerative nutritional factors, may contribute to the presentation of stroke associated depressive symptomatology.

8.2 Limitations and Recommendations for Future Research

The recommendations for future research relating to the current body of work derive from both the above outlined findings and from the limitations identified below.

Previous authors have argued that acute neurological scoring of motor functions, such as those used in the present thesis, provide a method by which to screen for and exclude animals that do not display the expected motor deficits (which are arguably presumed to reflect infarct induced disability). As the MCAo stroke model is associated with variability in terms of acute motor impairment and infarct size, this screening is argued to result in a final cohort that is more homologous, in terms of infarct size and related disability [447]. Indeed, a handful of studies demonstrate correlations between basic motor abilities, such as forelimb flexion and infarct size, several weeks, or even months after stroke induction [447, 448]. The vast majority of research however, indicates that in several animal models of brain injury, including the MCAo stroke model, a ‘spontaneous recovery’ of sensorimotor function (as indicated by measures such as reduced forelimb flexion, torso twisting and increased motor ability), occurs in the first few weeks following surgery [449-451]. Interestingly, this ‘spontaneous recovery’ is not dissimilar from clinical observations, where stroke survivors are observed to recover some motor function in the first few weeks following infarct [452], possibly due to early dissipation of ischemia related oedema and later, possibly due to compensatory neuroplastic changes in nearby/adjacent uninjured brain regions [453, 454].

In the present research, we were primarily interested in functional recovery in terms of behaviours designed to reflect clinical anxiety, depressive and hyperactive delirium symptomatology, and not sensory or motor impairments. The basic motor neurological testing conducted in the acute recovery phase, did provide an indication of consistency between our neurological outcomes, and those observed in previous research. Consistent with the majority of previous research, we found no correlation between infarct size, at six weeks post MCAo, and overall neurological function, as assessed during the first five days post-stroke. As motor impairments were seen to significantly decrease during the first five days post-surgery, our results are strongly indicative that basic and acute motor tests alone are neither adequate nor predicate longer term
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functional recovery, as defined by gross sensory or motor impairments. Additionally, these motor impairment behavioural tests were unable to localise the cause of the impairment [455, 456]. For example, in the lateral push test, MCAo affected rats may show weakened resistance when pushed toward the paralytic side, due to a number of impairments. Reduced resistance may result from a lack of basic motor control, or a failure to maintain balance, or a failure to receive and integrate sensory information (especially somatosensory, vestibular and proprioceptive information). This would be expected given the severe damage seen in the somatosensory cortex, (i.e. the rat may not perceive that they are being pushed). Indeed, the MCAo model of ischemic stroke affects many brain regions, as indicated by histology in chapters 5 and 6, and it is impossible to determine which of the affected regions are responsible for the acute deficits observed using these basic neurological ‘motor’ tests. Therefore, the simple motor impairment behavioural tests, used in the present thesis, are probably inadequate as a measure of complex stroke related functional impairment. Some other possibly more sensitive measures of motor impairment post MCAo include the Staircase test (during which the animals is placed on a narrow platform, from which it can reach down into a trough on the left, with its left forepaw, and a trough on the right, with its right forepaw, to retrieve food pellets) [457], the Rotorod Motor test (during which ambulatory function is assessed as animals must ambulate on the rotorod, which is set to a certain acceleration speed, in order to avoid a fall) [457], the Tactile adhesive-removal test (during which somatosensory function is assessed as a stimulus is attached to the animals forepaws and the latency of the animal to remove the stimuli from each forepaw, using the mouth, is recoded) [457]. Given that the whiskers are a primary channel through which rodents collect information from the nearby environment, and are topographically represented in the rodent brain [35], some form of whisker responsiveness could also serve as a measure of stroke induced somatosensory damage, that would be more sensitive than basic and acute measures of motor impairment.

Rodent models have been previously demonstrated to display affective and behavioural changes as late as 10 weeks post MCAo [458, 459]. The MCAo stroke model has been associated with behavioural changes, which the authors have interpreted to reflect depressive-like behaviours (specifically reduced sucrose consumption, argued to indicate desensitisation of the brain reward mechanism, a behaviour argued to reflect anhedonia, which is a symptom of clinical depression), for up to six weeks post-surgery.
in rodents. Indeed, in these animals the administration of a conventional antidepressant, citalopram, has been observed to increased sucrose consumption in MCAo operated animals, compared to MCAo operated animals not receiving citalopram [293, 294]. The MCAo stroke model has additionally been previously associated with decreased increased spontaneous locomotor activity and decreased time spent in the open arm of the elevated plus maze (which is widely interpreted to reflect anxiety like behaviour in rodents), at as late as 10 weeks post surgery [458, 459]. The effect of MCAo on time spent in the open arm of the elevated plus maze is diminished after administration with alofazol, a conventional anxiolytic medication [458]. Finally, rodents exposed to MCAo have been previously demonstrated to spend less time exploring novel objects than control animals, up to 30 days post surgery [460-462].

In animal models, patterns of physiological and behavioural responses to fear, stress or anxiety, that are linked to relevant neural circuits and hormone systems, have been argued to be the most appropriate means by which to study depressive-anxiety-like behaviours [309, 310]. In the present research, we employed a number of behavioural tests, selected to reveal animal responses to fear or anxiety, and studied changes in brain plasticity. A limitation of the present research however, is that we did not account for individual differences in coping styles between rats. More recently it has been argued that variability in coping styles to stress exists in both humans and animals, and that these differences, influence how people and animals respond to stressful, or anxiety provoking situations [307]. While differences in coping styles in groups of experimental animals have traditionally been ignored [463], guidelines have more recently been proposed, that aim to define ‘personality differences’ in rats [464], as well as models relating to differences in coping styles in rodents [465, 466]. In future research, it would be valuable to factor in baseline differences in animal coping styles, using methods outlined by previous authors [465, 466], when studying rodent behavioural response to stressful, or anxiety provoking situations.

One unavoidable limitation of examining morphological changes is the need to choose an appropriate marker to study cellular changes and this means prioritising the questions that can be asked. In the present research we chose to first establish if n-3-LC-PUFA dietary supplementation influences cell proliferation in general and used Ki-67 as general marker of cellular proliferation. As Ki-67 is a general marker of cell
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proliferation, it was not possible to determine what types of cells were proliferating in the ischemic brain, in the present research. Additionally, the data presented in the present thesis does not provide information regarding the site of origin of the newly born cells. For many years it was largely accepted that adult neurogenesis in the brain occurred only in the subgranular zone (SGZ) and the subventricular zone (SVZ) [467]. More recently, however, many authors have demonstrated that under conditions of experimentally induced stroke in animal models, these normal processes of neurogenesis are altered, and new, immature neurons proliferate to the site of ischemic damage [468]. Accordingly, the SGZ and SVZ show increased markers of cell proliferation post-stroke [14-16], and cells proliferating in the SVZ, have been observed to migrate to the area of tissue damage, in response to ischemia [468] (however cells proliferating in the SGZ, appear to continue to migrate to the granular cell layer of the dentate gyrus, even under ischemic conditions) [468].

It must be highlighted however that increased proliferation of cells from the SVZ, expressing neuronal markers such as –NeuN post ischemia, does not guarantee an increase in functional neurons in ischemic tissue [15, 405, 469, 470]. The newly born cells must first survive, and then form useful synaptic connections within the brain circuitry, in an environment that does not favour their survival [15, 405, 469, 470], presumably due to the persistent inflammation [471]. Rodent studies indicate that while cells appear to continue to migrate to damaged tissue for several months after injury [15, 472] that the newly born cells are seen to die within the ischemic tissue within a matter of weeks due to apoptosis [15, 473, 474]. Thus the role of the cell migration from the SVZ and the capacity of newborn neurons to replace damaged cells after stroke are still unclear. In the present thesis, we cannot provide information regarding the functional capacity of proliferating cells, or the possible effects of n-3 LC-PUFA supplementation, on the longer-term survival capacity of the newly proliferated cells. In future research, the functional capacity of new proliferating cells in the post ischemic brain, could be investigated using double labelling for proliferation and neurotransmitter markers, such as BrdU, GFAP, acetylcholine and NMDA etc [475].

In spite of the vast amount of research dedicated to understanding the role of adult cellular regeneration, induced by conditions such as ischemic stroke, it is important to note that the precise role of cellular proliferation, or even neurogenesis in functional
recovery post-stroke, is still unclear [470]. Previous research demonstrates that improved outcome, after pharmacological induction of neurogenesis, takes place in the very early post-stroke period, before neurons have had the opportunity to mature and integrate into functional networks [470]. These findings suggest that behavioural recovery post-stroke may relate to processes additional to neuronal replacement. For example, the proliferation of other cell types, such as astrocytes [476] and macrophages [476] that must occur first after damage to remove dead tissue, undoubtedly also contribute to recovery post-stroke. The relationship between different cell types in forming functional neural networks is a necessary area for future research. Experimentally, this would require a time course study designed to observe the patterns of cellular proliferation and survival, post-surgery, and the possible influence of n-3-LC-PUFA supplementation on these processes.

More recent research has begun to establish that post ischemic neurogenesis occurs also in cerebral cortex [17-21]. New cells, with the capacity for proliferation have also been seen directly in ischemia induced necrotic tissue, an area more traditionally believed to be populated only by inflammatory cells [17]. Other research has indicated that progenitor/stem cells appear in many other parts of the central nervous system also, including the striatum [477, 478] cortex [477-481] spinal cord [482, 483] and subcortical white matter [484]. It appears that neurogenesis may be limited to specific brain regions (such as SVZ and SGZ), under homeostatic conditions, but under pathological conditions such as stroke, regional neural stem/progenitor cells, may be mobilised with the purpose of removal of damaged cells and tissue and accelerating repair of damaged tissue and providing new cells to fill in the left space and replace the dead cells, additional to activating SVZ and SGZ neurogenesis [14]. Understanding the origin and migratory paths of certain types of proliferating cells, post ischemia, will undoubtedly contribute to a better understanding of the mechanisms driving cellular regeneration, and thus, the development of methodologies designed to facilitate this recovery process.

A further limitation of the present studies is that immunohistochemical analysis was only possible after behavioural testing was completed at six weeks post surgery and thus histology was only conducted at one time point. We speculated that ischemia associated cell proliferation, in the hippocampus, may have already occurred by the time the first
spatial displacement recognition test was conducted, at two weeks post-surgery, and corrected any spatial mapping disabilities, that may have been previously present. Indeed, we then demonstrated that there was in fact no difference in cell death in the regions CA1 and DG of the hippocampus, between surgery and diet conditions, at six weeks post-surgery. However, our studies were unable to determine if hippocampus cells had in fact repopulated by six weeks post MCAo, or simply if MCAo surgery did not induce hippocampal cell death initially. Answering this question would also require a time course study, designed to observe the patterns of cellular death and proliferation, in the hippocampus, post-surgery, and how this correlated with exploration time of a displaced object.

Another limitation of the present study is that we did not investigate exactly how much EPA and DHA was present in serum or vascular at different time points, between dietary conditions. Previous authors have demonstrated that six weeks of fatty acid supplementation results in higher levels of EPA, but not DHA, in red blood cells in the rat [344], in rats supplemented with a diet containing 12.36% of EPA [344]. By contrast the present study only supplemented the rats with 0.27% of EPA. We selected this more conservative supplementation percentage to model a more clinically plausible omega 3 fatty acid intake, and to avoid possible overdose oxidation effects. Optimal cellular function is dependent on the ratio of $\omega$-3: $\omega$-6 [172], and previous research has demonstrated that in cell cultures, $\omega$-3 PUFAs can be neurodegenerative when administered in large doses excess or incubated for an extended duration of time [277-280].

Finally, certain differences exist between animal models and clinical populations, which are relevant to the methodology of the current thesis, and need to be considered. For example, rodent models of stroke, such as MCAo, often render the hippocampus ischemic [467, 485-487]. In clinical populations however, the hippocampus is supplied by superficial branches of the posterior cerebral artery, and thus is not usually rendered ischemic by MCA stroke, as assessed using brain imaging at the time of admission to hospital [467, 485-487]. Additionally, a clinical study shows no difference in cell proliferation between depressed and non-depressed patients, at the time of post-mortem [488]. Human studies however are limited in that cellular genesis cannot be studied in the same way that it can be studied in animal models, making animal models
particularly valuable [489]. Animal research indicates conventional antidepressants result in increasing hippocampus neurogenesis [23]. One study has demonstrated that sub-chronic treatment with omega-3 alpha-linolenic acid (ALA) increases neural stem cell proliferation, which corresponds with increased BDNF protein levels, and reduced depressive symptomatology, at day ten post MCAo, in the rodent model [411]. Additionally, in the present research, large infarcts were produced in young rats, that were exposed to MCAo surgery, which is consistent with most rodent based stroke models, though not human strokes which generally affect elderly individuals [445, 446] and consists of a smaller infarct area [289]. While aged animal stroke models are associated with certain issues, such as increased cost and very high mortality rates [457], best practice guidelines recommend that in order to aid clinical translation, rodent models of stroke should use aged animals [445, 446]. Accordingly, in future research it would be valuable to study longer term MCAo related behavioural outcomes, cell degeneration, proliferation, and the possible mediating effects of dietary supplementation in aged animals.

8.3 Overall Conclusion

This thesis systematically reviewed the role of both pro-inflammatory cytokines and polyunsaturated fatty acids, in the processes of cell death; via regulation of intrinsic apoptosis inducing factors. In the experimental chapters, the present thesis demonstrated that MCAo results in gross cell death and proliferation, which is sustained at least until six weeks post-surgery. MCAo associated cell death is related to longer-term impaired functional outcome, as defined by anxiety-like behaviour and locomotor hyperactivity. Dietary supplementation with PUFA has been interpreted to reflect less obvious hyperactive locomotion and anxiety-like symptomatology in the rat model. Additionally, dietary supplementation with PUFA has now been associated with increased cellular proliferation in the thalamic region of the contralesional hemisphere. This thesis further demonstrated the potential risks even after very short term PUFA supplementation. Clinically elevated expression of homocysteine, which is capable of inducing cell death, was also shown to be associated with depressive symptomatology, in stroke survivors.

Together, findings from the work conducted in this thesis have enhanced understanding of the ischemia related biological mechanisms that are likely to contribute to such a
high prevalence of affective disorders post-stroke. Additionally, this thesis has highlighted the ability of nutritional factors to mediate these biological mechanisms and thus mediate associated recovery and well being. The findings of the present thesis support the hypothesis that ischemia results in the degeneration of cellular networks, and that this degeneration is closely linked to stroke associated behavioural and affective disorders.
References


Mechanisms of Stroke Affective Disorders


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Mechanisms of Stroke Affective Disorders


Mechanisms of Stroke Affective Disorders


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Appendices

Appendix I: Ethics Clearance Statement Animal Studies

Certificate Of Approval
To conduct research using animals or animal tissue

Principal Investigator: A/Prof David Howells
Department: Department of Medicine, Austin Campus
Heidelberg, Vic 3084
Project Title: Inflammation and depression, and post stroke depression in rats
Project No: A2010/03865

Approval date: 23 April 2010 Expiry date: 23 April 2013

☐ Animal Ethics Committee Approval date 23/04/2010
☐ Biosafety Committee Approval date .../.../... ☑ not required

I confirm that the Principal Investigator or Co-Investigators were not involved in the approval of this project.

The Austin Health Animal Ethics Committee (AEC) is constituted by Austin Health with authority to approve animal based research on the Austin Health campuses. It has been established within the guidelines prepared by:

The National Health and Medical Research Council of Australia (NHMRC)
Commonwealth Scientific and Industrial Research Organization (CSIRO)
Australian Research Council (ARC)
Australian Vice-Chancellor’s Committee (AVCC)
Bureau of Animal Welfare, Victoria

Signed: _______________________________ Date: 23/04/10

Prof Mauro Sandrin
Chairman
Animal Ethics Committee
Appendix II: Conditions of Ethics Clearance Animal Studies

Austin Health

Certificate Of Approval

To conduct research using animals or animal tissue

The Principal Investigator is the person who takes responsibility for the ethical and scientific conduct of the study and as the Principal Investigator you are reminded that you are responsible for all procedures carried out under this approval. Below are the conditions of approval that must be met for ethical approval to continue. If the below conditions are not met, ethical approval will no longer be valid.

The Principal Investigator is required to:

- Ensure that any researchers involved in this project are properly trained
- Request approval for any proposed change in study personnel
  (Request for Minor Amendment Form should be used - a template is available on the website)
- Request approval for any proposed change in protocol or any time extension, including the reasons for that change and any ethical implications
  (Request for Minor Amendment Form should be used - a template is available on the website)
- Advise, by letter to the Chair, any incident or unpredicted event that has animal ethical or welfare implications (Please use the adverse event report form - a template is available from the Research Ethics Unit or the AEC website.)

The Principal Investigator is required to submit

- A progress report every 12 months
  (Progress Report Form should be used - a template is available on the website)
- A detailed final report at the conclusion of the study

If the study does not commence within 12 months of the approval date on this certificate, this approval will lapse. A resubmission to the Animal Ethics Committee will be required.

I acknowledge that I have read the above conditions and agree to abide by them.

Signed: ............................................ Date: 31/1/2010
Name: ............................................
(please print)

Please sign both copies, keep one copy for your records and return the other copy to the Research Ethics Unit

Research Ethics Unit
Henry Buck Building, Studley Rd Heidelberg VIC 3084
Telephone 9496 4090
Fax 9496 4103
Email ethics@austin.org.au
Website www.austinmedicine.unimelb.edu.au/research/ethics.html
Appendix III: Ethics Clearance Clinical Study

Tillägg till tidigare ansökan Dnr 31-92

I en pågående randomiserad studie jämföra vård vid Strokeenhet inom vårdkedja ut mot strokeprofilerad vård i geriatrisk i en behandlingsgrupp och konventionell vård i andra behandlingsgruppen. Inklusion av patienter närmast sig sitt slut under våren 1994. I den planerade 1-årsuppföljningen görs en noggrann evaluering av neurologiskt tillstånd, funktionsnivå, hjälpmedelbehov m.m. enl tidigare ansökan.


För upp till 100 patienter planerar vi att komplettera med en MR-undersökning och CBF-undersökning. Risken och besväret för patienter bedöms som ringa i relation till den positiva effekt patienterna upplever av ökad klarhet kring sin sjukdom och dess effekter.


Ovanstående tillägg har granskats och godkänts av:

Lars Rönnbäck
Professor, Avd. för neurologi
Divisionschef, Neurologiska divisionen

Christian Blomstrand
Cheffläkare, Neurosjukvården
Docent,Inst.för klinisk neurovetenskap

Granskad och godkänd som ordförande av ledningen

Postadress: Sahlgrenska sjukhuset
Visigatan 31, S-413 45 Göteborg, Tfn 031-771 20 00, Faks 031-771 21 85

Sahlgrenska sjukhuset

413 45 Göteborg

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Appendix IV: Translated Version of Ethics Clearance Clinical Study

Amendments to previous submission Dnr 31-92

In an ongoing randomised study compare treatment in Stroke Unit including comprehensive care towards stroke concentrated geriatric treatment in one group and conventional treatment in the second group. Includes patients approaching the end during spring the year 1994. In the planned follow up after 1 year, a detailed review of neurological state, functional ability, required level of assistance, etc. will be carried out as per earlier submission.

We now wish to expand this research with a neuropsychiatric study aimed at the risk for dementia and other psychiatric diseases. The form to be used is taken from the H70-study in which they have been used since 1971 with an amendment in 1986. The use of this form have previously been accepted by the Ethical Committee 760322 § 8 application number 52 and amended 910320. The same study was also accepted for NK 75 900920 Dnr 263-90. An interview of relatives, to be used to diagnose dementia diseases, has also been accepted 890428 Dnr 123-89 and amended 910320.

The goal is to study the spectrum of psychiatric diseases associated with stroke. In the previous studies of more than 2000 people, no issues have been encountered in relation to the interviews. The patients aged 70 or above who have been exhaustively studied according to previous project submissions have experienced the follow up as very positive. Relatives have also been very positive to the follow up. Therefore, we expect that the additional study, carried out by doctors with good psychiatric and neurologic understanding, will be perceived as positive. We plan that up to 200 stroke patients be examined during the 1 year follow up with the additional study.

For up to 100 patients, we plan to complement the study with MR exams and CBF exams. The risks and discomfort for the patients are considered to be minor compared to the positive effects patients experience from better understanding of their condition and its effects.

We therefore ask for permission from the Ethical Committee for the above supplement to the already approved submission. The main responsible people for the study are Christian Blomstrand, Neurology Björn Fagerberg, Medicine, Ingmar Skoog, Psychiatry. Under the supervision of Christian Blomstrand and Ingmar Skoog, Thomas Lindén, Neurology department, is responsible for the interviews.

The above amendment has been reviewed and approved by

Christian Blomstrand    Lars Rönnbäck
Medical Director, Neurology    Professor of Neurology
Associate professor, Inst. for Clinical Neuroscience    Head of department of Neurology
# Appendix V: Ingredients, Calculated Nutritional Parameters of the Basal AIN93G Diet

## Diet Form and Features
- Semi pure diet. 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.

## Calculated Nutritional Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>AIN93G diet</th>
<th>Standard AIN93G Rodent Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>19.4%</td>
<td>200 g/Kg</td>
</tr>
<tr>
<td>Total Fat</td>
<td>7.0%</td>
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<tr>
<td>Total Carbohydrate</td>
<td>56.9%</td>
<td>70 g/Kg</td>
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<tr>
<td>Crude Fibre</td>
<td>4.7%</td>
<td>50 g/Kg</td>
</tr>
<tr>
<td>AD Fibre</td>
<td>4.7%</td>
<td>404 g/Kg</td>
</tr>
<tr>
<td>Digestible Energy</td>
<td>16.1 MJ / Kg</td>
<td>132 g/Kg</td>
</tr>
<tr>
<td>% Total calculated digestible energy from lipids</td>
<td>16.0%</td>
<td>3.0 g/Kg</td>
</tr>
<tr>
<td>% Total calculated digestible energy from protein</td>
<td>21.0%</td>
<td>13.1 g/Kg</td>
</tr>
<tr>
<td>Ash</td>
<td>4.5%</td>
<td>1.4 g/Kg</td>
</tr>
</tbody>
</table>

## Ingredients

- Casein (Acid) 200 g/Kg
- Sucrose 100 g/Kg
- Canola Oil 70 g/Kg
- Cellulose 50 g/Kg
- Wheat Starch 404 g/Kg
- Dextrinised Starch 132 g/Kg
- DL Methionine 3.0 g/Kg
- Calcium Carbonate 13.1 g/Kg
- Sodium Chloride 2.6 g/Kg
- AIN93 Trace Minerals 1.4 g/Kg
- Potassium Citrate 2.5 g/Kg
- Potassium Dihydrogen Phosphate 6.9 g/Kg
- Potassium Sulphate 1.6 g/Kg
- Choline Chloride (75%) 2.5 g/Kg
- AIN93 Vitamins 10 g/Kg
## Appendix VI: Ingredients, Amino Acids, Vitamins, and Minerals of the Basal AIN93G Diet

<table>
<thead>
<tr>
<th>Calculated Amino Acids</th>
<th>Calculated Total Vitamins</th>
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</thead>
<tbody>
<tr>
<td>Valine</td>
<td>Vitamin A (Retinol)</td>
</tr>
<tr>
<td>Leucine</td>
<td>Vitamin D (Cholecalciferol)</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Vitamin E (α Tocopherol acetate)</td>
</tr>
<tr>
<td>Threonine</td>
<td>Vitamin K (Menadione)</td>
</tr>
<tr>
<td>Methionine</td>
<td>Vitamin C (Ascorbic acid)</td>
</tr>
<tr>
<td>Cystine</td>
<td>Vitamin B1 (Thiamine)</td>
</tr>
<tr>
<td>Lysine</td>
<td>Vitamin B2 (Riboflavin)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Niacin (Nicotinic acid)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Vitamin B6 (Pyrondoxine)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Pantothenic Acid</td>
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</table>

<table>
<thead>
<tr>
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<th>Calculated Fatty Acid Composition</th>
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<tr>
<td>Calcium</td>
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<tr>
<td>Phosphorous</td>
<td>Palmitic Acid 16:0</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Stearic Acid 18:0</td>
</tr>
<tr>
<td>Sodium</td>
<td>Palmitoleic Acid 16:1</td>
</tr>
<tr>
<td>Chloride</td>
<td>Oleic Acid 18:1</td>
</tr>
<tr>
<td>Potassium</td>
<td>Gadoleic Acid 20:1</td>
</tr>
<tr>
<td>Sulphur</td>
<td>Linoleic Acid 18:2 n6</td>
</tr>
<tr>
<td>Iron</td>
<td>a Linolenic Acid 18:3 n3</td>
</tr>
<tr>
<td>Copper</td>
<td>Arachadonic Acid 20:4 n6</td>
</tr>
<tr>
<td>Iodine</td>
<td>EPA 20:5 n3</td>
</tr>
<tr>
<td>Manganese</td>
<td>DHA 22:6 n3</td>
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<td>Total n3</td>
</tr>
<tr>
<td>Zinc</td>
<td>Total n6</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>Total Mono Unsaturated Fats</td>
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<td>Selenium</td>
<td>Total Polyunsaturated Fats</td>
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<td>Cadmium</td>
<td>Total Saturated Fats</td>
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<td>Fluoride</td>
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<td>Lithium</td>
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<tr>
<td>Boron</td>
<td></td>
</tr>
<tr>
<td>Nickel</td>
<td></td>
</tr>
<tr>
<td>Vanadium</td>
<td></td>
</tr>
</tbody>
</table>
Appendix VII: Ingredients, Calculated Nutritional Parameters of the Fish Oil Enriched SF09-109 Diet

Diet SF09-109 5% Fat High N3 Semi-Pure Modification of AIN93G Rodent Diet

A 5% fat (high n3 fat) semi-pure diet formulation for laboratory rats and mice based on AIN-93G. This formulation satisfies the nutritional requirements for growth of rats and mice. Some modifications have been made to the original formulation to suit locally available raw materials.

<table>
<thead>
<tr>
<th>Calculated Nutritional Parameters</th>
<th>Ingredients</th>
</tr>
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<tbody>
<tr>
<td>Protein</td>
<td>Casein (Acid)</td>
</tr>
<tr>
<td>Total Fat</td>
<td>Sucrose</td>
</tr>
<tr>
<td>Crude Fibre</td>
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<td>AD Fibre</td>
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<td>(Copha)</td>
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<td>energy from protein</td>
<td>DL Methionine</td>
</tr>
<tr>
<td></td>
<td>Calcium Carbonate</td>
</tr>
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<td></td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td></td>
<td>AIN93 Trace Minerals</td>
</tr>
<tr>
<td></td>
<td>Potassium Citrate</td>
</tr>
<tr>
<td></td>
<td>Potassium Dihydrogen Phosphate</td>
</tr>
<tr>
<td></td>
<td>Potassium Sulphate</td>
</tr>
<tr>
<td></td>
<td>Choline Chloride (75%)</td>
</tr>
<tr>
<td></td>
<td>AIN93 Vitamins</td>
</tr>
</tbody>
</table>

|                  | 200 g/Kg       |
|                  | 100 g/Kg       |
|                  | 47 g/Kg        |
|                  | 3 g/Kg         |
|                  | 50 g/Kg        |
|                  | 424 g/Kg       |
|                  | 132 g/Kg       |
|                  | 3.0 g/Kg       |
|                  | 13.1 g/Kg      |
|                  | 2.6 g/Kg       |
|                  | 1.4 g/Kg       |
|                  | 2.5 g/Kg       |
|                  | 6.9 g/Kg       |
|                  | 1.6 g/Kg       |
|                  | 2.5 g/Kg       |
|                  | 10 g/Kg        |

Diet Form and Features

- Semi pure diet. 12 mm diameter pellets.
- Pack size 5 Kg, vacuum packed in oxygen impermeable plastic bags, under nitrogen. Bags are packed into cardboard cartons to protect them during transit. Smaller pack quantity on request.
- Diet suitable for irradiation but not suitable for autoclave.
- Lead time 2 weeks for non-irradiation or 4 weeks for irradiation.
### Appendix VIII: Ingredients, Amino Acids, Vitamins, and Minerals of the Fish Oil Enriched SF09-109 Diet

#### Calculated Amino Acids

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Percentage</th>
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<td>Valine</td>
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<td>1.80%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.90%</td>
</tr>
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<td>Threonine</td>
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<tr>
<td>Methionine</td>
<td>0.80%</td>
</tr>
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<td>Cystine</td>
<td>0.06%</td>
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<td>Lysine</td>
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</tr>
<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>1.00%</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.30%</td>
</tr>
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</table>

#### Calculated Total Minerals

<table>
<thead>
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<tr>
<td>Magnesium</td>
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<td>Sodium</td>
<td>0.15%</td>
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<td>Chloride</td>
<td>0.16%</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.40%</td>
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<tr>
<td>Sulphur</td>
<td>0.23%</td>
</tr>
<tr>
<td>Iron</td>
<td>75 mg/Kg</td>
</tr>
<tr>
<td>Copper</td>
<td>7.0 mg/Kg</td>
</tr>
<tr>
<td>Iodine</td>
<td>0.2 mg/Kg</td>
</tr>
<tr>
<td>Manganese</td>
<td>20 mg/Kg</td>
</tr>
<tr>
<td>Cobalt</td>
<td>No data</td>
</tr>
<tr>
<td>Zinc</td>
<td>50 mg/Kg</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>0.15 mg/Kg</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.3 mg/Kg</td>
</tr>
<tr>
<td>Cadmium</td>
<td>No data</td>
</tr>
<tr>
<td>Chromium</td>
<td>1.0 mg/Kg</td>
</tr>
<tr>
<td>Fluoride</td>
<td>1.0 mg/Kg</td>
</tr>
<tr>
<td>Lithium</td>
<td>0.1 mg/Kg</td>
</tr>
<tr>
<td>Boron</td>
<td>3.4 mg/Kg</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.5 mg/Kg</td>
</tr>
<tr>
<td>Vanadium</td>
<td>0.1 mg/Kg</td>
</tr>
</tbody>
</table>

#### Calculated Total Vitamins

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (Retinol)</td>
<td>4 000 IU/Kg</td>
</tr>
<tr>
<td>Vitamin D (Cholecalciferol)</td>
<td>1 000 IU/Kg</td>
</tr>
<tr>
<td>Vitamin E (a Tocopherol acetate)</td>
<td>75 mg/Kg</td>
</tr>
<tr>
<td>Vitamin K (Menadione)</td>
<td>1 mg/Kg</td>
</tr>
<tr>
<td>Vitamin C (Ascorbic acid)</td>
<td>None added</td>
</tr>
<tr>
<td>Vitamin B1 (Thiamine)</td>
<td>6.1 mg/Kg</td>
</tr>
<tr>
<td>Vitamin B2 (Riboflavin)</td>
<td>6.3 mg/Kg</td>
</tr>
<tr>
<td>Niacin (Nicotinic acid)</td>
<td>30 mg/Kg</td>
</tr>
<tr>
<td>Vitamin B6 (Pyradoxine)</td>
<td>7 mg/Kg</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>16.5 mg/Kg</td>
</tr>
<tr>
<td>Biotin</td>
<td>200 ug/Kg</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>2 mg/Kg</td>
</tr>
<tr>
<td>Inositol</td>
<td>None added</td>
</tr>
<tr>
<td>Vitamin B12 (Cyanocobalamin)</td>
<td>103 ug/Kg</td>
</tr>
<tr>
<td>Choline</td>
<td>1670 mg/Kg</td>
</tr>
</tbody>
</table>

#### Calculated Fatty Acid Composition

<table>
<thead>
<tr>
<th>Acid</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated Fat C12:0 and less</td>
<td>0.17%</td>
</tr>
<tr>
<td>Myristic Acid 14:0</td>
<td>0.18%</td>
</tr>
<tr>
<td>Palmitic Acid 16:0</td>
<td>0.90%</td>
</tr>
<tr>
<td>Stearic Acid 18:0</td>
<td>0.27%</td>
</tr>
<tr>
<td>Palmitoleic Acid 16:1</td>
<td>0.22%</td>
</tr>
<tr>
<td>Oleic Acid 18:1</td>
<td>0.63%</td>
</tr>
<tr>
<td>Gadoleic Acid 20:1</td>
<td>0.04%</td>
</tr>
<tr>
<td>Linoleic Acid 18:2 n6</td>
<td>0.05%</td>
</tr>
<tr>
<td>a Linoleic Acid 18:3 n3</td>
<td>0.02%</td>
</tr>
<tr>
<td>Arachadonic Acid 20:4 n6</td>
<td>0.08%</td>
</tr>
<tr>
<td>EPA 20:5 n3</td>
<td>0.27%</td>
</tr>
<tr>
<td>DHA 22:6 n3</td>
<td>1.19%</td>
</tr>
<tr>
<td>Total n3</td>
<td>1.66%</td>
</tr>
<tr>
<td>Total n6</td>
<td>0.23%</td>
</tr>
<tr>
<td>Total Mono Unsaturated</td>
<td>1.10%</td>
</tr>
<tr>
<td>Total Polyunsaturated Fats</td>
<td>1.91%</td>
</tr>
<tr>
<td>Total Saturated Fats</td>
<td>1.67%</td>
</tr>
</tbody>
</table>

---

200
Appendix IX: Post-Surgery Monitoring Sheet

* COMMUNICATION WITH BRF STAFF – notified of animal location
post-surgery (utilise whiteboard)

* ANIMAL LOCATION
<24h: Pre/post op room & on heating mat
> 24h: Exp holding room #2 & entered in log book

ANIMAL WELFARE:
- Date
- Day
- Time
- Investigator

BODY WEIGHT & B.A.R (bright, alert, responsive) SCORE (normal is 0, score 1, 2, 3 for ↑ severity)
- Weight yesterday
- Weight today
- Weight change
- B.A.R.
- Approach response

GENERAL CLINICAL SIGNS (normal is 0, score 1, 2, 3 for ↑ severity)
- Inactive
- Hunched posture
- Coat rough – fur on end
- Red eye/ nose discharges
- Dehydration (as per skin pulling)

BEHAVIOURAL SIGNS OF PAIN IN RATS (normal is 0, score 1, 2, 3 for ↑ severity)
- Back arch (hunched up with arched back)
- Belly press (presses belly to cage floor)
- Writhe (twisting of body or flank)
- Stagger (sudden loss of balance / gait)
- Twitch (sudden spasm of flank muscles)
- Fall (rat falls over)

WATER CONSUMPTION
- Pre-bottle weight (A)
- Post-water weight (B)
- Water intake (A-B) (mls)

FOOD CONSUMPTION
- Pre-food weight (A)
- Post-food weight (B)
- Food intake (A-B) (mg)

OPERATION SITE – HEAD
- Wound OK
- Bleeding
- Sutures OK

OPERATION SITE – NECK/THROAT
- Wound OK
- Bleeding
- Sutures OK

POST-OPERATIVE SUPPORT (shaded boxes represent days where this must be conducted)
- Paracetomol (120mg/kg bottle)
- Saline (3ml SC)
- Soft food/ sunflower seeds
### Appendix X: Guide for Acute Neurological Impairment Testing

<table>
<thead>
<tr>
<th>FORELIMB FLEXION</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>No flexion – Forelimbs extend equally outstretched toward the bench</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Mild – Left forelimb at approximately 45° angle consistently. OR If angle is greater than closer to 90° but is non consistent.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Moderate to severe – Left forelimb at 90° or greater. Forelimb flexion is consistent.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TORSO TWISTING</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>No Signs – Body elongated and extended toward bench</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Mild – Half twist</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Moderate to severe – Consistently strong twisting. Head and forelimbs brought toward hindlimbs.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LATERAL PUSH</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>Equal resistance – Animal will resist being pushed</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Weakened resistance – Animal will show weakened resistance whilst trying to correct</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>No resistance – Animal has severely weakened resistance. Will tend to “roll” with left legs collapsing after being pushed to left.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MOBILITY</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>Normal mobility – Animal is able to freely walk etc</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Spontaneous movement reduced</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Needs stimulus to move</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Unable to walk</td>
</tr>
</tbody>
</table>
### Appendix XI: Infarct Analysis Drawings

<table>
<thead>
<tr>
<th>PUFA MCAo # 1 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PUFA MCAo # 2 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PUFA MCAo # 3 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PUFA MCAo # 4 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
</tr>
</tbody>
</table>
Appendix XI: Infarct Analysis Drawings Cond.

<table>
<thead>
<tr>
<th>PUFA MCAo # 5 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PUFA MCAo # 6 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Basal MCAo # 1 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Basal MCAo # 2 Distance From Bregma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
</tr>
</tbody>
</table>
## Appendix XI: Infarct Analysis Drawings Cond.

**Basal MCAo # 3 Distance From Bregma**

<table>
<thead>
<tr>
<th>1</th>
<th>-2.3</th>
<th>-4.52</th>
<th>-5.8</th>
<th>-7.3</th>
</tr>
</thead>
</table>

![Infarct Analysis Drawings Basal MCAo # 3](image1)

**Basal MCAo # 4 Distance From Bregma**

<table>
<thead>
<tr>
<th>1</th>
<th>0.2</th>
<th>-1.8</th>
<th>-4.52</th>
<th>-6.8</th>
</tr>
</thead>
</table>

![Infarct Analysis Drawings Basal MCAo # 4](image2)

**Basal MCAo # 5 Distance From Bregma**

<table>
<thead>
<tr>
<th>1</th>
<th>-0.26</th>
<th>-2.3</th>
<th>-4.52</th>
<th>-6.04</th>
</tr>
</thead>
</table>

![Infarct Analysis Drawings Basal MCAo # 5](image3)

**Basal MCAo # 6 Distance From Bregma**

<table>
<thead>
<tr>
<th>1.2</th>
<th>0.2</th>
<th>-2.3</th>
<th>-4.8</th>
<th>-7.64</th>
</tr>
</thead>
</table>

![Infarct Analysis Drawings Basal MCAo # 6](image4)
Appendix XII: Summary of Significant Findings.

<table>
<thead>
<tr>
<th>Summary of Significant Findings Presented in the Thesis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemorrhage Bleeding</strong></td>
</tr>
<tr>
<td>n-3-LC-PUFA supplementation associated with risk of reperfusion related haemorrhage at time of surgery.</td>
</tr>
<tr>
<td><strong>Food and Water</strong></td>
</tr>
<tr>
<td>MCAo operated animals consumed less food than sham operated animals</td>
</tr>
<tr>
<td><strong>Fatty Acids in Brain Tissue</strong></td>
</tr>
<tr>
<td>n-3-LC-PUFA fed animals showed less n−6 in whole brain tissue than animals fed the basal diet at 6 weeks post surgery</td>
</tr>
<tr>
<td><strong>Infarct Volume</strong></td>
</tr>
<tr>
<td>MCAo affected animals showed a greater volume (mm³) of damaged tissue, when compared to sham operated animals that did not show any infarct damage.</td>
</tr>
<tr>
<td><strong>Motor Impairment</strong></td>
</tr>
<tr>
<td>Sham operated animals consistently scored very low on motor impairment measures at all-time points compared to MCAo operated animals.</td>
</tr>
<tr>
<td><strong>Emergence Frequency</strong></td>
</tr>
<tr>
<td>MCAo affected animals made more emergences from the hide box into the open-field than sham affected animals at weeks four and six</td>
</tr>
<tr>
<td><strong>Emergence Duration</strong></td>
</tr>
<tr>
<td>n-3-LC-PUFA fed animals spent fewer seconds out in the open field area than basal fed rats at week six post-surgery</td>
</tr>
</tbody>
</table>
### Percentage of Emergence Duration in Centre of Open Field

- n-3-LC-PUFA fed spent significantly more time in the centre of the arena at week four and week six compared to basal diet fed animals.

### Novel Object Exploration

- MCAo affected animals explored the novel object less than sham operated animals at week four post-surgery.
- n-3-LC-PUFA supplemented animals in each surgery condition spent more time exploring the novel object than the basal fed animals at week six.

### Caspase-3 and Ki-67 Expression in the Thalamic Region of the Ipsilesional Hemisphere

- MCAo operated rats show higher Caspase-3 and Ki-67 expression compared to shams.

### Caspase-3 and Ki-67 Expression in the Thalamic Region of the Contralesional Hemisphere

- MCAo operated animals showed more Caspase-3-ir labelled cells than shams.
- Animals supplemented with n-3-LC-PUFA showed fewer Caspase-3-ir labelled cells than basal diet fed animals.

### Caspase-3 and Ki-67 Expression in the Thalamic Region of the Ipsilesional vs. Contralesional Hemisphere

- MCAo operated animals showed more Caspase-3-ir cells in the thalamic region of the ipsilesional hemisphere, compared to the thalamic region of the contralesional hemisphere.
- MCAo operated animals showed more Ki-67 in the thalamic region of the ipsilesional, compared to the thalamic region of the contralesional hemisphere.

### Correlations Between Caspase-3 and Ki-67 Expression and Behavioural Outcomes, at Six Weeks Post-Surgery

- Spearman correlations showed a positive correlation between the number of Caspase-3-ir labelled cells in the thalamic region of the ipsilesional hemisphere and the total emergence duration in the open-field.
- The number of Caspase-3-ir labelled cells in the thalamic region of the ipsilesional hemisphere positively correlated with the number of times that the rat emerged from the hide box, into the open-field, in the free CA1 region of the hippocampus and the total emergence duration in the open-field.
- The number of Ki-67-ir labelled cells in the contralesional thalamic region negatively correlated with number of times the rat emerged from the hide box into the open-field arena.
- A negative correlation between the number of Ki-67-ir labelled cells in the contralesional thalamic region and the duration of time spent moving in the open-field arena approached significance.
exploration test, at six weeks post-surgery

**Rates of Depression and Vitamin Deficiency among Stroke Survivors**

Individuals with elevated tHcy or MMA were almost twice as likely to be classified as clinically depressed, than were individuals who did not show elevated homocysteine or methylmalonic acid
Ethics Statement

I Michaela Pascoe, hereby declare that all conditions pertaining to ethics clearance were met including the submission of all required annual and final reports.

Miss Michaela Pascoe.
List of Publications Produced as a Result of the Project

Chapter 2:


Chapter 3:


Chapter 5:


Chapter 7: