

**The effects of chronic multivitamin supplementation  
on neurocognition in the elderly**

**Doctor of Philosophy**

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2012**

## **Abstract**

Age-related decline occurs across the lifespan and is a feature of the normal ageing process. With an ageing global population there is an increasing scientific interest in the potential of health and lifestyle interventions to improve cognitive function in the elderly. Evidence from epidemiological and randomised controlled trials has indicated that vitamins and other dietary substances may be particularly important for cognitive function. Heterogeneous findings have been obtained from Randomised Controlled Trials which have investigated the effects of multivitamin supplements on cognition, in older adults. This study represents the first trial to apply neuroimaging techniques to investigate the effects of multivitamin supplementation on brain electrical activity. When combined with behavioural measures the examination of brain electrical activity may provide additional insights into the neurocognitive effects of chronic multivitamin supplementation.

This thesis reports the findings from a 16 week, randomised, placebo-controlled, double-blind trial which investigated the effects of chronic multivitamin supplementation on cognitive performance and the steady state visually evoked potential (SSVEP) measure of brain electrical activity, shown previously to be sensitive to fast occurring changes in cognition. The multivitamin contained a combination of vitamin, mineral and herbal components. Participants were elderly women aged between 64 and 82 years. 28 participants were allocated to the multivitamin treatment and 28 to placebo. Cognitive performance was assessed using a validated battery of computerised memory and attentional tasks. It was predicted that chronic treatment with a combined multivitamin and herbal supplement would enhance the cognitive domains most vulnerable to age-related decline. Changes in the SSVEP were assessed during the performance of a spatial working memory delayed response task. Biochemical and cardiovascular mechanisms of potential cognitive enhancements were also examined.

The results indicated that multivitamin supplementation improved spatial working memory speed of response, a cognitive process which is known to be compromised with age. There were no

multivitamin-related treatment effects for measures of attention or verbal memory. Multivitamin supplementation significantly increased SSVEP latency, interpreted as an increase in inhibitory neural processes. The multivitamin increased concentrations of vitamin B<sub>6</sub>, B<sub>12</sub>, E and lowered homocysteine. Multivitamin supplementation did not benefit other measures of cardiovascular health, indicating the observed cognitive improvements were not mediated by cardiovascular mechanisms.

Findings from this study indicate that the SSVEP measure of brain activity is useful for examining the neurocognitive effects of multivitamins. Furthermore, it is suggested that chronic multivitamin supplementation may be effective to improve neurocognition in the elderly.

## Acknowledgements

Firstly I must thank my Mum Elaine for her support on every level throughout my years of study. You have taught me the skills of persistence and determination and have raised me with the belief that I can achieve anything I set my mind to.

I would like to thank my primary supervisor Dr Andrew Pipingas for his expertise across all aspects of this thesis. I am very thankful to have had such a supportive supervisor who was willing to place so much time and energy into this project. Good PhD supervisors are hard to come by and I am very lucky to have found one! I am also grateful to Prof Richard Silberstein for sharing his insights into the SSVEP and for providing the Matlab scripts I required to complete the SSVEP statistical analysis. Special thanks to Associate Prof David Liley for conducting the participant medical examinations for this study.

Thank you to Prof Andrew Scholey for your feedback on this thesis, and to Prof Con Stough and the rest of the Nutra research group for establishing a supportive and resourceful research environment.

I would like to thank Doreen Carbuhn and Tiffany Keele for proof reading numerous drafts of this thesis and improving the overall readability, spelling and grammar.

I would like to thank my friends for their support, especially the other PhD students from the BSI who looked after me through some difficult times and generally made the whole PhD process more enjoyable. I am especially grateful to Angela Mayes for all our chats about the SSVEP and those handy Matlab scripts, and to Renee Castell for helping out with data collection.

I would like to acknowledge and thank Swisse Vitamins for providing funding for this trial.

Thank you Paul for your patience and encouragement and for reminding me that life isn't all about hard work 😊

## **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 this thesis. This thesis contains no material previously published or written by another person except where due reference is made. Where the work is based on joint research or publications, discloses the relative contributions of the respective authors.

Signed

Dated

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## Chapter 1 Introduction and overview

The concept of cognitive health is gaining importance as rapid population ageing is taking place in the Western world. This is particularly evident in the United States and the United Kingdom, where those aged over 65 are at or approaching 15% of the population (Crews & Zavotka, 2006). As a greater percentage of the population moves towards old age, their medical and primary care needs will be exacerbated, resulting in increased financial pressure on the health care system. Furthermore, an ageing population will result in an increased prevalence of age-related neurodegenerative disorders such as dementia. In 2006 there were 190,000 people in Australia with dementia, and this figure is expected to grow to 465,000 by 2031 (AIHW, 2007). The projected growth in dementia rates is a global issue. In 2005 it was estimated that 24 million people worldwide were living with dementia, it is predicted that this quantity will double every 20 years to reach 81 million by the year 2040 (Ferri et al., 2005). The cost of caring for those with dementia imposes a significant financial burden on tax payers and an emotional burden for family members.

With an ageing population and escalating rate of dementia, there is an increasing need to identify preventative strategies to delay the age of dementia onset and reduce the projected number of dementia cases in future years (Jorm, Dear & Burgess, 2005). Alzheimer's disease (AD) is the most common form of dementia and is characterised by the DSM-IV as a marked decline from previous functioning in short term memory, and a severe disruption to language, planning or visual processing (American Psychiatric Association, 2000). Age constitutes the major risk factor for AD, and it is estimated that in developed countries such as the United States, approximately 13% of individuals over the age of 65, and 48% of persons over the age of 85 are affected by this disease (Thies & Bleiler, 2011).

Cognitive deterioration occurs across the lifespan and is a feature not only of AD, but also the normal ageing process. In many cases the experience of cognitive decline may be a precursor to the development of AD (Goedert & Spillantini, 2006). Consequently, in order to delay the onset or rate of dementia, it may be necessary to target interventions to individuals prior to the appearance of cognitive decline. Currently there is scientific interest in the potential of health and lifestyle interventions to improve cognitive function, or slow the rate of decline in the elderly. There is evidence from randomised controlled trials (RCT) to indicate that aerobic exercise programs (Baker et al., 2010), mental training (Valenzuela & Sachdev, 2009), and dietary supplements consisting of omega-3 fatty acids (Yurko-Mauro et al., 2010), herbal preparations (Mix & Crews, 2002; Pipingas et al., 2008) or vitamins (Durga et al., 2007) may be capable of improving cognitive function, and potentially serve as interventions against cognitive decline.

Age-related cognitive decline is believed to be the consequence of a range of structural and physiological changes in the brain including loss of grey and white matter and alterations in levels of neurotransmitters. Cognitive domains prone to age-related deterioration include working memory, episodic memory and attention (Ronnlund et al., 2005). Findings from electrophysiological and neuroimaging studies have revealed that the neural correlates of memory and attention also decline with age (Cabeza, 2001; Reuter-Lorenz & Lustig, 2005). Alterations to the function of brain regions including the prefrontal cortex (PFC), parietal and temporal lobes have been implicated in decline to these cognitive processes.

Observations from prospective and epidemiological studies have demonstrated that maintaining adequate vitamin and nutritional status may be particularly important for cognitive function in the elderly. Vitamin depletion has been shown to precede cognitive decline (Kado et al., 2005), and both intake of specific nutrients, and circulating levels of vitamins in the blood have been correlated with cognitive function in healthy elderly (Perrig, Perrig & Stähelin, 1997; Maxwell et al., 2005).

With advanced age, increased levels of oxidative stress and homocysteine contribute to neuropathology, and consequently cognitive deterioration (McCaddon, 2006; Obeid & Herrmann, 2006). A range of vitamins are known to contribute to the regulation of healthy levels of homocysteine and oxidative stress in the body. The antioxidant and B vitamins have been of particular interest to researchers due to their dual roles in the maintenance of neural and cardiovascular health (Cantuti-Castelvetri, Shukitt-Hale & Joseph, 2000; Rao & Balachandran, 2002; Mariani et al., 2005; Bourre, 2006; Ng & Ye, 2006). In the body these vitamins do not operate in isolation and may exert more potent effects when administered in combination as in the form of a multivitamin. In this thesis a multivitamin will be defined as a supplement containing a minimum of three vitamins, and not comprised solely of B vitamins or antioxidant vitamins.

Dietary supplementation with multivitamins is common practice (Radimer et al., 2004; Nahin et al., 2006) and the trend of multivitamin use is growing, especially in the elderly (Rock, 2007). In Australia, multivitamins have been demonstrated to be the most frequently used form of vitamin supplement in seniors over 65 years of age (Goh et al., 2009).

A growing number of trials have been conducted to investigate the potential nutraceutical effects of multivitamin supplementation (Grima et al., 2011). Nutraceuticals are dietary substances which possess health or medicinal benefits, and in this thesis the term will be used to describe vitamin, mineral or herbal substances with purported cognitive effects. Despite an increase in the number of trials conducted to investigate the potential cognitive effects of multivitamin supplementation, trials conducted in non-demented, community dwelling elderly have yielded heterogeneous results. Numerous antioxidant herbal extracts including *Ginkgo biloba* and *Bacopa monniera* have been demonstrated to exert cognitive enhancing effects (Stough et al., 2001a; Stough et al., 2001b), therefore it may be anticipated that greater cognitive benefits could be obtained from a multivitamin containing both vitamin and herbal constituents.

This thesis presents the results from a 16 week, randomised, double-blind, placebo-controlled, clinical trial which investigated the neurocognitive effects of a combined multivitamin, mineral and herbal supplement in 56 elderly women. The women who volunteered in the trial were aged between 64 and 82 years and reported subjective memory complaints. Due to their age and memory status, this sample was classified as being „at risk“ of cognitive decline.

In this study, the effects of multivitamin supplementation on cognition were assessed using an age-sensitive, computerised cognitive assessment battery (Pipingas et al., 2010). It has been suggested that in the elderly, the cognitive processes most susceptible to cognitive deterioration demonstrate the greatest improvements from nutraceutical intervention (Pipingas et al., 2008; Ryan et al., 2008). Potential mechanisms of the multivitamin were examined using a range of biochemical and cardiovascular parameters. The mechanisms by which multivitamins may influence neurocognition in the elderly are poorly understood. It is conceivable that multivitamin supplementation is capable of exerting cognitive enhancements via influence on the cardiovascular system. Alternatively there may be an impact on cognition due to direct actions on the brain. To date no studies have investigated the effects of multivitamins on brain activity.

Examination of brain activity may enable greater insights into the neurocognitive effects of chronic multivitamin supplementation than can be obtained from neuropsychological or other behavioural measures alone. Brain electrical measures possess the ability to capture and monitor the timing of cognitive improvements, and enable the inspection of specific cognitive sub-processes. The inclusion of measures of brain activity in trials designed to investigate the cognitive enhancing effects of nutraceuticals is not a new phenomena, and the effects of a range of herbal extracts have been studied in this manner (Semlitsch et al., 1995; Kennedy et al., 2003; Page, Findley & Crognale, 2005; Dimpfel et al., 2006; Dimpfel et al., 2007).

In the current study the effects of multivitamin supplementation on brain activity were examined using the steady state visually evoked potential (SSVEP). The SSVEP is a measure of brain electrical activity which possesses the ability to assess both transient, fast occurring cognitive

processes and more sustained task related processes associated with memory (Silberstein, 1995). The specific paradigm utilised to record the SSVEP is known as steady state topography (SST), a technique in which the SSVEP is elicited by a 13Hz uniform flicker, delivered via LED goggles, and superimposed on any visual cognitive task undertaken by the participant. The SSVEP has been shown to be a sensitive measure of variations in performance and task demands across a range of cognitive domains (Silberstein et al., 1990; Silberstein et al., 2000; Ellis, Silberstein & Nathan, 2006). A further advantage of SST is the responsiveness of this technique to neuropharmacological manipulation during cognitive activation (Thompson et al., 2000). In this study a spatial working memory delayed response task was selected as the cognitive activation task as the neural correlates of working memory are known to be vulnerable to age related decline (Rypma et al., 2001), and thus may benefit from nutraceutical intervention. As this thesis represents one of the first studies to utilise SST to assess nutraceutical effects on brain activity, an important component of this body of work was to establish the suitability of SST for this purpose.

This thesis contains 9 chapters. Chapter 1 presents the thesis introduction and outline. Chapter 2 provides an overview of neurocognitive function in the elderly, with the purpose of identifying cognitive processes which decline with age and as a consequence may serve as targets for amelioration by nutraceutical intervention. Chapter 3 collates research findings linking cognition to vitamin status in the elderly, and discusses roles of vitamins in the body which may influence neurocognition. Evidence is then reviewed from RCTs which have examined the ability of nutritional interventions with either individual or combined B vitamins, antioxidant vitamins and multivitamins to enhance cognitive performance in healthy elderly. The thesis aims and hypotheses are presented at the end of the third chapter. Chapter 4 contains the clinical trial methods, followed by an introduction to the SSVEP and a description of the SST methodology.

Following the methods, there are three chapters which contain the thesis experiments. Chapter 5 investigates the effects of the multivitamin on the performance of cognitive tasks and a range of possible mechanisms of cognitive enhancements including nutrient measures, biochemical health

markers and cardiovascular parameters. Chapter 6 examines the neural underpinnings of a spatial working memory DRT, with the purpose of establishing the SSVEP as a suitable measure of nutraceutical effects and developing a framework in which to interpret the effects of multivitamin supplementation on the SSVEP. Chapter 7 investigates the effects of multivitamin supplementation on the SSVEP measure of brain electrical activity. The final thesis discussion is presented in Chapter 8.

## **Chapter 2 Neurocognitive function in the elderly**

This chapter will present an overview of potential mechanisms of cognitive decline including brain structural and physiological changes which accompany the ageing process, followed by a discussion of cognitive theories which have aided in the interpretation of age-related declines to cognitive function. The latter part of this chapter will describe age-associated changes to cognitive domains which appear to be preferentially vulnerable to the normal ageing process. The focus of this section will be on studies of attention, working memory and episodic memory. Functional changes to neural correlates of these processes, as illustrated from neuroimaging and electrophysiological studies, will be discussed. The aim of this chapter is not to provide an exhaustive review of the literature, rather a more generalised introduction to age-related cognitive change in healthy elderly. The terms „healthy elderly“ and „normal ageing“ will be used to describe individuals who are not suffering from dementia or other age-associated cognitive or neurodegenerative disorders.

### **2.1 Overview of cognitive changes across the lifespan**

Longitudinal studies have shown that cognitive change in healthy elderly is not a unitary process, and that advancing age is accompanied by greater inter-individual variance, particularly in the domains of memory (Rabbitt et al., 2004) and cognitive speed (Christensen, 2001; Wilson et al., 2004). Individual differences stemming from genetic risk factors (Alexander et al., 2007), education (Ardila et al., 2000), cardiovascular function (Beeri et al., 2009), physical activity (Larson et al., 2006) depression (Depp & Jeste, 2006), and nutritional status (Moreiras et al., 2007) represent a few of the more widely researched predictors of cognitive decline in the elderly. In some cases poorer memory performers may be in the prodromal stage of an age-related neurodegenerative disease such as Alzheimer’s disease (AD), as many of the same

cognitive domains to be affected by the normal ageing process are exacerbated in AD (Collie & Maruff, 2000).

Even in healthy people changes in cognition occur across the lifespan, with memory decline widely held to be one of the hallmarks of advanced age. The onset of memory decline may commence long before old age, from as early as 20 years of age (Nilsson, 2003). Towards the later stages of life, deficits can become particularly apparent across a wider range of cognitive domains. As a general rule, measures of fluid intelligence, which include a wide variety of abilities relying on memory, reasoning, and spatial abilities are most vulnerable to the effects of ageing (Craik & Bialystok, 2006). Decline occurs to processes which rely on complex, controlled, goal oriented behaviour, including performance monitoring, the generation of future goals, and the ability to adjust behaviour in response to feedback (Budson & Price, 2005). These cognitive operations are referred to as executive function, and are particularly important for effective working memory and episodic memory performance. In comparison, other forms of memory including vocabulary and verbal IQ remain relatively intact in older adults (Christensen, 2001). Crystallized intelligence, described as knowledge gained from cultural influences, and experience tends to increase until age 60 rather than decrease with age (Jones & Conrad, 1933). Beyond the age of 70, crystallized abilities decline, albeit to a lesser degree than fluid intelligence (Christensen et al., 1994).

## **2.2 Mechanisms of cognitive decline**

The concept of cognitive ageing is complex and it is not possible to attribute the changes which occur over the lifespan to a single mechanism. Cognitive ageing is not a uniform process and as such, the rate and extent of decline experienced by each individual will vary, as will the factors which instigate cognitive change (Christensen, 2001; Hess, 2005). Studies of the brain have revealed that a number of neuroanatomical and physiological changes occur over the lifespan (Dickstein et al., 2007; Tumeh et al., 2007). The following section aims to briefly describe some

of the brain structural and physiological changes which appear to be relatively common to the ageing process, and therefore represent potential contributors to cognitive decline.

### **2.2.1 Brain structural and physiological changes**

#### *Grey and white matter loss in ageing*

In the brain, grey matter volume declines over the lifespan (Good et al., 2001; Raz et al., 2005; Smith et al., 2007), primarily due to an age-related reduction in synaptic densities (Terry & Katzman, 2001). Neuronal loss also occurs as a result of mitochondrial dysfunction, changes in metabolic rate, oxidative stress and neuroinflammation (Floyd & Hensley, 2002). Regions encompassing the prefrontal cortex (PFC) are particularly vulnerable to ageing. In older adults aged between 59 and 85 years, grey matter shrinkage has been demonstrated to be most prominent in frontal and parietal regions when compared to occipital and temporal regions over a period of two to four years (Resnick et al., 2003). Specifically, grey matter shrinkage in the lateral PFC has been demonstrated to occur at a rate of over 5% per decade between the ages of 20 and 80 years, with other frontal regions also showing the greater decline in volume (Raz et al., 2004). Decrease in global brain volume in turn exerts effects upon cognitive function, with age related atrophy shown to be a predictor of cognitive decline on IQ measures (Rabbitt et al., 2008). Furthermore, shrinkage of specific brain regions has been correlated with decline to distinct cognitive functions (Raz et al., 1998). In a longitudinal investigation, Cardenas et al. (2009) measured brain volume in subjects aged 50 to 92 years and followed their cognitive change over a one year time period. The results of this study revealed that smaller frontal lobe volume was associated with greater impairments on a composite measure of executive functioning, whilst left temporal lobe volume was related to the extent of semantic memory decline. These findings support the premise that deterioration of anterior brain regions contribute to executive function deficits in the elderly (West, 1996).

Anterior white matter is also susceptible to age-related alterations (Raz et al., 2005; Firbank et al., 2007). Diffuse changes in white matter leads to volume loss, and small infarcts contribute to anterior white matter deterioration (Pugh & Lipsitz, 2002). There is evidence that white matter

lesions correlate with cardiovascular risk factors and cognition (Breteler et al., 1994). White matter hyperintensities are lesions located in the deep white matter and can be measured using Magnetic Resonance Imaging (MRI). Blood vessels affected by small vessel disease are presumed to be responsible for the formation of white matter hyperintensities, due to chronic hypoperfusion of the white matter, disruption of the blood-brain barrier and leakage of plasma into the white matter (DeBette & Markus, 2010). In addition to age (Longstreth Jr et al., 1996), one of the major predictors of the development of white matter lesions (Kuller et al., 2010) and white matter loss (Firbank et al., 2007) is untreated hypertension. Midlife hypertension has been demonstrated to be predictive of volume loss later in the lifespan (Swan et al., 1998) and associations with rate of white matter shrinkage and other vascular parameters such as levels of homocysteine in the body have also been identified (Firbank et al., 2010). In the elderly, the prevalence of white matter lesions has been associated with processing speed and performance on tests of executive functioning (Rabbitt et al., 2007), and individuals with lesions have demonstrated greater deficits on motor and attentional speed measures, than those without (Ylikoski et al., 1993). These findings indicate that white matter damage may exert detrimental effects on cognition in older adults.

Frontal white matter tract disruption may also represent an important contributor to age-related cognitive change (Persson et al., 2006). A cortical „disconnection“ theory has been proposed, whereby loss of white matter tract integrity leads to reduced cerebral connectivity, and subsequently cognitive decline in the elderly (O'Sullivan et al., 2001). Diffusion tensor imaging (DTI) enables the examination of white matter tracts *in vivo* and use of this methodology has contributed to the current understanding of white matter deterioration in ageing (for a review see Madden, Bennett & Song, 2009). Using this methodology the greatest age-related differences in volume have been identified in the anterior corpus callosum (Lebel, Caverhill-Godkewitsch & Beaulieu, 2010). In a cross-sectional study of adults aged 22 to 84 years, frontal white matter connections displayed the greatest decline in structural integrity with age, whereas limbic connections were relatively preserved (Michielse et al., 2010). The volume of most frontal white matter tracts was found to decrease from the age of 60 years, and this volume decrease was

maximal in individuals in their late 70s. This finding corresponds with the observation that memory and executive function deficits become most apparent in the seventh and eighth decade of life (Park et al., 1996; Ronnlund et al., 2005). Direct relationships between white matter integrity and cognition have been identified in the elderly. Using DTI, white matter tract disruption in normal ageing has been correlated with executive function as measured by the trail making test (O'Sullivan et al., 2001). There is also evidence to suggest that the effects of ageing on particular white matter tracts can influence specific cognitive functions. In one study, the integrity of white matter pathways extending from frontal regions to anterior temporal and other anterior regions were associated with performance on executive functioning and working memory tasks, and tracts connecting posterior regions of the cerebrum were associated with visual recognition memory performance in older adults (Davis et al., 2009). Similarly, Kennedy and Raz (2009) have identified a correlation between age-related degradation of anterior brain areas, decreased processing speed and working memory deficits. In contrast, posterior white matter decline was associated with reduced inhibition and greater task switching costs. These results indicate that changes to anterior white matter pathways are particularly important for executive function declines in the elderly, whilst integrity of posterior pathways may be equally essential to declines in other cognitive processes.

#### *Age-related changes in prefrontal and striatal dopamine*

It has been suggested that age-related changes in the striatum, a region with extensive connections to the PFC, may also have important consequences for cognition due to its role in dopaminergic function (Bäckman et al., 2000). The striatal nuclei are vulnerable to age-related shrinkage, with findings from a longitudinal study revealing volume loss of approximately 3% per decade (Raz et al., 2003). Concentrations of dopamine and D2 receptor density decrease with age (Wong et al., 1997), and it has been estimated from a positron emission tomography (PET) investigation that there is a loss of more than 10% of D2 receptors in the anterior cingulate cortex, frontal cortex, lateral temporal cortex and the hippocampus, per decade, from the age of 20 years (Kaasinen et al., 2000). It is likely that decreases in the number of these receptors influences cognitive function over the lifespan. Age-related decline of the dopamine system in

the PFC has been proposed to contribute to cognitive deficits in the elderly (Braver et al., 2001). Decreases in PFC dopamine activity have been associated with performance on cognitive tasks drawing on frontally mediated executive functions including the Wisconsin Card Sorting Test and Stroop Color-Word Test interference (Volkow et al., 1998). Dopaminergic dysfunction has also been implicated in neuropathological ageing. Loss of dopaminergic neurons and striatal dopamine depletion in Parkinson's disease is thought to contribute to the characteristic executive function deficits which accompany this age-associated neurodegenerative disorder (Owen, 2004).

### *Preclinical Alzheimer's disease pathology*

A further consideration which may influence the relationship between brain and cognitive changes is that some elderly individuals may be in a preclinical phase of AD. As the neurodegenerative process of AD occurs 20 to 30 years prior to the clinical onset (Goedert & Spillantini, 2006) in some cases, cognitive deficits will be due to the progression of AD related pathology and not to the normal ageing process. Currently, only a probable diagnosis of AD can be assigned prior to death, with a definite diagnosis confirmed by post-mortem clinical and histopathological evidence (Dubois et al., 2007). In the elderly, AD is the most common form of dementia and is characterized by the DSM-IV as a marked decline from previous functioning in short term memory, and a severe disruption to language, planning or visual processing (American Psychiatric Association, 2000). Those with AD also experience disturbances to global cognitive function and the ability to care for themselves. Individuals who have experienced a decline in cognition which is greater than expected for their age and education level, but is not severe enough to meet the criteria for dementia are classified as in the stage of Mild Cognitive Impairment (MCI) (De Mendonca et al., 2004). Activities of daily living are maintained in those with MCI, whereas they are impaired in AD (Petersen, 2004). In many cases MCI represents a preclinical stage of AD, particularly in those with the amnesic form of MCI who have been observed to develop dementia at a rate of approximately 10-15% per year compared with healthy controls who convert to dementia at a rate of 1-2% per annum (Petersen, 2007).

The major risk factor for AD is age (Carr et al., 1997). Other predictors of AD include vascular risk such as hypercholesterolaemia, hypertension, atherosclerosis, coronary heart disease, elevated homocysteine levels (Selhub, 2006; Duron & Hanon, 2008; Lange-Asschenfeldt & Kojda, 2008), oxidative stress (Polidori, 2004), obesity (Gustafson et al., 2003) and diabetes (Ott et al., 1999).

The neuropathological process of AD has been well documented. Computed tomography (CT) and MRI show enlargement of ventricles due to substantial loss of brain tissue, nerve cells, synapse and dendrites, caused by the presence of neurofibrillary tangles and beta amyloid plaques (A $\beta$ ) (Rusinek et al., 1991; Petrella, Coleman & Doraiswamy, 2003; Kidd, 2008). Pathologically, these AD-characterising features are caused by the deposition of abnormal proteins. Neutritic or senile plaques are extracellular deposits of A $\beta$  surrounded by dystrophic neurites, reactive astrocytes, and microglia, whereas tangles are intracellular aggregates formed by a hyperphosphorylated form of the microtubule-associated protein tau (Blennow, de Leon & Zetterberg, 2006). According to the amyloid cascade hypothesis, the pathogenic mechanism of AD is an imbalance between the production and clearance of A $\beta$  in the brain, leading to neuronal degeneration and dementia (Hardy & Higgins, 1992). Damage initially occurs to the large cortical neurons subserving cognition in the temporal lobe structures and pyramidal cells found in the cortical memory pathways, then later in the remaining neocortex and association areas (Braak & Braak, 1991; Norfray & Provenzale, 2004). Mechanisms such as neurovascular dysfunction, inflammatory processes, oxidative stress, and mitochondrial dysfunction are thought to elicit this neuropathology (Blennow et al., 2006).

To complicate the distinction between preclinical AD and normal ageing, some of the neuropathological correlates of AD have also been identified in healthy individuals. For instance, neurofibrillary tangles and plaques have been observed in the brains of individuals free from dementia (Davis et al., 1999; Price & Morris, 1999). Other forms of pathology such as diffuse plaques, consisting of amyloid aggregations free from altered neuritis and glia, also occur in normal ageing and degenerative conditions other than AD (Norfray & Provenzale, 2004).

Cholinergic dysfunction has been proposed to contribute to the progressive memory loss associated with AD (Bartus et al., 1982). In AD, degenerative changes including cell loss and down regulation of choline acetyltransferase occur in the cholinergic neurons of the basal forebrain complex (Schliebs & Arendt, 2006). These cholinergic neurons project from the basal forebrain complex to the cerebral cortex and hippocampus and have been shown to be important for human cognitive function (Everitt & Robbins, 1997). Impaired cholinergic neurotransmission has also been suggested to contribute to A $\beta$  plaque pathology and to increase phosphorylation of tau protein in neurofibrillary tangles, processes involved in the progression of AD pathology (Beach et al., 2000; Terry Jr & Buccafusco, 2003). In normal ageing, reductions in choline acetyltransferase in the hippocampus have been observed from 40 years of age (Perry et al., 1992), indicating that healthy ageing may also be associated with cholinergic disruption. When measured *in vivo*, however, only modest reductions in cholinergic terminals have been identified (Kuhl et al., 1996), suggesting that cholinergic loss may be more important for the progression of AD than for cognitive change as a result of the normal ageing process.

Apolipoprotein E (ApoE) has also been implicated in the neurodegenerative process of AD. ApoE is a plasma glycoprotein predominantly produced in the liver, with secondary sites in the brain (Mahley, Weisgraber & Huang, 2006). ApoE is involved in lipid homeostasis, particularly in determination of the levels of LDL and HDL cholesterol, which are directly and inversely correlated with risk for cardiovascular disease respectively (Smith, 2002). In the brain, ApoE has critical functions involving the redistribution of cholesterol during neuronal growth, nerve regeneration, maintenance of synaptodendritic connections, and scavenging of toxins (Mahley et al., 2006). Carriers of the ApoE e4 allele have increased coronary risk (Bennet et al., 2007) and e4 carriers also have a higher risk of developing late onset Alzheimer's disease (Corder et al., 1993). The e4 allele of the ApoE gene on chromosome 19 has been identified to be involved in the pathogenesis of both late-onset familial and sporadic AD (Saunders et al., 1993). ApoE can be inherited through one of three alleles (e3, e4 and e2), of which e3 is the most common and e2 is the least. It is suggested ApoE4 may contribute to the pathological process of AD by

modulating the aggregation of A $\beta$  and through the regulation of brain lipid metabolism through the ApoE receptors (Bu, 2009).

The ApoE e4 allele has also been associated with cognition in elderly without dementia (Kang et al., 2005). Over a period of 7 years the ApoE e4 allele has been associated with a greater rate of memory deterioration in elderly free from dementia (Hofer et al., 2002). Episodic memory may be particularly prone to decline with this memory domain demonstrating the greatest decline over 6 years (Wilson et al., 2002). However, not all trials have identified cognitive impairment in carriers of the ApoE e4 allele. Jorm et al. (2007) observed no association between presence of the e4 allele and cognitive ageing on the domains of episodic memory, working memory, mental speed, reaction time, or reading vocabulary in subjects between the ages of 20 and 64 years. Controlling for the effects of age and education has also diminished the relationship between the e4 allele and neurocognitive performance in those free from dementia (Welsh-Bohmer et al., 2009). Whilst some trials have revealed cognitive impairment in Apoe e4 carriers, the extent to which this may reflect preclinical dementia is not yet fully understood.

#### *Summary of brain structural and physiological changes*

The human brain is complex, and a single mechanism of brain ageing cannot adequately account for the range of cognitive changes which occur across the lifespan. In healthy ageing there is evidence that both grey and white matter loss contribute to cognitive decline. Volume loss is greatest in anterior brain regions and correlates with deficits in executive function. Decreases in dopamine in the striatum and PFC also represent brain physiological changes which may exert influence on cognitive function. It is probable that cognitive ageing represents a culmination of these brain physiological changes. Finally, it is difficult to separate the process of normal ageing from age-related degenerative disorders such as AD, which may possess a preclinical phase of 20 to 30 years prior to diagnosis. The neuropathological process of AD involves the formation of neurofibrillary tangles and A $\beta$  plaques, as well as cholinergic dysfunction, ultimately leading to loss of memory and other cognitive functions.

### 2.2.2 Theories of cognitive ageing

It is widely held that a broad array of cognitive functions decline with age, particularly those which rely on attention and memory. Whilst alterations to the structure and physiology of the brain contribute to age-related cognitive decline, there are also changes which occur at the level of mental processing which may have important consequences for cognition. Several theories of cognitive ageing have been explored by researchers who have endeavoured to delineate the underlying cause of age-related cognitive changes. These theorists have shared a common aim, to identify a mechanism of ageing, which can be used to explain decline across multiple cognitive domains. The following section will present the key theories of cognitive ageing which have been used by researchers from the fields of experimental psychology and neuroimaging to interpret observations of age-related cognitive change.

#### *Processing speed theory of cognitive ageing*

A popular theory of cognitive ageing proposed by Salthouse (1996), suggests increased age is associated with a decrease in the speed of which processing operations can be undertaken, and this alteration in speed is responsible for impaired cognitive functioning across a range of measures of fluid intelligence. A key hypothesis for this theory posits that substantial shared variance on measures of processing speed accounts for almost all the age-related variance on a number of cognitive tasks and domains. Evidence for this account of cognitive ageing has been obtained from statistical analyses which have revealed that controlling for the effects of speed reduces the relationship between age and cognitive performance across numerous cognitive tests (Salthouse, 1996; Salthouse, 2000; Verhaeghen & Cerella, 2002).

The processing speed theory is not without criticism, and has been described as overly descriptive rather than causative (Glisky, 2007). Other investigators have suggested that processing speed alone cannot account for age-related cognitive change. Analyses of several cross sectional data sets by Allen et al. (2001) indicated that age related decline could not be

attributed to a single processing speed mechanism. In an investigation conducted by Park et al. (1996), it was found that processing speed and working memory independently contributed to age-related changes in memory performance, and it was suggested by these researchers that working memory deficits are not solely the result of underlying speed of processing. In line with this premise, the results from a trial of young adults aged 18 to 35 years and older adults aged 60 to 80 years has indicated that processing speed, working memory and inhibitory control interactively contribute to age-related memory decline (Head et al., 2008).

*Inhibitory control and information processing accounts of cognitive ageing*

Two alternate views of cognitive ageing focus on attentional processes. The inhibitory control account posits that inhibitory processes at the level of selective attention become less efficient with advancing age (Hasher & Zacks, 1988; Hasher et al., 1991). Consequently, older adults experience reduced ability to suppress stimuli and inhibit responses not directly relevant to the goal of the specific task or process being undertaken. Essential to effective working memory is the ability to suppress irrelevant information and to enhance task relevant goals or input, modulated by top-down influence (Gazzaley et al., 2005). As individuals age, working memory processes become more susceptible to irrelevant distracters, with the presence of a visual distracter shown to impair performance to a greater extent in older than younger adults (West, 1999). The irrelevant content which enters working memory may consequently impair performance by taxing memory storage and maintenance processes (Milham et al., 2002).

The second attentional theory focuses on processing resources. This view of cognitive ageing proposes that elderly have reduced access to processing resources, and as cognitive demand or number of cognitive operations increase, so too does the competition for this limited pool of resources ( Craik & Byrd, 1986). Accordingly, deficits will be seen on tasks that require high memory loads, complex processing or division of attention. Dual task paradigms, requiring the division of attention between the performance of a primary and secondary task, have demonstrated that performance is affected to a greater degree in older than younger adults (Chen, 2000; Vivien Rekkas, 2006). In relation to memory performance this theory predicts that

deficient processing resources will result in a shallower, less elaborate processing of information or events ( Craik & Byrd, 1986).

Limitations of the inhibitory control and information processing accounts of cognitive ageing have also been recognized. Whilst failure to inhibit irrelevant information has been demonstrated to contribute to deficits in working memory in the elderly (Hedden & Park, 2001), this theory may be less useful in describing age-associated deficits to other cognitive processes. Other results have indicated that deficits to inhibition and interference do not account for age-associated variance in cognition, independent of the effects of processing speed (Salthouse & Meinzig, 1995; Earles et al., 1997).

#### *Frontal lobe hypothesis of cognitive decline*

A constraint which is common to the processing speed, inhibitory control, and processing resources accounts of cognitive ageing, is their failure to adequately describe any underlying neural mechanisms which may account for cognitive change across the lifespan. One theory which differs in this respect is the frontal lobe theory of ageing, which attempts to describe cognitive change within the context of the brain structural and functional changes which occur with age. According to the frontal lobe theory, the PFC is particularly vulnerable to the effects of ageing, with diminished anterior function thought to contribute to decline across a range of cognitive domains. The frontal lobe theory is based on the premise that the prefrontal cortex is the first region to show signs of malfunction in normal ageing (Dempster, 1992). Abilities which rely on this region are proposed to decline at an earlier point in time and to a greater degree than cognitive processes supported by other cortical areas. The application of this theory consequently should explain some of the age-related decline observed in tasks which rely on sustained attention, reasoning ability, working memory and executive function (West, 1996). Evidence for the frontal lobe hypothesis may also be obtained from studies of structural neuroimaging which have demonstrated that the prefrontal region is preferentially affected by grey and white matter shrinkage (Raz et al., 2005), white matter hyperintensities (Pugh & Lipsitz, 2002), and dopamine decreases with age (Kaasinen et al., 2000).

Since the original conceptualisation of the frontal lobe theory, a number of functional neuroimaging studies have indicated that some subsections of the PFC are more vulnerable to the effects of ageing than others. Work by Rypma and colleagues has demonstrated that functional decline is more prominent in the dorsolateral region involved in the manipulation of memory content, than the ventrolateral region required for maintenance of memory content, during performance of delayed response tasks (Rypma & D'Esposito, 2000; Rypma & D'Esposito, 2001; Rypma et al., 2001). Dopaminergic dysfunction in the dorsolateral prefrontal cortex (DLPFC) has been suggested to contribute to such deficits (Braver et al., 2001).

Criticism of the frontal lobe hypothesis of ageing has come from Greenwood (2000) who has challenged the idea that the frontal regions are selectively and differentially affected by ageing, arguing that the non-frontal cortical lobes are equally vulnerable to age-related decline. Evidence from functional neuroimaging suggests that in the elderly there is a loss of specificity of the posterior cortical regions which process the sensory aspects of memory input (Park et al., 2004). The dorsal and ventral streams are essential for processing visual memory and attentional content (Ventre-Dominey et al., 2005), thus any disruption to these pathways will have consequences for the efficiency of working memory organisation. The results from several studies indicate that age-related decreases in neural specialization occur in these posterior processing regions (Schiavetto et al., 2002), and this phenomena is commonly accompanied by the recruitment of additional frontal regions including the PFC (Grady et al., 1994; Payer et al., 2006). This occurrence is thought to reflect compensatory frontal recruitment to offset neuroanatomical declines of posterior regions associated with aging (Davis et al., 2008).

### *Summary of cognitive theories of ageing*

It has been theorised that age-related decreases in processing speed, attentional resources and inhibitory processes contribute to the decline of a range of cognitive functions in the elderly. The frontal lobe hypothesis of ageing provides an alternate account of cognitive ageing, proposing that decline to structure and function of the PFC influences the function of processes which rely

on this brain region. It is likely that a combination of these factors account for cognitive change in a range of domains, rather than a single mechanism. This section presented theories developed to account for generalised cognitive changes which occur with ageing. Section 2.3 will discuss in greater depth cognitive processes which appear to be preferentially vulnerable to the effects of ageing and consequently may serve as targets for amelioration.

### **2.3 Specific neurocognitive domains vulnerable to age-related decline**

Cognitive ageing is not a uniform process, and it is apparent that some cognitive faculties are more susceptible to age-related cognitive decline than others. It has been documented from both longitudinal and cross sectional investigations that there is a slowing of response time in the elderly, regardless of whether perceptual, or complex higher order tasks are performed (Sliwinski & Buschke, 1999; Deary & Der, 2005). This finding can in part be explained by the increased time taken to produce a motor response as a result of age-associated changes to nerve conduction speed (Rivner, Swift & Malik, 2001). Slowing of perceptual and motor speed has also been attributed to white matter changes in the brain (Ylikoski et al., 1993) and loss of myelin integrity as individuals age (Bartzokis et al., 2010). A further contributor to slower response time in the elderly is a trade-off between speed and accuracy during cognitive task performance, with speed sacrificed in order to enhance accuracy (Salthouse, 1979; Brébion, 2003). Even when task instructions emphasise speed, older adults do not demonstrate improved response time relative to younger adults (Brébion, 2001). Other processes susceptible to cognitive deterioration include attention, working memory and episodic memory (Ronnlund et al., 2005; Glisky, 2007). Electrophysiological and neuroimaging techniques have aided substantially in the understanding of age-associated decline to these cognitive processes. Specifically, event related potential (ERP) measures of brain electrical activity can be time-locked to specific task components, and have been used to uncover age-related changes in the fast occurring neural processes involved in cognitive task performance. Neuroimaging methodologies which possess high spatial resolution, such as positron emission tomography (PET) and functional magnetic resonance (fMRI) have

assisted researchers to determine the brain regions preferentially affected by ageing during a range of task demands and cognitive processes (Rossini et al., 2007).

The following section will describe the neurocognitive changes associated with the ageing process. Specifically the cognitive domains most vulnerable to age-related decline including attention, working memory and episodic memory (Budson & Price, 2005) will be described, taking into account functional brain changes which may be implicated in the deficits to these cognitive domains.

### **2.3.1 Attention**

Attention refers to the concentration of mental activity required for any conscious, effortful cognitive process and is an essential requirement for most cognitive operations. Divided and selective attention are particularly vulnerable to the effects of age (West, 1999; Verhaeghen & Cerella, 2002). Divided attention refers to the ability to attend to more than one source of information simultaneously, whereas selective attention involves attending to certain stimuli at the same time disregarding others. Sustained attention requires concentration on a task over an extended time period. Generally this form of attention is less affected by age (Glisky, 2007). In the brain, attentional processes are supported by a distributed network of structures including the anterior cingulate cortex, PFC, parietal cortex, extrastriate cortex, superior colliculus, thalamus, and the basal ganglia (Posner & Dehaene, 1994; MacDonald et al., 2000; Wager & Smith, 2003; Mayer et al., 2007). The direction of attention can be guided by bottom-up processes, which emphasise the processing of incoming information at a basic sensory level, or by top-down processes involving the integration of higher order mental functions such as prior exposure, expectation and strategy (Styles, 2004).

Ageing has been demonstrated to impair divided attention. In contrast to young adults, the elderly exhibit greater difficulties dividing their attention between more than one source of information or task (Chen, 2000). Age-related attentional deficits appear to be greatest during

dual task performance, when subjects must attend to two or more tasks at the same time, whilst also switching from the demands of one task to another (Verhaeghen & Cerella, 2002). Interestingly, dual tasks which rely on controlled processing, or have a substantial motor response component, appear to be particularly compromised by age (Riby, Perfect & Stollery, 2004). Evidence from a recent fMRI trial indicates that under dual task conditions there is an age-related reliance on prefrontal resources responsible for top-down management of task goals, whilst other regions comprising a lateral, frontal, parietal network involved in the division of attention were comparable for younger and older adults (Hartley, Jonides & Sylvester, 2011).

The construct of divided attention is closely related to the ability to process task requirements whilst ignoring distracters. This form of selective attention also appears to be compromised with age (West, 1999). It has been suggested that the difficulties with selective attention and increased distractibility which accompany ageing may be due to altered prefrontal cortex function (Chao & Knight, 1997). Other findings indicate that parietal regions may also contribute to heightened distractibility in the elderly. In an fMRI investigation of ageing, younger and older adults performed a task that required visual and auditory selective attention and cross-modal attention shifts (Townsend, Adamo & Haist, 2006). The results of this study showed that younger adults displayed a pattern of frontal and parietal activation whilst shifting attention between modalities which was not present during the selective attention conditions. In contrast, older adults demonstrated similar activation in frontal and parietal regions during both conditions, suggesting an age-related loss of processing selectivity and failure to inhibit task-irrelevant information.

The Stroop paradigm has also been used to investigate age-related changes in selective attention (Verhaeghen & De Meersman, 1998). In the congruent Stroop condition, subjects name or respond to colour names printed in the same coloured ink. In the incongruent Stroop condition, subjects respond to colour names printed in different coloured ink (MacLeod, 1991). The observation that subjects take longer to respond in the incongruent condition is known as the Stroop interference effect, and is thought to index executive function (Van Der Elst et al., 2006). In cross sectional investigations consisting of subjects across the adult lifespan, the Stroop

interference effect has been correlated with age, indicating that this task is sensitive to the effects of age-related decline (Van Der Elst et al., 2006; Pipingas et al., 2010). Whilst some researchers have attributed these changes solely to age-related slowing of processing speed (Verhaeghen & De Meersman, 1998), others have indicated that age-associated deficits to inhibitory control may also influence Stroop performance (Bugg et al., 2007).

Findings from an fMRI study indicate that deficits to top-down control may influence Stroop performance (Milham et al., 2002). During the incongruent trials of the Stroop task, where irrelevant input of the colour name was required to be suppressed, regions required for attentional/top-down control such as the DLPFC and parietal cortex (Posner & Dehaene, 1994) were less responsive in older than in younger adults. Greater activation was observed in temporal regions and the ventrolateral PFC, suggesting deeper processing of the irrelevant word and increased ability of the irrelevant representation to gain entry to working memory respectively. These findings support the proposal that deficiencies in inhibitory control processes contribute to impaired cognitive function in the elderly (Hasher et al., 1991).

Other neuroimaging studies have indicated that bottom-up processes are also affected in the ageing brain. Findings from an event-related fMRI study, which utilised a visual search paradigm, suggests that an age-related decline in the efficiency of bottom-up processes mediated by visual cortical regions is accompanied by a greater reliance on fronto-parietal top-down mechanisms (Madden et al., 2007). Similarly, from an investigation where young and elderly subjects performed a bi-field visual selective attention task, results revealed the young group recruited posterior brain regions such as visual processing areas, thalamus and hippocampus, whilst the older group engaged components of a frontal circuit (Solbakk et al., 2008). Conjointly, these findings suggest that when there is a decline in bottom-up attentional processes, an age-related increase in the reliance on frontally mediated top-down processes may occur.

### 2.3.2 Memory

Memory can be broadly characterised into the areas of working memory, declarative memory and non-declarative memory. Working memory refers to the short term maintenance and processing of material (Baddeley, 1992). Declarative memory can be characterised as the conscious component of long term memory and includes semantic and episodic memory. Semantic memory comprises general world knowledge or facts, and episodic memory consists of memories of particular events (Tulving, 1987). Non-declarative memory is more implicit and involves procedural memory, priming, conditioned and associative learning (Graf & Schacter, 1987). Working memory and episodic memory are vulnerable to age-related decline (Ronnlund et al., 2005; Pipingas et al., 2010), whilst semantic and implicit memory are less affected by ageing (Nilsson, 2003).

#### *Working memory*

Declines to working memory occur as a consequence of the ageing process. Specifically, working memory is invoked during the short term retention of information, when memory content is required to be held in mind in order to achieve a particular goal or outcome. Working memory requires goal-oriented, attentional processes. Consequently, it is not surprising that there is a large degree of overlap between the neuroanatomical regions implicated in working memory and attention (Mayer et al., 2007). Working memory involves a distributed network encompassing the lateral PFC, parietal and temporal lobes (Wager & Smith, 2003; Koch et al., 2005). Anterior regions of the network have been implicated in the executive functions of working memory (Wagner et al., 2001), and posterior regions in processing incoming sensory information (Zimmer, 2008).

In the 1950s a relatively simplistic concept that the short term maintenance of information in memory is restricted to only seven (plus or minus two) items at any one time was proposed by Miller (1956). A more comprehensive description of working memory has since been offered by Baddeley (1992) who has theorised that working memory is a multi-component system,

consisting of a central executive which directs attention, and controls complex goal-orientated behaviour. The central executive is aided by two slave systems, the visuo-spatial sketchpad, and phonological loop, which actively hold and manipulate visual images and speech-based information respectively. Baddeley (2000) has since updated this model of working memory to include the concept of an episodic buffer which provides a temporary interface between the slave systems and long term memory. The episodic buffer is assumed to be controlled by the central executive and to provide storage capabilities for episodic memory.

Working memory is one of the cognitive domains preferentially influenced by ageing, with this ability continuing to decline throughout life, especially once individuals enter their seventh decade (Park et al., 1996). Along with episodic memory, measures of working memory have demonstrated the greatest sensitivity to age when examined in a cross sectional investigation of individuals aged from 21 to 86 years (Pipingas et al., 2010).

The delayed match to sample, or delayed response task (DRT), is one of the most commonly used paradigms to investigate working memory alterations with age, within both behavioral and neuroimaging protocols. An early version of this task was utilized by Jacobsen in the 1930s to investigate memory deficits in monkeys with bilateral prefrontal lesions (Jacobsen, 1936). In studies of human working memory, a stimulus is presented which subjects are instructed to remember. The stimulus is then removed during a period of delay, after which a probe is shown and subjects indicate if this probe matches the initial stimulus. The Sternberg task is an example of a delayed response task used to test maintenance of a memory set usually consisting of 3 to 9 letters or numbers. The *n*-back task requires subjects to indicate whether a probe is the same as a stimulus presented “*n*” back (i.e. 1-back, 2-back, 3-back). The *n*-back task is ideal to study effects of manipulation of the content of working memory and variations in attentional load, whilst keeping the stimulus and response demands of the task constant (McEvoy, Smith & Gevins, 1998).

Studies of working memory have indicated that deficits to frontal neural resources may contribute to age-related changes to this cognitive faculty. Specifically, alterations of the functioning of the DLPFC have been identified in studies which have utilized fMRI to investigate working memory. Age-related under-activation of the DLPFC has been observed under conditions of increasing memory load, and has been correlated with processing speed (Rypma & D'Esposito, 2000; Rypma et al., 2001). This reduction in activity at high memory loads has been suggested to reflect a loss of neural efficiency of the DLPFC in older adults (Rypma, Eldreth & Rebbelchi, 2007). Interestingly, increased rather than decreased DLPFC activation has been demonstrated during complex manipulation of working memory content, leading to the proposal that older adults require additional executive functions to meet task storage and processing demands (Reuter-Lorenz et al., 2001; Emery et al., 2008).

In line with this premise, electrophysiological studies have revealed that during working memory performance there is a decrease in amplitude and increase in latency of the posterior components of the P300 ERP waveform in older adults (McEvoy et al., 2001; Knott et al., 2004), and this occurrence is usually accompanied by a more frontal P300 distribution (Muller & Knight, 2002). It is possible that elderly require additional frontally mediated executive functions either to constantly update the representation of the target stimuli in working memory, or to compensate for decline to posterior neural resources (Friedman, 2003).

### *Episodic memory*

Episodic memory refers to the explicit and declarative recollection of previously experienced personal events (Budson & Price, 2005). Episodic memory can be differentiated from other forms of declarative memory as it involves memory of specific experiences, or the context in which events were initially encoded into memory (Tulving, 1987). Regions of the medial temporal lobe encompassing the hippocampus (Squire, 2004), and prefrontal cortical areas are essential for episodic memory (Rugg et al., 1999; Rugg, Otten & Henson, 2002). Episodic memory has been shown to decline from the age of 60 onwards (Ronnlund et al., 2005) and is the domain most affected in AD (Dubois et al., 2007).

When studied in an experimental setting, episodic memory can be measured using a standard recognition paradigm, with an additional requirement to recollect specific details regarding the context in which the material was initially encoded into memory. Contextual recognition tasks are used for this purpose and may require individuals to recollect details such as the colour, spatial location, word list, or array from which the stimulus was initially presented during encoding. Within episodic memory, age-related decline in performance has been suggested to reflect deficits in the processes involved in the encoding of new material (Glisky, Rubin & Davidson, 2001) as well as the retrieval of information from memory (Spaan, Raaijmakers & Jager, 2003). Older adults also experience greater difficulties consciously recalling the context of where an event or experience was encoded into memory, than the item itself (Spencer & Raz, 1995; Fabiani et al., 1999; Friedman, 2000).

Event related potential studies have revealed that there are age-related reductions in parietal (Morcom & Rugg, 2004; Fjell, Walhovd & Reinvang, 2005) and frontal (Fabiani et al., 1999) waveforms associated with episodic retrieval. The ERP parietal effect has been thought to index a simple judgment of prior occurrence (Morcom & Rugg, 2004). In contrast, the frontal effect is involved in monitoring the outcome of a retrieval attempt (Morcom & Rugg, 2004), and integrating information about an item's previous occurrence with its initial contextual features (Rugg et al., 1999). The observed reductions of these waveforms in the elderly, suggest that less efficient retrieval processes may contribute to older adult's difficulties with episodic memory.

Findings from event-related fMRI studies indicate that some aspects of age-related episodic memory deterioration are due to alterations during encoding, rather than retrieval. In a study which investigated encoding processes in memory, older adults were found to display less activity in the anterior inferior temporal cortex and alterations in prefrontal activity when compared to young adults (Morcom et al., 2003). In a trial which examined the contributions of encoding and retrieval to the age-associated decline in memory, it was revealed that older adults demonstrated reduced activity in the left anterior medial temporal lobe during encoding

(Daselaar et al., 2003). It was suggested by these authors that it was this deficit, rather than a failure to recover information from memory during retrieval, which contributed to age-related differences in episodic memory performance.

### **2.3.3 Compensatory processes in the ageing brain**

Whilst traditional views of cognitive ageing have focussed on age-associated decline in cognition, neural processes and neuroanatomy, recent evidence from the field of neuroimaging suggests that even in old age the brain is flexible, and maintains the ability to functionally reorganise in the face of such losses. From this viewpoint the elderly brain possesses the ability to adapt, or compensate for other age-associated inefficiencies by recruiting alternate or additional neural resources to those utilized by the young. The PFC is one such region which has demonstrated flexibility of function, with a number of researchers identifying a relatively consistent occurrence of increased bilateral PFC activity in the elderly during episodic and working memory activation, which is not apparent in the young. Cabeza et al. (1997) reported that although activity of the PFC was right lateralized during performance of a cued-recall task in young adults, PFC activation was bilateral in older adults during performance of the same task. Similarly, during the retrieval phase of a verbal recognition task, regional cerebral blood flow (rCBF) activation occurred primarily in right prefrontal cortex in younger adults, whilst in older adults prefrontal activity was less lateralised (Madden et al., 1999). This same pattern of bilateral activity in elders has been identified for the anterior components of working memory circuitry during the maintenance of both spatial and verbal content, whereas in younger adults opposite patterns of laterality for verbal and spatial working memory were observed (Reuter-Lorenz et al., 2000). Further evidence for an age-related change in lateralisation has been obtained from studies of episodic memory, which have commonly identified a left lateralised prefrontal effect during encoding, followed by predominantly right lateralised activity during retrieval (Tulving et al., 1994; Habib, Nyberg & Tulving, 2003). In comparison, bilateral prefrontal activation has been observed in elderly during episodic encoding (Morcom et al., 2003) and retrieval (Grady et al., 2002) which was not seen in the young.

Based on this discovery of an age-related shift in hemispheric function of the PFC, Cabeza (2002) has conceptualised the hemispheric asymmetry reduction in older adults, or HAROLD model. Specifically, HAROLD is viewed by Cabeza as a general ageing phenomenon rather than a task or content specific occurrence, and this interpretation has been corroborated from further studies which have replicated this finding in the cognitive domains of working memory (Mattay et al., 2006), episodic retrieval (Payer et al., 2006), perception (De Sanctis et al., 2008) and inhibitory control (Nielson et al., 2001).

The standpoint that has been most widely adopted by researchers in the field of neurocognitive ageing seems to be that the overactivation observed in seniors reflects a compensatory function (Reuter-Lorenz et al., 2000; Friedman, 2003; Buckner, 2004; Reuter-Lorenz & Cappell, 2008) and these brain regions are “working harder” than the same region in younger adults (Reuter-Lorenz & Cappell, 2008). Thus the underlying assumption is that increased bilateral activity is somehow beneficial to cognitive processes (Cabeza et al., 2002). A contrasting view proposes that such alterations in functional activations reflect deficits in neurotransmission, characterized by a “noisier” system which in turn may result in less distinct neural representations (Li, Lindenberger & Sikström, 2001).

#### **2.3.4 Summary of neurocognitive changes with ageing**

When considered collectively, it is apparent that ageing results in the deterioration of basic cognitive processes including response time, attention, working memory and episodic memory. Evidence from the field of cognitive neuroscience suggests that age-related changes to many of these processes are associated with alterations to the function of specific brain regions, especially the PFC. Changes to the function of the parietal and temporal lobes have also been implicated in age-associated declines in attention and episodic memory respectively. There also appears to be a greater reliance by older than younger adults to engage executive processes either to manage task demands or compensate for loss of neural efficiency. Findings of increased bilateral prefrontal activity have also been observed during performance of attentional, working memory and episodic memory tasks. This pattern of activity has been interpreted as compensatory and

indicates that the ageing process should not solely be defined by declines to brain function and cognition.

## **Chapter 3 Micronutrients and neurocognition in the elderly**

### **3.1 Introduction**

With advancing age, there is evidence that dietary and lifestyle factors increasingly influence morbidity and mortality (De Groot & Van Staveren, 2010). The importance of adequate diet has been illustrated by epidemiological data linking a greater intake of fruit and vegetables with lower mortality rate, reduced risk of cancer and cardiovascular disease mortality in the elderly (Steinmetz & Potter, 1996; Joshipura et al., 2001). The consumption of a Mediterranean diet rich in plant foods has been associated with similar health benefits (Trichopoulou & Vasilopoulou, 2000; Knoops et al., 2004). The high concentrations of micronutrient, antioxidant and flavonoid components of these foods have been postulated to contribute to these health effects (Tapsell et al., 2006). In recent years, research has expanded to investigate the importance of diet for cognitive function, particularly as individuals age.

Growing evidence suggests that maintaining vitamin and nutritional status may be particularly important for cognitive function in the elderly. Vitamins are chemicals which do not belong to the major categories of fats, proteins or carbohydrates, and cannot be synthesized by the body in quantities necessary for normal requirements. Vitamins and mineral micronutrients must be introduced through the diet, and with reduced intake, impaired absorption or increased requirements, body stores may become depleted (Huskisson, Maggini & Ruf, 2007). The elderly represent a division of the population who are at risk of sub-optimum nutrient intake from their diet (Ulger et al., 2010). Physiological factors such as reduced caloric requirements, increased satiation due to hormonal changes, and alterations in absorption through the intestinal tract, contribute to nutrient deficiencies (Elmadfa & Meyer, 2008). Psychological influences including declines in the hedonic experience of food may also influence age-related declines in nutrient intake (Morley, 1997).

Vitamins, such as the B vitamins and antioxidants, are essential for normal neurophysiological and cognitive function due to their role in neurotransmitter production, as well as neuroprotective (Cantuti-Castelvetri et al., 2000; Floyd & Hensley, 2002; Ferrari, 2004; Bourre, 2006) and cardioprotective mechanisms (Diaz et al., 1997; Valko et al., 2007). The importance of vitamin status for cognition in the elderly has been established in many population studies which have identified a correlation between individual vitamins or micronutrients and cognitive function (Kado et al., 2005; Maxwell et al., 2005; Kang et al., 2009). Furthermore, low levels of B vitamins (Clarke et al., 1998) and antioxidants (Jonides et al., 1993; Rinaldi et al., 2003; Mecocci, 2004) have been identified in mild cognitive impairment (MCI) and Alzheimer's disease (AD), indicating that low vitamin status may be associated with the neuropathological process of AD and may possibly precede this age-related neurodegenerative disorder. Consequently it has been suggested that individuals at high risk of developing cognitive decline or dementia may benefit from dietary intervention consisting of vitamin supplements (Jelic & Winblad, 2003).

There are two main pathways via which vitamins may exert effects on cognition in the elderly. The first route is via direct effects on the brain. The second route is via indirect effects on the cardiovascular system. The ability of vitamins to regulate levels of homocysteine, oxidative stress and inflammation may be essential to both pathways (Diaz et al., 1997; Mattson & Shea, 2003; Solfrizzi et al., 2006). As discussed in the previous chapter of this thesis, brain structural and physiological changes represent important predictors of cognitive function in the elderly. There is also substantial literature to suggest that cardiovascular function may contribute to the health of the brain, and consequently cognitive function (Longstreth Jr et al., 1996; Kuller et al., 2010). Ageing has been associated with increases in blood pressure, cholesterol, arterial stiffness, cardiovascular disease and stroke (Wilson et al., 1998; Mitchell et al., 2004; Duron & Hanon, 2008). In turn, each of these conditions have been associated with poorer cognitive function in the elderly (Wang et al., 2002; Elias et al., 2004; Hanon et al., 2005; Knecht et al., 2009).

This first part of this chapter will discuss the findings from key epidemiological and correlational studies which have examined the relationship between cognition and B vitamins and antioxidant vitamin status. The role of selected vitamins and micronutrients in the body will be described, along with potential mechanisms by which these vitamins may influence cognition. All available studies which have examined the relationship between brain structural measures and vitamins conducted in humans will be included. Indications that dietary vitamin interventions are capable of reducing biomarkers, or risk factors, for cognitive decline in human subjects will then be presented. In this section, evidence will be reviewed individually for the B vitamins, and antioxidants. As the B vitamins and antioxidants have been researched most extensively for their relationship with cognition (Isaac, Quinn & Tabet, 2008; Malouf & Grimley Evans, 2008), these vitamins will be the primary focus of this chapter. Relevant findings related to zinc and vitamin D will also be discussed. Only micronutrients which have been examined in relation to cognition in elderly humans will be covered in this chapter.

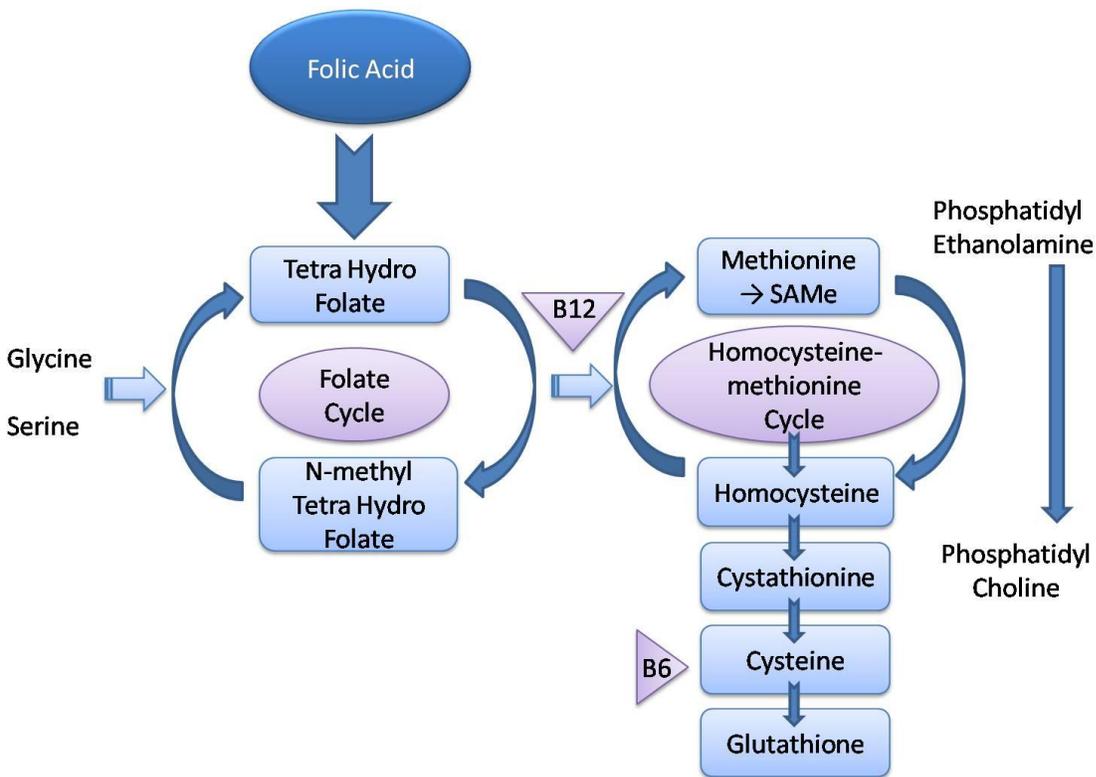
The second part of this chapter will review evidence from randomised controlled trials (RCTs) which have investigated the potential of B vitamins, antioxidant vitamins, zinc and multivitamins to enhance cognitive function in the elderly. This review will include all placebo-controlled studies which have examined the effects of chronic vitamin supplementation on cognition in healthy elderly. Following this review of the relevant literature, methodological difficulties with previous research will be discussed and future directions will be highlighted. This chapter will conclude with the formulation of the thesis aims and hypotheses.

## **3.2 Vitamins and cognition in the elderly: Correlational and epidemiological findings and mechanisms of function.**

### **3.2.1 Role of B vitamins in the body**

The B vitamins belong to a group of micronutrients essential for healthy functioning of the brain and nervous system, and are necessary for energy metabolism in the body. Members of the

vitamin B complex include B<sub>1</sub> (thiamine) which plays a role in the metabolism of carbohydrates, B<sub>12</sub> which is a cofactor for two enzymes: methionine synthase and L-methylmalonyl-CoA mutase and B<sub>6</sub> (pyridoxine) which is required for the synthesis of neurotransmitters including adrenaline, serotonin, dopamine, GABA and tyramine. Folic acid (folate) is involved in the metabolism of amino acids, the synthesis of nucleic acids and formation of blood cells and nerve tissue, while B<sub>2</sub> (riboflavin) is required for the conversion of B<sub>6</sub> and folic acid into their coenzyme forms (Huskisson et al., 2007). See Figure 3.1 for the folate and homocysteine-methionine cycle.



**Figure 3.1** Folate and homocysteine-methionine cycle. Adapted from Malouf and Evans (2008)

Vitamin B<sub>12</sub> depletion is common in old age (Wolters, Hermann & Hahn, 2003). This may be due to insufficient B<sub>12</sub> absorption in the elderly, rather than an inherent lack of the micronutrient

in the diet (Selhub et al., 2000). Along with vitamins B<sub>6</sub> and folate, B<sub>12</sub> lowers levels of homocysteine, a sulphur-containing amino acid. Concentrations of homocysteine increase with age (Joosten, Lesaffre & Riezler, 1996) and elevated levels have been linked to death caused by cardiovascular disease, increased incidence of stroke, dementia, AD, and a higher prevalence of chronic heart failure (Refsum et al., 2006; Selhub, 2006; Solfrizzi et al., 2006). Folate and vitamin B<sub>12</sub> are required for the methylation of homocysteine to methionine and B<sub>6</sub> is necessary for the metabolism of homocysteine to cysteine (refer to Figure 3.1). The amino acid methionine is essential to one-carbon metabolism, a series of biological processes crucial for DNA synthesis, DNA repair, and various methylation reactions (Mattson & Shea, 2003). Insufficient intake of folate or vitamin B<sub>12</sub> disrupts the methylation cycle and causes an intracellular accumulation of homocysteine which may be toxic to neurons (Sachdev, 2005).

Observations of psychiatric symptoms associated with B<sub>12</sub> deficiency were documented as early as 1849, and over the past 50 years low B<sub>12</sub> status has been related to memory impairment, personality change and psychosis (McCaddon, 2006), many symptoms of which overlap with dementia (American Psychiatric Association, 2000). There is also evidence that folate may be equally important for mental function, whilst elevated homocysteine levels may be detrimental. Studies have shown that in individuals with AD, vitamin B<sub>12</sub> and folate are lowered, and homocysteine elevated (Clarke et al., 1998; Selhub et al., 2000; Seshadri, 2006; Solfrizzi et al., 2006). Significantly, low serum folate levels (Quadri et al., 2004) and elevated plasma total homocysteine levels (Quadri et al., 2005) have been identified in individuals with MCI as well as those with AD. As MCI represents a condition which generally precedes the onset of dementia these researchers proposed that low folate and elevated homocysteine may predate dementia, and that maintenance of micronutrient levels through dietary supplementation may aid in dementia prevention. Whilst this is a promising assertion, there is still debate as to whether inadequate B<sub>12</sub> and folate may contribute to the neurodegenerative process associated with AD, or whether deficiencies may reflect a consequence of the disease process (Seshadri, 2006).

### **3.2.2 The relationship between B vitamins, homocysteine and cognitive function in healthy elderly**

There is evidence to suggest that a relationship exists between B<sub>6</sub>, folate, and cognitive function in healthy elderly. In the cross-sectional Maine-Syracuse study of 812 young through to elderly subjects, a relationship was identified between vitamin B<sub>6</sub> and multiple cognitive domains encompassing visual-spatial organization, working memory, scanning-tracking, and abstract reasoning (Elias et al., 2006). Findings from the Singapore Longitudinal Ageing study, which investigated 451 high functioning Chinese elders aged 55 years or older, revealed that higher levels of folate were associated with better scores on a verbal learning instrument (Feng et al., 2006). Reduced levels of folate have also been shown to be predictive of cognitive decline. Results from the MacArthur Studies of Successful Aging demonstrated that low folate levels in individuals aged in their seventies predicted cognitive decline 7 years later (Kado et al. 2005).

Despite the historical connection between B<sub>12</sub> and cognition, several epidemiological studies have failed to find an association between B<sub>12</sub> concentration and cognitive status (Kado et al., 2005; Mooijaart et al., 2005; Nurk et al., 2005; Feng et al., 2006). Moretti et al. (2004) proposes that measurement of methylmalonic acid (MMA), a product of amino acid metabolism, may provide a more useful diagnostic tool for B<sub>12</sub> deficiency. Results from the Oxford Healthy Aging Project showed that when holotranscobalamin, the biologically active fraction of vitamin B<sub>12</sub> and MMA was used as measures of vitamin B<sub>12</sub> status, each was associated with a more rapid cognitive decline on the mini mental state exam (MMSE) during a 10 year period in elderly aged 65 or older (Clarke et al., 2007).

Levels of homocysteine are closely related to concentrations of the other B vitamins (Huskisson et al., 2007). Homocysteine has been associated with cognitive deficits and conditions of pathological ageing such as AD (Quadri et al., 2004), and may be considered as a biomarker for cognitive decline. For example, elevated homocysteine and methylmalonic acid have been related to digit symbol and block design performance in elderly with an average of 76 years (Lewerin et al., 2005). Riggs et al. (1996) reported that men aged 54-81 years from the Boston

Veterans Affairs Normative Aging Study who had higher concentrations of plasma homocysteine and lower concentrations of B<sub>12</sub> and folate, also had poorer spatial copying skills. Similarly, outcomes from The Third National Health and Nutrition Examination Survey revealed that individuals aged over 60 years, with elevated homocysteine accompanied by low folate levels, demonstrated poorer story recall than those with normal levels of homocysteine (Morris et al., 2001).

Similar findings have been noted from trials which have focused on longitudinal decline. Further analysis from the Boston Veterans Affairs Normative Aging study revealed that in the middle aged to elderly men, plasma folate became the strongest predictor of spatial copying ability, independent of homocysteine at a three year follow up (Tucker et al., 2005). The results of this study also found that baseline homocysteine levels predict spatial copying performance, and this same finding has been replicated over a five year period in a small sample of 32 elderly (McCaddon et al., 2001). Nurk et al. (2005) found that greater levels of homocysteine at baseline predicted memory deficit after 6 years in those aged 65-67 years who participated in the Hordaland Homocysteine Study. Similarly, in this trial an increase in homocysteine or decrease in folate over this 6 year period was linked to a lower memory score (Refsum et al., 2006). Conflicting results have also been identified. In adults aged 65 years or older, greater intake of dietary folate has been associated with greater cognitive decline over 6 years on a composite measure of immediate and delayed recall, the MMSE and the symbol digit modalities test (Morris et al., 2005).

Collectively, the results from these studies indicate there is a relationship between levels of the B vitamins and both cognitive function and cognitive decline in the elderly. In particular, levels of folate and homocysteine appear to be the strongest predictors of cognition. As homocysteine represents a biomarker of cognitive decline, reducing levels of this amino acid may serve to enhance cognitive performance and slow the rate of decline experienced by the elderly.

### **3.2.3 Mechanisms of B vitamins relevant to cognition**

The effects of B vitamins on cognition may broadly be divided into direct influence on brain neurophysiology and indirect effects on vascular mechanisms. To maintain normal levels of homocysteine, Vitamin B<sub>6</sub>, B<sub>12</sub> and folic acid are required for the methylation of homocysteine to methionine. In turn, methionine is required for the synthesis of adenosylmethionine (SAM), the sole donor for methylation reactions in the brain. Products of these reactions include the neurotransmitters dopamine, noradrenalin and serotonin, as well as proteins, phospholipids, DNA, and myelin (Selhub et al., 2000). Additionally, SAM is critical for the maintenance of choline in the central nervous system, and for the generation of acetylcholine and the antioxidant glutathione (Tchantchou et al., 2008). According to the hypomethylation hypothesis, some neuropathology and loss of cognitive function in the elderly may be the result of a lower production of SAM and, consequently, methyl acceptors including neurotransmitters due to B<sub>12</sub> and folate deficiency (Rosenberg & Miller, 1992; Calvaresi & Bryan, 2001; Selhub, 2002). In turn, increasing the levels of B<sub>12</sub> and folate may be expected to benefit brain and cognitive function by increasing the production of neurotransmitters. See Figure 3.2 for vitamins involved in neurotransmitter production.

• Vitamin B <sub>1</sub> :	→	Glutamic acid	→ GABA (γ-aminobutyric acid)
• Vitamin B <sub>2</sub> :	→	Tyrosine	→ Noradrenaline → Serotonin → Benzylamine
• Vitamin B <sub>6</sub> :	→	Glutamic acid	→ GABA
		tyrosine	→ Dopamine → Adrenaline → Noradrenaline
		Tryptophan	→ 5-Hydroxytryptamine → Serotonin
		Histidine	→ Histamine
• Nicotinamide:	→	Tryptophan	→ 5-Hydroxytryptamine → Serotonin
• Vitamin C:	→	Tyrosine	→ Dopamine → Noradrenaline

**Figure 3.2** Role of selected water-soluble vitamins in neurotransmitter synthesis via their involvement in amino acid metabolism. Taken from Huskisson et al. (2007)

A second hypothesis posits that impaired neurocognitive function in the elderly may stem from the deleterious effects of elevated levels of homocysteine (Calvaresi & Bryan, 2001). The following section will describe evidence which has demonstrated an association between the B vitamins and homocysteine, with brain structural and cardiovascular parameters.

### *B vitamins, homocysteine and the brain*

In the brain, elevated homocysteine increases oxidative stress and DNA damage, triggers apoptosis and imparts excitotoxic effects (Sachdev, 2005). *In vitro*, homocysteine disrupts neuronal homeostasis by multiple routes including N-methyl-D-aspartate (NMDA) channel activation leading to excessive calcium influx and glutamate excitotoxicity (Ho et al., 2002). Hyperhomocysteinemia may also induce gene expression and interact with specific targets including cellular receptors, intracellular proteins and molecules of nitric oxide, leading to neuropathology (McCaddon, 2006). Raised homocysteine can disturb normal methylation,

promoting amyloid and tau protein accumulation (Obeid & Herrmann, 2006). Homocysteine has also been implicated in AD pathology (Seshadri, 2006) and has been found to be raised in these individuals (Clarke et al., 1998; Quadri et al., 2004) . When elevated, homocysteine has been correlated with the presence of white matter hyperintensities (Sachdev, 2005), a form of cerebrovascular damage which has been associated with decline in neurobehavioural functioning in the elderly (Swan et al., 1998).

Elevated homocysteine and low vitamin B<sub>12</sub> levels have been implicated in cortical atrophy. For instance, vitamin B<sub>12</sub> levels in 107 healthy\_community dwelling elderly have also been associated with brain volume loss over a five year period (Vogiatzoglou et al., 2008). The results of this study revealed that the decrease in brain volume was greater among those with lower vitamin B<sub>12</sub> and holotranscobalamin levels. Specifically, those in the bottom tertile for B<sub>12</sub> (<308 pmol/L) at baseline experienced the greatest rate of brain-volume loss. Based on these findings, the authors concluded that vitamin B<sub>12</sub> status may provide an early marker of brain atrophy, possibly representing a potentially modifiable risk factor for cognitive decline in the elderly. Increasing plasma homocysteine levels have been associated with atrophy of cortical and hippocampal regions, but not the amygdala, in over one thousand healthy elderly aged between 60 and 90 years (Den Heijer et al., 2003) and thinner hippocampal width in 156 seniors above 80 years of age (Williams et al., 2002). In a recent investigation by Firbank et al (2010), white matter atrophy rate and hippocampal atrophy rate over two years was correlated with homocysteine levels independent of B<sub>12</sub> levels in 80 hypertensive individuals aged 70-89 years. Conversely, there was no association between homocysteine and rate of grey matter atrophy. These findings may point to an effect of homocysteine on volume loss of selected brain structures. As brain atrophy has been shown to be an important predictor of cognitive function in the elderly (Rabbit et al., 2008), homocysteine-induced cortical volume loss may contribute to cognitive decline experienced by the elderly.

Findings from a recent randomised trial of 271 individuals indicated that two years of B vitamin supplementation was capable of slowing the rate of brain atrophy (Smith et al., 2010). In this

study, elderly with MCI were assigned to a treatment of vitamins B<sub>6</sub>, B<sub>12</sub> and folate or placebo. Volumetric magnetic resonance imaging (MRI) scans revealed that the treatment reduced the rate of atrophy by approximately 30%, and biochemical measures indicated that this was accompanied by a reduction in homocysteine. These findings indicate that reducing homocysteine via B vitamin supplementation may exert protective effects on brain structural parameters.

Erickson et al. (2008) have shown that greater intake of vitamins B<sub>6</sub> and B<sub>12</sub> is related to grey matter volume in specific cortical regions. In this study, 32 participants aged between 59 and 79 years underwent an MRI scan and a three day food diary was collected to determine dietary vitamin intake. Grey matter volume in the left and right parietal regions were associated with B<sub>12</sub>, whereas volume of the anterior and posterior cingulate, left parietal lobe and superior frontal gyrus were related to B<sub>6</sub>. Based on these findings, it was proposed that cognitive processes which rely on these regions are most susceptible to vitamin B deficiency and therefore may demonstrate the best response to vitamin supplementation.

Low levels of B vitamins, independent of homocysteine concentrations, have also been associated with neuropathology in the elderly, suggesting that mechanisms other than homocysteine-induced toxicity and vascular damage may contribute to detrimental neurophysiological changes. In a fascinating study conducted by Snowdon et al. (2000), blood nutrient levels were correlated with brain pathology in a group of elderly nuns who lived in the same convent, ate from the same kitchen, and consequently had comparable lifestyle and environmental factors. Blood was collected and analysed for nutrients, lipids and nutrient markers. Following the death of 30 nuns aged between 77 and 98, a neuropathologist examined the brains for signs of atrophy, AD lesions (neurofibrillary tangles, senile plaques and neuritic plaques) and atherosclerosis in the major arteries at the base of the brain. The results of this study showed that serum folate levels were negatively related to atrophy of the neocortex, particularly in those with a significant number of AD lesions in the neocortex. As folate was negatively correlated with subgroups of participants displaying minimal signs of vascular neuropathology

such as arteriosclerosis and brain infarcts, it was proposed that the association between low folate levels and atrophy may not be entirely due to the deleterious effects of vascular disease. Similarly, De Lau et al. (2009) have posited that neuropathology correlated with low B<sub>12</sub> levels may arise from other non vascular factors. Findings from this larger scale, population-based, Rotterdam Scan Study demonstrated that poorer vitamin B<sub>12</sub> status in the normal range was significantly associated with greater severity of white-matter lesions, in particular periventricular white-matter lesions in 1019 healthy elderly. However, as B<sub>12</sub> levels were not related to cerebral infarcts, the authors hypothesised that the association between B<sub>12</sub> levels and white matter lesions may be explained by effects on myelin integrity in the brain rather than through vascular mechanisms. The development of neuropathology in the brain associated with low vitamin B levels may, therefore be a contributing factor to cognitive decline or below optimum cognitive function in the elderly.

#### *B vitamins, homocysteine and the cardiovascular system*

Elevated homocysteine constitutes a risk factor for vascular disease (Clarke et al., 1991) and stroke (Selhub, 2006) and has demonstrated a relationship with cardiovascular risk factors such as cholesterol levels, blood pressure, and heart rate (Nygard et al., 1995). High concentrations of homocysteine have been associated with mortality in patients with coronary artery disease (Nygård et al., 1997). When raised, homocysteine exerts a detrimental effect on the cardiovascular system via pro-coagulant interactions with platelets and vascular endothelium (De Koning et al., 2003). Vascular damage may be incurred from reactive oxygen species, including superoxide and hydrogen peroxide, produced during the auto-oxidation of homocysteine (Welch & Loscalzo, 1998). Vascular insult also occurs due to reduced bio-availability of endothelial nitric oxide, a potent vasodilator (Obeid & Herrmann, 2006). Homocysteine-induced damage to endothelial cells can promote arteriosclerosis, a condition associated with poorer cognitive function (Breteler et al., 1994; Knopman et al., 2001; Seshadri, 2006). Collectively these findings suggest homocysteine may influence cognition in the elderly via direct effects on the brain or through indirect mechanisms operating on the cardiovascular system.

### 3.2.4 Vitamin interventions and homocysteine

Despite the vulnerability of the elderly to vitamin B depletion and age-associated increases in homocysteine (Joosten et al., 1996; McCaddon, 2006) in many instances these conditions can be rectified by appropriate dietary intervention. For instance, studies have demonstrated that supplementation with vitamin B<sub>6</sub>, B<sub>12</sub> and folate is capable of lowering homocysteine in the elderly over periods spanning from two years (McMahon et al., 2006) to as little as four months (Lewerin et al., 2005). In trials which used B<sub>12</sub> and folate, six months treatment in elderly over 70 years of age with mild B<sub>12</sub> deficiency successfully lowered homocysteine (Eussen et al., 2006), and one year's treatment reduced homocysteine in patients, 65 years or older, with vascular disease (Stott et al., 2005). Chronic use of multivitamins has also shown to be a simple and efficacious method to increase peripheral nutrient levels and decrease concentrations of homocysteine in the elderly. In an open label trial in individuals aged 24 to 79 years, multivitamin supplementation increased the plasma concentrations of folate, vitamins B<sub>6</sub>, B<sub>12</sub>, C, E, and  $\beta$ -carotene and reduced homocysteine by 1.2  $\mu\text{m/L}$  after 12 and 24 weeks (Earnest et al., 2002). These findings were replicated in a double-blind placebo-controlled trial after 24 weeks treatment with the multivitamin (Earnest, Wood & Church, 2003). Findings from another 24 week, double-blind, placebo-controlled trial in 220 women over 60 years of age, revealed that homocysteine was reduced by 1.45  $\mu\text{m/L}$  in those who received a daily multivitamin, whereas it was increased in those allocated the placebo (Wolters, Hermann & Hahn, 2005). Concentrations of methylmalonic acid (MMA), a marker of vitamin B<sub>12</sub> deficiency, were not reduced, possibly due to the low vitamin B<sub>12</sub> content of the multivitamin under investigation. These results indicate that dietary interventions with B vitamins or multivitamins can reduce concentrations of homocysteine, a biomarker of cognitive decline, over a period of months. Subsequently, lowering of homocysteine may represent a potential intervention against cognitive decline in the elderly.

*B vitamin summary*

The B vitamins exert important effects on neurophysiological function via methylation and the lowering of homocysteine concentrations in the body. Low levels of these vitamins and elevated homocysteine have been associated with pathological ageing conditions characterized by a loss of cognitive function, such as AD and MCI. Consequently, homocysteine represents a biomarker of cognitive decline. Epidemiological studies have revealed that in healthy elderly, levels of vitamin B<sub>6</sub>, B<sub>12</sub>, folate and homocysteine have been associated with cognitive function. In particular, levels of folate and homocysteine appear to be the strongest predictors of cognition. The B vitamins and homocysteine have been correlated with neuropathology in the brains of those with AD and healthy elderly subjects, as well as cardiovascular risk parameters. Dietary supplementation with B vitamins and multivitamins can lower levels of homocysteine, and via this action potentially improve neural, cardiovascular and cognitive function in the elderly.

### **3.3 Antioxidants**

Oxidative stress is also thought to contribute to the neuronal changes responsible for cognitive decline in normal and pathological ageing. Oxidative stress represents a disturbance in the equilibrium status of pro-oxidant reactions involving oxygen free radicals and those involving antioxidants (Valko et al., 2007). It is the maintenance of this pro-oxidant/antioxidant balance via redox homeostasis which is vital for healthy cellular function. Vitamins A, C, E, selenium and Co-enzyme Q10 exert antioxidant effects and protect neural tissue from aggression by free radicals (Bourre, 2006). Beta-carotene is a precursor to vitamin A and is a potent antioxidant. Vitamin C levels are particularly high in the brain, and this antioxidant is known to interact synergistically with B complex vitamins and is essential for the metabolism and utilization of folic acid (Huskisson et al., 2007). Vitamin C is also required for the transformation of dopamine into noradrenalin (Bourre, 2006). The function of this vitamin has been suggested to extend to neuromodulation of dopamine, regulation of acetylcholine and catecholamine release, and glutamate and GABA mediated neurotransmission (Harrison & May, 2009). Vitamin E is a lipid-soluble chain-breaking antioxidant, which exerts neuroprotective effects against reactive oxygen

species (Cantuti-Castelvetri et al., 2000). Specifically, vitamin E exerts its antioxidant activity in lipid-based cell membranes and can inhibit the process of lipid peroxidation (Isaac et al., 2008). Co-enzyme Q10 is another fat soluble molecule which acts as an antioxidant and co-enzyme in mitochondria (Boreková et al., 2008).

In addition to essential micronutrients, there is a growing body of literature which indicates that numerous herbal extracts containing phytochemicals deliver potent antioxidant actions in the body (Tapsell et al., 2006). Specifically, culinary herbs, as well as those used in traditional Chinese or Ayurvedic medicine have been investigated for their antioxidant activities (Zheng & Wang, 2001; Dragland et al., 2003) and potential neuroprotective mechanisms (Iriti et al.). Herbs contain several lipid-soluble tocopherols, carotenoids and sterols which may protect against lipid peroxidation in addition to water-soluble phenolic acids and flavonoids, which are capable of scavenging reactive oxygen species (Tapsell et al., 2006). Flavonoids constitute important polyphenolic compounds which have been investigated for their potential to reduce cardiovascular risk factors (Vita, 2005; Hooper et al., 2008) and improve cognitive function (Macready et al., 2009). Antioxidant actions of flavonoids include lowering of oxidative stress and neuro-inflammatory events (Schmitt-Schillig et al., 2005). Other important mechanisms of flavonoids and their metabolites in the brain have been proposed, including effects on long term potentiation (LTP), a process involved in memory formation. It has been suggested that flavonoids interact with neuronal signalling pathways to promote LTP and synaptic plasticity, actions which are likely to contribute to the beneficial effects of flavonoids on human cognitive performance (Spencer, 2008; Spencer, 2009).

### **3.3.1 The relationship between antioxidants and cognitive function**

A number of population studies have demonstrated that antioxidant intake may represent a dietary factor which has important consequences for cognitive function in the elderly. The Chicago Health and Aging Project investigated the dietary habits of 2,889 community residents, aged 65 to 102 using a food frequency questionnaire (Morris et al., 2002). The results of this

study revealed that higher intake of vitamin E from diet or supplements was associated with a slower rate of cognitive decline over three years, as measured by a combined cognitive score derived from tests of immediate and delayed story recall, a measure of perceptual speed and the MMSE. There was little evidence of association with vitamin C or carotene intake. In contrast, findings from the Rotterdam Study revealed that intake of beta-carotene, and not vitamin C or E, was associated with performance on the MMSE in 5182 elderly aged 55-95 years (Warsama Jama et al., 1996). In the PAQUID study of 1640 French elders, dietary intake of flavonoids was associated with cognitive performance as measured by a combined MMSE, visual retention and verbal fluency score. Similarly, after 10 years, individuals with a higher intake of flavonoids demonstrated a slower rate of decline on this measure (Letenneur et al., 2007).

Vitamin status in the blood has been associated with cognitive function in the elderly. The Third National Health and Nutrition Examination Survey investigated blood vitamin levels taken from a multiethnic sample of 4,809 elderly residents of the United States (Perkins et al., 1999). Findings from this trial revealed that higher serum levels of vitamin E were associated with better memory recall of a three sentence story. Interestingly, no correlations between vitamin A, beta-carotene, selenium, or vitamin C and memory performance were identified. By contrast, in a sample of Swiss elderly aged 64 to 95 years, both past and current levels of ascorbic acid and beta-carotene were associated with free recall, recognition and vocabulary performance (Perrig et al., 1997). In the Cognitive Change in Women study, a more comprehensive neuropsychological test battery was utilized to assess the domains of memory, executive function, language, attention, and visual function in 526 women aged 60 or above with cognitive impairment (Dunn et al., 2007). Baseline results from this study revealed that low serum alpha-tocopherol status was cross-sectionally associated with increased odds ratio of memory and mixed cognitive impairments. In contrast, previous vitamin E supplement intake was not associated with any type of cognitive impairment.

The use of combined vitamin C and E supplements appears to be associated with the rate or extent of longitudinal cognitive decline in the elderly, as measured by the MMSE and modified

versions of this assessment. For instance, use of both vitamin C and E supplements by elderly men aged 71 to 93 has been linked to better cognitive function three to five years later (Masaki et al., 2000). In a large sample of community dwelling elderly women who participated in the Nurse's Health Study, long term use (>10 years) of vitamin C and E supplements was associated with better performance on cognitive tests including verbal fluency, digit span backwards and a telephone administered version of the MMSE (Grodstein, 2003). Those taking both supplements displayed equivalent cognitive function to individuals two years younger. Similarly, elderly subjects over the age of 65 years from the Canadian Study of Health and Ageing using combined vitamin C and E supplements were less likely to experience significant cognitive decline on a modified version of the MMSE during a 5-year follow-up period (Maxwell, 2005). Outcomes from the Cache County Study revealed that participants aged 65 years or older with lower levels of intake of vitamins C, E and carotene had a greater acceleration of the rate of cognitive decline on the modified MMSE over seven years compared to those with higher levels of intake. It was concluded from this study that higher antioxidant dietary vitamins C, E, and beta-carotene may delay cognitive decline in the elderly (Wengreen et al., 2007). Separate analyses from this study demonstrated that those with the APOE e4 allele using vitamins C, E, or multivitamin supplements in combination with non steroidal anti-inflammatory drugs showed less cognitive decline over an eight year period than those with the e4 allele who were not using antioxidant supplements (Fotuhi et al., 2008). These results suggest that those at a higher genetic risk of developing AD may benefit from use of anti-inflammatory medication and antioxidant supplementation.

When considered together, evidence from epidemiological studies suggests that intake of selected antioxidant vitamins, as well as antioxidant vitamin level in the body, is related to cognition in the elderly. Long term use of vitamins C and E also appears to be protective against cognitive decline.

### 3.3.2 Mechanisms of antioxidants relevant to cognition

#### *Basic mechanisms of oxidative stress and inflammation*

The free radical hypothesis of ageing posits that detrimental age-related changes take place in the brain as the result of an inability to cope with oxidative stress that occurs throughout the lifespan (Beckman & Ames, 1998). In a physiological context, oxidative stress can be defined as an excessive bioavailability of reactive oxygen species (ROS) caused by an imbalance between production and destruction of ROS by antioxidants (Kregel & Zhang, 2007). ROS are products of normal cellular metabolism and exert a number of physiological roles when levels are maintained at low concentrations by the process of redox homeostasis (Valko et al., 2007). In aerobic cells, ROS are produced in the mitochondria and in excess levels cause damage to mitochondrial components and initiate degradative processes (Cadenas & Davies, 2000). The consequence of oxidative stress is damage to lipids, proteins and DNA (Floyd & Carney, 1992). Oxidative stress leads to lipid peroxidation, a degradative process which modifies the fluidity and permeability of neuronal membranes, leading to an alteration of cellular functioning and damaged membrane-bound receptors and enzymes (Mariani et al., 2005). The brain, in particular, is vulnerable to the effects of oxidative stress as it possesses reduced free radical scavenging ability and requires high quantities of oxygen (Floyd & Carney, 1992; Cantuti-Castelvetri et al., 2000). Alterations in membrane lipids, microvasculature changes and increases in oxidized proteins and lipids may contribute to the increased vulnerability of the brain to oxidative stress with ageing (Floyd & Hensley, 2002).

It has been suggested that inflammation may represent a unifying pathophysiological mechanism relating to a range of diseases associated with the ageing process (Libby, 2007). In a healthy state, inflammation is the first response of the immune system to infection or irritation. During acute inflammation, blood flow is increased and clotting factors enter the injured area. Neutrophils migrate to the site of inflammation and interact with the resident tissue cells. Reactive oxygen species, cytokines, procoagulants, and other molecules are then elaborated to amplify and sustain the inflammatory response (Libby, 2007). However, sustained inflammation

can also exert deleterious effects on the CNS and cardiovascular system (Berliner et al., 1995; Akiyama et al., 2000; Zipp & Aktas, 2006).

Oxidative stress is generally measured by biomarkers of lipid oxidation, protein carbonyls, F<sub>2</sub>-isoprosanes or thiobarbituric acid-reactive substances (TBARS). Oxidative stress has been implicated in neuropathological ageing with increased levels of oxidative damage to neurons and mitochondrial DNA reported in AD and MCI (Berr et al., 1998; Berr, 2002; Mecocci, 2004; Mecocci et al., 2004; Lovell & Markesbery, 2007). Subsequently markers of oxidative stress may also represent bio-markers of cognitive decline in the elderly. In addition to greater oxidative stress, low peripheral levels of vitamins A, C, E and carotenoids have been identified in MCI and AD patients in comparison to controls (Barabás, Nagy & Degrell, 1995; Rinaldi et al., 2003). Furthermore, intake of flavonoids, which exert powerful antioxidant actions, has been associated with dementia risk in over 1000 subjects aged above 65 years over a five year period (Commenges et al., 2000).

In AD, free radicals causing neuronal loss are considered to be produced as a result of the deposition of aggregated A $\beta$  peptide (Reynolds et al., 2007). It has been suggested that as a part of the disease process of AD, mitochondrial failure caused by hypoperfusion contributes to the generation of reactive oxygen species, resulting in oxidative damage to the brain, especially in the vascular endothelium and in neurons with high metabolic activity (Aliev et al., 2004). In response to this neural injury, immune cells such as microglia and macrophages then elicit a secondary inflammatory reaction, which contributes to ongoing damage (Zipp & Aktas, 2006).

In 1166 healthy elderly, higher levels of TBARS have been associated with the greatest levels of decline on the MMSE over a period of four years, indicating that oxidative stress may also be related to cognitive function in the normal ageing process (Bert et al., 2000). As antioxidants possess the ability to reduce the effects of oxidative damage to the brain (Rao & Balachandran, 2002), maintaining adequate antioxidant status may slow the rate of neuropathology.

*Oxidative stress in the brain*

To date, few studies have investigated antioxidant status in relation to brain structural or neuropathological correlates in humans. In one study, antioxidant levels were measured in frontal and occipital regions on post mortem examination. Higher concentrations of the major carotenoids, tocopherols, and retinol were identified in the frontal cortex than the occipital cortex, and an age-associated decline in these levels was apparent in the frontal cortex of 10 samples of brain tissue (Craft et al., 2004). Furthermore, postmortem examination of elderly with MCI or early AD has revealed that these individuals had elevated oxidative stress in the superior and middle temporal gyri as measured by TBARS, malondialdehyde and protein carbonyls (Keller et al., 2005). Interestingly, concentrations of TBARS were also correlated with neuritic plaques, indicating that this marker of oxidative stress may be important in the pathogenesis of AD. Recent findings published from the Adult Changes in Thought study examined oxidative stress in the brains of 66 elderly subjects at autopsy (Sonnen et al., 2009). The results of this study showed that increased free radical damage to cerebral, cortical neurons, as measured by F4 neuroprostanes, was apparent in AD cases, current smokers and those with microvascular brain injury. There was no relationship between free radical damage to the cortex and vitamins C, E or multivitamin use at the time of last evaluation before death. As the criteria for vitamin use only specified that supplements were used one week out of the month prior to evaluation, measurement of peripheral antioxidant levels may have been more indicative of antioxidant status in this elderly group. In a study which looked at plasma vitamin C levels in 13 patients with brain injury, vitamin C was lower in those individuals than in healthy control subjects. Furthermore, the major diameter of the brain lesion in those with brain injury was negatively correlated with vitamin C levels, indicating that damage to the brain may increase oxidative stress (Polidori, Mecocci & Frei, 2001). Further investigation of antioxidant status and oxidative stress in relation to neuropathology are required to ascertain the relationship between brain structure and antioxidant status in an elderly subject population.

*Inflammation in the brain*

There appears to be an important role of inflammatory processes in neuropathological ageing. In AD, damaged neurons, neurofibrillary tangles and beta amyloid plaques (A $\beta$ ) represent likely sources of inflammation in the brain, leading to further neuronal damage (Akiyama et al., 2000). Macrophage/microglia are the primary immune cells found in the CNS and in AD are activated by A $\beta$  proteins, mediating cellular damage and the production of neurotoxins (Minagar et al., 2002). Inflammation may also play a role in normal brain ageing and related cognitive decline. In the elderly, several studies have identified a relationship between the inflammatory marker high sensitivity reactive protein (hCRP) and cognitive function. In over 3000 elderly Americans, individuals with higher levels of hCRP and interleukin-6, but not tumour necrosis factor, was related to greater cognitive decline over two years on the 3MS measure of cognition (Yaffe et al., 2003). Similarly, in a sample of over 1000 elderly free from dementia, subjects in the highest hCRP tertile had higher odds of memory and visuospatial impairment. In this study hCRP was not related to executive or language impairment (Noble et al., 2010).

Inflammation as measured by hCRP has been associated with brain structural integrity. In a study of 321 healthy people with an average age of 63 years, higher levels of hCRP were related to worse executive function performance and white matter integrity of the frontal pathways (Wersching et al., 2010). Comparably, in 50-65 year olds, elevated inflammatory markers including hCRP were associated with loss of white matter integrity in corticosubcortical pathways and frontal and temporal association fibres (Miralbell et al., In press). These findings indicate inflammation may be related to cerebral microstructural disintegration, possibly contributing to cognitive decline in healthy elderly.

*Cardiovascular pathology: the role of oxidative stress, inflammation and nitric oxide*

Oxidative stress has been implicated in the pathophysiological process of cardiovascular disease and stroke (Mariani et al., 2005), conditions associated with cognitive impairment (Wang et al., 2002; Elias et al., 2004). According to the oxidative modification hypothesis of atherosclerosis, low density lipoprotein (LDL) accumulates in the sub-endothelial space in arteries where it becomes oxidized leading to foam cell formation, endothelial dysfunction and injury, ultimately

enhancing the progression of atherosclerotic lesions (Steinberg et al., 1989; Diaz et al., 1997). Inflammation also contributes to this process (Berliner et al., 1995; Libby, Ridker & Maseri, 2002; Packard & Libby, 2008) via interactions between platelets, leukocytes, and endothelial cells (Gawaz, Langer & May, 2005). Leukocytes generally adhere poorly to vascular endothelial cells, however in the presence of proinflammatory factors such as elevated cholesterol, obesity, insulin resistance and hypertension, chemokines and vascular cell adhesion molecules mediate the attachment of monocytes and lymphocytes to platelets on the endothelium (Libby & Theroux, 2005; Packard & Libby, 2008). Leukocytes migrate into the intima where they signal to endothelial and smooth muscle cells, eventually leading to lesion formation and rupture (Libby et al., 2002). When elevated, C-reactive protein (CRP) represents a marker of systemic inflammatory response and has been shown to be a strong predictor of the risk of cardiovascular events (Ridker et al., 2000) and cognitive impairment (Noble et al., 2010). Other biomarkers such as fibrinogen (Packard & Libby, 2008) and 11-dehydro-thromboxane, an indicator of platelet activation (Arnaud et al., 2007), represent markers of vascular-related inflammation in the body.

Endothelium-derived nitric oxide (NO), synthesized by the endothelial NO synthase (eNOS) is both a major mediator of endothelium-dependent vasodilation and possesses important anti-inflammatory and antithrombotic properties (Landmesser, Hornig & Drexler, 2004). As described above, leukocyte adhesion and infiltration into the intima, regulated by leukocyte-adhesion molecules and chemokines, occurs during the formation of atherosclerotic lesions (Libby et al., 2002). Endothelium-derived NO reduces leukocyte adhesion, and also limits platelet activation, adhesion, and aggregation (Landmesser et al., 2004). Elevated CRP levels have been associated with impaired endothelial vascular reactivity, demonstrating the link between the processes of inflammation and endothelial dysfunction (Fichtlscherer et al., 2000).

With the ageing process, blood vessels lose elasticity, leading to an increase in arterial stiffness (Mitchell et al., 2004). Although largely dependent on blood pressure (Laurent, Boutouyrie & Lacolley, 2005), endothelial function also represents an independent contributor to arterial stiffness. Specifically, a role for the release of vasoactive mediators such as NO has been

implicated in the control of arterial stiffness (Wilkinson et al., 2002). Arterial stiffness represents a risk factor for vascular disease (Gatzka et al., 1998; Qureshi et al., 2007) and cognitive decline (Hanon et al., 2005; Pase et al., 2010), as increases in pulse pressure, a measure of arterial stiffness, can induce damage to the microvascular structures of the brain (O'Rourke & Safar, 2005).

In the elderly, higher intake and peripheral levels of antioxidants appear to be associated with decreased risk of vascular disease (Jialal & Devaraj, 2003). Specifically, cellular antioxidants protect against the cytotoxic effects of oxidized LDL and endothelial dysfunction related to the formation of atherosclerotic lesions by preserving endothelial-derived NO (Diaz et al., 1997). Vitamin C increases the availability of nitric oxide (Taddei et al., 1998), a compound which prevents platelets from adhering to the vascular endothelium (Vita, 2005), and Gale et al. (1996) have identified that a high intake of vitamin C may be protective against cerebrovascular disease and related cognitive decline. Intake of vitamins C and E in men at age 70 have also been correlated with biomarkers of inflammation and oxidative stress, including F<sub>2</sub>-isoprostane, prostaglandin F<sub>2α</sub>, high sensitive C-reactive protein (hsCRP), interleukin-6 (IL-6) at a seven year follow up (Helmersson et al., 2009). Blood levels of selenium, another antioxidant, in 615 Swedish elderly men at baseline has been shown to predict levels of oxidative stress almost 30 years later, indicating this nutrient may likewise possess important cardiovascular protective mechanisms (Helmersson et al., 2005).

Flavonoids have been examined for their cardio-protective properties. Dietary intake of some foods rich in flavonoids and compounds including flavanones and anthocyanidins, have been associated with lower risk of death due to coronary heart disease and cardiovascular disease (Mink et al., 2007). The effects of flavonoids on the cardiovascular system may be due to their ability to reduce LDL lipid peroxidation (Lapointe, Couillard & Lemieux, 2006). This may be achieved via actions including the scavenging of reactive oxygen species, metal chelation and protection of LDL-associated antioxidants. Other mechanisms include a reduction in macrophage oxidative stress and activation of the glutathione antioxidant system (Fuhrman & Aviram, 2001).

Some flavonoids have also been demonstrated to lower blood pressure (Hodgson & Croft, 2006), an action potentially due to the ability of flavonoids to improve endothelial function (Vita, 2005) and to increase the availability of NO (Achike & Kwan, 2003). Benefits of dietary flavonoids to measures of arterial stiffness have also been attributed to alterations in endothelial function (Pase, Grima & Sarris, 2011). The capability of flavonoids to modify endothelial function and arterial stiffness, may in turn be associated with increased cerebral blood flow, enhancing the delivery of oxygen and glucose to the brain (Ghosh & Scheepens, 2009).

### **3.3.3 Vitamin supplementation, oxidative stress and inflammation**

Antioxidants combat oxidative stress in the body and vitamins E and C have been demonstrated to decrease markers for oxidative DNA damage (Boothby & Doering, 2005). Following supplementation with vitamin E for a period of two years, markers of oxidative stress including hCRP, LDL oxidation F<sub>2</sub>-isoprostanes and monocyte superoxide anion concentrations were reduced in patients with coronary artery disease (Devaraj et al., 2007). Shorter term benefits have been observed as well. In healthy young to middle aged subjects with low ascorbic acid levels, vitamin C supplementation has been demonstrated to reduce immunoglobulin protein carbonyl levels after ten weeks (Carty et al., 2000). Comparable findings have been observed following two months treatment of either vitamin C or E on plasma F<sub>2</sub>-isoprostanes in almost 400 participants with elevated oxidative stress at baseline (Block et al., 2008). Benefits of combined vitamin formulas on other markers of oxidative stress have also been documented. Cheng et al. (2001) reported that antioxidant vitamin status and enzymatic activities were improved in 34 healthy subjects following five weeks multivitamin treatment and that there was a reduction in the susceptibility of red blood cells to peroxidation by free radicals. After a period of only four weeks multivitamin supplementation, reduced DNA damage in lymphocytes and decreased H<sub>2</sub>O<sub>2</sub>-induced DNA breakage has been identified in 80 middle aged to elderly subjects (Ribeiro et al., 2007). Data from a population study has also revealed an association between greater frequency multivitamin use and longer telomere length in women aged 35 to 74 years (Qun et al., 2009). Telomeres are the protein-DNA structures at the ends of eukaryotic chromosomes and in

human cells, they shorten during ageing when exposed to the effects of oxidative stress and chronic inflammation (von Zglinicki, 2002; von Zglinicki & Martin-Ruiz, 2005).

Multivitamin supplementation has been demonstrated to be a straightforward way to increase the status of multiple antioxidants in the body. In a study of 24 weeks duration, serum vitamin E and  $\beta$ -carotene improved and levels of serum vitamin C were maintained in elderly women who received the multivitamin, whilst a decline in vitamin C was observed in the placebo group (Wolters, Hermann & Hahn, 2004). Evidence of a cardioprotective effect of combination vitamin formulas has also been obtained. In an open label trial in individuals aged 24 to 79 years, multivitamin supplementation decreased the susceptibility of LDL cholesterol to oxidation (Earnest et al., 2002). In a double blind placebo controlled replication of this study, the same blood parameters and outcome measures were found to improve after 24 weeks treatment with the multivitamin (Earnest et al., 2003).

Benefits to bio-markers of inflammation have also been observed following vitamin treatment. A trial conducted by Arnaud et al. (2007) revealed that two years treatment with combined ascorbic acid, vitamin E,  $\beta$ -carotene, selenium and zinc led to a reduction in platelet activation in healthy individuals and similar treatment effects of combined antioxidants have been observed after 20 weeks in those with low antioxidant status (Salonen et al., 1991). In a shorter duration trial in a sample of individuals with dementia or MCI, platelet activation was decreased after only 12 weeks supplementation with B vitamins or aspirin (Clarke, Harrison & Richards, 2003). In a study which measured C-reactive protein, an inflammatory marker which is used clinically to predict cardiovascular events (Ridker et al., 2000; Ridker & Silvertown, 2008), post hoc analysis was conducted on blood samples obtained from subjects who had participated in a six month trial (Church et al., 2003). The results from this double-blind placebo-controlled investigation into the effects of multivitamin supplementation demonstrated a reduction in C-reactive protein levels associated with the treatment.

Other cardiovascular improvements have been associated with antioxidant supplementation. Six months treatment with a combination antioxidant supplement has been shown to improve lipid metabolism and lower blood pressure in individuals with multiple cardiovascular risk factors (Shargorodsky et al., 2010). When combined with vitamin C, flavonoid extracts such as pine bark have demonstrated a trend of systolic blood pressure reduction after 12 weeks in smokers (Young et al., 2006) and five weeks in older individuals at risk of cognitive decline (Pipingas et al., 2008). The high antioxidant status of the pine bark extract (Rohdewald, 2002), combined with other vasorelaxant actions (Kwak et al., 2009) may be responsible for these effects.

### *Antioxidant Summary*

Antioxidant vitamins are essential for reducing oxidative stress, a condition which is exacerbated over the lifespan. Antioxidants such as vitamin C may also play an important role in neurotransmitter regulation. In addition to lowering oxidative stress, powerful herbal antioxidants known as flavonoids may influence cognitive function via interactions with neuronal signalling pathways. Findings from epidemiological studies indicate that there is a relationship between both antioxidant dietary intake and status in the body, and cognitive function. Specifically, chronic use of vitamin C and E supplements may help slow the rate of cognitive decline in the elderly. Antioxidant depletion and elevated oxidative stress have been associated with AD and MCI, indicating that markers of oxidative stress may also represent biomarkers of cognitive decline via neural death and dysfunction. Oxidative stress and inflammation also contribute to cardiovascular pathology, whilst antioxidants exert cardioprotective actions. Dietary supplementation with antioxidants has been demonstrated to reduce biomarkers of oxidative stress, inflammation and other cardiovascular risk parameters in the body. Such improvements to markers of cognitive decline and cardiovascular health may in turn influence cognitive function in the elderly.

### 3.3.4 Other micronutrients related to cognition

#### *Zinc and cognition*

In addition to the antioxidant vitamins, micronutrients including calcium, magnesium and zinc are known to be essential for optimum neural functioning, although their relationship to cognitive processes in the elderly has not been researched as extensively. Specifically, magnesium is essential for enzymes requiring vitamin B<sub>1</sub> as a cofactor, and is required for the synthesis and action of ATP (Bourre, 2006). Calcium regulates neurotransmission and zinc is required as a structural component of many proteins, hormones, hormone receptors and neuropeptides (Huskisson et al., 2007).

Zinc deficiencies are relatively common and even at a mild to moderate level they can impair neuropsychological function (Sandstead, 2000). Zinc increases antioxidant activity, with supplementation demonstrated to increase the activity of zinc dependant antioxidant enzymes in healthy elderly subjects (Mariani et al., 2008). Lower zinc concentrations have been associated with neuropathology in AD (Tully, Snowdon & Markesbery, 1995). In this study, fasting serum zinc concentrations, determined approximately 1 year before death of 12 AD subjects, were correlated with total senile plaques and diffuse plaques and were suggestive for neuritic plaques. These findings suggest that low zinc concentration may play a role in plaque formation in AD.

In a study of cognitive function in 850 healthy elderly recruited from European countries including Italy, Greece, Germany, France and Poland, plasma zinc status was correlated with global cognitive functioning as measured by the MMSE (Marcellini et al., 2006)(Marellini et al, 2006). The observation that inhabitants from countries who consumed a diet richer in zinc exhibited better cognitive function, suggests that zinc supplementation may exert beneficial effects on cognition in elderly who are zinc deficient.

*Vitamin D and cognition*

Research into the importance of vitamin D for cognition in normal and pathological ageing has only recently gained momentum. Factors including low sunlight exposure, age-related decreases in cutaneous synthesis, and diets low in vitamin D, contribute to the high prevalence of vitamin D inadequacy in the elderly (Holick, 2006). Vitamin D is a steroid hormone which maintains levels of calcium, phosphorus and bone mineralization in the body. More pertinent to cognition, vitamin D receptors are located in regions of the brain important for planning, processing and forming new memories, pointing to a biological role for vitamin D in neurocognitive function (Buell & Dawson-Hughes, 2008). It has been suggested that vitamin D may exert protective effects against cardiovascular and cerebrovascular disease, peripheral artery disease inflammation and promote neuronal health (Buell et al., 2010).

In recent study of 213 elderly, vitamin D concentrations were found to be lower in subjects with dementia than in healthy participants (Buell et al., 2010). Insufficient levels of peripheral vitamin D were associated with MRI indicators of cerebrovascular disease. Specifically, lower vitamin D was related to white matter hyperintensity volume and severity, pathology which is consistent with brain atrophy, impaired cerebral vascular function, and poorer frontal lobe cognition.

Levels of vitamin D with reference to cognitive function have been investigated in a study of 40 individuals with mild AD and 40 controls, with cognitive function assessed using the Short Blessed Test, MMSE, Clinical Dementia Rating and a factor score from a neuropsychometric battery. These results showed that over half the sample had abnormally low vitamin D levels, defined as less than 20 ng/mL, and that vitamin D deficiency was associated with poorer performance on the Blessed Test and higher Clinical Dementia Rating (Wilkins et al., 2006).

The relationship between cognition and vitamin D has also been investigated in healthy elderly. In a large sample of middle aged to elderly men free from dementia, levels of vitamin D were associated with digit symbol substitution performance (Lee et al., 2009). Recently published findings from the Nutrition and Memory in Elders study also revealed a positive association

between vitamin D and measures of executive function and attention processing speed parameters in over 1000 elderly subjects (Buell et al., 2009). As this relationship remained robust after adjustment for homocysteine, apoE4 allele, plasma B vitamins, and multivitamin use, it is possible that vitamin D may also represent an important predictor of cognitive function in the elderly.

In summary, there is evidence that zinc and vitamin D may be related to cognitive function in the elderly. A greater number of trials in individuals with AD and healthy elderly are required to determine their role in neuropathological ageing and mechanisms of cognitive improvement.

### **3.4 Vitamins and cognition in the elderly: Evidence from randomised controlled trials**

The identification of a relationship in the elderly between cognition and B vitamin and antioxidant status has led to a number of intervention trials designed to determine whether supplementation with these vitamins can improve cognitive function. The utilization of a randomised, double-blind, placebo-controlled methodology has allowed researchers to objectively investigate the effects of these micronutrients on cognition. The following section will review evidence from RCTs which have examined the effects of B vitamins, antioxidant vitamins, zinc and multivitamins on cognitive function. Studies will be limited to trials of healthy elderly, where such trials are available.

#### **3.4.1 B vitamins**

Perhaps the most promising results linking B vitamins to cognition have been obtained from the Folic Acid and Carotid Intimamedia Thickness (FACIT) trial (Durga et al., 2007). In this trial over 800 adults aged 50 to 60 years were randomly allocated to either 800 µg folic acid per day, or a placebo, for a period of three years. At the end of the study period, the folic acid treatment led to an improvement in information processing speed which was not evident in the placebo

group. A composite score, which included measures of information processing speed and memory, was also found to benefit from supplementation. In contrast, mixed results regarding the cognitive effects of 35 days of B<sub>6</sub>, B<sub>12</sub> and folate supplementation have been obtained from a cross sectional study of young, middle aged and older women (Bryan, Calvaresi & Hughes, 2002). In this study, participants who supplemented with B<sub>12</sub> or folate performed worse on a verbal fluency task than those who received B<sub>6</sub> or the placebo. The low dose of B<sub>12</sub> used in this study (15 µg) coupled with the short treatment period may account for these results. By contrast, memory performance improved for the oldest group who received folate, indicating that there were some benefits of the treatment for elderly women.

Conclusions derived from Bryan et al. (2002) specified that the oldest participants demonstrated cognitive improvements with folate supplementation. Thus it could be argued that the treatment effects of B vitamins may be specific to certain subgroups amongst the elderly, such as the very old, or those with cognitive impairment, but not dementia. Support for this premise has been obtained from a study of patients with depleted vitamin B<sub>12</sub> levels at baseline (Eastley, Wilcock & Bucks, 2000). In this trial, three months intervention improved verbal fluency for individuals with cognitive impairment, whilst the same benefits were not observed for those with established dementia. Several smaller trials have demonstrated minor improvements to memory in individuals at risk of cognitive decline due to low vitamin status. Deijen et al. (1992) identified improvements to long term memory in vitamin B<sub>6</sub> deficient men, aged in their 70s, following three months vitamin B<sub>6</sub> supplementation. It should be noted, however, that this effect could be partially attributed to a decrease in performance of the placebo group over this time period. Memory improvements were also observed in a small trial of elderly, aged 70 to 90 years, with low folate at baseline when treated for deficiency over a period of 2 months (Fioravanti et al., 1997). In this study cognitive benefits were observed on the Randt memory task which provides a measure of serial, paired, rote, and incidental learning.

In a study of patients aged over 65 years with ischemic vascular disease, an established risk factor for cognitive decline (Breteler et al., 1994; Nash & Fillit, 2006), there were no observed

cognitive improvements on the letter digit coding test or telephone interview for cognitive status following 12 months treatment with either folate and vitamin B<sub>12</sub>, or vitamin B<sub>6</sub> and riboflavin (Stott et al., 2005). This result is in opposition to the suggestion that individuals most vulnerable to cognitive decline may experience the greatest improvements from vitamin B supplementation. Cognitive assessment of the patients was limited to a letter digit coding test and a telephone interview for cognitive status, and for this reason it is unknown whether the treatment failed to impart any cognitive benefits or if the cognitive instruments may have been unsuitable to detect any nutraceutical effects of the treatment.

Other less positive findings have been reported. In a study into the effects of four months combined vitamin B<sub>6</sub>, B<sub>12</sub> and folate supplementation in community-dwelling elderly, there were no improvements in neuropsychological task performance (Lewerin et al., 2005). Furthermore, Eussen et al. (2006) failed to identify any effects of B<sub>12</sub> or combination B<sub>12</sub> and folate supplementation on cognition in older persons aged 70 or above with mild vitamin deficiency. In this trial, 195 participants who received either 1000 µg B<sub>12</sub> alone or 1000 µg B<sub>12</sub> combined with 400 µg folate daily for 24 weeks did not show any improvements on a comprehensive battery of neuropsychological tests of attention, visual, short and long term memory. Similarly, results from a two year trial did not support the hypothesis that lowering homocysteine with B<sub>6</sub>, B<sub>12</sub> and folate would improve performance on neuropsychological tests (McMahon et al., 2006).

A potential criticism of these investigations relates to the use of neuropsychological instruments to assess the nutraceutical effects of B vitamins. Neuropsychological tests initially designed to detect brain impairment or measure IQ may not be as responsive to cognitive benefits as cognitive tasks sensitive to the more subtle deficits that accompany the ageing process. In a review of randomized controlled trials, Balk et al. (2007) also concluded that there was little beneficial effect of B<sub>6</sub>, B<sub>12</sub> or folate supplementation on cognitive function, although these authors argue that there is still a need for larger-powered, longer-duration studies to properly assess whether vitamin B supplementation is effective in slowing cognitive decline or improving cognitive performance in the elderly. A similar conclusion was reached based on a Cochrane

review of trials which focused on folate, combined with or without vitamin B<sub>12</sub> (Malouf & Grimley Evans, 2008). On the basis of their review, Balk et al. (2007) proposed that in future studies, only cognitive function tests which adequately differentiate cognitive domains should be used to evaluate whether B vitamin supplementation affects each distinct cognitive domain under investigation. Due to the fact that low vitamin B intake can selectively compromise cortical structures (Erickson et al., 2008), the cognitive domains supported by these regions may prove to be more vulnerable to depleted B vitamin levels, or even respond better to dietary supplementation than others.

### **3.4.2 Antioxidants**

In a placebo-controlled study into the effects of 12 months combined beta carotene, vitamin C and vitamin E treatment, there were no identified cognitive benefits on a range of computerised memory and attention tasks in a healthy elderly sample of 195 individuals (Smith et al., 1999). Several other large scale placebo-controlled studies have investigated the effects of selected antioxidant supplementation on cognitive measures in elderly adults. The point must be raised that these studies were not primarily designed to measure cognitive performance changes with supplementation, and in some cases no cognitive data was available prior to antioxidant treatment. Participants in the Age-Related Eye Disease Study were randomly assigned to receive either daily antioxidants (vitamin C, 500 mg; vitamin E, 400 IU; beta carotene, 15 mg), zinc and copper only (zinc, 80 mg; cupric oxide, 2 mg), antioxidants plus zinc and copper, or a placebo. Treatment groups did not differ in any of the cognitive tests after approximately seven years of treatment (Yaffe et al., 2004). A cognitive testing component including assessment of general cognition, verbal memory, and category fluency was added to the Physician's Health Study, where elderly men supplemented their diet with 50mg beta carotene or a placebo on alternate days (Grodstein et al., 2007). Treatment with beta carotene for 3 years or less had no impact on cognitive performance, whereas treatment duration of at least 15 years provided significant benefits for verbal memory, cognitive status and a composite score of these measures. The findings from this study suggest that long term interventions, implemented at early stages of

brain ageing may provide cognitive benefits. Using the same outcome measures, participants in the Women's Health Study received either vitamin E supplementation (600 IU) on alternate days or a placebo (Kang et al., 2006). There were no differences in the global composite score between the treatment and placebo groups after nine and a half years and it was concluded that long-term use of vitamin E supplements did not provide cognitive benefits among generally healthy older women.

### **3.4.3 Zinc**

Few studies have utilised RCT methodology to explicitly evaluate the cognitive effects of trace minerals such as zinc. In a trial of 387 healthy adults aged 55-87 years, subjects received either 15 or 30 mg of zinc per day (Maylor et al., 2006). Treatment effects were assessed at three and six months using measures of visual memory, working memory, attention and reaction time from the Cambridge Automated Neuropsychological Test Battery (CANTAB). At the three month testing period, spatial working memory was improved at both dosages, however a detrimental effect of the lower dose was observed on an attentional measure, indicating that the beneficial treatment effects of zinc may be limited to specific cognitive domains.

### **3.4.4 Multivitamins**

Multivitamins contain a combination of the B vitamins and antioxidant vitamins described previously in this chapter, in addition to minerals such as calcium, magnesium, zinc and iron. The use of multivitamins is relatively common in the elderly, with data from the US National Health and Nutrition Examination Survey showing that 63% of individuals aged 60 years or above had used a dietary supplement in the past month and 40% had used a multivitamin supplement within the past month (Radimer et al., 2004). This figure appears to be somewhat higher in older adults, with another study reporting that over 59% of elderly aged above 75 years supplemented their diet with multivitamins (Nahin et al., 2006). In Australia, it has been reported by elderly over the age of 65 years that the primary reason for multivitamin use is as a dietary supplement and secondarily to maintain general health (Goh et al., 2009).

Despite the widespread use of these supplements in the elderly, relatively few researchers have used RCTS to investigate the chronic effects of multivitamin supplementation on cognitive function in healthy elderly. A summary of the basic methods and outcomes of trials which have used a randomised, double-blind, placebo-controlled, parallel groups design to investigate the chronic effects of multivitamins on cognition is shown in Table 3.1. Due to the small number of studies fitting these criteria, trials including both young and elderly subjects are summarised in the table and reviewed in text.

**Table 3.1** Summary of multivitamin and cognition randomised controlled trials

<b>Study</b>	<b>Participants</b>	<b>Treatment</b>	<b>Outcome Measures</b>	<b>Cognitive Treatment Effects</b>
Benton et al. 1995	127 males and females age range : 17-27 years	Multivitamin consisting of 10 vitamins. 12 months duration	Computerised tests : Simple and Complex Reaction Time Digit Symbol Substitution Continuous Attention	Significant treatment effect on attentional speed in females only
Cockle et al. 2000	139 males and females age range : 60-83 years	Multivitamin consisting of 10 vitamins. Up to 24 weeks duration	Critical Flicker Fusion Choice Reaction Time Sternberg Memory Scanning Word Scan Task	No treatment benefits
Wolters et al. 2005	220 females age range : 60-91 years	Multivitamin consisting of 13 vitamins. Six month duration	Pattern Recognition WAIS-III Symbol Search KAI Intelligence Score	No treatment benefits
McNeill et al. 2007	910 males and females age range : over 65 years	Multivitamin consisting of 11 vitamins and 5 minerals. Twelve month duration	Digit Span Forward Verbal Fluency	Small treatment benefit to verbal fluency in those aged over 75 years
Summers et al. 2010	113 males and females age range : 50-75 years	Multivitamin consisting of 34 antioxidant vitamins, minerals, amino acids, lipids and herbals. Four month duration	Mini-Mental Status Examination (MMSE) Names-Learning Paired Association Word Free Recall	Significant treatment effect on Names-Learning Paired Association and Word Free Recall measures
Haskell et al. 2010	226 females age range : 25-50 years	Multivitamin consisting of 25 vitamins and minerals. Nine week duration	Computerised Multi- tasking Framework : Mathematical Processing Stroop Colour-word Highest Number Task Memory Search Task	Significant treatment effect on accuracy for all tasks and response time for Mathematical Processing and Stroop

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<b>Study</b>	<b>Participants</b>	<b>Treatment</b>	<b>Outcome Measures</b>	<b>Cognitive Treatment Effects</b>
Kennedy et al. 2010	215 males age range : 35-55 years	Multivitamin consisting of 8 vitamins and 3 minerals. 33 day duration.	Computerised Cognitive Demand Battery : Serial Subtractions Rapid Visual Information Processing Stroop Task Peg and Ball Task Wisconsin Card Sort Subjective Mental Fatigue	Significant treatment effect for Serial 3s Subtractions task and Mental Fatigue rating
Harris et al. 2011	51 males age range : 50-74 years	Multivitamin consisting of 51 vitamins, minerals and herbal extracts. Eight week duration.	Swinburne University Computerised Cognitive Assessment Battery : Simple and Choice Reaction Time Stroop Congruent and Incongruent Immediate, Delayed and Contextual Recognition Spatial Working Memory N Back (1 and 2 back)	Significant treatment effect for Contextual Recognition, Memory accuracy and trend for Stroop Incongruent reaction time

The findings from several RCTs investigating the effects of multivitamins in the elderly have not uncovered cognitive benefits. For instance, Cockle et al. (2000) did not identify improvements to reaction time, short term memory or recognition memory following up to 24 weeks of multivitamin supplementation. Similarly, no benefits were found on measures of pattern recognition, IQ or symbol search after 24 weeks of multivitamin supplementation in German women aged 60 and above (Wolters et al., 2005). In a twelve month trial conducted by McNeill et al.(2007), there were no changes in verbal fluency or digit span performance in individuals aged 65 years or above. Once the age groups were separated, there was a small beneficial effect of the multivitamin treatment on the verbal fluency task for subjects aged over 75 years.

More recently, positive results have been obtained from trials which have investigated combined multivitamin and herbal supplements. Benefits to verbal fluency and recall have been identified from a study which examined the cognitive effects of a complex antioxidant blend consisting of 34 vitamins, minerals, amino acids, lipids and herbal extracts, all with antioxidant properties (Summers et al., 2010). Similarly, cognitive improvements were identified in males aged 50 to 75 years after a period of only two months treatment with a multivitamin supplement containing vitamins, minerals, and herbal antioxidant extracts (Harris et al., 2011). Benefits were observed on computerised measures of episodic memory and the stroop attentional task. Intake of herbs contributes significantly to levels of plant antioxidants (Dragland et al., 2003) and may also yield synergistic benefits when in the presence of other antioxidant vitamins (Cantuti-Castelvetri et al., 2000). Consequently it is conceivable that combined multivitamin and herbal supplements may exert greater effects on cognition than formulas consisting of vitamins alone.

Investigations in younger subject groups have also identified cognitive improvements following multivitamin supplementation. Following twelve months treatment with a multivitamin, benefits to speed of attention have been observed, although improvements were restricted to female participants (Benton, Fordy & Haller, 1995). Other findings indicate that the cognitive benefits of multivitamin supplementation in young adults may be limited to measures which require high levels of cognitive demand. In a nine week trial of young to middle aged females, multivitamin-related improvements were observed on a computerised

multitasking framework consisting of mathematical, stroop, numerical processing and memory search tasks (Haskell et al., 2010). Similarly, cognitive performance has been modulated on a highly demanding serial subtraction task following 33 days multivitamin supplementation in men of a comparable age range (Kennedy et al., 2010). A recent meta-analysis conducted by our research group identified memory enhancing effects of multivitamins across trials that included younger and elderly subjects (Grima et al., 2011). In this systematic review, benefits were observed to fluid intelligence processes including number facility and free recall. Combined, these findings indicate that cognitive benefits of multivitamin supplementation may not only be restricted to the elderly.

Several other smaller trials have examined the cognitive effects of multivitamin supplementation. In a study of only 20 subjects, there were no treatment-related improvements to the MMSE following 12 months supplementation with a multivitamin or placebo in elderly with cognitive impairments (Baker et al., 1999). A three month study of 47 young adults compared the cognitive effects of supplementation with a multivitamin, a poly-herbal formula and a placebo. Despite small, unequal treatment group sizes, there appeared to be some cognitive enhancements associated with the multivitamin and poly-herbal treatments (Shah & Goyal, 2010). Replication of this study with larger, equal group sizes may be required to confirm these results. In a trial of young to elderly subjects, improved performance on a digit memory task and the trails making test have been documented after time periods of two weeks and three months treatment with a dietary supplement consisting of folic acid, B<sub>12</sub>, vitamin E, S-adenosylmethionine, N-acetyl cysteine and Acetyl-L-carnitine (Chan et al., 2009). Interestingly, in an open label extension, memory benefits were diminished when the treatment was withdrawn for three months and re-emerged when treatment was reinstated for the final three months. As only a sub-set of elderly subjects were shown to respond to the treatment and only a fraction of the initial sample remained at the 12 month conclusion of this study, replication of this study may be necessary to generalize these findings to an elderly population.

### **3.4.5 Summary of findings from RCTs conducted to investigate the cognitive effects of vitamins in the elderly**

In summary, the findings from RCTs that have investigated whether dietary supplementation with vitamins, either in isolation or in the form of multivitamins, can enhance cognitive function in the elderly, have been inconsistent. Due to methodological disparities, particularly in relation to the cognitive outcome measures used in these trials, it is difficult to establish whether there are any; a) benefits to general cognitive function, or b) benefits to specific cognitive domains associated with vitamin supplementation. Variations in the vitamins under investigation, also contributes to this lack of clarity, particularly in relation to multivitamin formulas. Regardless of methodological inconsistencies, RCTs have provided some confirmation that vitamin supplementation can benefit cognitive function in the elderly. For example, there is limited evidence to suggest that supplementation with vitamin B<sub>12</sub> or folate may improve processing speed and memory. Advantages of long term beta carotene supplementation have also been identified, however the telephone administered cognitive tests used in a number of these trials may not be fully sensitive to the neurocognitive effects of antioxidant supplementation. Specific brain regions including the hippocampus, are preferentially vulnerable to oxidative damage (Lovell & Markesbery, 2007), and research focusing on the cognitive domains supported by regions vulnerable to age-associated pathology may provide more useful insights into the cognitive outcomes resulting from antioxidant supplementation. Zinc supplementation has been shown to enhance spatial working memory, indicating that the treatment effects of zinc may be limited to specific cognitive domains. Finally, the results from some RCTs have provided evidence that multivitamin supplements may modify selected memory and attentional processes in the elderly. The subsequent section of this chapter will provide a more in-depth discussion of methodological difficulties with previous research, and future directions for this field of study.

### **3.5 Methodological considerations and future directions**

The primary issue facing the interpretation of research findings from investigations which have examined the effects of vitamin supplementation on cognition in the elderly is the

selection of cognitive outcome measures used in these trials. In order to obtain clear evidence that dietary supplementation with micronutrients can improve cognitive performance or slow the rate of cognitive decline in the elderly, there needs to be greater forethought and justification of the cognitive domains under investigation in randomised controlled trials. For instance, global measures such as the MMSE provide only an overall estimate of an individual's level of functioning without reference to any specific cognitive domain. The use of such measures may result in difficulty distinguishing the effects of dietary interventions on memory or other more general cognitive processes (Benton, Kallus & Schmitt, 2005). Furthermore, these instruments have been primarily designed to diagnose dementia, and possess limited ability to detect cognitive change in healthy individuals due to ceiling effects (Reisberg, 2007). They also may not be sensitive to smaller cognitive alterations following short term nutritional intervention in healthy elderly (MacReady et al., 2011), as benefits of short term vitamin supplementation are more likely to result from improved neurotransmitter production, neural function, or blood flow to the brain due to improved cardiovascular health, rather than a reversal of pathology in the brain. In addition, such global instruments have demonstrated limited ability to detect treatment effects in healthy individuals (Summers et al., 1990) and even AD (Hogan, 2007). It has been argued that nutritional effects are likely to be subtle and that global measures may not be sensitive enough to capture improvements in performance in healthy individuals (Bryan et al., 2002). The same criticism may apply to neuropsychological or IQ measures which were not developed with the intention of facilitating the measurement of small nutraceutical benefits.

Interestingly, despite the criticism of global and neuropsychological instruments for the purpose of identifying cognitive benefits with vitamin supplementation, the same measures have demonstrated utility in detecting relationships between vitamin status, or deficiency and cognitive function and extent of decline. However the use of these measures has limited the ability of researchers to determine whether levels of particular vitamins are related to cognitive function in specific cognitive domains. In the elderly, neuropathological correlates of low vitamin status include neuronal death and dysfunction, white matter hyperintensities, and cortical atrophy - factors which over sufficient time duration may exert detrimental effects on an individual's general level of cognitive function. It is likely that cognitive

instruments such as the MMSE and other neuropsychological measures may be sensitive to this form of cognitive decline.

A recent meta-analysis of randomised controlled trials has concluded that dietary supplementation with B vitamins, antioxidants and multivitamins do not exert clinically important effects on global cognition in the elderly (Jia, McNeill & Avenell, 2008). Instead, it may be that cognitive domains related to fluid intelligence, which are most vulnerable to the deleterious effects of ageing (Salthouse, 1996; West, 1999; Christensen, 2001), will respond best to vitamin or nutraceutical intervention. For example, chronic folic acid supplementation has been shown to improve performance on tests that measure information processing speed and memory, domains that are known to decline with age (Durga et al., 2007). Recent studies have also shown that supplementation with powerful plant derived antioxidants, known as flavonoids, can enhance mental functions in older adults on working memory-related indices (Pipingas et al., 2008; Ryan et al., 2008). Further support for the premise that vitamin supplementation may improve the domains most susceptible to age-related decline has been obtained from a trial which investigated the effects of two months multivitamin supplementation on cognitive function in elderly men (Harris et al., 2011). In this study, the measures of episodic memory and attention, which had previously demonstrated sensitivity to age-related cognitive decline (Pipingas et al., 2010), displayed the greatest enhancements following multivitamin treatment. Thus, it is possible that focussing on fluid intelligence measures, which are susceptible to age-associated deterioration, may uncover benefits of vitamin supplementation which are not apparent when cognitive instruments, unresponsive to the effects of age are used.

A final consideration relates to the role of vitamins in brain function. Evidence reviewed in the first half of this chapter demonstrated that there is a relationship between intake or levels of B vitamins and antioxidant vitamins with brain structural parameters, including brain volume and neuropathology. In humans, the association between vitamin status and brain functional measures has not yet been investigated, nor have effects of vitamin supplementation on brain activity been examined in healthy individuals. In patients with severe vitamin B<sub>12</sub> deficiency, prolonged latency of the P300 component of the event related

potential (ERP) waveform has been shown to be normalised and MMSE score to be improved after vitamin B<sub>12</sub> therapy (Kalita & Misra, 2008). Similarly, five months B vitamin treatment concurrently improved cognitive performance on behavioural measures and modified relative quantitative electroencephalograph (qEEG) power in vitamin B<sub>12</sub> deficient elderly (Van Asselt et al., 2001). Whilst these studies were conducted in patients with severe B vitamin deficiencies, the findings of these trials provide evidence that treatment with vitamin therapy is capable of influencing brain function. It is possible that the integration of cognitive assessments with brain imaging techniques, such as functional magnetic resonance (fMRI), or electrophysiological measures of brain activity, may also provide greater insights into the ability of vitamin interventions to modulate cognition in healthy adults.

### **3.6 Thesis aims and experimental overview**

#### *Thesis Rationale*

The overall aim of this thesis was to investigate the effects of a multivitamin, mineral and herbal supplement on cognition and brain function in elderly women. A substantial body of epidemiological evidence demonstrates an association between vitamin status and cognitive function in the elderly. However, findings from RCTs which have investigated the effects of individual B vitamin or antioxidant supplements on cognitive function in the elderly have been less promising. Multivitamins contain combined vitamin and mineral components and for this reason may exert greater synergistic effects on the body than individual vitamins administered in isolation. Relatively few trials have investigated the effects of multivitamin supplementation on cognition in non-demented, community dwelling elderly, and findings from these studies have been inconsistent. Heterogeneity in the cognitive outcome measures used in these trials may have contributed to these discrepancies.

Multivitamin supplementation has been demonstrated to exert beneficial effects on a range of biochemical and cardiovascular health parameters including markers of oxidative stress, inflammation, cholesterol and endothelial function. It is possible that improvements to these health indices may be responsible for any cognitive enhancing effects of chronic multivitamin supplementation in the elderly.

It has recently been suggested that modifying vitamin and mineral levels in healthy individuals may provide beneficial influence to brain function (Haskell et al., 2010). To date, no studies have investigated the effects of multivitamins on brain activity. The steady state visually evoked potential (SSVEP) associated with a 13Hz light flicker, provides a measure of brain electrical activity. The 13Hz SSVEP enables the inspection of neural processes with a high temporal precision, and this characteristic has rendered the SSVEP a sensitive measure of variations in cognitive performance (Silberstein et al., 1990; Silberstein et al., 2000). Specifically, the SSVEP has demonstrated utility as a measure of neural processes related to memory and attention (Silberstein et al., 1990; Silberstein et al., 2001). A further advantage of the SSVEP is the responsiveness of this technique to neuropharmacological manipulation during cognitive activation (Thompson et al., 2000). These features indicate the SSVEP may be responsive to the potential cognitive enhancing and nutraceutical effects of multivitamin supplementation.

#### *Thesis Aims and Hypotheses*

This thesis presents the results from a 16 week, randomised, double-blind, placebo-controlled clinical trial which investigated the effects of a multivitamin supplement on cognition and brain electrical activity in 56 elderly women. To maintain consistency with several previous trials which have investigated the effects of combined vitamin or multivitamin formulations on cognition in a single gender (Bryan et al., 2002; Wolters et al., 2005; Haskell et al., 2010) this study was restricted to a female only sample. There were three aims of this thesis :

**The first aim of this thesis was to investigate the effects of chronic multivitamin supplementation on cognitive performance in the elderly.**

**The second aim was to examine the potential mechanisms of cognitive improvement due to chronic multivitamin supplementation in the elderly.**

**The third aim was to establish the SSVEP as a sensitive measure of the potential cognitive enhancing effects of multivitamin supplementation, and to examine the influence of chronic multivitamin supplementation on brain activity.**

There is evidence which indicates that in the elderly, the cognitive processes most susceptible to cognitive deterioration, in turn, demonstrate the greatest improvements from nutraceutical intervention (Durga et al., 2007; Pipingas et al., 2008; Ryan et al., 2008). Cognitive domains prone to age-related deterioration include working memory, episodic memory and attention (Ronnlund et al., 2005). In the first experiment of this thesis, cognitive performance was assessed using a battery of computerised memory and attentional tasks which have previously demonstrated sensitivity to age-related cognitive decline (Pipingas et al., 2010) and nutraceutical intervention (Pipingas et al., 2008). It was hypothesised that chronic treatment with a combined multivitamin and herbal supplement would enhance the cognitive domains most vulnerable to age-related decline. The mechanisms via which a combined multivitamin, mineral and herbal supplement may influence cognitive function in the elderly are poorly understood. Consequently, the secondary purpose of this experiment was to investigate potential mechanisms of the multivitamin which may have contributed to any cognitive enhancing effects. The effects of the multivitamin on vitamin blood levels, homocysteine, markers of inflammation and oxidative stress, and other cardiovascular parameters such as cholesterol, blood pressure and arterial stiffness were examined. Blood safety markers were included to assess the safety and tolerability of the multivitamin.

The cognitive domain of working memory is vulnerable to age-related decline (Salthouse, 1990; Rypma & D'Esposito, 2000; Rajah & D'Esposito, 2005), and for this reason it is possible that supplementation with a reputed cognitive enhancing multivitamin may improve the neural processes which subservise working memory performance. The aim of the second experiment was to develop a framework in which to interpret the treatment effects of a purported cognitive enhancing multivitamin on the SSVEP, associated with a spatial working memory task. To achieve this purpose, it was necessary to first gain a thorough understanding of the spatio-temporal pattern of SSVEP amplitude and latency in relation to the sub-processes elicited by a spatial working memory DRT, and to explore the SSVEP correlates of working memory performance in all elderly subjects. To date, these aspects of working memory have not been investigated in an elderly sample using SST.

The final experiment of this thesis was conducted to investigate the effects of chronic multivitamin supplementation on brain electrical activity, as measured by SST associated

with a spatial working memory DRT. It was predicted that the SSVEP would be responsive to the cognitive enhancing effects of multivitamin supplementation in the elderly. Specifically, it was hypothesized that the effects of the multivitamin on the SSVEP would reflect the pattern of SSVEP amplitude and latency identified in the previous experiment, associated with „good“ working memory task performance.

The clinical trial methodology is detailed in the subsequent chapter of this thesis. An overview of the SSVEP measure of brain activity will follow. The methodology chapter will conclude with a description of the SSVEP recording and analysis stages relevant to the SSVEP experimental chapters.

## Chapter 4 Methods

The methods chapter of this thesis will be divided into two sections. The first section will document the characteristics of all the subjects who participated in the experiments included in this thesis. Participant screening procedures, cognitive and mood screening measures will be described in this section, followed by the clinical trial methodology, including trial design and specifics of the multivitamin supplements. The clinical trial procedure, along with the cognitive assessment instruments, biochemical and cardiovascular measures used in the clinical trial will also be included in this section.

The second part of the methods chapter will provide an introduction to the steady state visually evoked potential (SSVEP) as a measure of brain electrical activity, and the steady state topography (SST) recording paradigm. Current understanding of the neurophysiological basis of the SSVEP amplitude and latency components will be discussed within the framework of investigations into the cognitive domains of attention and working memory. This chapter will conclude with a description of the spatial working memory task used with the SSVEP, the SSVEP recording procedure, signal processing stages required to extract and calculate the SSVEP, and the statistical analysis of the SSVEP.

### 4.1 Participant characteristics

#### 4.1.1 Screening

##### *Inclusion and exclusion criteria*

Participants were community-dwelling elderly females aged 64 years or over. The sample was restricted to an all-female demographic, as there is evidence to suggest that gender differences exist in the electroencephalograph (EEG) (Kemp et al., 2002) and it may be inappropriate to pool male and female EEG data. In addition, the action of pharmacological agents can differ depending on gender and hormonal influences (Tanaka, 1999). The sample was limited to right handed individuals. It has been suggested that handedness may represent

a potentially confounding variable when interpreting EEG data, as some differences in neural organization can exist in individuals who are left hand dominant (Knecht et al., 2000).

Exclusion criteria included a history of dementia, neurological disorder, stroke, epilepsy, Parkinson's disease, head trauma or excessive alcohol use. Smokers were excluded, as nicotine has been shown to modulate the EEG (Thompson et al., 2000) and withdrawal effects can be detrimental to cognitive performance (Hendricks et al., 2006). Further exclusion criteria comprised a history of mental illness, depression or anxiety disorders, or the use of anti-depressant or anti-anxiety medication. Those using medications with a cognitive enhancing effect including stimulants and anticholinergics, and individuals taking high dose anticoagulants were ineligible for participation. Individuals who were currently using a multivitamin supplement or any products containing Ginkgo biloba or St John's Wort at the time of telephone screening were permitted to participate in the study following a 30 day wash out period.

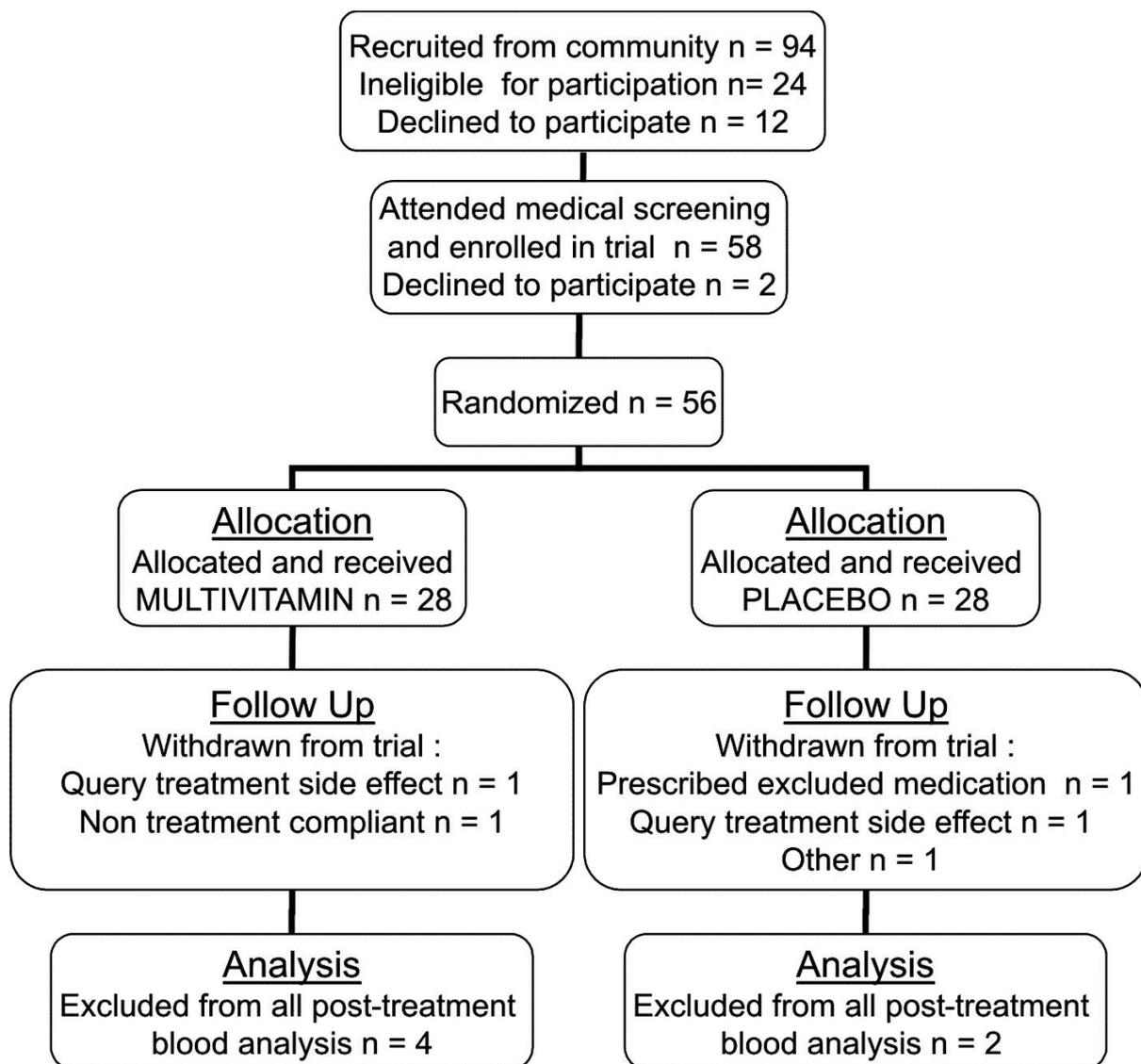
### *Recruitment*

Participants were recruited from the community by way of newspaper advertisements and posters. Advertisements were placed in local newspapers and state wide senior publications. The advertisements asked for women who were „concerned about their memory“ or „experiencing memory difficulties“, and were right handed, non smokers, aged over 65 years, and were interested in participating in memory research. Posters were placed on public notice boards in libraries, community centres and supermarkets in areas local to Swinburne University, Hawthorn campus. Individuals contacted the researcher by phone to express interest in participating in the study and were read a summary of the research project, including time demands prior to undergoing a telephone screening interview. In order to meet the criteria for participation, individuals were required to answer “yes” to the question “do you feel like your memory is becoming worse?” Participants were also asked four questions pertaining to their subjective experience of memory loss. Prior to attending the medical screening and consent session all eligible participants were mailed information about the study, the disclosure document and a form on which to record their medical history.

*Screening procedure*

Prior to enrolment in the study, potential participants attended a one hour screening session designed to assess their suitability to participate in the study. Individuals were given the opportunity to discuss the study requirements with the investigator prior to signing the consent form. Written informed consent was obtained from all participants. A separate form was used to obtain consent for an optional apolipoprotein E (ApoE) status test. Participants were screened for cognitive impairment using the mini mental state examination (MMSE), and depression using the short form of the Geriatric Depression Scale (GDS). Verbal IQ was assessed using the contextual Aus-NART. A brief memory measure constructed by Jorm et al. (1997) was used to screen for subjective memory complaints.

Participants then underwent a medical examination with a Medical Practitioner. Participants provided the Medical Practitioner with a summary of their medical history, current medication, and underwent a brief physical examination during which height, weight, pulse rate and blood pressure were measured. The overall aim of the medical screening was to determine whether the individual was generally in good health and free from any medical conditions which may preclude participation in the study. A further aim was to exclude any individuals using contraindicative medication, or to exclude any individuals taking medication which may interact with the multivitamin supplement under investigation. The final purpose of the medical screening was to ensure that participants were free from dementia. The Medical Practitioner achieved this by reviewing MMSE scores, questioning individuals as to their ability to carry out activities of daily living and assessing their orientation to time and place. The numbers of participants recruited and screened for this trial are shown in Figure 4.1.



**Figure 4.1** Participant recruitment flowchart

#### 4.1.2 Screening Measures

##### *Mini-Mental State Examination*

The Mini-Mental State Examination (MMSE; Folstein, Folstein & McHugh, 1975) was used as a brief measure of participant's cognitive status. The MMSE briefly assesses an individual's orientation to time and place, attention, recall and language, and is commonly used as a dementia screening instrument (Tombaugh & McIntyre, 1992). The MMSE is scored out of 30, with scores below 24 possibly indicative of dementia (Lezak, Howieson &

Loring, 1994). This was used as a screening measure and participants were required to obtain a score 24 or higher in order to be eligible to participate in the trial.

#### *Geriatric Depression Scale (Short form)*

The short form of the Geriatric Depression Scale (GDS) was used as a screening test for depression and consists of 15 items which require a yes/no answer. Scores between 0 and 4 are considered normal, scores between 5 and 9 may indicate mild depression, and scores ranging from 10 to 15 indicate moderate to severe depression (Sheikh & Yesavage, 1986). The short form of the GDS has been shown to be highly correlated with the full version ( $r = 0.89$ ,  $p < .001$ ), indicating it is an appropriate substitution for the full 30 item scale (Leshner & Berryhill, 1994). The GDS was used as a screening measure and participants were required to obtain a score below 9 to be eligible to participate in the trial.

### **4.1.3 Demographic Measures**

#### *Subjective Memory Complaint Scale*

A brief memory measure constructed by Jorm et al. (1997) was used during the telephone interview to screen for subjective memory complaints. There were four items on this measure: „Do you have more trouble remembering things that have happened recently?“, „Are you worse at remembering where belongings are kept?“, „Do you have trouble recalling conversations a few days later?“, and „Do you have more trouble remembering appointments and social arrangements?“. Responses were rated as: 0, „No, not much worse“; 1, „Yes, a bit worse“; 2, „Yes, a lot worse“. Scores ranged from 0 to 8. A co-efficient alpha of 0.71 has been reported for this instrument (Jorm et al., 1997).

#### *Contextual Australian National Adult Reading Test*

The Contextual Australian National Adult Reading Test (C-AusNART) was used as an estimate of verbal IQ. The standard version of the National Adult Reading Test (NART) has been used to estimate baseline intelligence in comparable studies of the effects of vitamin supplementation in the elderly (Cockle et al., 2000; McMahon et al., 2006). The NART has been validated as an estimator of pre-morbid ability in mild to moderate dementia (McGurn et al., 2004), and consequently is suitable for use as an IQ estimate in elderly who may be

experiencing cognitive decline. The C-AusNART represents a modification of the standard NART and consists of 60 target words placed into the context of simple meaningful sentences to be read aloud by the participant. Each incorrect pronunciation of a target word within these sentences is scored as an error. The C-AusNART is used to estimate verbal IQ using the number of pronunciation errors. The following formula was used to estimate Weschler Adult Intelligence Scale verbal IQ =  $110.51 - 0.48 (\text{C-AusNART errors}) + 2.97 (\text{education code; } 1 = \text{less than 9 years, } 2 = 9 \text{ to } 10 \text{ years, } 3 = 11 \text{ to } 12 \text{ years, } 4 = 13 \text{ to } 15 \text{ years, } 5 = 16 \text{ or more years})$ . The C-AusNART has been shown to be a useful predictor of verbal IQ in the Australian population and is suitable for individuals with an English speaking background, with an internal consistency of 0.93 and inter-rater reliability of 0.98 (Lucas, Carstairs & Shores, 2003).

#### 4.1.4 Participant demographics

The same subject group participated in all experiments included in this thesis. As shown in Table 4.1 the average age of participants was 71.1 years, with an average of 12 years of education. The mean IQ as assessed by the contextual Aus-NART was 108.7. Cognitive status as measured by the MMSE suggested that participants were free from dementia, with an average score of 28.7 obtained for this instrument. The average score obtained on the GDS was 1.4. Amongst the 56 included participants, 20 reported a family history of dementia. From the 50 participants who provided consent to undergo ApoE status testing, 5 subjects possessed the e3/2 genotype, 30 possessed the e3/3 genotype, 14 subjects were of the e3/4 genotype and one subject was e4/4.

**Table 4.1** Participant demographics

<b>Demographics</b>	<b>N</b>	<b>M</b>	<b>SD</b>	<b>Range</b>
Age	56	71.1	4.6	64-82
Years education	55	12.0	3.3	6-20
MMSE	56	28.7	1.3	24-30
IQ	52	108.7	6.0	95-118
GDS	56	1.4	1.6	0-8
Subjective memory	56	2.5	1.5	0-6

### *Concurrent medication use*

All but two participants were using medication at the time of the study. Of the participant sample, 30 women were using hypertensive medication, 17 were using lipid lowering medication and 10 were using a fish oil supplement. Several participants reported using an individual vitamin supplement, with five participants using vitamin C, four participants using vitamin D, two participants using vitamin E, three participants using folate and one using vitamin B6. Other medication used by participants was not deemed to be related to the study outcomes. Participants were requested to discontinue individual vitamin supplementation throughout the trial duration.

## **4.2 Clinical trial design, randomisation and blinding**

The trial was a randomised, placebo-controlled, double-blind, parallel group, 16 week study. Eligible participants were randomly allocated the Swisse Women's Ultivite 50 plus™ supplement or a placebo with an allocation ratio of 1:1. Randomisation was conducted using a computer generated random sampling set. The treatments were allocated in blocks of four by the supplier, Swisse Vitamins™.

Placebo and treatment were packaged in identical blister packs and contained in boxes numbered according to the randomisation schedule. Participants were allocated the next sequential number upon enrolment to the study. The blinding list was held by an investigator not involved in data collection. Data was unblinded at the end of data collection.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki. Approval for the trial was approved by the Swinburne Human Research Ethics committee.

### **4.2.1 Treatment**

The multivitamin treatment used in this study was the Swisse Women's Ultivite 50 plus™. These multivitamins are available over the counter in Australia and are registered with the

TGA (ARTG Number: 140130). The treatment is a multivitamin, antioxidant and mineral formula with added herbal and antioxidant plant extracts. Ingredients of the multivitamin and recommended daily intake of the vitamin and mineral components are shown in Table 4.2. The placebo tablets were identical in appearance to the multivitamin. Placebo tablets contained starch and 2mg of Riboflavin (vitamin B<sub>2</sub>) to provide tablets with a similar smell and to produce a similar colouration of the urine. Supplements were packaged in blister packs containing seven tablets and were labelled with each day of the week. Each participant received a box containing 17 sheets of supplements, yielding a total of 119 supplements. Participants were instructed to take one supplement each day, with breakfast, for 16 weeks. An extra seven tablets were provided to allow for blood to be collected after the post-treatment appointment.

#### *Compliance*

Participants were required to return all packaging at the post-treatment appointment. Remaining supplements were counted to determine treatment compliance.

#### *Calculation of Sample Size*

Due to the exploratory nature of this trial, a formal power analysis was not possible. Instead the sample size calculation was based on the findings of a previous trial which identified cognitive improvements with a moderate effect size, in 42 males on the same battery of cognitive tasks, following five weeks supplementation with a nutraceutical formula (Pipingas et al., 2008). A sample size of 60 participants was determined to be adequate for this purpose. A total of 60 participants provides a more than adequate number of subjects for an electroencephalogram (EEG) study.

#### **4.2.2 Statistical analysis**

All statistical analyses are detailed within the methods sections of each experimental chapter. For each experiment, cognitive and health data were analyzed using the Statistical Package for the Social Sciences (SPSS). Data was screened for out of range and outlier values. Data values 3 standard deviations from the mean, or greater, were designated as outliers and removed from the relevant analysis. The assumption of normality was assessed using the Shapiro-Wilks statistic and via examination of frequency histograms. Variables skewed in a

positive direction were subjected to a log X transformation, and the X<sup>2</sup> transformation was applied to variables skewed in a negative direction.

**Table 4.2** Ingredients of the multivitamin and nutrient recommended daily intake for elderly women

Component	Daily Dose	RDI	Component	Daily Dose
Retinyl Acetate (2500 IU of vitamin A)	862.5 µg	700 µg	Lactobacillus rhamnosus	80 million organisms
Folic Acid	500 µg	400 µg	Lactobacillus acidophilus	80 million organisms
Thiamine Hydrochloride (vitamin B1)	30 mg	1.1 mg	Bifidobacterium longum	35 million organisms
Riboflavine (vitamin B2)	30 mg	1.1 - 1.3 mg	Citrus Bioflavonoids Extract	20 mg
Nicotinamide (vitamin B3)	20 mg	900 mg	Tunera Diffusa Leaf Dry (Damiana)	500 mg
Calcium Pantothenate (vitamin B5) (equiv. pantothenic acid 64.13 mg)	70 mg	4 mg*	Ginkgo Biloba Leaf Dry (Maidenhair tree) (equiv. Ginkgo flavonglycosides 4.8mg and ginkgolides and bilobalide 1.2 mg)	1000 mg
Pyridoxine Hydrochloride (vitamin B6) (equiv. pyridoxine 24.68 mg)	30 mg	1.5 mg	Silybum Marianum Dry Fruit (St. Mary's thistle)(equiv. flavanolignans : silybin 17.14 mg)	1500 mg
Cyanocobalamin (vitamin B12)	115 µg	2.4 µg	Scutellaria Lateriflora Herb Dry (Skullcap)	50 mg
Cholecaliferol (vitamin D3) (equiv. vitamin D 200 IU)	5 µg	10 - 15 µg*	Vitis Vinifera Dry Seed(Grape seed) (equiv. procyanidins 7.9 mg)	1000 mg
Biotin (vitamin H)	150 µg	25 µg*	Urtica Dioica Leaf Dry (Nettle)	100 mg
d-alpha-tocopheryl acid succinate (equiv. vitamin E 24.2 IU)	20 mg	7 mg*	Ubidecarenone Co- Enzyme Q10) (from patented Ultrasome CoQ10)	2 mg
Calcium Ascorbate Dihydrate (vitamin C) (equiv. ascorbic acid 165.3 mg)	200 mg	45 mg	Cimicifuga Racemosa Root & Rhizome dry (Black cohosh)	200 mg
Phytomenadione (vitamin K1)	60 µg	60 µg*	Cynara Scolymus Leaf Dry (Globe artichoke)	50 mg
Zinc Amino Acid Chelate (equiv. zinc 15mg)	75 mg	8 mg	Curcuma Longa Rhizome Dry (Turmeric)	100 mg
Calcium Orotate (equiv. calcium 10 mg)	100 mg	1300 mg	Withania Somnifera Root Dry (Ashwagandha)	500 mg
Magnesium Apartate Dihydrate (equiv. magnesium 6.74 mg)	100 mg	320 mg	Crataegus Monogyna Fruit Dry (Hawthorn)	100 mg
Selenomethionine (equiv. selenium 26 µg)	65 µg	60 µg	Silicia Colloidal Anhdrous (equiv. silicon 9.35 mg)	20 mg
Molybdenum Trioxide (equiv. molybdenum 45 µg)	67.5 µg	50 µg	Bacopa Monnieri Whole Plant Dry (Bacopa)(equiv. bacosides calculated as bacoside A 1.125 mg)	50 mg
Chromium Picolinate (equiv. chromium 50 µg)	402 µg	25 µg*	Lecithin Powder -Soy Phosphatidylserine Enriched Soy (equiv. phosphatidylserine 2 mg)	10 mg
Manganese Amino Acid Chelate (equiv. manganese 3 mg)	30 mg	5 mg*	Spearmint Oil	2 mg
Ferrous Fumerate (equiv. iron 5 mg)	16.01 mg	8 mg	Vaccinium Myrtillus Dry (Bilberry) (equiv. anthocyanosides 324 µg)	100 mg
Copper Gluconate (equiv. copper 1.2 mg)	8.57 mg	1.2 mg*	Tagetes Erecta Flower Dry (Marigold) (Lutein esters calculated as lutein (of Tagetes erecta) 1 mg)	100 mg
Potassium Iodide (equiv. iodine 149.83 µg) (equiv. potassium 46.18 µg)	196 µg	150 µg	Vaccinium Macrocarpon Fruit Dry (patented cranberry pacran)	800 mg

RDI = recommended daily intake for Australian women aged 51 or above. Where two values are shown higher value refers to separate RDI for women >70 years of age.

\* = Adequate intake where RDI values were not available. RDI and adequate intake values obtained from National Health and Medical research Council (2006).

### **4.2.3 Baseline and post-treatment testing procedure**

Participants attended the baseline testing session at the Brain Sciences Institute at 1030 or 1130 hours. Participants were requested to refrain from consuming tea or coffee for two hours prior to attending this appointment. During the baseline testing session, participants completed verbal and computerised cognitive tasks. The average duration of the baseline testing appointment was approximately 2.5 hours. Participants first completed a modified version of the Swinburne University Computerised Cognitive Assessment Battery (SUCCAB). The time duration to complete the SUCCAB ranges from approximately 25-30 minutes. The California Verbal Learning Task II (CVLT-II) was then administered to assess immediate verbal recall, recognition and verbal learning. There was a 20 minute interval between the immediate and delayed memory components of the CVLT-II. During this delay, measurement of blood pressure and arterial stiffness was conducted. Delayed verbal recall and recognition were then measured using the CVLT-II. Once cognitive testing was complete, participants were prepared to undergo an electroencephalogram (EEG). Participants performed several computerised cognitive tasks during the recording of their brain electrical activity. On completion of the testing session participants were provided with a pathology request to attend a local pathology collection centre for blood samples to be taken. Participants were requested to attend the blood collection centre within four days of the testing session. Participants were asked to attend in the morning and to fast from 2200 hours the night before.

Participants returned for the post-treatment appointment approximately 16 weeks after the baseline testing session. Participants were allowed up to four days variation from the scheduled appointment date. All subjects returned at the same time of the morning as their baseline testing session. The same testing procedure from baseline was followed at the post-treatment appointment. Alternate forms of the SUCCAB, CVLT-II and EEG cognitive tasks were utilized.

#### 4.2.4 Cognitive Battery

##### *SUCCAB*

The standard Swinburne University Computerised Cognitive Assessment Battery (SUCCAB) consists of eight tasks designed to assess processing speed, attention and memory processes in healthy adult populations. Tasks from this battery have previously demonstrated sensitivity to the effects of age (Pipingas et al., 2010) and nutraceutical intervention (Pipingas et al., 2008). Specifically, in adults aged 21 to 86 years, the spatial working memory and contextual recognition subtests demonstrated the greatest sensitivity to age (Pipingas et al., 2010). Within this investigation, good re-test reliability and validity of these subtests was identified.

In the current study, several modifications were made to the SUCCAB to reduce the difficulty of tasks and to increase accuracy in the elderly sample. The maximal time during which participants could produce a valid response was increased from 450ms to 750ms for the simple and complex reaction time tasks. The maximum valid response time was extended to 5950ms for the stroop congruent, stroop incongruent, immediate recognition, contextual recognition and delayed recognition memory tasks. These tasks were modified so that stimuli requiring a response would remain on the computer screen for a maximum of 5950ms, or until a button press was delivered by the participant. The purpose of this modification was to enable individual participants to perform the task at their own pace, and to reduce the difficulty of the tasks. In the elderly, a speed versus accuracy trade off has been observed, whereby older adults are required to perform cognitive and neuropsychological tasks at a slower rate than their younger counterparts, in order to achieve an equivalent level of performance (Brébion, 2001). The simple and choice reaction time and working memory tasks allowed shorter times to respond and time out responses on these tasks were classed as incorrect responses.

Pilot testing in elderly approaching the upper age limit of the subjects in the current study, indicated that the spatial working memory task may require additional practice to enable elderly subjects to perform the task at a sufficient level of accuracy (i.e greater than 50%). The standard version of the SUCCAB spatial working task has been demonstrated to be one of the tasks most sensitive to age (Pipingas et al., 2010), and for this reason it was important

to use the standard form of this computerised measure. A less demanding version of the spatial working memory task was developed to provide extra practice for the elderly subjects. In this task a 4 x 4 grid was presented on the screen, with five, rather than six grid locations filled with a white square. Subjects were required to remember the location of three white squares, instead of the four included in the standard task version. The practice spatial working memory task was performed immediately prior to the standard SUCCAB working memory task at baseline and post-treatment.

### *Task specifics*

The SUCCAB is run in Pipscript04, a DOS based program which provides millisecond accuracy for response times. The SUCCAB tasks were presented via computer and a hand held button box was used to deliver all responses. Participants were seated approximately one metre from the computer monitor and were instructed to hold the button box with two hands. Four buttons were positioned on the box at opposite vertical and horizontal locations. The word „yes“ was printed next to the right button and the word „no“ was positioned next to the left button. The right button was coloured in red, the left in blue, the top button in green and the bottom button in yellow. Instructions were presented on the computer screen for each test and the investigator was available to answer any task-related questions. A practice trial was performed immediately prior to each task under the supervision of the investigator. The following tasks were performed by participants:

*Simple Reaction Time:* In this task a white square was displayed on the screen for 750ms. Participants responded by pressing the „yes“ response button as quickly as possible each time the square was presented. The square was presented a total of 20 times with an inter-stimulus interval between 1 and 4 seconds.

*Complex Reaction Time:* Participants were shown either a blue triangle or a red square displayed for 750ms. In this task participants responded each time the triangle or square was presented by pressing the corresponding coloured button on the button box. There were 20 trials with an inter-stimulus interval ranging from 1 to 3.7 seconds.

*Immediate Recognition Memory:* During memory encoding, a series of 30 abstract images were presented on the screen for 4 seconds each. Participants were instructed that their memory would be tested on these images immediately after they had viewed all of the images and again after approximately 20 minutes. During memory response, a second series of 30 images were then shown, where 15 images were the same as the ones previously presented during encoding and 15 were new. A „yes“ button press indicated that the image had been recognized, whereas a „no“ button press indicated the image was new. The images were presented for a maximum of 5950ms, or until a response was made, with an inter-stimulus interval of 450ms.

*Stroop Congruent:* In this task, the words „red“, „blue“, „green“ or „yellow“ were presented on the screen in corresponding coloured ink. Participants were required to respond to the word by pressing the same coloured button. A total of 40 stimuli were shown for a maximum of 5950ms or until a response was made, with an inter-stimulus interval of 450ms.

*Stroop Incongruent:* Similar to the stroop congruent task the words „red“, „blue“, „green“ or „yellow“ were presented on the screen, however, this time the word was different to the ink colour. Participants were required to ignore the written word and to respond to the colour of the ink by pressing the same coloured button. A total of 40 stimuli were shown for a maximum of 5950ms or until a response was made, with an inter-stimulus interval of 450ms.

*Spatial Working Memory:* In this task, a 4 x 4 grid was presented on the screen for 2950ms, with 6 grid locations filled with a white square. The white squares then disappeared and the grid was empty for 1950ms. A single white square was then shown in the grid for 1950ms. A „yes“ response indicated that the white square was in the same position as one from the initial presentation, and a „no“ response indicated it was in a different location. This procedure was repeated four times. In total there were 14 trials in this task.

*Contextual Recognition Memory:* A series of 20 pictures of everyday objects were presented at a location either at the top, bottom, right or left of the screen for a total of 4 seconds each. A second series of the same images were then presented in centre of the screen. Participants responded by pressing the button which corresponded to the original location of the picture.

Images were presented for 5950ms or until a response was made, with an inter-stimulus interval of 450ms.

*Delayed Recognition Memory:* This task was the follow-up to the immediate recognition memory task. A series of 30 images were then shown, where half of the images were the same as the ones previously seen during the initial recognition memory task and the other half had not been previously presented. A „yes“ button press indicated that the image had been recognized, whereas a „no“ button press indicated the image was new. The images were presented for a maximum of 5950ms or until a response was made, with an inter-stimulus interval of 450ms.

#### *SUCCAB outcome measures*

The primary cognitive outcome measures were a memory composite measure consisting of averaged response times from the immediate, delayed, and contextual recognition and working memory subtests, and an attention composite measure comprised of the averaged response times from the reaction time and stroop tasks. The development of these measures will be described in Chapter 5 (Section 5.2.5).

#### *California Verbal Learning Task- II*

The CVLT-II is a commonly used neuropsychological measure of verbal memory (Delis et al., 2000). Reliable change indices indicate that an alternate form of the CVLT-II should be used in longitudinal trials or those with repeated assessments to reduce practice effects (Woods et al., 2006). In the current study, the CVLT-II was administered in line with standard procedures. Briefly, a list of 16 items taken from four semantic categories was presented aurally to participants five times. Immediately after each list presentation, the participants were asked to repeat as many words as possible, in any order. A distracter list consisting of a different 16 words was then read to participants, and again they were asked to repeat as many words as possible. Participants were then assessed on their free recall of the original word list. The number of words recalled at this point was used as a measure of immediate recall in this study. To assess cued recall, participants were then provided with the semantic categories. After a delay of 25 to 30 minutes, free recall of the initial word list

was assessed, followed by cued recall and finally forced choice (yes/no) recognition was measured. Only scores for immediate and delayed recall were analysed in this study.

#### **4.2.5 Biochemical measures**

Blood sample collection and analysis was performed by a pathology clinic with collection centres around Melbourne. Eight vials of blood were collected via venipuncture. Blood samples were analysed according with the pathology centre's standard protocol. The following content was analysed:

##### *B vitamins*

Homocysteine, vitamin B<sub>6</sub>, B<sub>12</sub> and folate were used to provide measures of B vitamin status. To maintain normal levels of homocysteine, vitamin B<sub>6</sub>, B<sub>12</sub> and folic acid are required in the methylation of homocysteine to methionine (Huskisson et al., 2007). For this reason, when measured in the body, the levels of these vitamins have a tendency to be relatable. As reviewed in Chapter 3, the B vitamins and homocysteine have been associated with cognitive function and implicated in the health of the brain and cardiovascular system.

Homocysteine was measured in serum. The reference range provided by the pathology company indicated that normal values for homocysteine ranged from 5 to 15  $\mu\text{mol/l}$ . In women, it has been suggested that levels above 10.4  $\mu\text{mol/l}$  may be indicative of elevated homocysteine (Selhub et al., 1999).

Vitamin B<sub>12</sub> was measured in serum. The reference range provided by the pathology company indicated that levels below 150 pmol/l may indicate B<sub>12</sub> deficiency, and concentrations above 180 pmol/l were classified as falling within the normal range. It has been suggested that when considering an elderly sample, serum levels of vitamin B<sub>12</sub> should be approaching 279 pmol/l in individuals aged 65 to 70 years and 268 pmol/l in those aged 75 to 80 years in order to completely rule out the possibility of deficiency (Wahlin et al., 2002).

Vitamin B<sub>6</sub> was determined from whole blood using high performance liquid chromatography (HPLC) direct analysis of PLP (Pyridoxal-5'-phosphate form). The

reference range provided by the pathology company indicated that normal values for vitamin B<sub>6</sub> ranged from 35 to 110 nmol/l. Data from several population studies have indicated that using a cutoff of 35 nmol/l may result in an erroneous B<sub>6</sub> deficiency classification for some elderly individuals with normal B<sub>6</sub> levels (Bailey et al., 1997; Bates et al., 1999).

#### *Antioxidants*

Antioxidant status was measured using plasma vitamin C and serum vitamin E (total tocopherol). The importance of these vitamins for cognitive function, brain and cardiovascular health was discussed in Chapter 3. The reference values for vitamin E provided by the pathology company indicated that normal levels ranged between 12 and 46  $\mu\text{mol/L}$ .

#### *Lipids*

Lipids were assessed using total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides. LDL cholesterol represents the most harmful form of cholesterol and, when elevated, contributes to build up of cholesterol in the arteries, leading to artery blockage. HDL aids in prevention of cholesterol build-up and blockage of the arteries, and is described as a „good“ form of cholesterol in the body. Triglycerides are another form of fat found in the body. These lipid measurements represent strong predictors of cardiovascular disease (Wilson et al., 1998; Ingelsson et al., 2007). In accordance with the reference range provided by the pathology company, levels of HDL greater than 1  $\mu\text{mol/L}$ , levels of LDL below 2.5  $\mu\text{mol/L}$  and levels of triglycerides below 1.5  $\mu\text{mol/L}$  are considered normal.

#### *Inflammation*

High sensitivity C-reactive protein (hCRP) was used as a measure of inflammation. Of the inflammatory markers, hCRP has been shown to be the strongest univariate predictor of the risk of cardiovascular events (Ridker et al., 2000). This widely used inflammatory biomarker constitutes a risk factor for future vascular events with levels of hCRP <1, 1 to 3, and >3 mg/l indicative of lower, average, and higher relative risk respectively (Ridker & Silvertown, 2008). Higher levels of hCRP have also been associated with poorer cognitive function in the elderly (Yaffe et al., 2003; Noble et al., 2010), indicating that hCRP may represent a

biomarker of cognitive decline. The reference range provided by the pathology company indicated that levels of hCRP less than 11.1 mg/l were in the normal range.

### *Oxidative Stress*

Protein carbonyls provide a biomarker of oxidative stress. The benefits of protein carbonyls for use in intervention studies are that they are stable, allow the detection of dose-response relationships, and reflect meaningful biological disease endpoints (Collins, 2005). Specifically, protein carbonyls have been used to measure oxidative modification of proteins in diabetes, neurodegenerative diseases and ageing (Stadtman, 1992; Chevion, Berenshtein & Stadtman, 2000). In the current study the 2,4-dinitrophenylhydrazine (DNPH) reaction was used to measure protein carbonyl concentration in plasma. The quantity of protein-hydrozone produced during the DNPH reaction was quantified spectrophotometrically at an absorbance between 360-385nm.

Protein carbonyls were analysed by Southern Health using a Cayman analysis kit. The final unit of measurement obtained from this analysis was nmol/ml. As most human studies have reported carbonyl levels in the form of nmol/mg protein (Buss et al., 1997; Chevion et al., 2000; Korolainen & Pirttilä, 2009; Polidori et al., 2009), it is not possible to directly compare the baseline levels and multivitamin treatment effects identified in the current trial, with those of prior studies. One exception was an investigation of oxidative stress markers in ageing which revealed that in women aged 60 to 69 years, levels of protein carbonyls were in the range of 20 to 25 nmol/ml (Kasapoglu & Özben, 2001).

### *Fibrinogen*

Fibrinogen was used to provide a marker of hemostasis and endothelial function. Fibrinogen is a plasma glycoprotein, and when elevated, represents a cardiovascular risk factor. Levels exceeding the 7.0 g/L range related to a high risk of developing cardiovascular disease (Kannel et al., 1987). Levels of fibrinogen have also been associated with cognitive decline in the elderly, indicating fibrinogen may also represent a risk factor for cognitive decline (Rafnsson et al., 2007). The reference range provided by the pathology company indicated that values between 2-4 g/L were in the normal range.

### *Blood Safety*

Urea and electrolytes were used as measures of kidney function. Elevated urea is an indicator of kidney function failure or dehydration. The electrolytes are the various salts in the bloodstream including sodium, potassium, chloride and bicarbonate.

The Liver Function Test (LFT) was used to provide an indication of whether liver function was adequate, and as a measure of active liver damage. Certain herbal preparations and botanicals have been associated with hepatotoxic effects (Stickel, Egerer & Seitz, 2000). Consequently, in the current study, the LFT was conducted to establish that the multivitamin and herbal formulation under investigation was safe and well tolerated by the elderly participants.

The LFT consists of total protein, albumin, globulin, bilirubin, gamma glutamyl transpeptidase (GGT), alkaline phosphatase (ALK Phos), Alanine Transaminase (ALT) and aspartate transaminase (AST). Albumin represents a marker of protein synthesis, globulin represents a marker of immunologic response, and bilirubin provides a measure of organic anion metabolism. There are two categories of cell damage which characterize liver injury: cell necrosis and sluggish bile flow (cholestasis). ALT and AST are used as indicators of cell necrosis. ALK Phos and GGT provide indicators of cholestasis (Burke, 2002).

### *ApoE status*

Apolipoprotein E (ApoE) is a plasma glycoprotein predominantly produced in the liver, with secondary sites in the brain (Mahley et al., 2006). ApoE is involved in lipid homeostasis, particularly in determination of the levels of LDL and HDL cholesterol, which are directly and inversely correlated with risk for cardiovascular disease respectively (Smith, 2002). Carriers of the ApoE e4 allele have increased coronary risk (Bennet et al., 2007) and e4 carriers also have a higher risk of developing late onset Alzheimer's disease (Corder et al., 1993). In the current study ApoE status was measured at baseline and served as a demographic variable.

### *Excluded tests*

Tests of vitamin C and folate were excluded from analysis in the present investigation. The reference range provided by the pathology company indicated that values between 28-120  $\mu\text{mol/L}$  were in the normal range. Values of vitamin C indicated that oxidation of a large number of samples occurred during blood collection/analysis. As numerous samples were considered to be compromised, it was necessary to exclude all vitamin C data. Analysis of the folate data revealed that it was unreliable when compared to previous data sets collected for other projects at the Brain Sciences Institute. Specifically there was no relationship between baseline and post-treatment levels of folate in the placebo group. As a consequence, folate data was also excluded from the present investigation.

## **4.2.6 Cardiovascular measures**

### *Blood Pressure*

Blood pressure was measured using an automated blood pressure sphygmomanometer, from the left arm, in a seated position. Hypertension, or elevated blood pressure, refers to systolic blood pressure greater than 140 mmHg or diastolic blood pressure greater than 90 mmHg. Hypertension constitutes the precursor to most types of cerebrovascular events (Wilson et al., 1998) and the risk of hypertension tends to increase with age (McEnery, Wilkinson & Avolio, 2007). The prevalence of hypertension in the Australian population has been estimated at 28.6 per 100 in those aged above 25 years, with 15.2 cases per 100 untreated (Briganti et al., 2003).

### *Arterial Stiffness*

With the ageing process, blood vessels lose elasticity, leading to an increase in arterial stiffness (Mitchell et al., 2004). Arterial stiffness has been associated with cognitive impairment in the elderly (Hanon et al., 2005; Elias et al., 2009) and represents a risk factor for coronary artery disease (Gatzka et al., 1998). Pulse wave velocity (PWV), an evaluation of arterial distensibility has been shown to be strongly correlated with atherosclerosis in hypertensive individuals (Blacher et al., 1999). In the current study, arterial stiffness was measured by central pulse pressure, peripheral pulse pressure and central augmentation index

using the Sphygmocor system (SphygmoCor; AtCor Medical, Sydney, Australia), a valid determinant of arterial stiffness (El Assaad, Topouchian & Asmar, 2003).

Measures were obtained by means of applanation tonometry, applied at the radial artery. From the radial signal, the SphygmoCor software calculates aortic pulse wave by means of a validated and population based generalized transfer function. The aortic pulse wave was calculated as the difference between the first and second systolic peak, given as a percentage of the aortic pulse pressure. Central and peripheral pulse pressure were calculated as the difference between systolic and diastolic blood pressure, measured from the sphygmomanometer and from the aortic pulse wave respectively. Central augmentation index was calculated by dividing the augmentation pressure by the pulse pressure, multiplied by 100. An operator index of 78 or above on the SphygmoCor was required to be obtained in order for data to be included in analysis.

### **4.3 SSVEP measure of brain activity**

The field of cognitive neuroscience has risen from a multitude of approaches and methodologies adopted by researchers to investigate the neural processes which underlie human cognition. EEG recordings, particularly event related potentials (ERPs), which can be time-locked to specific task components, have been used to uncover the fast occurring neural processes involved in cognitive task performance. Functional magnetic resonance (fMRI), which enables the mapping of regional blood flow in specific neural locations and the advent of repetitive transcranial magnetic stimulation (rTMSs), have aided researchers in determining the brain regions related to specific task demands and cognitive processes (Rossini et al., 2007).

Each neuroimaging or brain mapping technique possesses both advantages and disadvantages and for this reason must be carefully selected for the appropriate purpose. Regional metabolic methods possess excellent spatial resolution, but provide only limited temporal resolution. The opposite is true of ERP methodologies, which are ideal for the investigation of fast occurring neural processes, yet offer poor spatial localization of brain activity. The SSVEP is

an electrophysiological measure which possesses the ability to assess both transient, fast-occurring cognitive processes and more sustained task-related processes associated with memory (Silberstein, 1995). Whilst still susceptible to the same spatial limitations as other electrophysiological measures, the SSVEP is relatively insensitive to artefact and can be used to provide a continuous measure of time-variable cognitive processes (Silberstein et al., 1990).

The following section will provide an overview of the steady state topography (SST) recording paradigm and the SSVEP measure of brain electrical activity. The neurophysiological basis of the SSVEP will be discussed within the framework of investigations into the cognitive domains of attention and working memory. This will be followed by a description of the SSVEP methodology used in this thesis, including the spatial working memory task used with the SSVEP, the SSVEP recording procedure, signal processing stages and statistical analysis of the SSVEP.

#### **4.3.1 Introduction to the SSVEP**

The „steady state evoked potential“ has been described by Regan (1989) as a repetitive evoked potential whose constituent discrete frequency components remain constant in phase and amplitude over an infinitely long period of time. Steady state evoked potentials can be driven by auditory or visual stimulation. The SSVEP is elicited by flashing visual input. Based on the criteria adopted by Regan, the SSVEP can be distinguished from a transient evoked potential (EP) based on several important characteristics. Firstly, the transient EP will return to baseline following stimulation, whereas the SSVEP is a continuous response which does not return to a resting state. Secondly, transient EPs are best described in the time domain where each peak of the waveform is well defined. In contrast the SSVEP is poorly represented in the time domain, as individual components of the wave form cannot be associated with any specific stimulus cycle. Instead, it is appropriate to describe the SSVEP in the frequency domain where the spectral distribution remains constant over time.

The SSVEP can be elicited by flickering lights or flickering images on a computer screen, which move or flash at a constant frequency. When driven by unstructured stimulation, three

components of the SSVEP have been identified, with amplitude peaks occurring at frequencies of approximately 10Hz, 20Hz and 40Hz (Regan, 1989; Silberstein, 1995). When stimulated at these frequencies, the human visual cortex will respond to flickering stimuli more strongly than to stimuli flickering at adjacent frequencies (Herrmann, 2001). The latency of the response to stimulation in the range of 4 to 15Hz is approximately 200 to 275ms. Higher frequency stimulation of 18 to 30Hz are associated with a latency of 85 to 200ms, whilst those elicited above 40Hz have a shorter latency of approximately 30 to 60ms (Silberstein, 1995) and reveal less inter-individual variability than those at lower frequencies (Vialatte et al., 2010). The amplitude of the low frequency SSVEP is maximal in the occipitoparietal region, and is consistent with the notion that the SSVEP is generated in occipital cortical regions (Silberstein, 1995; Pastor et al., 2003), however frontal sources of the SSVEP have also been identified (Srinivasan et al., 2007; Vialatte et al., 2010).

#### *Artefact and the SSVEP*

A particular advantage of using the SSVEP as a measure of brain activity is the relative insensitivity of this response to noise and artefact produced by mains interference, eye blinks, electro-oculograph (EOG) (Regan, 1989) and electromyography (EMG) (Gray et al., 2003). Using traditional EP recording methodologies, the power distribution of interference caused by artefact is generally distributed throughout the EEG frequency band. This can lead to high levels of signal contamination, and consequently loss of data. In comparison, all of the power of the SSVEP is focused at the stimulus frequency or its harmonics (Nunez & Srinivasan, 2006). Fourier analysis acts as a narrow band filter, enabling the properties of the SSVEP to be maintained at the target frequency, whilst attenuating artefact produced by biological noise (Silberstein, 1995). Resistance to biological artefact represents a favourable characteristic of the SSVEP, particularly when a repeated measures experimental design is used and the margin for data loss is minimal.

#### *Steady state topography (SST)*

Over the past 30 years the SSVEP has grown in popularity as a measure of the neural processes which underlie cognition. One of the initial studies to uncover a relationship between human cognition and the SSVEP was conducted in 1986 by Wilson and O'Donnell. In this investigation the relationship between the SSVEP and several reaction time tasks,

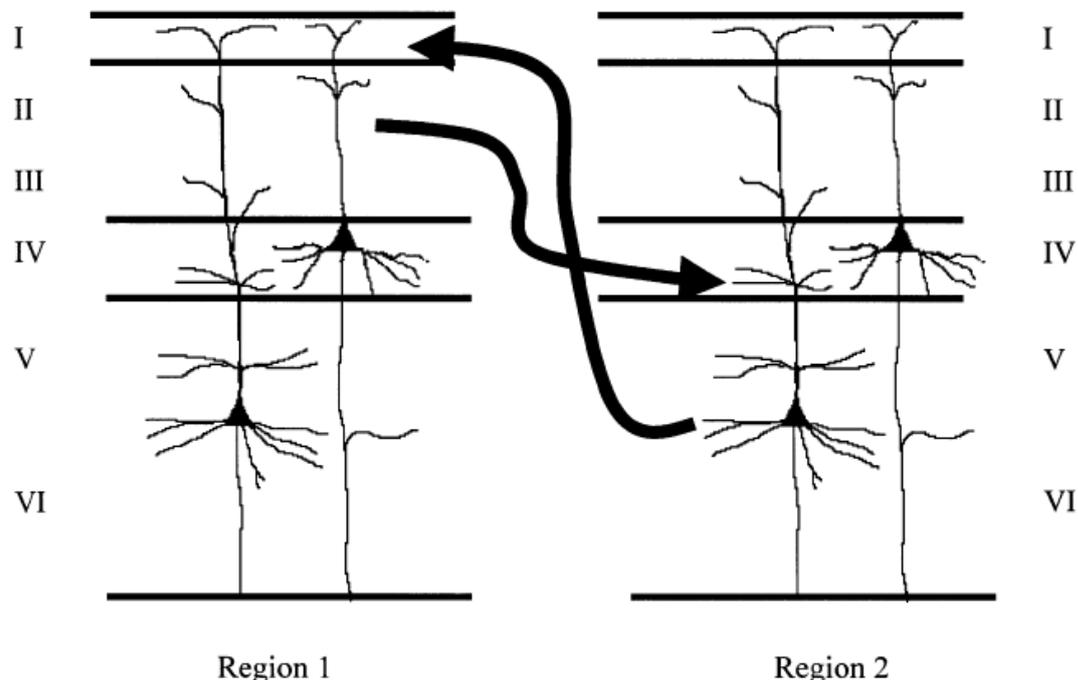
including a memory scanning task, was examined. The key finding of this study was that the SSVEP response to different frequencies was associated with different cognitive task components. Specifically, when elicited by medium frequency (15-23Hz) light flickers, SSVEP latency was related to the cognitive processing aspects of the memory scanning task. Conversely, when stimulated at higher frequencies (40-59Hz), latency was related to the sensory input aspects of the task. The findings of this study provided early evidence that the SSVEP can be used to index the processes which underlie cognitive function.

Since this initial discovery of a relationship between aspects of the SSVEP and cognition, the SSVEP has been validated as a useful technique to investigate attentional mechanisms in both healthy individuals (Silberstein et al., 1990) and clinical groups (Silberstein et al., 1998; Silberstein et al., 2000). Other mental functions including memory (Silberstein et al., 2000; Van Rooy et al., 2001; Ellis et al., 2006), executive function (Silberstein, Ciorciari & Pipingas, 1995) and emotional processing (Kemp et al., 2002; Gray et al., 2003; Mayes et al., 2009) have also been investigated using this technique. The specific paradigm adopted in these studies is known as steady state topography (SST). Silberstein (1995) characterized SST (previously described as steady state probe topography) as a technique in which the stimulus eliciting the SSVEP is a uniform flicker irrelevant to the cognitive tasks performed, with brain electrical activity recorded from either 64 or 128 scalp electrodes.

Many SST studies have relied on a task-irrelevant diffuse light flicker set to a frequency of 13Hz. The flickering light is delivered via light emitting diode (LED) goggles and is consequently superimposed on any visual cognitive task undertaken by the participant. A 13Hz light flicker has been selected for use with this methodology due to the close proximity of this frequency to alpha activity. The EEG alpha band has previously been linked to cognition and cortical activation (Ray & Cole, 1985). When stimulated at a frequency of 10Hz, the SSVEP exerts a maximal response, however, a spontaneous peak in the alpha range also occurs at approximately 10Hz (Regan, 1989; Herrmann, 2001). This spontaneous 10Hz activity can be considered as noise that contributes to a low signal-to-noise ratio. The 13Hz frequency was chosen to maximize this signal to noise ratio, whilst maintaining the properties of stimulation in the high alpha frequency band.

*Neurophysiological basis of the SSVEP*

A model regarding the neurophysiological basis of the SSVEP has been described in detail by Silberstein et al. (2001). To summarize, the thalamo-cortical and cortico-cortical feedback loops are regarded as fundamental to neural processing. Ascending or feedforward cortico-cortical fibres and descending or feedback thalamo-cortical loops have been studied extensively (Felleman & Van Essen, 1991; Mountcastle, 1997) and are shown in Figure 4.2. It has been suggested by Silberstein et al. (2001) that these feedforward and feedback re-entrant loops contribute to the SSVEP in the relevant frequency range. Several overlapping characteristics of the cortico-cortical feedback loops with the SSVEP provide support for this premise. Specifically, the thalamo-cortical and cortico-cortical feedback loops play an important role in the genesis of driven 8-18Hz EEG rhythms, a frequency range which corresponds to the maximal resonance of the SSVEP (Regan, 1989). In addition, the velocity of the SSVEP phase has been measured at 7-11 metres/sec (Burkitt et al., 2000) and this range is similar to the action potential speeds of 6-9 metres/sec in cortico-cortical fibres (Nunez, Wingeier & Silberstein, 2001).



**Figure 4.2** Cortico-cortico Feedback Loop. Feed-forward and feedback cortico-cortico fibers that constitute the re-entrant loops. Taken from Silberstein et al. (2006)

The SSVEP is determined by two components, amplitude and phase. The amplitude represents the magnitude of the SSVEP, and the phase is considered to represent the latency or speed of transmission through the brain. In terms of the neurophysiological significance of the SSVEP, amplitude has been suggested to be analogous to upper alpha band EEG activity, and to reflect transmission efficiency of the thalamo-cortical and cortico-cortical feedback loops (Silberstein et al., 2001). In keeping with prior investigations of the SSVEP, and for greater ease of interpretation, phase variations will be described as latency variations from this point onwards. Latency has been described as reflecting neural processing speed (Kemp et al., 2002). Alterations in 13Hz SSVEP activity have been suggested to reflect neuronal activity within cortical pyramidal cells (Kemp et al., 2004). A reduction of the SSVEP latency has been interpreted as increased post-synaptic excitation of these pyramidal neurons (Silberstein et al., 2000). Latency increase has been suggested to reference decreased excitation, or an increase in post-synaptic inhibition (Silberstein et al., 2000).

#### **4.3.2 SSVEP and cognition**

##### *SSVEP and alpha*

Using the SST methodology, Silberstein et al. (1995) initially interpreted reduced SSVEP amplitude and increased phase lag to be associated with increased regional brain activity. From this perspective, changes in the SSVEP due to cognitive activation were likened to event related desynchronisation (ERD) in the alpha band. ERD refers to a decrease in power in the EEG, related to a decrease in synchrony of underlying neural populations (Pfurtscheller & Lopes Da Silva, 1999). Such transient reductions in alpha activity have been observed with increased task demands and attention requirements during perceptual intake (Ray & Cole, 1985; Dujardin et al., 1993). Specifically, ERD of the lower alpha band has been implicated in attentional processes, whilst upper alpha oscillations have been described in the search and retrieval processes from semantic long term memory (Klimesch, 1999). It has been suggested that EEG frequencies within the alpha band, related to semantic memory, involve the thalamus, and are generated by thalamo-cortical and cortico-cortical feedback loops (Klimesch, 1997; Klimesch, 1999).

*SSVEP and Attention*

Evidence that variations in the magnitude of the SSVEP response occurs in a similar manner to rhythmic alpha oscillations, was primarily obtained from a study of visual vigilance, where participants passively viewed a sequence of 60 squares, followed by 60 circles, followed by another 60 squares. The subsequent trial followed the same format, with the exception that participants were required to identify a modified circle amongst the 60 presented circles. The results revealed that when compared to the passive task, the SSVEP amplitude was attenuated in the central-parietal region when subjects were anticipating the appearance of the target, and over right prefrontal and occipital-parietal regions when the target was detected (Silberstein et al., 1990). In a further investigation by Silberstein et al. (1995), participants performed a computerised version of the Wisconsin Card Sort Task (WCST) with the aim of determining the criterion for sorting cards into separate groups. In the early interval, after presentation of the cue to change sort criterion, it was found that amplitude was reduced in the prefrontal, central and right parieto-temporal regions. Both studies demonstrate that amplitude reductions were greatest in the period of increased visual attention. SSVEP magnitude changes were interpreted as an increase in regional cortical activity related to increased attention and mirror the findings of transient changes in alpha activity associated with increased attentional or cognitive demands (Silberstein, 1995).

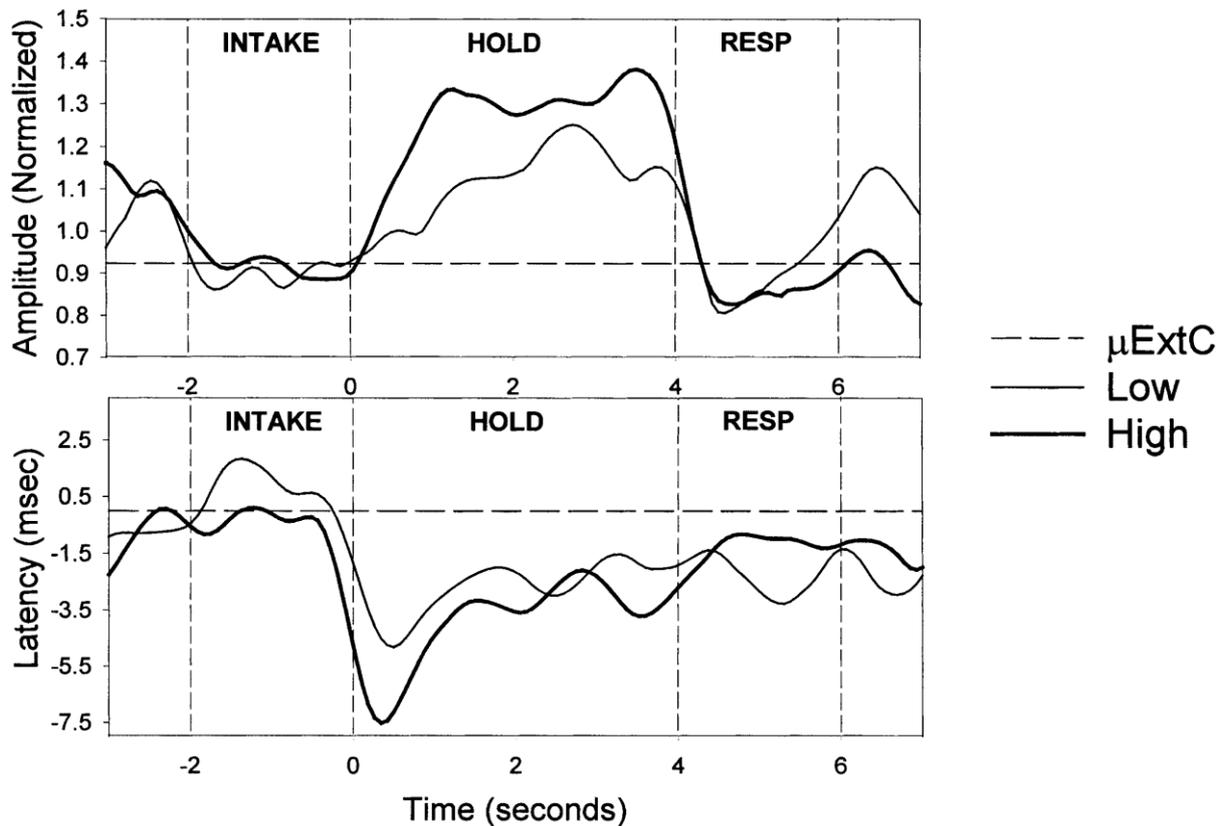
The functional significance of the latency component of the SSVEP has been obtained from investigations using an „A“, „X“ continuous performance task, where participants respond to the target letter „X“, but only when it is preceded by the letter „A“. This task has been used to examine the neural underpinnings of schizophrenia (Silberstein et al., 2000) and attention deficit/ hyperactivity disorder (ADHD) (Silberstein et al., 1998). In normal control subjects, SSVEP latency reduction has been identified at bilateral parietal and temporal sites coinciding with the appearance of the „A“, and at parietal and prefrontal sites on the presentation of the „X“ (Silberstein et al., 2000), as well as right prefrontal sites on appearances of the „A“ and „X“ and disappearance of the „A“ (Silberstein et al., 1998). It has been suggested by these authors that the latency reduction may index increased neural processing speed as a result of increased functional coupling between neural networks corresponding to either an increase in excitatory processes or decrease in inhibitory

processes. The identification of a correlation between prefrontal SSVEP latency and response time during the continuous performance task appears to support the interpretation that latency is related to processing speed (Silberstein et al., 2000).

### *SSVEP and working memory*

As introduced in Chapter 2, working memory is invoked during the short term retention of information and requires goal-oriented, attentional processes (Baddeley, 1992). During working memory retention, information is required to be held „online“ and either maintained or manipulated across a time delay. It is this feature of working memory which lends itself to measurement with the SSVEP. The ability of the SSVEP to monitor brain activity across a working memory delay represents an advantage over traditional ERP techniques which do not possess the temporal continuity required to directly measure maintenance processes associated with the delay period (Shucard et al., 2009).

Silberstein et al. (2001) have developed a model of the SSVEP which proposes that cognitive processes requiring information to be held on-line will be associated with a decrease in latency and increase in amplitude, reflecting increases in the transmission efficiency of the re-entrant loops in regions known to participate in working memory, such as the PFC and parietal regions. To empirically test this model, Silberstein et al. (2001) used a graded object working memory task to investigate the effects of holding either one or two objects in memory across a 4.2 second delay. Results from this study revealed that the hold period was associated with increased amplitude at occipital and prefrontal sites. This finding of a frontal SSVEP amplitude increase associated with the hold period of a working memory task has since been replicated using light flickers set to frequencies of 8.3 and 20Hz (Wu & Yao, 2007), and also using an alternate methodology consisting of stimuli presented over a 10Hz flickering diffuse light/dark contrasting background (Perlstein et al., 2003). Figure 4.3 displays time series data, demonstrating the frontal amplitude increase, from the trial conducted by Silberstein et al. (2001).



**Figure 4.3** Example of SSVEP amplitude and latency waveforms during performance of an object working memory task.

The bold trace demonstrates activity at electrode site Fz for a high demand condition relative to a control task and the other trace represents a lower demand condition. Taken from Silberstein et al. (2001).

A separate profile of SSVEP activity can be anticipated during the encoding, or perceptual intake component of a working memory task. These effects have been shown to parallel the findings of reduced SSVEP amplitude associated with performance of a visual vigilance task (Silberstein et al., 1990). Reduced SSVEP amplitude has been a feature of the encoding of both object and spatial material into working memory. For example, during encoding of non verbal objects, the results from Silberstein et al. (2001) demonstrated a significant reduction in amplitude at the left parietal region. Furthermore, during the encoding stage of a spatial working memory *n*-back task, amplitude and latency reduction were apparent across bilateral frontal sites (Ellis et al., 2006).

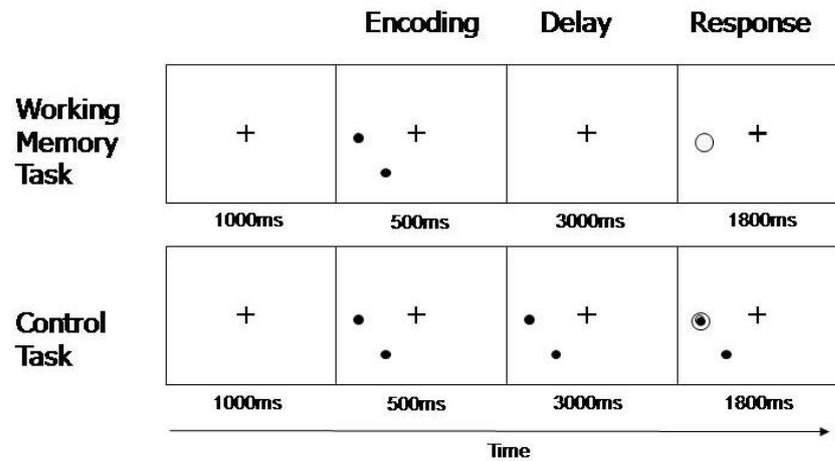
The pattern of SSVEP amplitude and latency associated with working memory also appears to be influenced by the difficulty of the task or condition performed. Graded effects were apparent for both the encoding and hold components of the working memory tasks in the investigations conducted by Silberstein et al. (2001) and Ellis et al. (2006). For the more difficult task conditions, graded effects generally occurred in the form of larger amplitude and latency reductions at frontal sites during encoding, and larger amplitude increases and latency reductions during the hold period. An added feature of the spatial *n*-back task was a shift from prefrontal amplitude increase to decrease over the 3 second delay, suggested to reflect a reallocation of the PFC from maintenance of memory content to the engagement of frontally-mediated executive processes (Ellis et al., 2006). This prefrontal alteration in the SSVEP was largest in more difficult *n*-back conditions, lending support to the premise that task difficulty may represent an important consideration when interpreting the pattern of the SSVEP associated with cognitive task performance.

Studies of the SSVEP have indicated that individual differences in performance related to aptitude or mental proficiency impact on the neural processes related to working memory. SSVEP latency has been correlated with performance on a musical DRT in musically-trained participants, with higher performers displaying larger latency decreases, with SSVEP latency decreases over the left temporal area during encoding, and left frontotemporal areas during retention (Harris & Silberstein, 1999). In a spatial working memory task, individuals with a higher intelligence quotient (IQ) as measured by the Weschler Adult Intelligence Scale – Revised (WAIS-R), demonstrated greater SSVEP latency increases frontally, and amplitude and latency decreases across occipital, parietal and temporal regions than those with an average IQ (Van Rooy et al., 2001). It was suggested by the authors of this study that differences in neural processing between higher and lower performers may reflect diverging cognitive strategies, or variations in the quality of frontal executive and posterior rehearsal processes. Comparably, when divided into high and low IQ groups based on WAIS performance, higher IQ has also been associated with greater SSVEP latency increases frontally during performance of the Raven’s Progressive Matrices (Song, 2005). Increased latency in this study was interpreted as a greater involvement of inhibitory processes in those with higher IQ, indicating that inhibitory processes may be important for intelligent behaviour.

The following section will provide a description of the spatial working memory task used with the SST paradigm, the SSVEP recording procedure, signal processing stages required to calculate and analyse the SSVEP, and the statistical analysis of the SSVEP.

#### **4.3.3 SSVEP spatial working memory task**

The spatial working memory task used during the recording of the SSVEP was a variation of Jonides et al. (1993) delayed-match-to-sample paradigm. Individual trials were separated by a 1 second fixation period. A fixation cross remained on the screen for the entire 6.3 second task duration. For each trial, subjects encoded the location of either two or three stimulus dots displayed on the circumference of an imaginary circle for 0.5 seconds, and then retained the location of the dots in memory across an unfilled 3 second interval. A probe circle was then presented for 1.8 second and participants determined whether or not the probe circle was located in the same position as one of the stimulus dots shown during encoding. Subjects responded with a right hand (yes) button press if the probe circle location matched the stimulus dot location and a left hand (no) button press if it was a non-match. The task was designed to elicit grading effects with either two or three dots presented during encoding. Maintaining the location of three dots in memory was intended to be more difficult than two dots. For non-match trials, the probe circle could be located either one, two or four positions away from one of the stimulus dots. The control task was identical with respect to stimulus encoding and response requirements, but did not require maintenance of cue representations across a delay period. That is, the dots remained on the screen throughout the hold period. Subjects responded with a right hand (yes) button press if the probe circle enclosed a dot and a left hand (no) button press if the probe circle did not enclose a dot. Task timing specifics are displayed in Figure 4.4. Participants completed two blocks of 40 trials of the spatial working memory task and one block of 40 trials of the control task. Each block lasted approximately four minutes. Fewer trials of the control task were required to achieve an optimum number of correct responses, as the control task was demonstrated to be easier than the working memory task during pilot testing. This task has been used previously by Van Rooy et al. (2001).



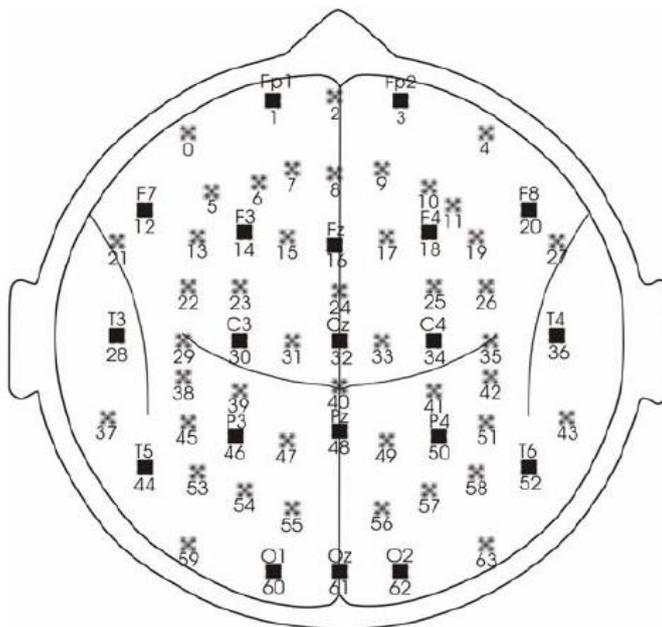
**Figure 4.4** Spatial working memory delayed response task

#### 4.3.4 SSVEP Recording Procedure

EEG was recorded from 64 tin, monopolar scalp electrodes, embedded in a lycra cap. Electrodes were positioned in accordance with the international 10/20 electrode system, with additional electrode locations shown in Figure 4.5. The cap was placed with FpZ and OZ an equal distance from the nasion and inion respectively, and fastened to a chest strap to prevent movement during cognitive task performance. Linked earlobes were used for reference and the nose was selected for the ground as per standard SST recording procedure (Silberstein, 1995). Impedance was below 10k $\Omega$  for reference electrodes. Brain electrical activity was amplified and band-pass filtered 3dB down at 0.1 and 80Hz prior to digitization to 16-bit accuracy at a rate of 500Hz. The raw EEG signal and power spectra was inspected manually for the presence of mains and biological artefact. Where the signal quality could not be enhanced during EEG setup, artefact-contaminated electrodes were documented.

Participants were fitted with goggles comprised of two sets of LED arrays viewed through half-silvered mirrors. The goggles were designed to superimpose a flickering white light on the participant's visual field, whilst still preserving normal vision. The SSVEP was evoked by a 13Hz sinusoidal flicker subtending a horizontal angle of 160° and a vertical angle of 90°. The visual flicker covered the majority of the visual field with a modulation depth of the stimulus against the background of 45%.

During cognitive task performance, participants were seated approximately 1.3 metres from the task computer. Cognitive task stimuli were presented on a computer with a 14 inch liquid crystal display (LCD) monitor. Subject response data was stored on the task computer. EEG data was acquired using custom instrumentation developed at the Brain Sciences Institute for use with the SST methodology. A separate computer was used to record and store the EEG data. EEG was later synchronized with task responses during offline signal processing.



**Figure 4.5** Location of EEG electrode positions

#### 4.3.5 Signal processing

For the subsequent signal processing stages involving time series calculation, averaging based on stimulus characteristics and averaging across subjects were applied to the SSVEP associated with the working memory task and the control task. Firstly, the SSVEP was determined from the 13Hz Fourier sine and cosine coefficients evaluated over 10 stimulus cycles at the 13Hz stimulus frequency, resulting in a temporal resolution of 770ms. A window width of this duration provides a sufficient signal-to-noise ratio, whilst enabling the tracking of rapid changes in the SSVEP which accompany mental processing (see Kemp, 2003 for a similar approach). For each electrode, the 10 cycle evaluation period was then shifted one stimulus cycle and the sine and cosine Fourier co-efficients recalculated for this

period. This process was repeated until all data in the initial file had been evaluated. As a result, a series of Fourier coefficients were produced for every electrode site, for the duration of each task.

Following the calculation of time series data, 6.3 second epochs of data centered on the presentation of the fixation cross and associated with correct task responses were extracted from the Fourier time series and averaged. Prior to the next stage of averaging the SSVEP across subjects, amplitude and phase were subjected to normalization. Amplitude normalization was conducted to prevent subjects with large amplitudes from dominating the group average (Silberstein et al., 1990). To achieve normalization, the mean amplitude was calculated for each electrode for the duration of the 6.3 second control task epoch. The values for each electrode were then averaged to create a normalization factor. This process was repeated for all subjects, yielding a unique normalization factor for each individual. For each electrode, the amplitude values in the 6.3 second epoch were then divided by the subject's unique normalization factor. In line with standard analysis procedures, phase values were also adjusted to reduce the likelihood of phase cancellation (Pipingas, 2003). SSVEP phase was normalized by calculating the average phase for each electrode across the epoch for the control task. These values were then subtracted from the phase of the corresponding electrode sites for the working memory task. The SSVEP epochs were then averaged across subjects. Phase was converted to latency using the formula  $(\text{change in phase}/2\pi) \times -(1000/13)$ . A delay in phase corresponds to an increase in latency.

In order to examine working memory task effects, SSVEP amplitude and latency differences were calculated by subtracting the 6.3 second epoch corresponding to the control task from the 6.3 second epoch associated with the working memory task.

Several prior investigations have examined the mean of the working memory delay period as a method of isolating the SSVEP activity associated with holding information „online“ in working memory (Silberstein et al., 2001; Macpherson, Pipingas & Silberstein, 2009). In this study, the 3 second working memory delay was also examined in this manner. The same signal processing procedure detailed above was followed for a shorter epoch encompassing the 3 second delay component of the working memory and corresponding period of the

control task. Rather than averaging *time series* data for the correct task responses, the *mean* Fourier coefficients for the 3 second epoch were averaged for correct responses, producing a pair of Fourier sine and cosine coefficients for each electrode and each task. Amplitude and latency normalization was conducted and data was averaged across subjects.

To isolate the activity associated with the 3 second delay period of the working memory task, SSVEP differences were calculated by subtracting the mean SSVEP of the 3 second control task epoch from the mean of the 3 second working memory task delay period.

#### *Artefact Rejection*

An advantage of the SSVEP is its resistance to artefact (Regan, 1989; Gray et al., 2003). Despite the relative insensitivity of this methodology to biological noise, several processes were used to detect artefact. Prior to signal recording, the power spectra of each electrode was examined and electrodes demonstrating excessive 50Hz mains interference were documented. Activity recorded from electrodes which were not consistently phase locked to the 13 Hz were designated as being contaminated by artefact. The average of the four closest electrodes was used to replace contaminated electrodes.

#### **4.3.6 SSVEP statistical analysis and topographical mapping**

Statistical analysis of the SSVEP was primarily carried out in BrainSci (SSPT Analysis Software, version 2). Data was presented using topographical maps to display specific time points and task components. Statistical significance of the SSVEP differences were determined using the bivariate Hotelling's  $T$ . This test was used to estimate the probability of falsely rejecting the null hypothesis (type-1 error) associated with task differences in the SSVEP latency and amplitude. Spatial principal components analysis has shown the SSVEP forms five independent factors (Silberstein & Cadusch, 1992), consequently the Hotelling's  $T$  statistic  $p$  values (2 tailed) were divided by five to correct for multiple electrodes. The  $p$  value was further adjusted to a more stringent level where appropriate. To maintain consistency, specific time points of interest were identified in the spatial working memory task (see Chapter 6) and the same time points were used for all analysis included in this thesis.

Correlations between the SSVEP and performance parameters were conducted in Matlab (Version 7.8) using custom developed scripts. Specificities of this analysis will be discussed in Chapter 6. Mixed between and within-subjects repeated measures analysis of variance (ANOVA) of the SSVEP amplitude and latency components were carried out using the Statistical Package for the Social Sciences (SPSS). Details of this analysis are described in Chapter 7.

The following chapters detail the thesis experiments. Chapter 5 presents the findings of an investigation into the cognitive effects of multivitamin supplementation and potential mechanisms via which any cognitive improvements may be mediated.

## **Chapter 5 An investigation into the effects of multivitamin supplementation on cognition in the elderly.**

### **5.1 Introduction**

Nutraceuticals are dietary substances which offer health or medicinal benefits. Selected vitamins, minerals, plant and animal extracts fall within this category of functional foods (Ferrari, 2004). As discussed in Chapter 3 of this thesis, the antioxidant and B vitamins have been of particular interest to researchers due to their dual roles in the maintenance of neural and cardiovascular health (Cantuti-Castelvetri et al., 2000; Rao & Balachandran, 2002; Mariani et al., 2005; Bourre, 2006; Ng & Ye, 2006). Antioxidants are known to reduce oxidative stress and the B vitamins to decrease levels of homocysteine (McCaddon, 2006; Møller & Loft, 2006). In the body, these vitamins do not operate in isolation and may exert more potent effects when administered in combination, as a multivitamin supplement.

A number of epidemiological and prospective studies have identified a relationship between B vitamin and antioxidant status and cognition in the elderly (Riggs et al., 1996; Masaki et al., 2000; Nurk et al., 2005; Tucker et al., 2005; Dunn et al., 2007). Their findings suggest that dietary supplementation, either with individual vitamins or combined vitamin and mineral formulas, may be capable of improving cognitive function. Despite this assertion, results from trials which have investigated the ability of multivitamin supplementation to improve cognition in the elderly have been less encouraging (Jia et al., 2008). Relatively few studies have investigated the cognitive effects of multivitamins in the elderly free from dementia, using a double-blind placebo-controlled methodology and these are summarized in Table 3.1 (see Section 3.4.4). In one study of healthy seniors, there was no improvement on digit span forward and verbal fluency measures associated with 12 months multivitamin treatment, although a small effect on elderly above 75 years and those at risk of vitamin deficiency was observed (McNeill et al., 2007). As the primary aim of this particular study was to investigate infection, only two secondary measures of cognitive assessment (limited to the domains of short term memory and executive function) were included in the protocol.

Similarly, six months multivitamin treatment in a group of elderly German women did not lead to any cognitive benefits (Wolters et al., 2005). However, once again, cognitive assessment was restricted to estimates of verbal intelligence and symbol search, instruments which have been suggested to be less sensitive to subtle nutraceutical effects than computerised measures of fluid intelligence (Haskell et al., 2008).

By contrast, findings from our research group have demonstrated improved performance on computerised memory and attention measures sensitive to the effects of age in elderly men, following eight weeks multivitamins (Harris et al., 2011). Recent investigations conducted with young to middle-aged adults have also identified multivitamin-related benefits on a computerised multi-tasking framework (Haskell et al., 2010) and a computerised mental subtraction task (Kennedy et al., 2010), lending support to the premise that computerised measures may be more responsive to the cognitive enhancing effects of multivitamins.

A second methodological consideration of past studies may relate to the specific multivitamin formulations under investigation. It is possible that dietary supplementation with multivitamins, consisting of vitamins and minerals alone, may not be sufficient to elicit cognitive improvements in the elderly. A study by Cockle et al. (2000), which utilised a relatively simple formulation of ten vitamins, failed to identify improvements within 12 to 24 weeks in computerised measures of processing speed and memory response time in elderly subjects. Conversely, positive results have been obtained from a recent four month trial which examined the effects of a complex antioxidant blend consisting of 34 vitamins, minerals, amino acids, lipids and herbal extracts, all with antioxidant properties specifically designed to impart cognitive enhancement (Summers et al., 2010). Herbal components of this formula included flavonoids, *Ginkgo biloba*, grape seed and gotu kola - ingredients which have gained interest as potential modulators of cognitive function (Ferrari, 2004; Kumar, 2006). Enhancements to cognition associated with the treatment were identified for measures of verbal memory. The treatment also reduced serum homocysteine levels in a subset of the participant sample.

Although the cognitive improvements identified by Summers et al. (2010) were promising, without the examination of treatment effects on other biological indices such as blood measures of vitamin status, oxidative stress, inflammation or cardiovascular parameters, the mechanisms of action can only be speculated. A two year study which used a treatment containing folate, vitamins B<sub>6</sub> and B<sub>12</sub>, with the sole purpose of lowering homocysteine, did not identify improvements on neuropsychological tasks including the Rey Auditory Verbal Learning Test (McMahon et al., 2006). These findings indicate that reductions in homocysteine may not be solely responsible for cognitive improvements associated with multivitamins, especially those which contain a range of vitamin and herbal ingredients, such as the formula utilised by Summers et al. (2010). For this reason it may be important to investigate other mechanisms of action when cognitive enhancements associated with nutraceutical treatment are identified.

Numerous benefits to cardiovascular health have been identified in users of multivitamins, including higher serum nutrient concentrations, and lower homocysteine, C-reactive protein, high-density lipoprotein (HDL), cholesterol, triglycerides, blood pressure and risk of diabetes (Block et al., 2007). In randomised trials, supplementation with multivitamins has been demonstrated to be an efficacious method of reducing homocysteine over a period of 8 to 24 weeks (Earnest et al., 2003; Wolters et al., 2004; Wolters et al., 2005; Harris et al., 2011). Multivitamins have also been shown to reduce oxidative stress by improving markers of DNA damage in lymphocytes (Ribeiro et al., 2007) and reducing oxidative damage in red blood cells (Cheng et al., 2001). Benefits to endothelial function and inflammatory markers have also been observed, with a reduction in platelet activation (Salonen et al., 1991; Arnaud et al., 2007), and concentrations of the C-reactive protein marker of inflammation (Church et al., 2003) following multivitamin intervention. Improvements to other cardiovascular risk factors, such as HDL cholesterol, have also been documented (Shargorodsky et al., 2010).

Given that multivitamin supplementation is capable of improving biomarkers of oxidative stress, inflammation, homocysteine, and endothelial function, parameters which have also been associated with cognitive decline (Schafer et al., 2005; Solfrizzi et al., 2006; Reynolds et al., 2007; Noble et al., 2010), it is conceivable that these cardiovascular actions may also contribute to cognitive enhancing effects in the elderly. For this reason, it may be prudent to

investigate cardiovascular mechanisms of action alongside the cognitive outcomes associated with a multivitamin intervention. Evidence suggests that vitamins and herbal flavonoids, which exert benefits to endothelial function, may also improve measures of arterial stiffness (Pase et al., 2011), an important predictor of cardiovascular health (Blacher et al., 1999). No studies have examined the effects of a combined multivitamin, mineral and herbal formula on arterial stiffness.

In order to address this issue, the current study investigated the effects of a multivitamin supplement with added antioxidant herbs on both cognition and cardiovascular measures, including arterial stiffness, in elderly women. Herbal components of this formula included *Bacopa monniera* (Brahmi), and *Ginkgo biloba*, along with other flavonoids known to possess numerous neurophysiological, pharmacological, and cardiovascular actions (Mantle, Pickering & Perry, 2000; Fuhrman & Aviram, 2001; Ross & Kasum, 2002; Youdim, Shukitt-Hale & Joseph, 2004; Hodgson & Croft, 2006; Kumar, 2006; Ramassamy, 2006; Spencer, 2008; Spencer, 2008).

*Bacopa monniera* is a traditional Ayurvedic medicine which has historically been used to reduce inflammation and fever, and to possess analgesic, sedative, antiepileptic and memory enhancing properties (Russo & Borrelli, 2005). In humans, improvements to learning and memory have been observed following 12 weeks treatment, but not following an acute dosage (Nathan et al., 2001). In a study of elderly participants aged 55 years or older, 12 weeks treatment with *Bacopa monniera* extract led to improvements in verbal memory measures (Morgan & Stevens, 2010), and similar findings have been identified in participants over 65 years of age in measures of delayed auditory verbal learning recall and stroop response time (Calabrese et al., 2008), and verbal memory in elderly with subjective memory complaints (Raghav et al., 2006).

*Ginkgo biloba* has received attention over the past 15 years as a potential cognitive enhancer in those with Alzheimer's disease (Kanowski et al., 1996; Le Bars et al., 1997; Mantle et al., 2000). Cognitive effects of *Ginkgo biloba* have also been observed in healthy individuals (Stough et al., 2001a). Clinical trials have revealed benefits to declarative episodic memory after 6 weeks treatment (Mix & Crews, 2002) and verbal memory following 12 weeks

treatment (Burns, Bryan & Nettelbeck, 2006), however not all investigations have uncovered cognitive improvements (Van Dongen et al., 2003). Cognitive actions of *Ginkgo biloba* may involve direct effects on the cholinergic neurotransmitter system essential for the regulation of cognition (Di Renzo, 2000), or indirect nitric oxide vasodilation-mediated increases in blood flow (Mehlsen et al., 2002; Achike & Kwan, 2003).

When combined with *Ginkgo biloba* and other herbal extracts and minerals, the cognitive and cardiovascular effects of vitamin supplementation are relatively unknown. Consequently, the current randomised, double-blind, placebo-controlled-trial, aimed to investigate both the cognitive and cardiovascular effects of 16 weeks treatment with a combined multivitamin, mineral and herbal supplement in healthy, community dwelling, elderly women.

Recent evidence indicates that in the elderly, the cognitive processes most susceptible to cognitive deterioration, in turn, demonstrate the greatest improvements from nutraceutical intervention (Pipingas et al., 2008; Ryan et al., 2008). Cognitive domains prone to age-related deterioration include working memory, episodic memory and attention (Ronnlund et al., 2005). Conversely, other more declarative forms of memory including vocabulary and verbal IQ remain relatively intact in older adults (Christensen, 2001), and may be less responsive to nutraceutical benefits. Consequently, in the current study, a validated battery of computerised, age-sensitive memory, attention and processing speed tasks (Pipingas et al., 2010) were utilized to test the hypothesis that the cognitive domains most vulnerable to the effects of age would benefit from multivitamin supplementation. It has been suggested that amongst the elderly there are some subgroups who will experience the greatest cognitive benefits from dietary interventions, and these may include individuals displaying early signs of cognitive impairment (Jelic & Winblad, 2003; Balk et al., 2007). In the current study the participants were healthy, community-dwelling elderly women with subjective reports of memory decline.

### **5.1.2 Aims and hypotheses**

There were two aims of this experiment. The first was to examine the effects of 16 weeks supplementation with a combined multivitamin, mineral and herbal formula on cognitive

performance in elderly women. A trial duration of 16 weeks was selected as cognitive improvements in the elderly have previously been identified after a comparable time period of combined multivitamin and herbal supplementation (Summers et al., 2010) and biochemical benefits after even shorter periods (Harris et al., 2011). It was predicted that improvements would be observed for speeded measures of memory and attention from the computerised cognitive battery, as these measures represent the processes most vulnerable to the effects of age. As enhancements to verbal memory have also been identified following nutraceutical treatment (Calabrese et al., 2008; Summers et al., 2010), the California Verbal Learning Task II (CVLT-II) was included to assess treatment effects on immediate and delayed verbal memory.

The second aim was to investigate the mechanisms contributing to the potentially cognitive enhancing effects of the multivitamin. To ascertain the role of individual vitamins and cognitive function, levels of vitamin B<sub>6</sub>, B<sub>12</sub>, E and homocysteine were first correlated with cognitive performance at baseline. This analysis was carried out to identify which nutrient or biochemical markers were associated with specific cognitive processes in this sample of elderly women. As described previously, multivitamin supplementation has been demonstrated to exert beneficial effects on a range of biochemical and cardiovascular health parameters including markers of oxidative stress, inflammation, cholesterol and endothelial function. In the current study it was anticipated that the multivitamin would be capable of increasing blood nutrient levels, whilst lowering homocysteine and markers of inflammation and oxidative stress. The effects of the multivitamin treatment on other cardiovascular parameters such as cholesterol, blood pressure and arterial stiffness were also examined. Blood safety markers were included to assess the safety and tolerability of the multivitamin in this sample of the elderly.

## 5.2 Method

A full description of the clinical trial methods, cognitive instruments, and biochemical and cardiovascular measures was provided in Section 4.2. The following methods section will provide a summary of the methodology applicable to this experiment and a more detailed description of the relevant statistical analysis.

### *Study Design*

The trial was a 16 week, randomised, placebo-controlled, double-blind, parallel group investigation. Eligible participants were randomly allocated to receive the Swisse Women's Ultivite 50 plus™ supplement or a placebo with an allocation ratio of 1:1 .

### *Treatment*

The treatment was a multivitamin, antioxidant and mineral formula with added herbal and antioxidant plant extracts (Swisse Women's Ultivite 50 plus™). Ingredients are shown in Table 4.2 (see Section 4.2.2). The placebo tablets were identical in appearance to the multivitamin. Placebo tablets contained starch and 2mg of Riboflavin (vitamin B<sub>2</sub>) to provide tablets with a comparable smell and to produce a similar colouration of the urine. Participants were instructed to take one supplement each day with breakfast for 16 weeks.

## **5.2.1 Participants**

Participants in the study were 56 community-dwelling elderly females aged between 64 and 82 years. Eligible participants were screened from an initial sample of 94 women (see Figure 4.1 for details). Participants were recruited from the community by way of newspaper advertisements and posters which asked for women who were „concerned about their memory“, or „experiencing memory difficulties“ and were right handed, non smokers and aged over 65 years, interested in participating in memory research.

## **5.2.2 Procedure**

### *Screening*

Prior to enrolment in the study, potential participants were initially screened by telephone interview and subsequently attended a one hour screening session designed to assess their suitability to participate in the study. Individuals were given the opportunity to discuss the study requirements with the investigator and ask questions prior to signing the consent form. A separate form was used to obtain consent for the optional ApoE status test. The researcher

then administered the mini mental state examination (MMSE) to provide a brief indication of the participants cognitive status and to exclude any individuals obtaining a score (<24) possibly indicative of dementia (Folstein et al., 1975). Verbal IQ was assessed using the contextual Aus-NART (Lucas et al., 2003). The Geriatric Depression Scale (GDS) was used to establish that individuals were free from depression. A brief memory measure constructed by Jorm et al. (1997) was used to screen for subjective memory complaints. Participants then underwent a medical examination with a Medical Practitioner to ensure they were healthy and suitable to participate in the trial.

### *Baseline testing*

Participants attended the baseline testing session at the Brain Sciences Institute at 1030 or 1130 hours. Participants were requested to refrain from consuming tea or coffee for two hours prior to attending this appointment. During the baseline testing session, participants completed verbal and computerised cognitive tasks. The total duration of the baseline testing appointment was approximately 2.5 hours. Participants first completed a modified version of the Swinburne University Computerised Cognitive Assessment Battery (SUCCAB). The SUCCAB takes approximately 25-30 minutes to complete. The California Verbal Learning Task – II (CVLT-II) was then administered to assess immediate verbal recall, recognition and verbal learning. There was a 20 minute interval between the immediate and delayed memory components of the CVLT-II. During this delay, measurement of blood pressure and arterial stiffness was conducted. Delayed verbal recall and recognition were then measured using the CVLT-II. Following this, participants underwent an electroencephalograph (EEG) recording whilst performing a working memory task. Details of this experiment are provided in Chapters 6 and 7.

On completion of the testing session, participants were provided with a pathology request to attend a local pathology collection centre (Gribbles Pathology) for blood samples to be taken. Participants were requested to attend the blood collection centre within four days of the testing session. Participants were also asked to attend in the morning and to fast from 2200 hours the night before. Participants were allocated 112 supplements for the 16 week study duration, with an additional seven tablets provided to allow for blood to be collected after the

post-treatment appointment. The participants were asked to take one supplement each day with breakfast, commencing after blood collection.

#### *Post treatment testing*

Participants returned for a post-treatment testing appointment 16 weeks after baseline testing. Subjects who could not attend on the scheduled return date were allowed a time window of five days to attend the testing session. Remaining supplements were counted by the investigator to determine treatment compliance. The testing procedure from baseline was repeated with alternate forms of the cognitive tests. Participants followed the same procedure for blood sample collection, with all valid blood samples collected within seven days of the post-treatment appointment.

### **5.2.4 Baseline and post-treatment measures**

#### *Cognitive tests*

##### *Swinburne University Computerised Cognitive Assessment Battery (SUCCAB)*

The SUCCAB consists of eight tasks including simple reaction time, complex reaction time, immediate recognition, stroop congruent, stroop incongruent, working memory, contextual recognition and delayed recognition. Instructions were presented on the computer screen for each test and the investigator was available to answer any task-related questions. A practice trial was performed immediately prior to each task. A full description of this cognitive battery is provided in the Chapter 4 (see Section 4.2.5).

##### *California Verbal Learning Task II (CVLT-II)*

The CVLT-II is a word-list learning task consisting of 16 items divided in to four semantic categories. Words were presented aurally to participants at a rate of 1.5 seconds. After each word-list presentation, participants repeated as many words as possible. This procedure was carried out five times. Immediate free recall was assessed after a distracter list consisting of a different 16 words was presented. Delayed free recall was assessed after a delay of 25 to 30 minutes.

### *Mood Measure*

While mood data was collected as part of this trial it was not examined as part of this thesis. A description of the mood task and results is included in Appendix C.

### *Biochemical analysis*

Blood sample collection and analysis was performed by a pathology clinic with collection centres located around Melbourne. Homocysteine and vitamin B<sub>12</sub> were measured in serum and vitamin B<sub>6</sub> was determined from whole blood using high performance liquid chromatography (HPLC) direct analysis of PLP (Pyridoxal-5'-phosphate form). Antioxidants were measured using serum vitamin E. Lipids were assessed using fasting serum total cholesterol, tryglicerides, high density lipids (HDL) and low density lipoprotein (LDL). High sensitivity C-reactive protein (hsCRP) was used as a measure of inflammation. Protein carbonyls were used to quantify oxidative stress. Kidney function and liver function tests were used as measures of blood safety.

### *Cardiovascular measures:*

#### *Blood Pressure*

Blood pressure was measured using an automated blood pressure sphygomanometer upon the left arm in a seated position.

#### *Arterial stiffness*

Arterial stiffness was measured by central pulse pressure, peripheral pulse pressure and central augmentation index using the Sphygmocor system (SphygmoCor; AtCor Medical, Sydney, Australia). Measures were obtained by means of applanation tonometry, applied at the radial artery of the left arm.

## **5.2.5 Statistical analysis**

### *Calculation of memory and attention composite measures*

Factor analysis was conducted using the baseline response time data from the eight SUCCAB subtests with the intention of reducing the number of cognitive outcome variables by developing composite cognitive measures. Principal components and factor analyses yielded

two factors with eigenvalues greater than one. Using a criterion of orthogonally rotated (Varimax) factor pattern scores greater than 0.42, all cognitive outcome variables were found to load on one of these two factors. The first factor possessed an eigenvalue of 3.46, and consisted of all the memory tasks including the immediate recognition, contextual recognition, delayed recognition and spatial working memory sub-tests. The second factor possessed an eigenvalue of 1.60 and was comprised of the simple reaction time, complex reaction time, stroop congruent and stroop incongruent tasks. Response times for the memory tasks were summed and then divided by four (the number of tasks) to establish the memory composite measure. The same procedure was followed using the attention tasks to develop the attention composite measure.

#### *Baseline correlations*

Pearson's product correlations were conducted at baseline to examine the relationship between individual nutrients and also to identify any associations between biochemical measures and cognitive performance on the SUCCAB and CVLT-II cognitive assessments. Variables which were not normally distributed were transformed using the log X transformation for data skewed in a positive direction and the X<sup>2</sup> transformation was applied to data skewed in a negative direction.

#### *Baseline and post-treatment comparisons*

For all analyses, the assumption of normality was assessed and variables skewed in a positive direction were subjected to a log X transformation, and the X<sup>2</sup> transformation was applied to variables skewed in a negative direction. Data values 3 standard deviations from the mean or greater were designated as outliers and removed from the relevant analysis. A *p* value of 0.05 was used to determine statistical significance, unless indicated otherwise.

*Cognitive data:* The primary cognitive outcome measures were a memory composite measure consisting of averaged response times from the immediate, delayed, and contextual recognition and working memory subtests, and an attention composite measure comprised of the averaged response times from the reaction time and stroop tasks.

As ceiling effects for accuracy were anticipated for simple and choice reaction time and stroop tasks (Pipingas, Harris et al., 2010), only response time was analysed. The composite measures were not corrected for multiple comparisons as these measures represent separate cognitive domains as confirmed by factor analysis conducted on baseline SUCCAB data.

For each subject, the change in response time from baseline to post-treatment was calculated. Univariate analysis of co-variance (ANCOVA) was used to examine the effect of treatment group on change in response time, with the relevant baseline score included as a covariate. On the identification of a significant treatment group effect, or a trend for this effect, post hoc t-tests were used to examine the difference between baseline and post-treatment measures for each group individually. Individual subtests of the SUCCAB composite measures were only explored on the identification of a consistent pattern of change from baseline on all sub-tests of the composite measure.

*Biochemical and cardiovascular data:* Biochemical data was analysed using the same ANCOVA procedure as the cognitive data, with the relevant baseline measure included as a covariate.

As several measures of blood nutrient levels were examined including vitamin B<sub>12</sub>, B<sub>6</sub> and E, a Bonferroni correction was applied to adjust for multiple comparisons. For these tests, a corrected *p* value of *p* <.02 was used to indicate statistical significance. Measures of homocysteine, hsCRP and protein carbonyls represent distinct biological parameters and were, therefore not adjusted for multiplicity. Other biochemical and cardiovascular parameters were explored as secondary outcomes, and as such, were not adjusted for multiple comparisons.

To investigate whether a relationship existed between multivitamin-induced changes in blood nutrient levels and cognitive improvements, Pearson's product correlations were conducted between change in biochemical levels and change in cognitive measures from baseline to post-treatment. Correlations were conducted only for measures which demonstrated statistical improvement or a trend for this effect after multivitamin supplementation.

### 5.3 Results

#### *Participant demographics*

51 of the 56 original participants completed the entire trial. Of the participants who did not return to the post-treatment testing session, two participants reported potential treatment side effects and discontinued the treatment. A further participant was prescribed anti-depressant medication by her General Practitioner during the study supplementation period, one participant was non-compliant with the treatment and the final participant was unable to return to the post-treatment appointment within the necessary time frame. Participant demographics for the multivitamin and placebo group are shown in Table 5.1. One-way analysis of variance indicated participant groups did not differ on the demographic characteristics of age, Aus-NART IQ, years education, MMSE score, geriatric depression score, nor subjective memory complaint rating ( $p > 0.1$ ).

**Table 5.1** Baseline multivitamin and placebo group demographics

<b>Demographic</b>	<b>Treatment</b>	<b>N</b>	<b>M</b>	<b>SD</b>
Age	Multivitamin	26	71.7	4.5
	Placebo	25	70.1	4.3
AUS-NART IQ	Multivitamin	23	109.5	6.0
	Placebo	24	108.0	5.7
Years Education	Multivitamin	26	12.2	3.4
	Placebo	24	11.9	3.3
MMSE	Multivitamin	26	28.8	1.6
	Placebo	25	28.6	1.3
Subjective Memory	Multivitamin	26	2.7	1.6
	Placebo	25	2.5	1.6
Geriatric Depression	Multivitamin	28	1.5	1.3
	Placebo	28	1.3	1.9

#### *Concurrent medication use*

In total, five participants from the multivitamin group and five from the placebo group were supplementing their diet daily with fish oil. All participants had been using fish oil supplements for a minimum of six months. The frequency of anti hypertensive medication use differed significantly between the groups ( $\chi^2 (1,56) = 4.60, p = .03$ ) with 17 participants in the multivitamin group using antihypertensive medication and 13 in the placebo group. The frequency of lipid lowering medication did not differ significantly with 10 participants in the multivitamin group and 7 in the placebo group using lipid lowering medication.

#### *Compliance*

Remaining tablets were counted at the post-treatment appointment. Compliance as determined by the number of remaining tablets was shown to be high, with an average of 3 of the 112 supplements remaining at the end of the 16 week period.

#### *Treatment Estimate*

Of the 51 subjects, 39 were asked which treatment believed that they had received. A total of 18 participants were uncertain which treatment they were allocated, 6 individuals correctly guessed they had received the multivitamin, 8 correctly guessed the placebo and 7 participants made an incorrect judgment. Fishers exact test revealed that the number of uncertain, correct and incorrect estimates of which treatment was received did not differ between the multivitamin and placebo groups ( $p = .70$ ).

#### *Treatment side effects*

One participant in the treatment group experienced nausea and vomiting after commencing multivitamin supplementation. The participant was referred to her General Practitioner and the event was reported to the Swinburne Human Research Ethics Committee. A participant in the placebo group developed a mild rash during the supplementation period. Both subjects discontinued treatment and were withdrawn from the trial.

### *Excluded participants*

*Cognitive data:* Participants with response times 3 or more standard deviations from the mean were excluded from the relevant cognitive analysis.

*Biochemical data:* Participants who were supplementing with vitamin B<sub>12</sub> prior to commencing the trial were excluded from the relevant vitamin post-treatment analysis. Two participants were excluded as outliers from vitamin B<sub>6</sub> analysis, two participants were excluded from hsCRP analysis, two participants were excluded from the LFT tests, and one participant was excluded from the ALP, bilirubin, GGT, AST and ALT blood safety tests for values over 3 standard deviations from the mean. A further six participants were excluded from all biochemical analysis for failing to attend the post-treatment blood collection appointment within seven days of the post-treatment cognitive testing session.

*Missing data:* Missing biochemical values are due to blood collection and processing errors. Reliable blood pressure and arterial stiffness measurements could not be obtained for all subjects.

### **5.3.1 Baseline biochemical results**

#### *Baseline correlations*

Baseline levels of vitamin B<sub>12</sub> were found to correlate with homocysteine ( $r(55) = -0.46$ ,  $p < .001$ ) and vitamin B<sub>6</sub> ( $r(52) = 0.29$ ,  $p = .03$ ). Homocysteine was significantly associated with vitamin B<sub>6</sub> ( $r(52) = -0.28$ ,  $p = .04$ ) and hsCRP ( $r(53) = 0.28$ ,  $p = .04$ ). Fibrinogen also correlated with hsCRP ( $r(53) = 0.44$ ,  $p = .001$ ). Vitamin E and protein carbonyls did not correlate with other blood nutrient variables at baseline. There were no significant associations between any of these blood markers and participant age. As a consequence, these correlations were not statistically adjusted for the effects of age.

### **5.3.2 Cognitive results**

To investigate the importance of individual vitamins for cognition, vitamin E, B<sub>6</sub>, B<sub>12</sub>, and homocysteine were correlated with performance on the computerised battery at baseline.

Greater levels of vitamin B<sub>12</sub> correlated with better spatial working memory accuracy on the SUCCAB ( $r(54) = .31$ ,  $p = .03$ ). A negative association with accuracy was identified for the

delayed recognition memory SUCCAB measure ( $r(55)=-0.30, p=.03$ ), indicating that higher B<sub>12</sub> levels were associated with worse performance on this task. Controlling for the effects of age did not alter the statistical significance of the relationship with working memory ( $r(51)=.31, p=.03$ ), or recognition memory ( $r(51)=-0.32, p=.02$ ).

Higher levels of vitamin E were associated with better performance on measures of spatial working memory accuracy ( $r(54)=0.30, p=.03$ ) and a trend for immediate recognition memory response time ( $r(55)=-0.27, p=.05$ ). When the effects of age were controlled, a linear trend was observed for spatial working memory ( $r(51)=-0.24, p=.05$ ) and immediate recognition memory response time ( $r(51)=-0.24, p=.09$ ).

Lower levels of LDL cholesterol ( $r(55)=-.28, p=.04$ ) and total cholesterol ( $r(56)=-0.29, p=.03$ ) were correlated with slower response time on the immediate recognition memory task. When the effects of age were controlled the effects for LDL cholesterol ( $r(52)=-.22, p=.09$ ) and total cholesterol ( $r(53)=-0.25, p=.07$ ) were no longer statistically significant. There were no significant correlations between vitamin B<sub>6</sub>, homocysteine, protein carbonyls, hsCRP, fibrinogen or any other lipid markers with any of the cognitive variables.

#### *Treatment effects of the multivitamin*

The means and standard deviations for accuracy and response times on the SUCCAB at baseline and post-treatment testing are shown in Table 5.2 and Figure 5.1 displays the changes in response time and accuracy from baseline to post-treatment. Due to ceiling effects, task accuracy was not analysed statistically for the attention measures, but is presented for fullness of information. A series of one way ANOVAs were conducted to investigate group differences at baseline, with significant differences identified for the composite memory measure ( $F(1,53)= 6.22, p =.02$ ), delayed recognition response time ( $F(1,54)= 4.14, p =.047$ ) and immediate recognition memory response time ( $F(1,54)= 8.35, p =.006$ ). On each of these measures, response time was slower for the multivitamin than placebo group.

**Table 5.2** Means and standard deviations for SUCCAB response times and number of words recalled on the CVLT-II at baseline and post-treatment

Cognitive Task	Treatment Group	N	Baseline		Post Treatment	
			Mean	SD	Mean	SD
<i>SUCCAB</i>						
Simple Reaction rt	Multivitamin	26	308.8	56.0	305.6	52.0
	Placebo	25	307.6	50.2	303.5	40.6
Simple Reaction %	Multivitamin	26	98.7	3.0	99.0	2.5
	Placebo	25	99.0	2.0	98.8	5.1
Complex Reaction rt	Multivitamin	26	518.2	63.7	497.0	74.6
	Placebo	25	503.7	67.2	500.0	62.4
Complex Reaction %	Multivitamin	26	85.4	9.4	91.2	17.4
	Placebo	25	87.4	9.7	92.8	13.2
Stroop Congruent rt	Multivitamin	26	862.3	171.2	855.2	200.4
	Placebo	25	799.4	161.8	792.8	119.2
Stroop Congruent %	Multivitamin	26	98.3	2.5	97.8	3.4
	Placebo	25	99.1	1.9	99.0	1.4
Stroop Incongruent rt	Multivitamin	26	1251.2	454.3	1280.9	506.7
	Placebo	24	1198.3	357.2	1096.9	203.9
Stroop Incongruent %	Multivitamin	26	97.8	3.7	96.6	6.9
	Placebo	24	97.2	3.6	97.5	2.9
Contextual Recognition rt	Multivitamin	26	1426.2	418.8	1240.4	256.1
	Placebo	25	1191.8	151.5	1116.7	234.3
Contextual Recognition %	Multivitamin	26	75.2	13.8	79.2	9.5
	Placebo	25	76.8	11.8	78.8	9.9
Immediate Recognition rt	Multivitamin	26	1527.9	393.5	1242.9	271.9
	Placebo	25	1236.5	287.3	1036.7	250.3
Immediate Recognition %	Multivitamin	26	70.5	9.2	79.2	9.5
	Placebo	25	73.6	12.3	83.1	5.4
Delayed Recognition rt	Multivitamin	26	1243.9	269.3	1159.0	210.9
	Placebo	25	1081.4	202.7	1036.2	185.5
Delayed Recognition %	Multivitamin	26	71.8	7.7	74.7	10.7
	Placebo	25	74.0	10.4	76.0	10.1
Working Memory rt	Multivitamin	25	<b>1064.2</b>	<b>131.6</b>	<b>1007.3</b>	<b>89.2</b>
	Placebo	25	<b>1021.5</b>	<b>77.9</b>	<b>1040.1</b>	<b>102.3</b>
Working Memory %	Multivitamin	25	64.4	13.3	64.1	12.8
	Placebo	25	62.9	12.6	63.2	14.1
Memory Composite rt	Multivitamin	25	1320.3	244.4	1160.6	157.9
	Placebo	25	1132.8	144.0	1057.4	149.3
Information Processing rt	Multivitamin	26	735.1	162.6	734.7	181.0
	Placebo	24	702.0	135.5	673.3	82.1
<i>CVLT-II</i>						
Immediate Free Recall	Multivitamin	26	11.5	3.3	11.7	3.6
	Placebo	24	11.4	2.5	12.0	2.8
Delayed Free Recall	Multivitamin	26	10.9	3.3	10.8	4.2
	Placebo	24	10.4	3.4	11.3	2.9

Bold font indicates significant time x treatment interaction

*Composite measures*

Examination of Figure 5.1 demonstrates that response time decreases were larger for the multivitamin group than the placebo group for all memory tasks in the SUCCAB and changes in the SUCCAB subtests included in the attention composite measure were much smaller than the memory changes over the four month period. ANCOVA, with adjustment for baseline response time, revealed there was no significant effects of treatment. Similarly, for the attention composite measure, the treatment effect did not reach statistical significance.

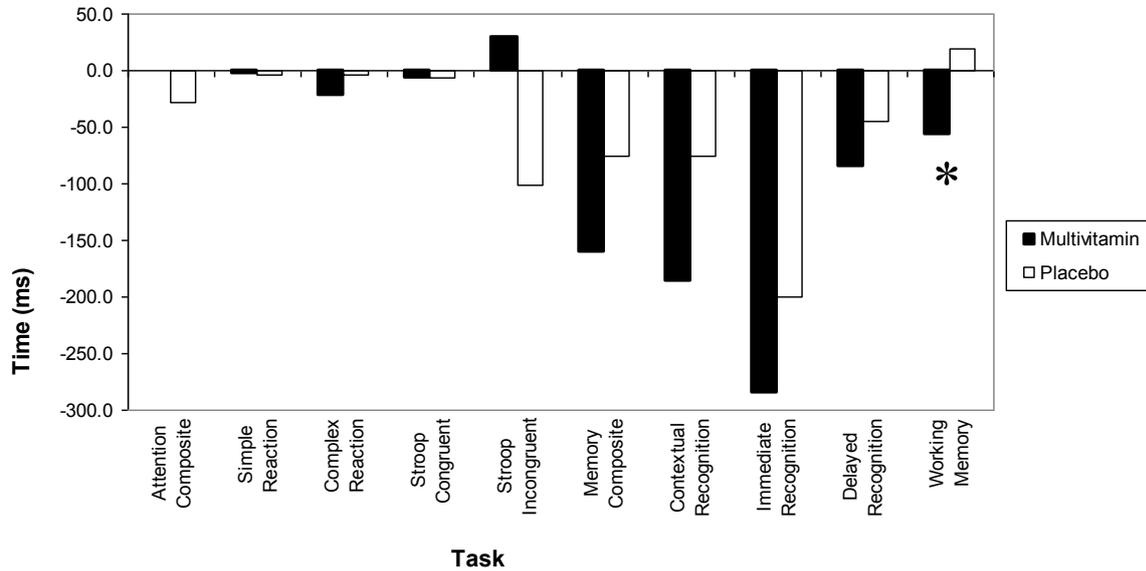
*SUCCAB subtests*

As shown in Figure 5.1, there was a greater reduction in memory response time for all the for the multivitamin treatment than the placebo in all the memory sub-tests of the SUCCAB. Subsequently, individual memory measures were examined for possible multivitamin treatment effects. ANCOVA, with adjustment for baseline response time for the working memory task, revealed a significant effect of treatment group ( $F(1,47) = 4.09, p=.049, \eta^2 = 0.08$ ). Post hoc t-tests revealed there was a significant change from baseline to post-treatment for the multivitamin group ( $p=.025$ ) which was not significant for the placebo group ( $p = .32$ ). There were no significant treatment effects for the immediate, contextual or delayed recognition memory tasks. There were no significant treatment effects for any of the accuracy measures.

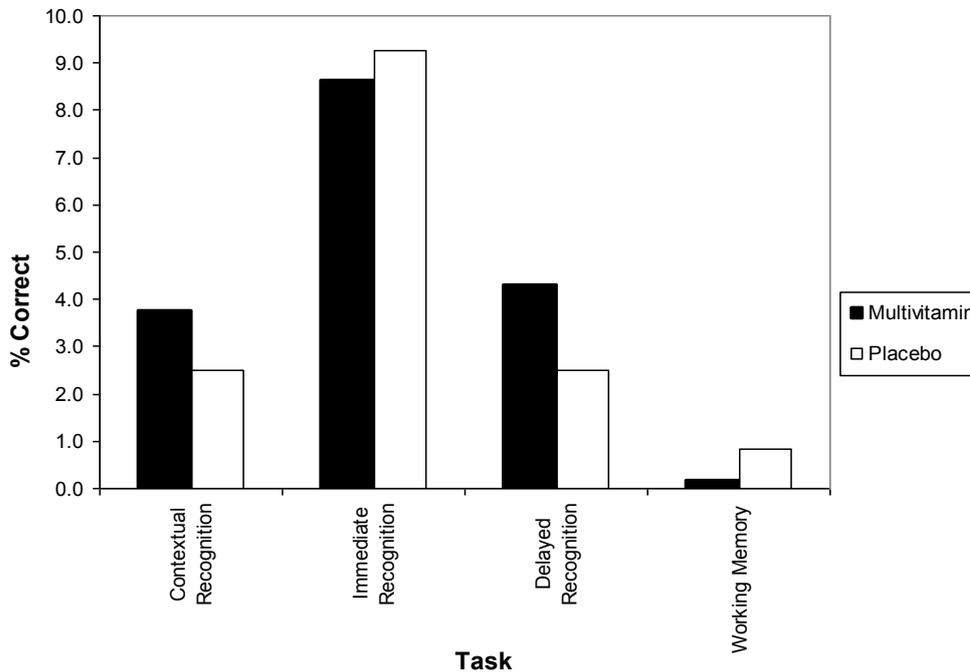
*Verbal Memory*

As shown in Table 5.2, performance on the CVLT-II was similar at baseline and post-treatment testing for the multivitamin and placebo treatment groups. There were no significant interactions for the CVLT-II measures.

In summary, the results demonstrate that four months supplementation with a multivitamin reduced memory response time for the working memory task of the SUCCAB. Whilst there appeared to be a greater reduction in response time for composite memory measure for the multivitamin than placebo treatment, this effect was not supported statistically, possibly due to large baseline differences. There were no significant improvements in SUCCAB attention measures or verbal memory associated with the multivitamin treatment.



\* = significant time x treatment interaction



**Figure 5.1** Changes from baseline to post-treatment for response time and accuracy.

The top figure shows changes in reaction time for attention tasks: simple and complex reaction time, congruent and incongruent stroop; and memory tasks: contextual, immediate and delayed recognition and working memory from baseline to post-treatment. The bottom figure shows accuracy changes for the memory measures.

### 5.3.3 Biochemical results

Means and standard deviations for the baseline and post-treatment assessment are shown in Table 5.3 for biochemical variables. A series of one-way ANOVAs were conducted to investigate group differences at baseline, with levels of vitamin B<sub>6</sub> shown to be significantly higher in the multivitamin than placebo ( $F(1,39)= 4.79, p =.04$ ). No other biochemical variables differed significantly at baseline.

**Table 5.3** Means and standard deviations for biochemical blood measures from baseline and post-treatment sessions.

<b>Blood Measure</b>	<b>Treatment Group</b>	<b>N</b>	<b>Baseline</b>		<b>Post Treatment</b>		<b>Baseline Change</b>
			<b>M</b>	<b>SD</b>	<b>M</b>	<b>SD</b>	
<i>Biochemical</i>							
<b>Homocysteine <math>\mu\text{mol/L}</math></b>	Multivitamin	21	<b>14.1</b>	<b>2.8</b>	<b>12.9</b>	<b>2.9</b>	<b>-1.2</b>
	Placebo	23	<b>15.1</b>	<b>2.8</b>	<b>15.4</b>	<b>3.6</b>	<b>+0.3</b>
<b>Vitamin B12 <math>\mu\text{mol/L}</math></b>	Multivitamin	21	<b>337.4</b>	<b>117.5</b>	<b>448.0</b>	<b>139.4</b>	<b>+110.6</b>
	Placebo	21	<b>318.7</b>	<b>103.9</b>	<b>293.4</b>	<b>103.8</b>	<b>-25.3</b>
<b>Vitamin B6 <math>\text{nmol/L}</math></b>	Multivitamin	19	<b>228.1</b>	<b>205.5</b>	<b>656.2</b>	<b>239.1</b>	<b>+428.1</b>
	Placebo	20	<b>117.6</b>	<b>92.0</b>	<b>165.8</b>	<b>193.5</b>	<b>+48.3</b>
<b>Vitamin E <math>\mu\text{mol/L}</math></b>	Multivitamin	21	34.9	8.0	37.3	6.9	+2.4
	Placebo	23	39.1	10.8	36.1	9.4	-3.0
<b>Fibrinogen</b>	Multivitamin	21	3.1	0.6	3.2	0.5	+0.2
	Placebo	23	3.1	0.5	3.3	0.7	+0.2
<i>Inflammation</i>							
<b>HsCRP</b>	Multivitamin	19	3.2	4.6	3.1	3.9	-0.1
	Placebo	23	2.2	2.3	1.8	1.6	-0.4
<i>Oxidative Stress</i>							
<b>Protein Carbonyls <math>\text{nmol/ml}</math></b>	Multivitamin	19	19.9	6.3	19.9	7.5	0
	Placebo	20	19.5	5.0	21.6	6.5	+2.2
<i>Lipids</i>							
<b>Total Cholesterol <math>\text{mmol/L}</math></b>	Multivitamin	21	5.3	0.8	5.4	0.7	+0.1
	Placebo	23	5.7	1.0	5.5	0.8	-0.2
<b>LDL Cholesterol <math>\text{mmol/L}</math></b>	Multivitamin	21	2.9	0.7	3.0	0.7	+0.1
	Placebo	23	3.3	1.0	3.2	0.8	-0.1
<b>HDL Cholesterol <math>\text{mmol/L}</math></b>	Multivitamin	21	1.8	0.4	1.8	0.5	0
	Placebo	23	1.8	0.5	1.8	0.4	0
<b>Tryglicerides <math>\text{mmol/L}</math></b>	Multivitamin	21	1.2	0.5	1.2	0.5	0
	Placebo	23	1.4	1.1	1.3	1.1	-0.2

Bold font indicates significant time x treatment interaction

### *Homocysteine*

Homocysteine was reduced in the multivitamin group, but not the placebo group and this treatment effect was significant ( $F(1,41) = 4.81$ ,  $p = .03$ ,  $\eta^2 = 0.11$ ). Post hoc t-tests indicated there was a trend for homocysteine to be reduced from baseline to post-treatment for the multivitamin treatment ( $p = .05$ ), but not the placebo ( $p = .67$ ).

### *Vitamin B<sub>12</sub>*

A significant treatment group effect ( $F(1,39) = 25.1$ ,  $p < .001$ ,  $\eta^2 = 0.39$ ) was identified for vitamin B<sub>12</sub> levels. Post hoc t-tests revealed there was a significant increase in levels of vitamin B<sub>12</sub> from baseline to post treatment testing ( $p < .001$ ), whilst there was no significant change for the placebo group ( $p = .13$ ).

### *Vitamin B<sub>6</sub>*

The results for vitamin B<sub>6</sub> levels revealed that there was a significant treatment group effect ( $F(1,36) = 43.91$ ,  $p < .001$ ,  $\eta^2 = .55$ ). Post hoc t-tests indicated that there was a significant increase in levels of vitamin B<sub>6</sub> from baseline to post treatment testing for the multivitamin group ( $p < .001$ ), whilst there was no significant change for the placebo group ( $p = .33$ ).

### *Vitamin E*

The results for vitamin E demonstrated a trend for an effect of treatment group ( $F(1,41) = 4.21$ ,  $p = 0.047$ ,  $\eta^2 = 0.09$ ). Post hoc t-tests revealed the change from baseline to post-treatment vitamin E levels did not reach statistical significance for the multivitamin ( $p = .07$ ) or placebo groups ( $p = .08$ ).

### *Fibrinogen, Inflammation and oxidative stress*

There were no significant treatment effects identified for fibrinogen, the hsCRP marker of inflammation, or the protein carbonyl measure of oxidative stress.

### *Lipids*

Table 5.3 shows that the lipid levels were similar at the baseline and post-treatment assessment. There were no significant treatment effects for these measures.

*Blood safety parameters*

The results for blood safety parameters are shown in Table 5.4. A series of one way ANOVAs were conducted to investigate group differences at baseline, with albumin significantly different at the  $p < .05$  level. The multivitamin treatment was not found to influence blood safety parameters. There were no significant treatment effects for the electrolytes, urea or liver function tests.

**Table 5.4** Means and standard deviations for blood safety measures at baseline and post-treatment assessment.

Biochemical Measures	Treatment Group	N	Baseline		Post Treatment		Baseline Change
			M	SD	M	SD	
Sodium mmol/L	Multivitamin	22	141.0	2.7	141.2	2.4	+0.2
	Placebo	21	141.1	2.5	141.3	2.2	+0.1
Potassium mmol/L	Multivitamin	22	4.2	0.4	4.2	0.4	0
	Placebo	21	4.4	0.3	4.4	0.3	+0.1
Chloride mmol/L	Multivitamin	22	105.1	2.7	105.3	2.2	+0.1
	Placebo	21	104.5	2.8	104.4	3.4	-0.1
Bicarb mmol/L	Multivitamin	22	30.0	2.3	30.0	2.9	-0.1
	Placebo	21	29.2	2.9	29.8	2.1	+0.5
Urea mmol/L	Multivitamin	22	5.8	1.4	5.6	1.3	-0.2
	Placebo	21	6.2	1.0	6.2	1.4	0
Creatine $\mu$ mol/L	Multivitamin	22	67.0	11.2	69.3	11.1	+2.2
	Placebo	21	69.9	9.4	72.0	9.9	+2.1
eGFR mL	Multivitamin	22	75.6	10.9	73.5	11.1	-2.1
	Placebo	21	72.5	10.4	70.0	9.7	-2.5
Tprotein g/L	Multivitamin	22	71.7	4.2	72.3	4.7	+0.5
	Placebo	23	72.3	3.9	72.5	3.9	+0.2
Albumin g/L	Multivitamin	22	44.3	1.9	44.2	2.4	0
	Placebo	23	45.3	1.4	45.7	1.5	+0.4
ALP U/L	Multivitamin	20	70.6	10.5	70.9	13.0	+0.3
	Placebo	20	67.7	16.3	68.0	17.2	+0.3
Bilirubin $\mu$ mol/L	Multivitamin	20	11.8	5.4	10.6	4.2	-1.2
	Placebo	23	12.3	4.5	12.4	5.6	+0.1
GGT U/L	Multivitamin	19	18.8	7.3	17.9	6.8	-0.9
	Placebo	22	20.3	9.9	19.1	11.7	-0.8
AST U/L	Multivitamin	20	22.8	4.9	23.4	5.0	+0.6
	Placebo	23	24.0	8.7	24.0	6.1	+0.1
ALT U/L	Multivitamin	20	22.7	8.1	23.6	5.2	+0.9
	Placebo	23	22.6	12.2	22.7	8.8	+0.1

*Correlations between cognitive and biochemical change*

To investigate whether a relationship existed between multivitamin-induced changes in blood nutrient levels and cognitive improvements, correlations were investigated for measures which demonstrated statistical improvement after multivitamin supplementation. For the multivitamin group, correlations were conducted to examine the relationship between changes in response time from baseline to post-treatment for the spatial working memory measure, and changes in blood levels of vitamin B<sub>6</sub>, B<sub>12</sub>, E and homocysteine. There were no significant correlations identified for these parameters.

In summary, the results from the biochemical analysis revealed that four months supplementation with the multivitamin increased blood levels of vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, and vitamin E. A reduction in homocysteine was observed but did not meet statistical significance. Supplementation with the multivitamin did not reduce the hsCRP marker of inflammation, protein carbonyl measure of oxidative stress, nor influence any of the lipid assessments. The multivitamin did not have any effect on measures of blood safety, indicating that the treatment was safe and well tolerated.

#### **5.3.4 Cardiovascular results**

Means and standard deviations for the cardiovascular baseline and post-treatment assessment are shown in Table 5.5. Results revealed there was a significant treatment effect for systolic blood pressure ( $F(1,43) = 6.04$ ,  $p = .02$ ,  $\eta^2 = 0.12$ ), which involved a significant decrease from baseline to post treatment for systolic blood pressure for those who received the placebo ( $p = 0.02$ ), but not the multivitamin ( $p = 0.41$ ). There were no significant changes in diastolic pressure. Changes in augmentation index, central and pulse pressure also did not reach significance.

**Table 5.5** Means and standard deviations for cardiovascular measures for baseline and post-treatment assessment

Cardiovascular Measure	Treatment Group	N	Baseline		Post Treatment		Baseline Change
			M	SD	M	SD	
Systolic BP	Multivitamin	23	148.8	20.7	151.5	24.2	+2.7
	Placebo	23	146.4	17.0	140.1	13.3	-6.3
Diastolic BP	Multivitamin	23	89.0	14.7	89.6	13.8	+0.5
	Placebo	23	86.3	10.6	83.3	10.0	-3.0
Central augmentation index	Multivitamin	19	36.0	6.6	31.5	11.7	-4.5
	Placebo	16	38.9	6.5	39.2	9.6	+0.2
Central pulse pressure	Multivitamin	19	52.2	10.7	51.0	14.9	-1.2
	Placebo	16	54.3	9.4	49.6	8.1	-4.7
Peripheral pulse pressure	Multivitamin	19	59.4	11.8	61.4	14.5	+1.9
	Placebo	17	60.5	10.3	56.7	7.6	-3.8

#### 5.4 Discussion

The current study investigated the effects of 16 weeks supplementation of a multivitamin, mineral and herbal formula on cognition in community-dwelling, elderly women. In line with the hypothesis that the multivitamin treatment would exert cognitive enhancing effects on the processes most vulnerable to ageing, the results revealed there was a significant reduction in spatial working memory response time. Improvements to response time were also identified for a composite measure comprised of all memory sub-tests of the SUCCAB, however this was not statistically significant. There were no observable treatment effects for measures of attention or verbal memory using the CVLT-II. In terms of biochemical changes, vitamins B<sub>6</sub>, B<sub>12</sub> were found to increase with multivitamin supplementation, and there was a trend for homocysteine to be reduced and vitamin E to be increased. No cardiovascular improvements were identified for the multivitamin, although a decrease in systolic pressure was observed for the placebo treatment.

##### *Baseline correlations with cognition*

The results at baseline revealed that cognitive performance correlated with selected vitamin concentrations. In particular, serum vitamin B<sub>12</sub> and vitamin E were associated with indices of short term memory, with higher levels of both vitamins related to more accurate performance on the spatial working memory subtest from the SUCCAB. After controlling for

the effects of age there was also a trend for higher levels of vitamin E to be associated with faster immediate recognition memory response time. These results are comparable with prior studies which have identified relationships between the baseline blood levels of specific nutrients and cognitive measures (Perkins et al., 1999; Cockle et al., 2000; Elias et al., 2006). Unexpectedly, higher levels of vitamin B<sub>12</sub> were also associated with lower simple recognition memory performance, a finding that is inconsistent with literature that suggests lower, rather than higher, levels of vitamin B<sub>12</sub> should be detrimental to cognitive performance (Moretti et al., 2004; McCaddon, 2006; Vogiatzoglou et al., 2008).

The success of identifying relationships between vitamin status and cognition in the elderly may depend on whether individuals are in the normal range or are deficient in the vitamins under investigation. In the current study, there were no subjects deficient in vitamin E and only one participant was deficient in vitamin B<sub>12</sub> using standard criteria. Based on the findings of a population-based sample of 1000 subjects, it has been suggested that serum levels of vitamin B<sub>12</sub> should be approaching 279 pmol/L in individuals aged 65 to 70 years and 268 pmol/L in those aged 75 to 80 years, in order to completely rule out the possibility of deficiency (Wahlin et al., 2002). According to this classification, one third of the participants had vitamin B<sub>12</sub> levels below 270 pmol/L, indicating they may be at risk of B<sub>12</sub> deficiency. Consistent with the finding that cognitive measures most sensitive to age correlate with biochemical vitamin indices (La Rue et al., 1997), levels of vitamins B<sub>12</sub> and E were associated with spatial working memory performance. As this subtest of the SUCCAB has demonstrated the greatest age effects (Pipingas et al., 2010), these results indicate that the spatial working memory measure may be suitable to detect cognitive changes related to subtle vitamin deficiency, or even low levels of vitamins within the normal range.

The influence of vitamin B<sub>12</sub> on memory may be due to its importance in the production of neurotransmitters such as dopamine, noradrenalin and serotonin, as well as proteins, phospholipids, DNA, and myelin (Selhub et al., 2000). Vitamin B<sub>12</sub> is further required for the synthesis of sadenosylmethionine (SAM), which is critical for maintenance of choline in the CNS, and generation of acetylcholine and the antioxidant glutathione (Tchantchou et al., 2008). It is possible that the association between vitamin E and memory may be attributable to the effects of long term dietary intake. For example, use of both vitamin C and E

supplements in seniors has been linked to better cognitive function when followed up three to ten years later (Masaki et al., 2000; Grodstein, Chen & Willett, 2003; Maxwell et al., 2005), indicating that these antioxidants may have a protective effect on cognition. These mechanisms are potentially due to the action of vitamin E as a lipid-soluble, chain-breaking antioxidant, exerting neuroprotective effects and decreasing oxidative stress (Cantuti-Castelvetri et al., 2000; Boothby & Doering, 2005; Devaraj et al., 2007).

In contrast to other studies, the present investigation did not uncover any associations between cognitive measures and either vitamin B<sub>6</sub> or homocysteine (Wolters et al., 2005; Elias et al., 2006). Specifically, Elias et al. (2006) identified a relationship between vitamin B<sub>6</sub> and multiple cognitive domains, ranging from visual-spatial organization to working memory, scanning-tracking, and abstract reasoning in community dwelling adults ranging in age from 26 to 98 years. This finding was in part attributed to the higher prevalence of vitamin B<sub>6</sub> deficiency than for other vitamins within this cohort of participants. It is possible that in the current study, the absence of such a relationship may be due to the adequate vitamin B<sub>6</sub> status of this participant group. Whilst participants were not deficient in vitamin B<sub>6</sub>, in general, the sample was classified as mildly hyperhomocystemic. In past studies, higher levels of homocysteine have been linked to poorer cognitive function at baseline (Mooijaart et al., 2005; Schafer et al., 2005; Feng et al., 2006), and when elevated, homocysteine constitutes a predictor of longitudinal cognitive decline (McCaddon et al., 2001; Nurk et al., 2005; Tucker et al., 2005). In our investigation, there was no evidence that individuals with elevated serum homocysteine demonstrated inferior cognitive performance to those with lower levels, however, the inclusion of a small sample size may have precluded the detection of any such relationship.

In summary, a clear pattern is yet to emerge correlating baseline vitamin status with specific cognitive domains, however, the results from this small sample provide some evidence that higher levels of vitamin B<sub>12</sub> and E are preferentially related to short term memory measures which are known to decline with age.

*Effects of multivitamin supplementation on cognition*

Whilst there appeared to be an effect of the multivitamin on the memory composite measure, the greater reduction in response time exhibited by the multivitamin than placebo group was not supported statistically. The discovery that spatial working memory response time was improved by the multivitamin treatment, provides some support for the hypothesis that in the elderly, the cognitive domains most affected by the ageing process, in turn, experience the greatest benefits from nutraceutical intervention (Durga et al., 2007; Pipingas et al., 2010). Working memory has been demonstrated to be particularly vulnerable to the effects of ageing (Salthouse & Meinz, 1995), and such age-related detriments to performance have been observed in a cross-sectional lifespan investigation using the same measure of spatial working memory as the current study (Pipingas et al., 2010). Participants in the current study reported subjective memory complaints, a condition which may relate to early manifestations of memory impairment (Jorm et al., 2001). It has been suggested that amongst the elderly, there are some subgroups who will experience the greatest cognitive benefits from dietary interventions (Jelic & Winblad, 2003; Balk et al., 2007). By focussing on a participant group experiencing below optimum cognitive functioning, combined with age-sensitive cognitive measures, the current study may have been more capable of identifying cognitive improvements with multivitamin supplementation than trials which identified negative treatment effects (Wolters et al., 2005; McNeill et al., 2007).

Interestingly, the observed cognitive benefits were restricted to the domain of non-verbal memory, and these results also support the predictions of other researchers. In particular, measures of fluid intelligence may be responsive to treatment with B vitamin or multivitamin formulations due to the role of these nutrients in the maintenance of central nervous system function and production of neurotransmitters relevant to memory function (Cockle et al., 2000; Bryan et al., 2002; Haskell et al., 2008). The findings also support the identification of multivitamin treatment effects on computerised measures of fluid intelligence in both younger (Haskell et al., 2010; Kennedy et al., 2010) and older participant samples (Harris et al., 2011).

Working memory represents an important component of fluid intelligence, with the ability to actively maintain information for the purpose of achieving a goal or outcome, essential for a

range of higher order cognitive functions (Baddeley, 1992; Engle et al., 1999). Growing evidence indicates that working memory may represent a cognitive domain which benefits preferentially from nutraceutical intervention (Pipingas et al., 2008; Ryan et al., 2008; MacReady et al., 2011). Performance on the same spatial working memory task included in the current study has demonstrated improvements following five weeks supplementation with a flavonoid blend (Pipingas et al., 2008), indicating that this measure is sensitive to both multivitamin and flavonoid intervention.

#### *Potential multivitamin-related mechanisms of cognitive improvement*

In the current study, treatment improvements were specific to working memory. Whilst it is probable that synergistic effects of the multivitamin were responsible for the cognitive enhancing effects, individual mechanisms may also have been important. Vitamins B<sub>6</sub> and B<sub>12</sub> were found to increase in concentration following the multivitamin treatment and thus may have contributed to the observed memory benefits. At baseline, approximately one third of the sample was at risk of B<sub>12</sub> deficiency and some cognitive benefits may have resulted from correcting existing deficiencies. Specifically, the hypomethylation hypothesis proposes that reduced folate and B<sub>12</sub> can lead to inhibition of SAM, which in turn restricts the production of myelin, neurotransmitters, and membrane phospholipids (Rosenberg & Miller, 1992). Based on this proposition, it is conceivable that increasing these nutrients via dietary supplementation may benefit the function and integrity of the nervous system. A recently published investigation into the effects of a vitamin B complex with vitamin C and minerals on cognitive performance in younger males, provides evidence for this premise (Kennedy et al., 2010). Following one month of supplementation, performance was improved on a mental calculation task which taxed the processes of psychomotor function, attention, working memory and executive function. However, in the current study, if a generalized improvement to the nervous system was to account for the improvements to speed of memory response, similar effects would be anticipated for the measures of processing speed and attention – a theory which was not observed.

Intake of vitamins B<sub>6</sub> and B<sub>12</sub> has also been related to brain volume of cortical regions important for working memory. In a study which utilised magnetic resonance imaging (MRI),

it was demonstrated that in healthy seniors, grey matter volume in the left and right parietal regions was associated with short term B<sub>12</sub> intake, and volume of the anterior and posterior cingulate, left parietal lobe and superior frontal gyrus were related to B<sub>6</sub> intake (Erickson et al., 2008). The frontal and parietal regions are part of a distributed network of brain areas required for working memory (Smith & Jonides, 1997; Wager & Smith, 2003), and the integrity of these regions may exert important influence on memory function. Despite this suggestion, it is more likely that the function rather than the structure of such brain regions would be altered by dietary intervention over a period of only 16 weeks. Consequently, other mechanisms may also have been important contributors to memory enhancements.

Lowering of homocysteine concentrations may represent one such factor. The multivitamin was shown to decrease homocysteine by 1.2 µmol/L, the same figure previously reported after 12 weeks multivitamin supplementation (Earnest et al., 2002) and slightly lower than the 1.57 µmol/L reduction observed by Summers et al. (2010) after 16 weeks. Results from trials which have investigated the cognitive effects of decreasing homocysteine in individuals with baseline levels of at least 13 µmol/L. have been mixed. In one study, reducing homocysteine via three years of folate supplementation demonstrated improvements to memory as assessed by a verbal learning task, information processing speed and sensorimotor speed in seniors aged 50-70 years (Durga et al., 2007). In contrast, two years supplementation with vitamins B<sub>6</sub>, B<sub>12</sub> and folate for the purpose of lowering homocysteine did not improve performance on verbal learning or any other neuropsychological assessments (McMahon et al., 2006). Similarly, six months treatment with vitamin B<sub>12</sub> and folate in elderly over 70 years of age and experiencing mild B<sub>12</sub> deficiency lowered homocysteine, but did not lead to improvements on neuropsychological task performance (Eussen et al., 2006). The same finding has been observed after four months combined vitamin B<sub>6</sub>, B<sub>12</sub> and folate supplementation (Lewerin et al., 2005).

When elevated, homocysteine exerts neurotoxic effects on the brain and is related to the presence of white matter hyperintensities (Sachdev, 2005). It has been suggested that homocysteine also affects specific brain structures, with higher levels of this amino acid associated with atrophy and smaller width of the hippocampus (Williams et al., 2002; Den Heijer et al., 2003). The hippocampus is a region important for long term potentiation (LTP),

a process involved in the formation of memories (Bliss & Collingridge, 1993). Based on findings specific to this brain region, there is suggestion that reducing homocysteine-induced hippocampal damage may contribute to memory-specific benefits. Currently it is still unclear whether the detrimental effects of homocysteine on the brain can be reversed (McCaddon, 2006), with cognitive improvements associated with lowering homocysteine only apparent after a period of three years (Durga et al., 2007). Future studies incorporating longitudinal homocysteine-lowering interventions with brain imaging techniques may be beneficial in answering this question.

In the current study, there was a trend for levels of vitamin E to increase following multivitamin supplementation, whilst there was a trend for vitamin E to decrease in those allocated to the placebo. Vitamin E is considered to be a powerful antioxidant with the ability to protect the brain from the harmful effects of oxidative stress (Gómez-Pinilla, 2008). In terms of a multivitamin formulation, vitamin E interacts synergistically with selenium (Bourre, 2006) and other plant derived antioxidants to increase overall antioxidant capacity (Fuhrman et al., 2000). In addition to lowering oxidative stress, it has been suggested that fat-soluble antioxidants such as vitamin E may also exert direct effects on the brain (Sen & Khanna, 2010). In rodents, vitamin E prevents ischemia-induced neuronal death in hippocampal cells (Hara, Kato & Kogure, 1990) and has been shown to facilitate LTP in neurons (Xie & Sastry, 1993). Thus it is possible that increases in vitamin E may also have contributed to cognitive improvements in the current study.

#### *Effects of the multivitamin on biochemical and cardiovascular parameters*

In the present study, a number of health outcomes, including biochemical measures of oxidative stress, inflammation and cardiovascular measures, were investigated in order to reveal indirect actions of the multivitamin on cognitive improvement. Whilst the multivitamin was found to increase levels of vitamins B<sub>6</sub>, B<sub>12</sub> and E, there were other biochemical measures which did not improve with multivitamin treatment. These findings differ from past studies which have identified benefits of multivitamin or combined antioxidant supplementation with respect to biochemical markers of oxidative stress and inflammation (Salonen et al., 1991; Church et al., 2003; Arnaud et al., 2007; Shargorodsky et

al., 2010). Differences in treatment composition, duration, and participant health characteristics may have contributed to disparity in these treatment outcomes.

Despite multivitamin treatment-related increases to levels of vitamin E, there were no significant changes in levels of protein carbonyls, a measure of oxidative stress. Studies examining dietary patterns have indicated that diets high in antioxidants may be related to lower levels of oxidative stress in the body. In middle aged to elderly subjects, a high intake of fruit and vegetables has been associated with higher blood nutrient levels, lower oxidative stress and better cognitive function (Polidori et al., 2009), and intake of antioxidants has been associated with levels of oxidative stress seven years afterwards in elderly adults (Helmersson et al., 2009). In further contrast to the present study, prior intervention studies have shown that four weeks multivitamin treatment reduced levels of DNA oxidative stress in a group of 80 elderly subjects (Ribeiro et al., 2007) and urinary isoprostanes were attenuated after 12 weeks supplementation with combined vitamins C and E in individuals at risk of cognitive decline (Clarke et al., 2003).

Some evidence also indicates that the influence of dietary supplementation on oxidative stress measures may be limited to those who have a low antioxidant status or elevated baseline oxidative stress. For example, in healthy subjects, 400mg daily of vitamin C was found to reduce immunoglobulin protein carbonyl levels at 10 and 15 weeks, but only in subjects with low baseline ascorbic acid (Carty et al., 2000). Other research has found that the benefits of two months treatment of either 1000mg daily of vitamin C or 800IU of vitamin E on plasma F<sub>2</sub>-isoprostanes were limited to individuals with elevated oxidative stress (Block et al., 2008). Reductions in protein carbonyls have also been identified following 12 weeks treatment of a combined vitamin C and flavanoid blend in smokers, a group exposed to large quantities of free radical and pro-oxidant compounds (Young et al., 2006). At baseline, levels of protein carbonyls in the present study were comparable to those previously measured in healthy elderly women (Kasapoglu & Özben, 2001) and may not represent an elevated state of oxidative stress.

In elderly free from dementia, C-reactive protein is amongst cardiovascular risk factors which have been linked to poorer cognitive function (Beeri et al., 2009; Noble et al., 2010) and

predictive of cognitive decline (Yaffe et al., 2003). Similar to the present study, in a trial of middle aged men there were no reductions to the C-reactive protein marker of inflammation following eight weeks B vitamin, antioxidant or combined B vitamin and antioxidant treatment (O'Doherty et al., 2010). In contrast to the results of the present study, reductions to this marker have been demonstrated following six month multivitamin supplementation in subjects aged 30 to 70 years (Church et al., 2003). Two months supplementation with vitamin C, but not vitamin E were shown to reduce C-reactive protein levels by 24% in individuals passively and actively exposed to cigarette smoke (Block et al., 2004) and these findings were replicated in healthy non-smokers (Block et al., 2009). The levels of vitamin C, investigated in the studies which obtained positive treatment effects, were 1000mg daily. In the current trial, a smaller dosage of only 200mg was included in the multivitamin. It is possible that the much higher dosage of vitamin C investigated previously may have been responsible for the observed reduction in inflammation. Effects of vitamin E on C-reactive protein have also been observed but appear to be more selective to groups with existing cardiovascular pathology (Devaraj et al., 2007).

In terms of other cardiovascular parameters, the findings from the present study also indicate that there was no effect of the multivitamin on lipid metabolism in elderly women. This result is not entirely unexpected as most evidence linking vitamin supplementation to cholesterol is limited to individuals with metabolic disorders or diabetes. For example, over three months, combined magnesium, zinc, vitamin C and E supplementation increased HDL in type 2 diabetic patients (Farvid et al., 2004), and 26 weeks treatment with a multivitamin has also shown to increase HDL and reduce LDL cholesterol in young to middle aged obese women (Li et al., 2010). Some evidence suggests that eight weeks folic acid supplementation may increase HDL levels in postmenopausal women (Villa et al., 2005), although these findings were based on a very small sample and require replication with a larger sample.

Furthermore the multivitamin treatment used in the current trial was not found to lower blood pressure, with a greater reduction in blood pressure observed in the placebo group. Changes in blood pressure medication were not measured over the course of the study so it is not possible to determine whether such alterations were due to changes in medication. On average, half the participant sample, with four more participants in the multivitamin than

placebo group, were already receiving treatment for hypertension. In the elderly, hypertension has been associated with increased rates of progressive whole brain atrophy, greater white matter hyper-intensities and poorer cognitive performance (Knopman et al., 2001; Raz et al., 2005; Fjorland et al., 2007; Knecht et al., 2009; Kuller et al., 2010). As oxidative stress is known to contribute to elevated blood pressure (Houston, 2005), antioxidants which combat oxidative damage have been investigated for their antihypertensive properties. In prior studies, combined treatment for eight weeks with zinc, ascorbic acid,  $\alpha$ -tocopherol and  $\beta$ -carotene has been shown to reduce systolic blood pressure in hypertensive and normotensive adults (Galley et al., 1997), and treatment with a multivitamin led to a decrease in blood pressure after 26 weeks in a sample of obese women (Wang et al., 2009).

Generally plant extracts, antioxidants, alpha-lipoic acid, co-enzyme Q-10 and flavonoids have been examined for their ability to lower blood pressure (Hodgson & Croft, 2006; Shay et al., 2009). The multivitamin used in the present investigation contained a range of herbal extracts and flavonoid components, and for this reason was anticipated to lower blood pressure. Flavonoids protect against endothelial dysfunction and promote vasorelaxation mediated by endothelial nitric oxide synthase activation, which possibly aids their antihypertensive actions (Kwak et al., 2009). When combined with vitamin C, flavonoid extracts such as pine bark have demonstrated a trend for systolic blood pressure to be reduced in smokers (Young et al., 2006) and older individuals at risk of cognitive decline (Pipingas et al., 2008).

There were no effects of the multivitamin on other cardiovascular indices of endothelial function, such as measures of arterial stiffness or fibrinogen. Age and elevated blood pressure are related to aortic stiffness (McEniery et al., 2007). Acute administration of vitamin C has been demonstrated to reduce arterial stiffness (Wilkinson et al., 1999), possibly due to preservation of endothelial derived nitric oxide (Diaz et al., 1997). Flavonoids with powerful antioxidant properties have also been suggested to improve arterial stiffness, although further evidence is required to fully substantiate this claim (Pase et al., 2011). Findings from the present study indicate that in elderly women, the combined multivitamin, mineral and herbal supplement investigated did not improve measures of arterial stiffness.

#### **5.4.1 Limitations and future directions**

There were several limitations to this experiment. Due to biochemical analysis errors during the processing of folate and vitamin C, these blood samples were unable to be used for either baseline or post-treatment analysis. As a consequence, it is not known whether 16 weeks supplementation with the multivitamin led to improvements in these nutrient levels, or whether they exerted any influence on cognitive or health parameters. In a trial of a comparable multivitamin supplement formulated for men, a statistical increase in folate was observed following only eight weeks treatment (Harris et al., 2011). Prior studies have also demonstrated significant increases in levels of vitamin C with multivitamin supplementation, although larger quantities were included in these products than in the multivitamin under investigation in our study (Cockle et al., 2000; Earnest et al., 2002). Of the antioxidants, vitamin C appears to exert the strongest effects on measures of oxidative stress and inflammation (Block et al., 2004; Block et al., 2008; Block et al., 2009), therefore, its absence in this investigation presents as a considerable constraint. The inclusion of blood tests measuring other antioxidants such as vitamin A and assessments of polyphenol status in future investigations may also aid in understanding the mechanisms of the multivitamin treatment which underlie cognitive improvement.

Another potential shortfall of this investigation was the absence of information regarding participant diet and antioxidant intake from food, prior to and over the course of the trial. It is possible that changes in dietary habits can also alter health indices, for instance, increasing fruit and vegetable intake over a period of three months has been demonstrated to improve nutrient levels in healthy individuals (Polidori et al., 2009). Regardless of dietary intake, measurement of blood markers did indicate that levels of all blood nutrient and biochemical markers, other than vitamin B<sub>6</sub>, were equivalent at baseline for the placebo and multivitamin groups. Whilst blood nutrient levels of B vitamins and vitamin E were shown to increase for the multivitamin group from baseline to post-treatment, there were no statistical changes in blood nutrient or biochemical markers for the placebo group over the 16 week period. Cholesterol levels over the treatment duration for both the placebo and treatment groups indicate that, in this regard, dietary lipid metabolism also remained stable. These findings, within the context of a randomised controlled trial, provide assurance that there were no

major dietary changes over the course of the study. However, dietary intake was not standardised on the testing days in this study and future studies may benefit from standardising dietary intake on the assessment days to further reduce any variability due to the potentially confounding influence of diet.

A further consideration relates to the cognitive assessment tools utilised in the present study. Alternate forms of cognitive tests are used to diminish practice effects in studies with repeated measures designs (Benedict & Zgaljardic, 1998), yet in this study practice effects of the placebo and treatment groups were still apparent on multiple measures, despite the use of alternate re-test forms of all cognitive assessments. Practice effects are a concern in studies such as this one, as the expected cognitive improvements are relatively small. As only minimal training was provided on the SUCCAB, the inclusion of a separate practice session may be necessary to eliminate practice effects in future studies.

In the current study, a degree of caution must be applied when concluding that multivitamin supplementation in the elderly preferentially improves memory, but not other cognitive domains. In a recent investigation in young adults, it was revealed that a high level of cognitive demand was necessary to capture the nutraceutical effect of a cognitive enhancing polyphenol (Scholey et al., 2010). Response time data from our study indicates that the memory sub-tests included in the cognitive battery may have been more difficult than the attention measures, and therefore more responsive to nutraceutical effects. In future studies, the addition of attention tasks of higher or graded difficulty may help to determine whether the cognitive enhancing effects of multivitamins in the elderly are specific to memory or are, instead related to a wider range of cognitive processes.

#### **5.4.2 Summary and conclusion**

In brief, the results of this trial indicate that 16 weeks supplementation with a combined multivitamin, mineral and herbal formula is capable of improving cognitive performance in elderly women. Specifically, the multivitamin treatment improved speed of response on a spatial working memory task. Although not reaching statistical significance, multivitamin

treatment led to a greater improvement to the memory composite measure and all memory sub-tests of the SUCCAB, indicating that the benefit to working memory was a genuine effect and unlikely to be due to chance. There were no multivitamin-related benefits to measures of attention or verbal memory.

The current study revealed that multivitamin supplementation significantly increased levels of vitamins B<sub>6</sub>, B<sub>12</sub>, with a trend for vitamin E to be increased and homocysteine to be decreased. Levels of vitamin B<sub>12</sub> and vitamin E were also found to correlate with cognitive performance at baseline, indicating that these vitamins may represent potential regulators of cognitive function.

Findings from this study provide some support for the hypothesis that the cognitive processes most vulnerable to age, such as working memory, demonstrate the greatest improvements in the elderly, following nutraceutical intervention. As no improvements were identified for markers of inflammation, oxidative stress or cardiovascular health, it is plausible that the observed improvements to cognition may be due to the synergistic effects of the vitamin, mineral and herbal components on the brain. The effects of multivitamin supplementation on brain activity have not previously been investigated.

The subsequent experiment of this thesis was conducted to examine the SSVEP correlates of a spatial working memory delayed response task. The purpose of this experiment was to establish the SSVEP as a useful measure of nutraceutical effects. The final experiment was carried out to investigate the effects of chronic multivitamin supplementation on brain activity as measured by the SSVEP.

## **Chapter 6 Establishing the SSVEP correlates of working memory performance in the elderly**

### **6.1 Introduction**

In the previous experimental chapter, it was revealed that 16 weeks multivitamin supplementation was capable of enhancing cognitive processes in a group of elderly women, as evidenced by improvements to speed of spatial working memory response. The cognitive domain of working memory is vulnerable to age-related decline (Salthouse, 1990; Rypma & D'Esposito, 2000; Rajah & D'Esposito, 2005), and for this reason it is possible that supplementation with a reputed cognitive enhancing multivitamin may improve the neural processes which subserve working memory performance. In order to assess the effects of the multivitamin on brain function, a spatial working memory delayed response task (DRT) was selected as a neural activation task, and steady state topography (SST) was utilised to track rapid changes in neural activity associated with the performance of this task. The effects of multivitamin supplementation on brain activity will be examined in Chapter 7. Prior to investigating the potential cognitive enhancing effects of the multivitamin on the steady state visually evoked potential (SSVEP), it was necessary to first establish the neural underpinnings of the spatial working memory DRT and the SSVEP correlates of working memory performance, in all elderly subjects at baseline. The final purpose of this chapter was to determine whether the SSVEP, associated with a spatial working memory DRT, would be sensitive to the potential cognitive enhancing and nutraceutical effects of multivitamin supplementation.

#### *The neural correlates of working memory*

Working memory can be described as the process invoked when memory content is required to be held in mind, in order to achieve a particular goal or outcome (Baddeley, 1992). The

neuroanatomy of working memory was initially studied in non-human primates (Fuster & Alexander, 1971) and more recently, using neuroimaging techniques such as positron emission tomography (PET), and functional magnetic resonance imaging (fMRI) (Linden, 2007). Working memory involves a distributed network encompassing the lateral prefrontal cortex (PFC), and parietal and temporal lobes (Wager & Smith, 2003; Koch et al., 2005). Within working memory, processing of visual information is mostly lateralised to the right hemisphere (Smith & Jonides, 1997). Spatial input is known to activate a dorsal pathway, and object memory to activate a left hemisphere ventral pathway (Courtney et al., 1996; Oliveri et al., 2001; Ventre-Dominey et al., 2005). During spatial working memory, posterior regions including the parietal lobe are important for processing sensory input, maintaining representations and directing attention (Zimmer, 2008). The ventral PFC then receives input from posterior association areas, and plays a role in the active maintenance of information, whereas the dorsolateral region is recruited to monitor and manipulate memory content (D'Esposito et al., 1999; Wagner et al., 2001; Glahn et al., 2002; Veltman, Rombouts & Dolan, 2003; Altamura et al., 2007).

Whilst PET and fMRI have been valuable resources regarding the functional localisation of working memory networks, the temporal resolution of these regional metabolic measures do not approach the millisecond accuracy of event related potential (ERP) brain electrical techniques (De Haan & Thomas, 2002). The SSVEP, elicited by a 13Hz light flicker, has demonstrated the ability to delineate the spatio-temporal characteristics of the working memory sub-processes of encoding and maintenance across a delay (Silberstein et al., 2001; Ellis et al., 2006). Advantages of the SSVEP over standard ERP paradigms include the capacity of the SSVEP to monitor brain activity associated with ongoing processing demands (Silberstein, 1995; Kemp et al., 2002) and the superior signal-to-noise ratio of this methodology (Gray et al., 2003; Vialatte et al., 2010).

Modulation of the 13Hz SSVEP during performance of a cognitive task indicates that the SSVEP responds in a manner specific to individual cognitive modes or domains. Consequently, the SSVEP is ideal for investigating the working memory sub-processes of encoding, maintenance and retrieval. Studies of the SSVEP in young adults have revealed that there is a distinct profile of neural activity associated with each of the encoding and

maintenance working memory task components (Silberstein et al., 2001; Ellis et al., 2006). During encoding of non-verbal objects, reduced SSVEP amplitude has been identified at left parietal regions (Silberstein et al., 2001) and across bilateral frontal sites during encoding of a spatial *n*-back task (Ellis et al., 2006). This profile of encoding-related SSVEP activity appears to mirror the findings of reduced SSVEP amplitude associated with performance of a visual vigilance task (Silberstein et al., 1990), and has been equated to the transient alpha reductions which accompany attentional processes (Ray & Cole, 1985; Silberstein, 1995).

In contrast, the hold period of an object working memory task has been associated with increased amplitude at occipital and prefrontal sites (Silberstein et al., 2001). Increased frontal amplitude during the hold period of a working memory task has since been replicated by other research groups, using alternate methodologies (Perlstein et al., 2003; Wu & Yao, 2007). The functional significance of the SSVEP amplitude increase has been considered to reflect increases in the transmission efficiency of the cortico-cortical re-entrant loops associated with holding information „on-line“ (Silberstein et al., 2001).

The retrieval element of a working memory task involves processes related to the retrieval of information from memory. This component of working memory has not been the focus of previous studies of the SSVEP. Consequently, examination of the sub-processes of an entire working memory DRT is required to provide a more complete understanding of the temporal dynamics of the SSVEP associated with working memory. Furthermore, previous studies (Silberstein et al., 2001; Ellis et al., 2006) have focussed on younger subjects, and the SSVEP working memory encoding/maintenance distinction has not been investigated in an elderly participant group.

#### *The influence of age on the neural correlates of working memory*

As summarized in Chapter 2, working memory is vulnerable to the effects of age and alterations to the neural correlates of working memory occur across the lifespan (Babcock & Salthouse, 1990; Salthouse, 1990; Rypma & D'Esposito, 2000). Numerous electrophysiological and neuroimaging studies have demonstrated that patterns of brain activity differ between young and elderly subjects during performance of working memory tasks (see Rypma & D'Esposito, 2000; Friedman, 2003; Rajah & D'Esposito, 2005). In brief,

these investigations have demonstrated that there is an age-related alteration in the activity of the dorsolateral region of the PFC (Rypma & D'Esposito, 2000; Rypma et al., 2001), potentially due to a loss of neural efficiency of this region (Rypma et al., 2007). Other working memory studies have revealed that the posterior neural processes which direct sensory processing and attention (Zimmer, 2008) are attenuated in the elderly, and these posterior reductions are coupled with a greater recruitment of frontal neural resources (McEvoy et al., 2001; Muller & Knight, 2002; Friedman, 2003).

Increased reliance on frontally-mediated executive processes may represent a compensatory mechanism which is beneficial for cognitive performance (Reuter-Lorenz & Cappell, 2008), indicating that not all age-related differences in brain activation are detrimental to cognitive performance. Functional reorganization of the PFC in the form of increased bilateral activity has been identified in studies of working memory (Reuter-Lorenz et al., 2000; Mattay et al., 2006) and other memory processes (Cabeza, 2002; Grady et al., 2002), and has been demonstrated to be conducive to successful task performance (Cabeza et al., 2002). Currently it is recognized these age-related increases in activation represent both a loss of neural specialization in some brain regions and concomitant compensatory processes in others (Rossi et al., 2004; Rajah & D'Esposito, 2005; Davis et al., 2008).

Evidence of compensatory processes in the ageing brain has also been obtained from a study of the SSVEP (Macpherson et al., 2009). In this study, older (59 to 67 years) and younger (20 to 30 years) adults performed a low demand object working memory DRT and a more difficult contextual recognition task. During working memory maintenance, older adults demonstrated smaller frontal SSVEP amplitude and latency differences when compared to a control task, than younger adults, and this was interpreted as an age-related decrease in neural processes. In particular, the characteristic working memory-related amplitude increases (Silberstein et al., 2001) were attenuated in the elderly. In contrast, during the memory retrieval component of the more difficult contextual recognition task, older adults demonstrated larger, more extensive, posterior SSVEP differences, possibly indicative of age-associated compensatory processes. Whilst these findings point to age-related effects upon the neural substrates of memory as measured by SSVEP, only the three second hold period of the DRT was investigated, with response-related neural activity examined for only

the contextual memory task. To date, our study (Macpherson et al., 2009) has been the only SSVEP investigation of working memory in the elderly.

*The neural correlates of performance*

The observed SSVEP amplitude reductions during memory encoding and attentional processes have been equated to event related desynchronisation (ERD) in the upper alpha band (Silberstein, 1995). ERD refers to a decrease of power in the electroencephalogram (EEG), related to a decrease of synchrony of underlying neural populations (Pfurtscheller & Lopes Da Silva, 1999). The magnitude of ERD corresponds to the extent of regional cortical activation (Klimesch, Doppelmayr & Hanslmayr, 2006), and tends to increase with task difficulty (Stipacek et al., 2003). Importantly, event-related changes in alpha ERD are highly dependent on the cognitive task or operation performed. For instance, ERD of the lower alpha band has been implicated in attentional processes, whilst upper alpha oscillations have been observed during search and retrieval processes from semantic long term memory (Klimesch, 1999). In terms of working memory, alpha ERD has been postulated to reflect memory search processes (Krause et al., 1996), potentially via the transient reactivation of long term memory (Ruchkin et al., 2003; Klimesch et al., 2006).

Notably, upper alpha ERD has demonstrated correlations with IQ measures of cognitive performance. Several studies have reported that during memory activation, better performing individuals display larger alpha ERD, corresponding to greater cortical activation (Klimesch et al., 1997; Doppelmayr et al., 2005). However, not all trials have demonstrated the same pattern of results. Investigations utilizing tasks that do not require semantic processing have identified smaller ERD in better performers (Grabner et al., 2004; Grabner, Neubauer & Stern, 2006; Riečanský & Katina, 2010), with the reduction in ERD thought to reflect the suppression of the task-irrelevant semantic memory system (Klimesch et al., 2006). Findings of smaller ERD in higher-ability individuals have also been described in terms of a „neural efficiency“ hypothesis, whereby higher ability individuals display less brain activation while performing cognitive tasks (Neubauer, Freudenthaler & Pfurtscheller, 1995). It must be noted that the relationship between complex factors such as the type of cognitive operation, task difficulty and participant gender appear to influence whether performance is related to

increased or decreased alpha ERD (Neubauer, Fink & Schrausser, 2002; Grabner et al., 2004; Neubauer & Fink, 2009).

Given that the 13Hz SSVEP is thought to reflect cortical activation in a similar manner to that of upper alpha ERD (Kemp et al., 2004), it may be anticipated that SSVEP amplitude would also correlate with measures of cognitive performance. Such a relationship between task performance and SSVEP amplitude has been identified in a study of working memory, which identified a positive correlation between working memory behavioural performance, as measured by a ratio of correct to incorrect responses, and frontal 10Hz power (Perlstein et al., 2003). In this trial, the SSVEP was elicited by a 10Hz, flickering diffuse light/dark contrasting background, and not by a 13Hz light flicker. To date, there have been no studies which have focussed on the association between individual performance and the 13Hz amplitude component of the SSVEP. Due to differences in the functional interpretation of the upper and lower alpha frequencies (Ray & Cole, 1985; Klimesch, 1999), it is not known whether stimulation with a 13Hz frequency would result in the same performance/amplitude relationship demonstrated for the lower frequency in the trial conducted by Perlstein et al. (2003).

In comparison to 13Hz SSVEP amplitude, several trials in young adults have examined the relationship between 13Hz SSVEP latency and cognitive performance. Faster responses on a continuous performance task have been associated with greater latency reductions at prefrontal sites, leading to the interpretation that SSVEP latency reduction may index increased processing speed in the brain (Silberstein et al., 2000). On the basis of these findings, decreased latency has been interpreted as an increase in the speed of neural information transmission (Silberstein et al., 2001). Conversely, increased latency is thought to be the consequence of a reduction in excitation or an increase in inhibitory processes in these networks (Silberstein et al., 2000). Frontal SSVEP latency increases have been identified in individuals with higher IQ, during performance of a working memory task (Van Rooy et al., 2001) and the raven progressive matrices (Song, 2005), indicating that inhibitory processes may also be important determinants of cognitive performance. Whilst these studies have revealed important associations between behavioural performance and the SSVEP in

younger subject groups, it is not known whether the same effects would be identified in an elderly cohort.

### **6.1.2 Aims and Hypotheses**

The current study was designed to examine the neural underpinnings of a spatial DRT and the SSVEP correlates of working memory performance in the elderly. To date, these aspects of working memory have not been investigated in an elderly sample using the SSVEP. The first aim of this experiment was to examine whether the same distinction between perceptual encoding processes and holding information „online“ in the DRT, an observation in younger adults (Silberstein et al., 2001; Ellis et al., 2006), is maintained in the elderly. In line with these previous studies, it was hypothesised that the encoding stage of the spatial DRT would be associated with a decrease in amplitude and latency over frontal regions, whilst the hold period would be associated with amplitude increases.

The second aim of this experiment was to investigate the SSVEP correlates of working memory performance using a spatial DRT. In order to achieve this, behavioural measures of response time and accuracy on the spatial DRT were correlated with the SSVEP components of amplitude and latency.

## **6.2 Method**

### **6.2.1 Participants**

In total, 53 elderly women participated in the SSVEP recording component of the clinical trial. Participants were aged between 64 and 82 years ( $M = 71.3$  years,  $SD = 4.6$  years), right-handed, non-smokers, with no history of stroke, epilepsy, dementia, Parkinson's disease, head trauma, excessive alcohol use, mental illness, depression, anxiety disorders and were not using anti-depressant, anti-anxiety medication or any medications with a cognitive enhancing effect. All participants had a mini mental status exam (MMSE) score of 24 or above ( $M = 28.7$ ,  $SD = 1.3$ ).

## 6.2.2 Procedure

### *Screening*

A full description of participant eligibility criteria, screening procedures and instruments is provided in Section 4.1. Prior to enrolment in the study, potential participants were screened over the phone and then attended a one hour screening session designed to assess their suitability to participate in the study. During this screening session, consent was obtained, cognitive status was determined using the MMSE (Folstein et al., 1975), and verbal IQ was assessed using the contextual Aus-NART (Lucas et al., 2003). Participants underwent a medical examination with a Medical Practitioner prior to enrolment in the study.

### *Baseline Testing*

Participants attended the baseline testing session at the Brain Sciences Institute at 1030 or 1130 hours. Participants were requested to refrain from consuming tea or coffee for two hours prior to attending this appointment. During the baseline testing session, participants first underwent verbal and computerised cognitive assessment, followed by cardiovascular measurements. Finally, EEG was recorded whilst participants performed a spatial working memory task and a control task.

EEG was recorded from 64 tin, monopolar, scalp electrodes, embedded in a lycra cap. Linked earlobes were used for reference and the nose was selected for the ground, as per standard SST recording procedure (Silberstein, 1995). Impedance was below 10k $\Omega$  for reference electrodes, measured with respect to the ground. Brain electrical activity was amplified and band-pass filtered 3dB down at 0.1 and 80Hz prior to digitization to 16-bit accuracy at a rate of 500Hz. The raw EEG signal and power spectra was inspected manually for the presence of mains and biological artefact. Where the signal quality could not be enhanced during EEG setup, artefact contaminated electrodes were documented.

Participants were fitted with goggles comprised of two sets of light emitting diode (LED) arrays viewed through half-silvered mirrors. The goggles were designed to superimpose a flickering white light on the participant's visual field, whilst still preserving normal vision. The SSVEP was evoked by a 13Hz sinusoidal flicker subtending a horizontal angle of 160°

and a vertical angle of 90°. The visual flicker covered the majority of the visual field with a modulation depth of the stimulus against the background of 45%.

During cognitive task performance, participants were seated approximately 1.3 metres from the task computer. Cognitive task stimuli were presented on a computer with a 14 inch liquid crystal display (LCD) monitor. Brain electrical activity was recorded whilst participants completed a spatial working memory task and control task with the 13Hz flicker, superimposed on the visual field. Both tasks were preceded by practice trials to reduce novelty effects and to ensure participants were comfortable with the task requirements.

### **6.2.3 Spatial working memory DRT task**

The spatial working memory task used during the recording of the SSVEP was a variation of Jonides et al. (1993) delayed-match-to-sample paradigm. Individual trials were separated by a 1 second fixation period. A fixation cross remained on the screen for the entire 6.3 second task duration. For each trial, subjects encoded the location of either two or three stimulus dots displayed on the circumference of an imaginary circle for 0.5 seconds, and then retained the location of the dots in memory across a 3 second fixation interval. A probe circle was then presented for 1.8 seconds and participants determined whether or not the probe circle was located in the same position as one of the stimulus dots shown during encoding. Subjects responded with a right hand (yes) button press if the probe circle location matched the stimulus dot location and a left hand (no) button press if it was a non-match. The task was designed to elicit grading effects, with either two or three dots presented during encoding. Maintaining the location of three dots in memory was intended to be more difficult than two dots. For non-match trials the probe circle could be located either one, two or four positions away from one of the stimulus dots. The control task was identical with respect to stimulus encoding and response requirements, but did not require maintenance of cue representations across a hold period. Task timing specifics are displayed in Figure 4.4. Participants completed two blocks of 40 trials of the spatial working memory task and one block of 40 trials of the control task. Each block lasted approximately four minutes. Fewer trials of the control task were required to achieve an equivalent number of correct responses as the control task was demonstrated to be easier than the working memory task during pilot testing.

#### **6.2.4 SSVEP signal processing**

SSVEP signal processing was conducted using BrainSci (SSPT Analysis Software, version 2). The procedure was described in Chapter 4, section 4.3.5. In brief, time series SSVEP data was calculated, and 6.3 second epochs of data, centred on the presentation of the fixation cross and associated with correct task responses, were extracted from the Fourier time series and averaged. SSVEP amplitude and latency were subjected to normalization and then averaged across participants. SSVEP differences were then calculated by comparing the SSVEP amplitude and latency time series associated with the working memory task to the amplitude and latency time series data associated with the control task. In order to isolate the activity associated with the hold period of the working memory task, SSVEP differences for the 3 second hold period were calculated by comparing the 3 second mean amplitude and latency related to the working memory task hold period, with the mean SSVEP amplitude and latency associated with the equivalent 3 second period from the control task. This process was repeated for all electrode sites.

#### **6.2.5 Statistical analysis**

##### *Behavioural data analysis*

Behavioural data was analyzed using SPSS version 17. Paired sample t-tests were used to compare task difficulty between the working memory task and the control task.

##### *SSVEP data analysis*

In order to examine the time course of amplitude and latency differences across the task, cluster plots were generated using Matlab (Version 7.8). A corresponding cluster plot presenting Hotelling's T data was produced to display the statistical significance of the amplitude and latency differences. Cluster plots have previously demonstrated utility in studies of the SSVEP (Gray et al., 2003; Ellis et al., 2006) and it has been suggested that clusters of statistical significance are more likely to reflect real statistical effects than numerous point-wise t-tests, which are prone to randomly distributed type 1 error (Murray et al., 2002). Hotelling's T was used to estimate the probability of falsely rejecting the null

hypothesis (type-1 error), associated with task differences in the SSVEP latency and amplitude. Spatial principal components analysis has shown that the SSVEP forms five independent factors (Silberstein & Cadusch, 1992) and, consequently the Hotelling's T statistic  $p$  values (2 tailed) were divided by five to correct for multiple electrodes. To account for multiple time points, a more stringent alpha level of .01 was adopted to determine statistical significance. Contours correspond to  $p$  values of  $p < .05$ , .01, .005, and .001. Repeated measures analysis of variance (ANOVA) was used to statistically compare the average of differences for each stage of encoding, hold and retrieval at electrode sites Fz, Cz and Pz.

In order to display task components and corresponding time points of interest more specifically, data was also presented using Hotelling's T maps. Individual maps were generated for an individual time point during encoding, the mean of the 3 second hold period and an individual time point during the presentation of the probe. The last time point corresponds to the period of memory retrieval and occurs earlier in time than the motor response. In line with the significance values displayed in the cluster plots, contours correspond to  $p$  values of  $p < .05$ , .01, .005, and .001. To correct for the inclusion of multiple time points, an alpha level of 0.01 was used to determine statistical significance.

To investigate the relationship between performance and the SSVEP, correlation analysis was carried out using a custom developed script in Matlab. In order to investigate the relationship working memory response time and SSVEP latency and amplitude, Pearson product-moment correlation coefficients were calculated for each 77ms time point of the 6.3 second epoch, at each electrode site. For this exploratory analysis, correlations were considered to be statistically significant at the  $p < .01$  level when they occurred at electrodes within the same region for three successive time points. The same time points examined in the Hotelling's T maps were used for the encoding and response period. Working memory „hold“ related activity was examined for the peak correlation within this 3 second period.

SSVEP amplitude and latency were correlated with a combined accuracy and response time measure. Response time was divided by accuracy, a method which has demonstrated utility in

neuroimaging studies to create a composite performance measure when accuracy levels are low (Mevorach, Humphreys & Shalev, 2006). A lower score on this measure was indicative of better overall task performance.

### 6.3 Results

#### *Excluded participants*

Three participants were excluded from EEG analysis due to low behavioural performance (below 40% accuracy) on the working memory task. SSVEP data from three further participants was unable to be extracted from the EEG files, however, behavioural data from these individuals was still included in the relevant analysis.

#### 6.3.1 Behavioural results

Paired sample t-tests were used to investigate task difficulty and graded effects. Average response times and performance accuracy for all participants are shown in Table 6.1.

**Table 6.1** Spatial working memory DRT response time and performance accuracy

Working Memory Trial	Response Time (ms)		Performance (% Correct)	
	M	SD	M	SD
Spatial WM Control	931.5	118.3	96.2	3.8
Spatial WM Task	1103.3	132.4	62.7	8.3
Spatial WM 2 Dot	1092.5	126.4	56.2	10.0
Spatial WM 3 Dot	1103.1	135.5	70.0	8.9
Spatial WM match	1067.5	166.0	80.0	13.0
Spatial WM non match 1	1173.7	162.7	31.1	16.8
Spatial WM non match 2	1218.1	160.5	45.8	16.9
Spatial WM non match 4	1087.2	122.8	79.1	5.6

N = 50

Paired sample t-tests revealed that participants performed the control task at a higher level of accuracy ( $t(49) = 26.70, p < .001$ ) and responded faster to trials on this task than on the working memory task, ( $t(49) = -11.21, p < .001$ ) indicating that the working memory task was more difficult to complete than the control task.

A straightforward pattern of graded effects was not apparent for trials containing two dots as opposed to three dots. The three dot trials were performed approximately 13% more accurately than the two dot trials and paired sample t-tests indicated that this difference in performance was statistically significant ( $t(49) = -10.04, p < .001$ ). There were no statistical differences in response times for trials containing three versus two dots. As the expected grading effects were not elicited by the task (i.e. three dot trials more difficult than two dot trials) no further analysis of the SSVEP, related to trial difficulty, was conducted.

To investigate whether the distance of the probe circle from the stimulus dots presented at encoding may have influenced task difficulty, correct match responses were compared to non-match responses. In trials where the probe circle was located one position away from one of the dots, accuracy was significantly lower ( $t(49) = 13.33, p < .001$ ) and response time longer ( $t(49) = -3.97, p < .001$ ) than on match trials. When the probe circle was located two positions away, the same pattern of findings was apparent for accuracy ( $t(49) = 10.93, p < .001$ ) and response time ( $t(49) = -5.86, p < .001$ ). There were no statistical differences when the probe circle was located four positions away. Whilst these results point to grading effects related to the distance of the probe from the stimulus, due to the small number of correct responses for these individual trials, no further SSVEP analysis was carried on this data.

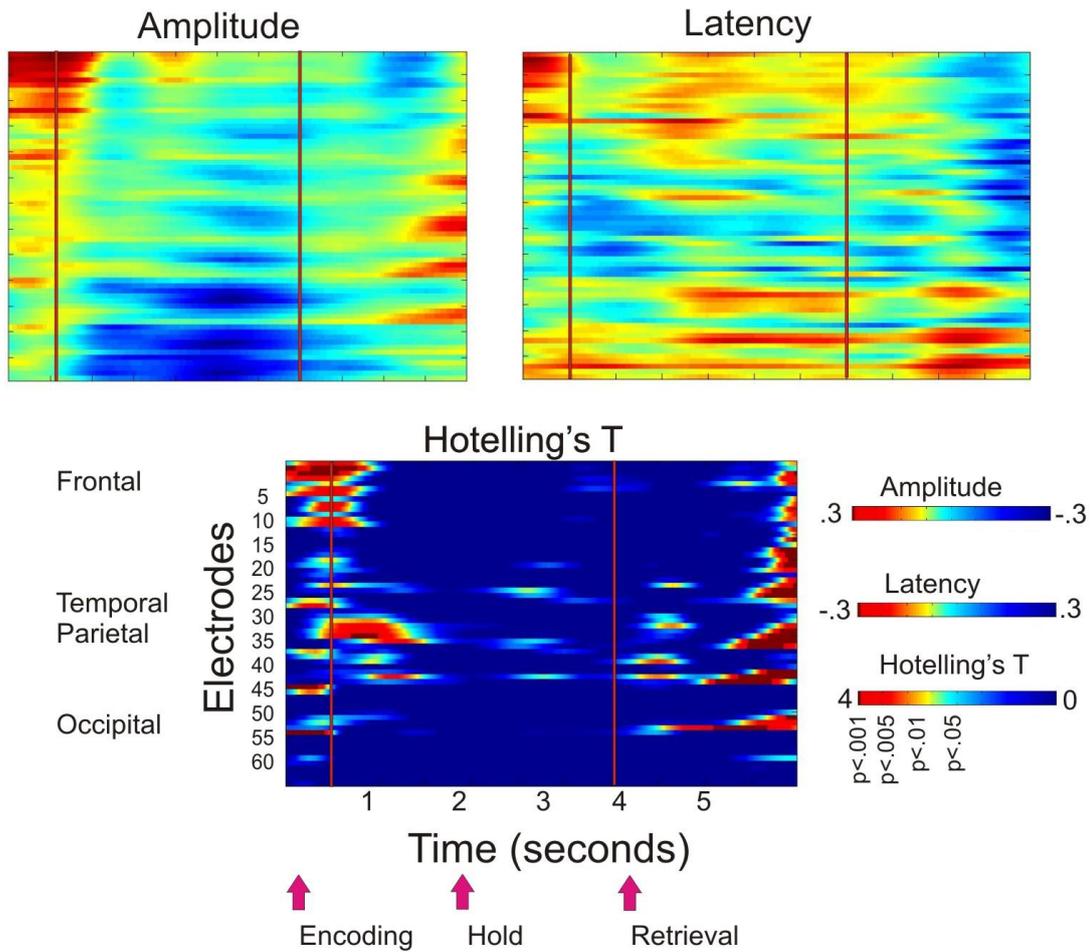
Bivariate correlations were performed to assess the relationship between working memory performance, age and IQ. Accuracy on the working memory task was significantly correlated with response time ( $r = -.29, p < .05$ ) and IQ ( $r = .47, p < .01$ ). There were no significant correlations between working memory performance and age. As a consequence age was not included as a covariate in the analyses conducted in this chapter.

### **6.3.2 SSVEP results: Working memory task effects**

In order to examine the temporal nature of the SSVEP associated with a spatial working memory task, cluster plots were generated to display SSVEP amplitude and latency differences for all electrodes (y axis) over time (x axis). Figure 6.1 shows that across the task duration, amplitude differences were clearly segregated according to the encoding, hold and response components of the spatial working memory task. Amplitude was reduced at frontal

sites during encoding, increased during the hold across parietal and occipital sites, and reduced at temporal and parietal sites during response. Latency was less variable over the 5.3 seconds displayed. Latency was increased over temporal parietal regions. Latency was decreased at occipital electrodes for the whole task and frontally until 1 second into response. Figure 6.1 demonstrates that Higher Hotelling's T values occur firstly at frontal electrodes during encoding, secondly at central sites at the beginning of the hold period, and thirdly at frontal, temporal and parietal sites from 1 second into presentation of the probe, until subjects made their response. Interestingly, these statistical effects were maximal during the encoding and response task components, but not during the 3 second hold.

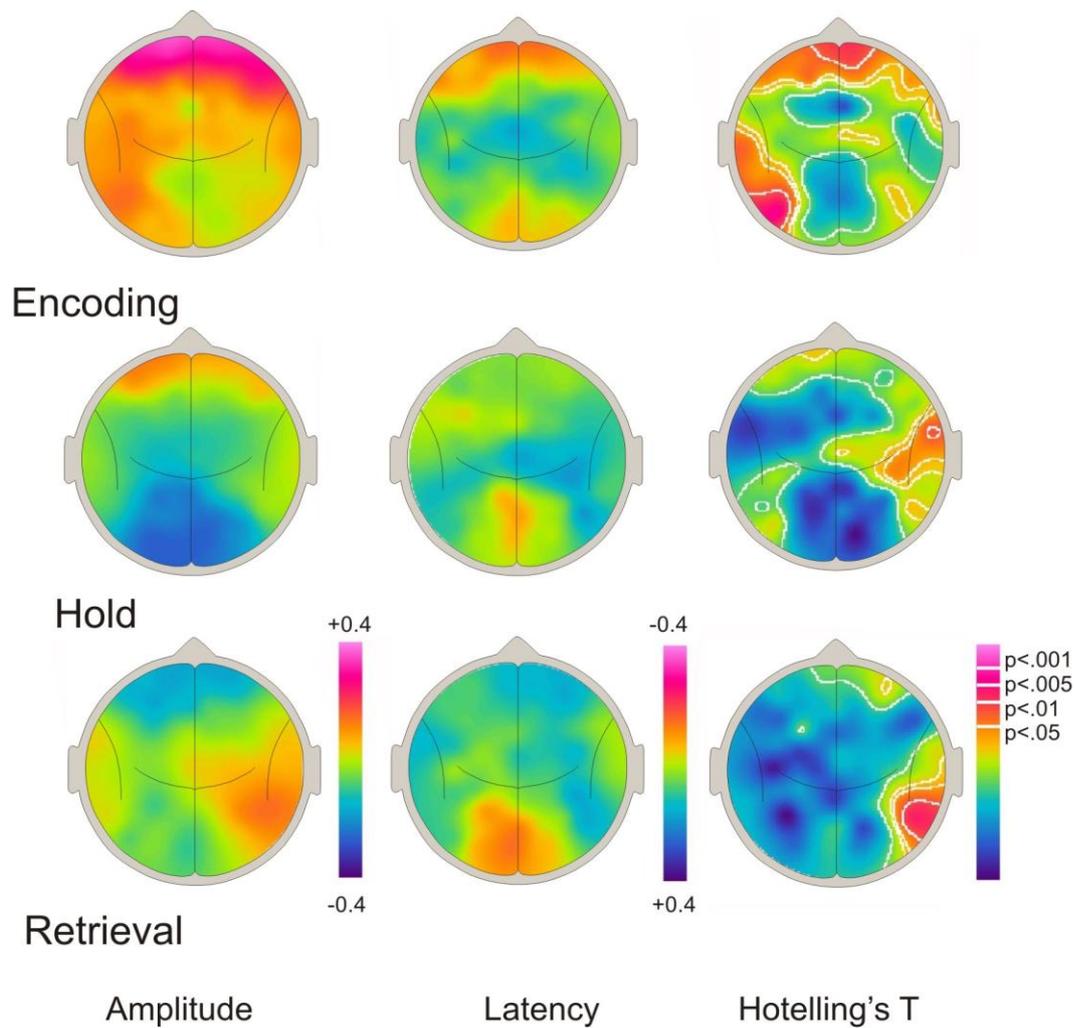
To compare the three stages of the working memory task repeated measures analysis of variance (ANOVA) was used to statistically compare the average of differences for each stage of encoding, hold and retrieval at electrode sites Fz, Cz and Pz. Whilst there were no significant differences between task components for latency there was a trend for amplitude ( $F(2,49) = 2.57, p = .08, \eta^2 = .05$ ). Repeated measures planned contrasts revealed amplitude differences during the hold and retrieval differed significantly ( $p = .02$ ), but there was no significant difference between the encoding and hold components ( $p = .18$ ).



**Figure 6.1** Cluster plots displaying SSVEP amplitude, latency and Hotelling's T for the spatial working memory DRT.

Time is presented on the  $x$  axis and electrodes on the  $y$  axis. Electrodes are approximately separated into frontal (electrodes 0 – 20), temporal parietal (electrodes 21 – 52), and occipital (electrodes 53 – 63) locations. Time points shown in the cluster plots represent the beginning of encoding through to the end of the response of the DRT. Cluster plots show amplitude on the left and latency on the right. Warmer colours represent amplitude or latency reductions relative to the control task, and cooler colours indicate amplitude or latency increases. Corresponding Hotelling's T statistics are shown at the bottom. Warmer colours represent greater statistical significance. Vertical lines separate sub-processes of encoding, hold and retrieval.

The subsequent Hotelling's T maps (Figure 6.2) present the SSVEP differences for specific task components of the DRT.



**Figure 6.2** Hotelling's T maps for the encoding, hold and retrieval periods of the DRT.

SSVEP amplitude is shown on the left, latency in the centre and Hotelling's T on the right. Warmer colours indicate amplitude decreases and latency decreases for the spatial working memory task relative to the control task. Conversely, cooler colours indicate amplitude and latency increases for the working memory task relative to the control task. Contours correspond to  $p$  values of  $p < .05$ ,  $.01$ ,  $.005$ , and  $.001$ .

*Encoding:* The top row of maps in Figure 6.2 represents a time point approximately 250ms into the 500ms perceptual encoding period of the task, whilst the stimulus dots were still visible on the screen. Encoding of the stimulus was associated predominantly with a frontal amplitude decrease relative to the control. Latency was decreased across frontal sites and

increased centrally. Statistical significance ( $p < .01$ ) was reached at bilateral frontal and left temporal sites for a pattern of decreased amplitude and latency, and these effects were maximal at left prefrontal and a left temporal site ( $p < .005$ ).

*Hold:* To investigate SST working memory effects associated with maintaining the location of the stimulus dots in memory, the mean SSVEP recorded during the 3 second „hold” period of the working memory task was compared to the mean SSVEP recorded during the corresponding 3 second period in the control task. The middle row of maps shown in Figure 6.2 displays bilateral amplitude decreases over temporal, parietal and frontal regions, with an increase in amplitude observed across central sites. Corresponding regions demonstrate an increase in latency, however, some prefrontal regions display a latency decrease. Hotelling’s T values reveal that these patterns of amplitude and latency differences were statistically significant over right frontal and temporal sites ( $p < .01$ ).

*Retrieval:* The lower row of maps represents a time point approximately 800ms after the presentation of the probe and two standard deviations prior to the average response on the working memory task. Amplitude is shown to be increased at prefrontal sites and decreased posteriorly. Latency is decreased at occipital sites and increased across all other regions. Hotelling’s T values indicate that increased latency and decreased amplitude reached statistical significance at a right temporal parietal region ( $p < .01$ ).

In summary, perceptual encoding was predominantly characterised by decreased amplitude and latency across bilateral frontal regions, maximal at left PFC and left parietal sites. Throughout the 3 second hold period, amplitude was decreased and latency increased at right frontal and temporal sites and at a more posterior right temporal site during retrieval.

### **6.3.3 SSVEP results : Correlations with performance**

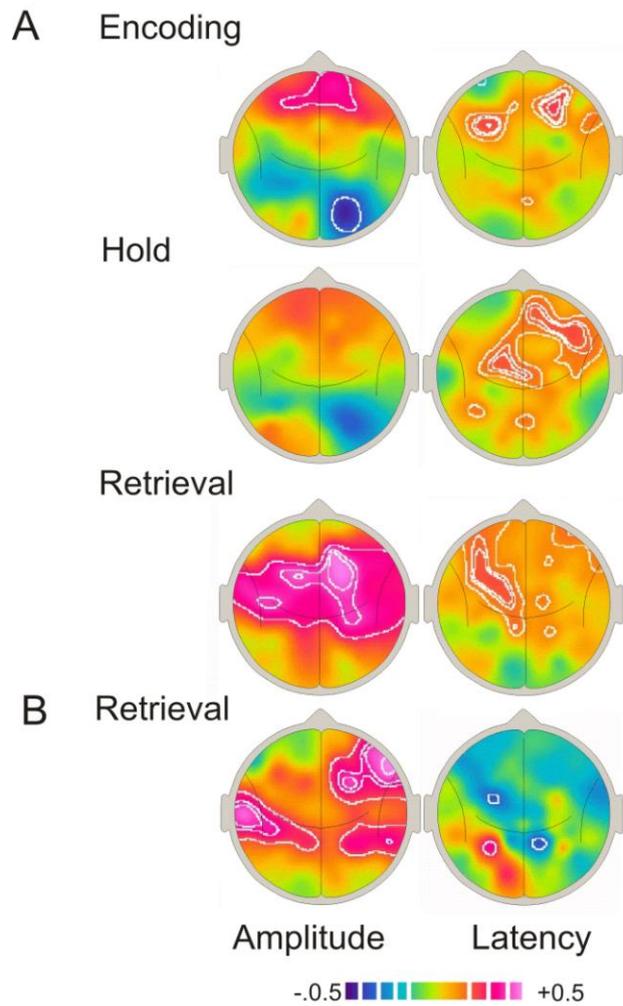
#### *Latency*

In order to investigate the SSVEP correlates of performance on the spatial working memory task, topographical maps were produced to display statistically significant correlations between SSVEP latency and response time. The same time points were used as those

displayed in the Hotelling's maps for encoding and response. As shown in Figure 6.3, the map for the hold period of the working memory task represents the peak correlation corresponding to a time point 2.5 seconds into the 3 second hold. To reduce the likelihood of making a type-1 error, correlations were only considered to be statistically significant if they occurred at electrodes within the same region for three successive time points. During encoding, a positive correlation was observed at left prefrontal sites encompassing F3 and surrounding sites, and right prefrontal sites. The same relationship was apparent at right prefrontal sites, including a cluster of electrodes around F4 and Cz, during the hold period. Retrieval was characterised by a correlation encompassing electrodes from C3 through to F3, and surrounding left prefrontal sites. These findings indicate that faster responders on the spatial working memory task demonstrated larger frontal latency decreases than slower performers. There were no correlations between SSVEP latency and the combined accuracy/response time measure which met the criteria for statistical significance.

#### *Amplitude*

Identical time points were investigated for SSVEP amplitude and are displayed in Figure 6.3. A significant positive correlation was identified over frontal regions, demonstrating that faster responders showed larger amplitude decreases relative to their slower counterparts. This relationship was apparent at F4 and surrounding electrodes during the retrieval task component only and did not reach statistical significance during the encoding or hold task components.



**Figure 6.3** Correlations between spatial working memory DRT performance and the SSVEP.

A) presents correlations between SSVEP amplitude and latency and response time on the DRT. B) displays correlations between SSVEP amplitude and latency and the combined response time and accuracy measure. Maps are only shown for time points when either amplitude or latency significantly correlated with performance. Warmer colours represent positive correlations and cooler colours represent negative correlations. Contours shown in both colours correspond to  $p$  values of  $p < .05$ ,  $.01$ ,  $.005$ , and  $.001$ .

Similarly, as shown in Figure 6.3, amplitude was related to the combined accuracy/response time performance measure during the retrieval component of the task only. A significant positive correlation was identified over left frontal and right temporal regions, demonstrating that better performers showed larger amplitude decreases relative to poorer performers. This

relationship was apparent at F4, F6, F8 and surrounding electrodes, and for temporal regions at T3 and surrounding sites.

There were no significant relationships between performance accuracy on this task and either SSVEP amplitude or latency. Correlations were investigated between subject's age and verbal IQ as measured by the Aus-NART, however there were no associations which met the criteria for statistical significance.

#### **6.4 Discussion**

The current study investigated the spatio-temporal dynamics of a spatial working memory task and the neural correlates of performance in elderly women using the SSVEP. The results indicate there was a clear distinction between the encoding stage of the spatial DRT and the hold period. Amplitude, in particular, was differentiated according to the task requirements involved in perceptual intake, holding information in memory and producing an appropriate response. In support of the hypothesis, amplitude and latency were decreased over frontal regions during encoding, and amplitude was increased over posterior brain sites during the hold period of the DRT. However, the dominant statistical effect constituted amplitude decreases and latency increases at right temporal sites and this pattern of activity was continued into the response.

In terms of the neural correlates of performance, a key finding of this study was the identification of a relationship between working memory behavioural performance and the SSVEP. Superior performance, as measured by faster responses, was associated with larger latency decreases at prefrontal and frontal sites throughout the entire spatial DRT. During the retrieval component of the DRT, SSVEP amplitude at frontal sites was positively correlated with response time, and latency was positively correlated with the combined accuracy and response time measure over right frontal and left temporal regions. Performance did not correlate with the SSVEP amplitude associated with the encoding or hold periods of the DRT.

*SSVEP working memory task effects in the elderly*

In the current study, the encoding period was associated with decreased amplitude and latency over bilateral prefrontal and left parietal regions. These amplitude and latency decreases during working memory encoding are comparable to the pattern of activity displayed previously by younger individuals (Silberstein et al., 2001; Ellis et al., 2006) and are consistent with perceptual processes observed during visual vigilance (Silberstein et al., 1990; Silberstein et al., 2000). These findings indicate that the SSVEP correlates of perceptual intake, involving the encoding of spatial information into working memory are maintained in old age.

In comparison, during the hold period, smaller statistical effects associated with posterior amplitude increase and frontal decrease were identified. In a prior study of the SSVEP, Ellis et al. (2006) identified two distinct patterns of SSVEP amplitude during the hold period of a spatial *n*-back task. A frontal amplitude increase characterized the early hold period, whilst the later hold component was associated with a topography of amplitude reductions in the PFC. The authors suggested that the shift from prefrontal amplitude increase to decrease may reflect a reallocation of the PFC from maintenance of memory content to the engagement of executive processes. During the hold in the present study, an amplitude reduction was observed right frontally, and approached statistical significance at bilateral prefrontal sites. An extensive body of electrophysiological and functional imaging literature has demonstrated a greater anterior activation in older than younger adults, generally interpreted as an increased reliance by the elderly on frontally mediated executive processes (Grady et al., 1994; Rypma & D'Esposito, 2000; McEvoy et al., 2001; Reuter-Lorenz et al., 2001; Muller & Knight, 2002; Daselaar et al., 2003; Friedman, 2003; Davis et al., 2008; West, Schwarb & Johnson, 2010).

The frontal SSVEP amplitude reduction during the hold period may reflect the greater reliance of this elderly subject group on the recruitment of compensatory executive processes during the simple maintenance of spatial working memory. Comparatively these same processes may only be required by younger adults during graded or more complex memory

manipulation tasks (Rypma et al., 2001; Missonnier et al., 2004). Decline to posterior cortical regions may require older adults to recruit more frontal cortical networks to compensate for loss of processing specificity (Grady et al., 1994; Park et al., 2004). For instance, the results from one study (Payer et al., 2006) revealed that older adults demonstrated decreased neural specialization in the ventral visual cortex, relative to younger adults, when they were required to remember either faces or houses. Interestingly, the decline in posterior specificity occurred at the same time as an increase in activity of the PFC. In the present study, the smaller accompanying posterior amplitude increases, which have previously been described as the dominant SSVEP working memory effect (Silberstein et al., 2001), may be indicative of an age-associated decline to posterior cortical resources. Whilst this suggestion cannot be confirmed in the current study due to the absence of a younger comparison subject group, findings from a prior study of ageing and the SSVEP indicate that neural processes in the elderly are reduced during the working memory hold component (Macpherson et al., 2009).

#### *SSVEP correlation with performance*

In the current study the correlations between the SSVEP and performance were apparent at frontal, prefrontal and temporal sites. Relationships have previously been demonstrated between comparable regions and speed of response in healthy subjects and those with schizophrenia, during response on an AX continuous performance task (Silberstein et al., 2000). Interestingly, these correlations were centered around the pre-motor and motor cortices, possibly reflecting a preparatory motor state. The dorsolateral prefrontal cortex (DLPFC) plays a role in cognitive control and motor preparation (MacDonald et al., 2000; Cole & Schneider, 2007), processes important for performance of DRTs. However, this interpretation can only be speculative, as the limited spatial resolution of this electrophysiological technique does not enable a definitive conclusion that the activity recorded from these sites was generated by the underlying brain regions.

In this study the SSVEP was correlated with measures of response time, but not accuracy, on the spatial working memory task. The finding that SSVEP latency correlated with response time, supports the premise that latency is related to neural processing speed (Silberstein et al., 2000). Conversely, amplitude was associated not only with response time, but also with a

combined accuracy and response time performance measure. These findings indicate that individually, measures of amplitude and latency may provide unique insights into the neural processes relevant to cognitive performance in the brain.

### *Latency*

In accordance with prior investigations of the SSVEP performance correlates in younger subject groups (Harris & Silberstein, 1999; Silberstein et al., 2000), topographical maps indicate that participants with faster response times display larger frontal latency decreases than their slower responding counterparts. This correlation with response time was observed across frontal and prefrontal sites during the encoding, hold and response components of the spatial working memory task. For the faster responders, this observation is indicative of an increase in neural processing speed in these regions possibly due to increased excitatory processes in the cortico-cortical feedback loops (Silberstein et al., 2001; Kemp et al., 2002; Gray et al., 2003). These results are consistent with the proposal by Rypma and Prabhakaran (2009) that performance speed is mediated by the efficiency of PFC connections to other cortical regions. These authors have suggested that faster performers benefit from rapid direct connections between brain regions, which may be particularly relevant to working memory DRT tasks by facilitating storage and strategies for successful performance. In the present study, evidence for increased excitatory processes in faster responders was not only limited to retrieval, but was also apparent at bilateral prefrontal sites during the intake and maintenance of spatial information. Given that latency was correlated with performance across the duration of the task, these results suggest that the larger latency reductions displayed by better performers may not be related to the individual task-related processes such as memory search or response preparation, but rather, may be due to the overall efficiency of working memory.

Shorter latency of the P300, another electrophysiological measure of processing speed (Polich, 1996), has also been associated with better performance on neuropsychological tests, including block design, matrices and digit span (Walhovd & Fjell, 2003). These findings are consistent with the premise that processing speed is related to cognitive ability (Deary, 2000). Differences in processing speed amongst the participants in this study may also be due to the effects of age. Processing speed decreases with age and has been described as a significant

contributor to working memory decline (Salthouse, 1990; Salthouse, 1996). In a study of the P300 across the adult life span, latency differences between the cognitively high and average performing groups were largest for the oldest subjects, suggesting that diverging patterns of cognitive ageing may lead to differential effects on processing speed in the brain (Riis et al., 2008). In the present investigation, the degree of cognitive decline experienced by each individual may also have exerted influence on neural processing speed.

Neuroimaging studies of working memory have identified age-associated detriments to the neural correlates of speed-related processing efficiency in the DLPFC (Rypma & D'Esposito, 2000; Rypma & D'Esposito, 2001). Decreases in white matter integrity may contribute to decreases in perceptual speed in the elderly (Madden et al., 2007; Madden et al., 2009). Age-related loss of white matter can disrupt the cortico-cortical pathways, leading to working memory disruption (Charlton et al., 2010) and slowing of mental processing (Ylikoski et al., 1993; Rabbitt et al., 2007). In the current study there was no relationship between SSVEP latency and age, although this may be due to the restricted age range of the sample.

### *Amplitude*

In terms of the SSVEP amplitude correlates of performance, larger amplitude reductions, suggested to correspond to greater cortical activation, were associated with superior task performance over frontal and temporal sites. As this relationship was only apparent during the retrieval stage of the DRT, it is possible that this pattern of activity is related to the efficiency of retrieval processes. In a prior study of working memory in which the SSVEP was elicited by a 10Hz, flickering diffuse light/dark contrasting background, better performance, as measured by a ratio of correct to incorrect responses, was correlated with larger 10Hz amplitude (Perlstein et al., 2003). In the current study, the specificity of the observed amplitude/performance relationship to memory retrieval, rather than the hold period of the DRT, may account for differences in the pattern of SSVEP amplitude identified.

SSVEP amplitude reductions have been likened to ERD in the upper alpha frequencies (Silberstein, 1995; Kemp et al., 2004). Alpha amplitude decreases with increased mental effort (Dujardin et al., 1993; Pfurtscheller & Lopes Da Silva, 1999) and analogously larger

SSVEP amplitude reductions have been observed at high task demands for working memory and recognition memory in young adults (Pipingas & Silberstein, 1995; Ellis et al., 2006). In a study conducted by Stipacek and colleagues (2003), memory load was increased across five levels of difficulty for a working memory task. The results of this study demonstrated greater alpha ERD at frontal, but not posterior, electrode sites for ascending difficulty, potentially indicating that frontal regions are more sensitive to increasing task demands. Alpha ERD has also been observed at parieto-occipital electrodes from 200-2000ms following the presentation of the probe in an auditory Sternberg task (Krause et al., 1996), and is likely to reflect the process of memory search and activation of lexical-semantic memory representations (Klimesch, 1997). In the current study the memory-scanning processes are more efficient in faster responders, as the relationship between SSVEP amplitude and response time is maximal, approximately 800ms, following the presentation of the probe during the response task component.

Cognitive ability has also been related to upper alpha ERD. In a study by Doppelmayr et al. (2005), subjects who performed better on measures of intelligence demonstrated larger ERD whilst undertaking a difficult verbal-semantic task. For high performers, ERD was maximal over left hemisphere centro-parietal regions relevant to verbal-semantic processing, as oppose to right posterior sites for low performing subjects. Whilst these results support the role of upper alpha ERD in semantic memory, they may also indicate that upper alpha reductions in higher performers should be evident across brain regions relevant to successful performance of the particular cognitive task under investigation. In the present study, SSVEP amplitude was correlated with performance at frontal and temporal regions, maximal at sites corresponding to the right PFC. The role of the right PFC, particularly the premotor area has been implicated in the rehearsal of spatial content in working memory (Smith et al., 1995; Smith & Jonides, 1997). Consequently, differences in performance-related SSVEP amplitude may be due to variations in the efficiency of the spatial rehearsal and retrieval processes mediated by this region.

Other studies have shown that cognitive performance is related to smaller, rather than larger, ERD. This occurrence has been interpreted in terms of a „neural efficiency“ hypothesis, which proposes that higher-ability individuals require less cortical activation while

performing cognitive tasks (Neubauer et al., 1995). Recently it has been suggested that when performing difficult cognitive tasks, particularly those which tax working memory, greater ERD will be observed in superior performers (Neubauer & Fink, 2009). Task accuracy in the current study indicates that participants found the spatial DRT to be challenging, thus the larger SSVEP amplitude reductions in better performers could possibly represent the ability of these individuals to invest more cortical resources, when necessary, to enable successful task performance.

Age-related changes in ERD in the alpha frequency range have also been documented. In contrast to younger adults, reduced alpha ERD has been observed in the elderly during auditory working memory retrieval, suggestive of age-related alterations in oscillatory responses in the later stages of the life-span (Karrasch et al., 2004). In a subsequent study of dementia, these changes were found to be particularly prominent in individuals with Alzheimer's disease (AD), for frequencies in the 7–17Hz range across frontal, central and left temporal sites, where ERD was present in healthy controls and absent in the AD group (Karrasch et al., 2006). Less ERD in the lower alpha band has been observed in individuals with mild cognitive impairment, a condition which usually represents a transitional stage of AD (Petersen, 2004), than in age-matched controls, during performance of a picture memory task (van der Hiele et al., 2006). The findings from these studies imply that alpha reductions related to working memory are affected by normal ageing and this may be exacerbated in pathological ageing. In the current study, there was no relationship between age and either component of the SSVEP, nor was there an association between age and task accuracy. The absence of such relationships may be due to the restricted age range of participants (64-82 years) within the elderly group.

#### **6.4.1 Limitations and future directions**

There are several limitations to the current study. Whilst the primary aim of this investigation was to establish the SSVEP correlates of performance and to gain an understanding of the SSVEP profile of working memory task effects in an elderly sample, the inclusion of a younger subject group would have enabled a more precise examination of age effects on the SSVEP. This may be especially relevant as the sample of elderly participants reported

subjective memory complaints, a condition which may correspond to early stages of memory impairment (Jorm et al., 2001). By indirectly comparing the results from these elderly participants to previous findings from younger adults, methodological differences between tasks and study designs cannot be fully accounted for.

Despite the relatively large subject numbers by neuroscientific standards, the inclusion of an all female sample may restrict the generalization of these findings. Differences in brain volume and neurochemistry have been identified between the sexes (Cosgrove, Mazure & Staley, 2007). Gender has been shown to influence performance on tasks of spatial, verbal, autobiographical, and emotional memory (Andreano & Cahill, 2009), and activation of brain regions including the primary sensorimotor, premotor cortex, superior parietal and lateral sulcus during visually guided movements (Gorbet & Sergio, 2007). A finding of particular importance to the current study is that gender may also impact on the relationship between fluid intelligence and alpha ERD during working memory performance (Neubauer et al., 2002; Grabner et al., 2004; Neubauer & Fink, 2009). In the study conducted by Grabner et al., (2004), a negative correlation between intelligence and ERD was identified in males, whereas in females there was a trend for higher intelligence to be associated with larger ERD. By focussing on a female only sample, the influence of gender on the SSVEP correlates of performance cannot be determined. For this reason, a replication of this study in elderly males may be beneficial to increase the applicability of these results.

Due to the observation that the spatial working memory task did not to elicit a clear pattern of grading effects, analysis of the corresponding SSVEP data could not be carried out. Comparison of different memory loads (i.e. holding the location of two dots or three dots in memory) may also have provided valuable insight into the effects of task difficulty and cognitive effort. This is especially relevant given that cognitive task difficulty has been shown to influence the relationship between performance and cortical activation (Doppelmayr et al., 2005). Obtaining subjective ratings of task or trial difficulty from participants may also be a valuable method of ascertaining cognitive effort.

The approach taken to assess cognitive performance in the current study was to correlate measures of behavioral performance on the spatial DRT with the brain activity recorded from the same task. Whilst some studies have assessed performance in a similar manner (Rypma & D'Esposito, 2000; Rypma et al., 2007), others have focused on IQ measures later correlated with neural activity recorded from a separate brain activation paradigm (Neubauer et al., 1995; Grabner et al., 2004; Grabner et al., 2006). Working memory represents an important component of fluid intelligence (Engle et al., 1999), thus performance on a working memory task should still reflect general ability. Future studies could benefit from including a full scale IQ assessment such as the Weschler Adult Intelligence Scale (WAIS), or the Raven's Progressive Matrices, rather than the more basic Aus-NART measure of verbal IQ, which was primarily used for screening purposes, and was not found to correlate with the SSVEP. This may be particularly relevant as higher IQ, as measured by the WAIS, has been associated with greater SSVEP latency increases frontally during performance of the Raven's Progressive Matrices (Song, 2005), and similar results have been observed on a working memory task (Van Rooy et al., 2001). These findings indicate that inhibitory processes as measured by the SSVEP may also be important for cognitive performance, but may not be apparent when response time measures of lower order cognitive processes are correlated with brain activity.

#### **6.4.2 Application of SSVEP findings to neuropharmacological interventions**

The sensitivity of both the amplitude and latency components of the SSVEP to inter-individual differences in performance, indicate that these measures of brain activity may also be appropriate to investigate improvements in neurocognitive performance following neuropharmacological intervention. Other indices of brain electrical activity, described within this chapter to be differentially associated with variations in cognitive performance, have previously been used for such a purpose. For instance, superior cognitive performance has been associated with shorter P300 latencies (Walhovd & Fjell, 2003), and substances with cognitive enhancing effects have been shown to decrease latency on this measure. Specifically, a reduction in P300 latency has been identified in healthy adults following administration of the cognitive enhancing substance methylphenidate, a dopamine reuptake blocker that increases the availability of dopamine at receptor sites (Cooper et al., 2005).

P300 latency has also featured as a neurocognitive measure in studies of nutraceuticals including *Ginkgo biloba* and ginseng, purported cognitive enhancers (Kumar, 2006), believed to exert actions on the cholinergic neurotransmitter system (Benishin et al., 1991; Di Renzo, 2000). In one study, P300 latency was reduced in memory impaired elderly following both acute and two month's *Ginkgo biloba* treatment (Semlitsch et al., 1995). A trend towards decreased P300 latency was identified following four weeks Ginkgo administration (Page et al., 2005), and similarly a trend for reduced P300 latency was observed in an acute trial of combined *Ginkgo biloba* and ginseng ingestion (Dimpfel et al., 2006). In addition to reducing P300 latency, acute dosage of ginseng has been found to modulate alpha activity (Kennedy et al., 2003), an EEG frequency band closely linked to cognitive performance. Comparatively, acute administration of nicotine, has been demonstrated to modify the amplitude of the 13Hz SSVEP (Thompson et al., 2000).

It is possible that behavioral improvements obtained from nutraceuticals may occur as a result of an increase in processing speed in the brain. The studies described above provide evidence for this premise by demonstrating that some cognitive enhancers increase neural processing speed, as measured by reductions in P300 latency. The current chapter revealed that superior working memory performance was associated with larger SSVEP latency reductions, also indicative of faster neural processing speed (Silberstein et al., 2000). Therefore, it may be anticipated that improved performance on a working memory task due to the effects of a cognitive enhancer may be associated with a decrease in SSVEP latency in regions of the brain identified in the current study and literature (Smith & Jonides, 1997; Wager & Smith, 2003; Zimmer, 2008) to be relevant to performance of a working memory task. As both larger latency and amplitude reductions were associated with better performance indices during a working memory task in this experiment, it may be expected that faster neural processing (i.e latency reduction) and increased cortical activation (i.e amplitude reduction) represent the neural signature of improved brain function during spatial working memory.

### 6.4.3 Summary and conclusions

In summary, the results of this study demonstrate that in a sample of elderly women, the profile of neural activity is differentiated for the task components of encoding, holding information „online“ and retrieval. These findings demonstrate that the SSVEP is a sensitive measure of working memory sub-processes in an elderly sample. Consistent with prior studies in younger adults (Silberstein et al., 2001; Ellis et al., 2006), amplitude and latency were decreased over frontal regions during encoding and amplitude was increased over posterior brain regions during the hold period of the DRT task. Amplitude increases during the hold were smaller than those identified in younger adults (Silberstein et al., 2001) and this reduction in neural processes supports previous findings which demonstrate that in the elderly, the SSVEP related to holding information „online“ is attenuated during the hold period of a working memory DRT (Macpherson et al., 2009). The pattern of SSVEP amplitude reductions and latency increases observed during the retrieval task component at right temporal parietal regions was similar to that observed during the hold period. However, it was during retrieval that the SSVEP demonstrated the most prominent relationship with task performance.

Examination of the SSVEP correlates of performance indicate that superior spatial working memory performance was related to greater latency reductions at frontal and prefrontal sites, indicating that faster performers demonstrated an increased rate of neural processing, corresponding to greater neural excitation at these sites. Better spatial working memory performance was also associated with larger amplitude reductions, suggestive of an increase in cortical activation, at prefrontal, frontal and temporal sites. The relationship between performance and the SSVEP was most prominent during the response stage of the working memory task and may reflect greater memory search efficiency.

Findings from this experiment indicate that the SSVEP is a useful measure of working memory sub-processes, and is sensitive to subtle inter-individual differences in working memory performance. These features indicate the SSVEP may be responsive to the potential cognitive enhancing and nutraceutical effects of multivitamin supplementation. Specifically, the pattern of SSVEP amplitude and latency associated with „good“ task performance may aid

The SSVEP correlates of working memory

in the interpretation of changes in neural processes which accompany multivitamin supplementation.

## **Chapter 7 The effects of multivitamin supplementation on brain electrical activity as measured by the SSVEP**

The findings reported in Chapter 5 indicated that chronic supplementation with a multivitamin, mineral and herbal supplement was capable of enhancing spatial working memory. In the prior experimental chapter, the spatio-temporal dynamics of a spatial working memory delayed response task (DRT) were examined using the steady state visually evoked potential (SSVEP). In this chapter it was established that the SSVEP is a suitable measure of working memory sub-processes in the elderly, and is sensitive to inter-individual differences in working memory performance. In the current experiment, the same DRT was used as a neural activation task and steady state topography (SST) was utilized to investigate the effects of the multivitamin on brain electrical activity. This represents the first study to examine the effects of multivitamin supplementation on measures of brain function in the elderly.

### **7.1 Introduction**

As individuals enter the later stages of the life span, adequate nutritional intake becomes pertinent to healthy neurological function. Selected vitamins and micronutrients are known to exert direct effects on neurophysiological parameters, and thus represent potential regulators of cognitive function (Huskisson et al., 2007). Via their role in the methylation cycle, vitamins B<sub>6</sub>, B<sub>12</sub> and folate are required for the production of neurotransmitters including dopamine, noradrenalin and serotonin (Selhub et al., 2000). These vitamins are also responsible for lowering levels of homocysteine, a sulphur-containing amino acid, which when elevated, can induce DNA strand breakage, increase oxidative stress, and trigger apoptosis in the brain (Mattson & Shea, 2003).

The brain requires large quantities of oxygen and as a consequence, lipids, proteins and nucleic acids in neurons are vulnerable to the effects of oxidative stress (Floyd & Carney,

1992; Mariani et al., 2005). It is understood that vitamins A, C, E, selenium and co-enzyme Q10 impart antioxidant effects and protect neural tissue from aggression by free radicals (Bourre, 2006). A range of mechanisms in the brain unrelated to antioxidant activities have also been proposed (Zingg & Azzi, 2004; Linnane, Kios & Vitetta, 2007; Gómez-Pinilla, 2008). Vitamin E may augment synaptic plasticity by protecting synaptic membranes from the deleterious effects of oxidative stress (Wu, Ying & Gomez-Pinilla, 2004). Vitamin C is required for the transformation of dopamine into noradrenalin (Bourre, 2006). Additionally the function of this vitamin has been suggested to extend to neuromodulation of dopamine, regulation of acetylcholine and catecholamine release, and glutamate and GABA-mediated neurotransmission (Rebec & Pierce, 1994; Harrison & May, 2009).

Neuroprotective effects and nootropic properties of a range of antioxidant herbal extracts have been proposed (Mantle et al., 2000; Kennedy & Scholey, 2006; Kumar, 2006; Tapsell et al., 2006). *Ginkgo biloba* represents one such herbal with putative actions including inhibition of neuronal apoptosis (Ahlemeyer & Kriegstein, 2003), enhanced synaptic plasticity and neuronal excitability (Williams et al., 2004), and increased cerebral blood flow due to vasodilation (Mantle et al., 2000). Actions of *Ginkgo biloba* may also involve direct effects on the cholinergic neurotransmitter system essential for the regulation of cognition (Di Renzo, 2000). Mechanisms of *Bacopa moniera*, curcumin (turmeric) and grape seed also indicate these botanicals may influence neural function, although cognitive effects in humans are still poorly understood (Frautschy et al., 2001; Ramassamy, 2006; Sarkaki, Farbood & Badavi, 2007; Wang et al., 2008).

Despite reputed neurophysiological mechanisms of B vitamins, antioxidants and herbal extracts, the cumulative effects of these substances on brain function have not been investigated. Evidence from behavioural studies, including the experimental data from Chapter 5 of this thesis, indicates that dietary supplementation with combined multivitamin and antioxidant herbal formulas can enhance cognition (Summers et al., 2010; Harris et al., 2011). However, examination of brain electrical activity may enable greater insights into the neurocognitive effects of chronic multivitamin supplementation than can be obtained from neuropsychological or other behavioural measures alone. Brain electrical measures possess the ability to capture and monitor the timing of cognitive improvements, and enable the

inspection of specific cognitive sub-processes. To date, no studies have been conducted to investigate the effects of such multivitamin preparations on brain function. Subsequently the current study examined the effects of chronic supplementation with a multivitamin, mineral and herbal formula on brain function, using the SSVEP measure of brain electrical activity.

SST enables the inspection of neural processes with high temporal precision, and this characteristic has rendered the SSVEP a sensitive measure of variations in performance and task demands across a range of cognitive domains (Silberstein et al., 1990; Silberstein et al., 2000; Ellis et al., 2006). A further advantage of SST is the responsiveness of this technique to neuropharmacological manipulation during cognitive activation. For example, during performance of a low demand visual vigilance task, nicotine administration was observed to increase the amplitude of the 13Hz SSVEP at right parietal regions, and to reduce latency across bilateral frontal regions (Thompson et al., 2000). These alterations in neural activity were suggested to potentially reflect alterations in cortical arousal. In another trial, citalopram, a selective serotonin re-uptake inhibitor was demonstrated to augment the SSVEP amplitude in parieto-occipital cortices during the viewing of images with pleasant valence and to suppress anterior–frontal and occipital SSVEP amplitude and latency for the viewing of negative valence images (Kemp et al., 2004). Conjointly, these findings point to the utility of the SSVEP in neuropharmacological research.

The current study explored the effects of 16 weeks treatment of a combined multivitamin, mineral and herbal supplement on the SSVEP measure of brain electrical activity, associated with the performance of a spatial working memory DRT. Evidence from Chapter 6 of this thesis, together with additional studies (Pipingas et al., 2008; Ryan et al., 2008), have indicated that in the elderly, cognitive domains such as working memory, which decline with age, may benefit from nutraceutical intervention. As the neural substrates of spatial working memory have also been demonstrated to decline with age (Reuter-Lorenz et al., 2000; McEvoy et al., 2001; Rypma et al., 2001), it may be anticipated that the neural processes which subserve working memory performance may also be enhanced following nutraceutical intervention.

Working memory is composed of higher-order cognitive processes which are subserved by a network of neural regions encompassing the prefrontal cortex (PFC), parietal and temporal lobes (Wager & Smith, 2003). Findings from the previous chapter of this thesis confirmed the importance of these regions for spatial working memory in the elderly. Specifically, it was revealed that superior spatial working memory performance was related to greater SSVEP latency reductions at frontal and prefrontal sites across the task duration. Such latency decreases were suggestive of an increased rate of neural processing across these regions. Better performance was also associated with larger amplitude reductions, indicative of an increase in cortical activation at prefrontal, frontal and temporal sites during working memory retrieval. Based on these findings it was suggested that the pattern of SSVEP amplitude and latency characteristic of „good“ task performance during working memory may aid in the interpretation of changes in neural processes which accompany multivitamin supplementation.

### **7.1.2 Aims and hypotheses**

The aim of the current experiment was to investigate the effects of chronic multivitamin supplementation on the SSVEP associated with a spatial working memory DRT in elderly women. In this double-blind, placebo-controlled, 16 week trial, it was predicted that the SSVEP would be a sensitive measure of the cognitive enhancing effects of multivitamin supplementation in the elderly. Specifically, it was hypothesized that during working memory hold and retrieval, greater SSVEP latency reductions indicative of increased processing speed, would be observed across frontal, temporal and parietal brain regions after chronic multivitamin supplementation. It was further anticipated that latency reductions would be accompanied by amplitude reductions, suggestive of greater cortical activation.

## 7.2 Method

### *Study Design*

The trial was a 16 week, randomised, placebo-controlled, double-blind, parallel group investigation. Eligible participants were randomly allocated the Swisse Women's Ultivite 50 plus™ supplement or a placebo, with an allocation ratio of 1:1. Full details of the clinical trial methods are described in Chapters 4 and 5.

### *Participants*

In total, 49 elderly women completed the baseline and post-treatment SSVEP recording component of the clinical trial. Participants were right-handed, non-smokers, with no history of stroke, epilepsy, dementia, Parkinson's disease, head trauma, excessive alcohol use, mental illness, depression, anxiety disorders and were not using anti-depressant, anti-anxiety medication or any medications with a cognitive enhancing effect. The participant flowchart is shown in Figure 4.1.

### **7.2.1 Procedure**

#### *Baseline and Post-treatment Testing*

Participants attended the baseline testing session at the Brain Sciences Institute at 1030 or 1130 hours. Participants were requested to refrain from consuming tea or coffee for two hours prior to attending this appointment. During the baseline testing session, participants first underwent verbal and computerised cognitive assessment, followed by cardiovascular measurements. Finally, the SSVEP was recorded whilst participants performed a spatial working memory task and a control task. Participants returned for a post-treatment testing appointment 16 weeks after baseline testing. Subjects who could not attend on the scheduled return date were allowed a time window of five days to attend the testing session. The SSVEP recording procedure from baseline was repeated with alternate forms of the working memory and control tasks.

The same SSVEP recording procedure documented in Chapter 6 (Section 6.2.2) applies to the current chapter. The spatial working memory DRT used in this experiment was described in Section 6.2.3.

### **7.2.2 SSVEP signal processing**

SSVEP signal processing was conducted using BrainSci (SSPT Analysis Software, version 2). This procedure was described in Chapter 4, section 4.3.5. In brief, time series SSVEP data was calculated, and 6.3 second epochs of data centred on the presentation of the fixation cross and associated with correct task responses were extracted from the Fourier time series and averaged. SSVEP amplitude and latency were subjected to normalization and then averaged across participants. In order to isolate the activity associated with the hold period of the working memory task, epochs consisting of the mean activity across the 3 second hold period of the DRT were averaged, whilst SSVEP amplitude and latency were subjected to normalization and then averaged across participants.

SSVEP data was also analysed using a mixed between and within-subjects repeated measures analysis of variance (ANOVA) design in SPSS Version 17. Each individual participant's SSVEP data, for all 64 electrodes, was extracted from BrainSci using custom designed scripts in Matlab. Prior to extraction, SSVEP amplitude was normalised for each individual and latency was rotated with reference to the control task.

### **7.2.3 Statistical analysis**

#### *Behavioural data*

In order to investigate changes spatial working memory and control task response time following the four month supplementation period, 2 time (baseline, post-treatment) by 2 treatment (multivitamin, placebo) repeated measures analysis of variance (ANOVAs) were conducted on the data, with time as the within subjects variable. Repeated measures ANOVAs were also used to investigate the effects of the multivitamin on the corresponding measures of accuracy.

To correct for the effects of potential differences in performance at baseline, and to maintain consistency with the statistical analysis adopted in Chapter 5 of this thesis, univariate analysis

of co-variance (ANCOVA) was used to examine the effect of treatment group on change in response time and accuracy. The relevant baseline measure was included as a covariate.

#### *SSVEP baseline spatial working memory DRT effects*

SSVEP differences were calculated to compare the baseline working memory DRT with the baseline control task. These differences are presented separately for the multivitamin and placebo groups in the form of statistical cluster plots. Hotelling's T presents the statistical strength of these differences. These plots represent the same data presented in Chapter 6, Figure 6.1, however in the current chapter, participants have been divided into the multivitamin and placebo groups, and relevant participants have been excluded. The cluster plots in this chapter demonstrate the working memory effects across the task duration for the multivitamin and placebo groups, and enable the SSVEP activity displayed by the two groups at baseline to be compared qualitatively. For greater detail regarding cluster plot presentation of SSVEP data, refer to Section 6.2.5.

#### *SSVEP treatment effects*

To investigate the effects of the multivitamin treatment on the brain electrical activity, differences between the post-treatment spatial working memory task and the corresponding baseline spatial working memory SSVEP were examined for the hold period of the DRT and for retrieval. To calculate differences for the 3 second hold period of the DRT, the mean SSVEP amplitude and latency associated with the post-treatment working memory task was compared to the equivalent mean 3 second period from the baseline working memory task. To calculate differences for retrieval, a single time point during retrieval was extracted from the time series data for the post-treatment DRT, and was compared to the same time point during retrieval for the baseline DRT. This same retrieval time point was used in all analyses included in this thesis, and represents the time during retrieval at which the greatest correlations between the SSVEP and spatial working memory performance were observed in Chapter 6 (see Figure 6.3). Differences comparing the post-treatment SSVEP with the baseline SSVEP during the hold and retrieval periods of the DRT are presented in the form of

difference and Hotelling's T topographical maps. This analysis was carried out separately for the multivitamin and placebo groups.

For both cluster plots and topographical maps, Hotelling's T was used to estimate the probability of falsely rejecting the null hypothesis (type-1 error) associated with task differences in the SSVEP latency and amplitude. Spatial principal components analysis has shown the SSVEP forms five independent factors (Silberstein & Cadusch, 1992), consequently the Hotelling's T statistic  $p$  values (2 tailed) were divided by five to correct for multiple electrodes. For all Hotelling's T maps, contours correspond to  $p$  values of  $p < .05$ ,  $.01$ ,  $.005$ , and  $.001$ . A more stringent alpha level of  $.005$  was used to determine statistical significance in this experiment to correct for the inclusion of two treatment groups.

#### *SSVEP analysis of variance*

Hotelling's T represents a within subjects measure and, for this reason, cannot be used to statistically determine between subjects effects in study designs where there is more than one treatment group. Hotelling's T maps only provide a qualitative comparison of the treatment groups, which in the field of neuroscience, is not sufficient to determine if a treatment effect exists (Nieuwenhuis, Forstmann & Wagenmakers, 2011). To overcome this potential limitation, a mixed between and within-subjects repeated measures analysis of variance (ANOVA) was used as a confirmation of the multivitamin effects on the SSVEP. In this analysis, only midline electrode sites including FZ, Cz and PZ were examined. This approach has the advantage of reducing type-1 error (Kemp et al., 2004) by focusing only on pre-determined cortical sites.

SSVEP amplitude and latency data, representing the average of each of the working memory encoding, hold and retrieval periods, were examined using mixed between and within-subjects repeated measures ANOVAs. Statistical analysis was conducted to compare the SSVEP related to the working memory task at baseline and post-treatment. SSVEP data was entered into a 2 time (baseline, post-treatment) x 2 treatment (multivitamin, placebo) x 3 electrode (FZ, CZ, PZ) repeated measures ANOVA, with treatment as the between subjects factor. To correct for the 3 stages of the working memory task, an alpha level of  $0.05/3$  ( $p < .016$ ) was used to determine statistical significance.

### 7.3 Results

The final participant sample consisted of 22 participants in the multivitamin group and 19 in the placebo group. One-way analysis of variance indicated that participant groups were matched on the demographic characteristics of age, Aus-NART IQ, years of education, MMSE score and subjective memory complaint rating. The same participants were included in all analyses within this experiment.

#### *Excluded participants*

Three participants were excluded from EEG analysis due to low behavioural performance on the working memory task. SSVEP data from a further five participants was unable to be extracted either from the EEG, or from the SSVEP data analysis program Brainsci. These individuals were excluded from all analysis within this chapter.

#### **7.3.1 Behavioural results**

The means and standard deviations for working memory response time and accuracy, corresponding to the two treatment groups at baseline and post-treatment are presented in Table 7.1. One way ANOVA revealed that at baseline, performance did not differ significantly between the multivitamin and placebo groups on the working memory or control task.

To examine multivitamin treatment effects and changes in response time on the spatial working memory task and control task following the four month supplementation period, 2 time (baseline, post-treatment) by 2 treatment (multivitamin, placebo) mixed design ANOVAs were conducted.

For working memory response time it was revealed there was a significant main effect of time ( $F(1, 39) = 6.28, p = .02, \eta^2 = .14$ ), however there were no significant interactions with treatment. Equivalent reductions in response time of approximately 50ms were identified for both groups.

For the control task, there was a statistical trend for a main effect of time, representing a reduction in response time for both groups ( $F(1, 39) = 3.47, p = .07, \eta^2 = .07$ ). There were no

significant interactions or main effects of time identified for the corresponding measures of accuracy.

Univariate ANCOVA, with the relevant baseline measure added as a covariate was used to examine the effect of treatment group on change in response time and accuracy. There were no significant effects of the treatment on response time or accuracy for the DRT or control task.

**Table 7.1** Means and standard deviations for baseline and post-treatment working memory DRT and control task performance

Cognitive Task	Treatment Group	Baseline		Post Treatment	
		Mean	SD	Mean	SD
Working Memory RT	Multivitamin	<b>1143</b>	<b>143</b>	<b>1094</b>	<b>171</b>
	Placebo	<b>1091</b>	<b>126</b>	<b>1041</b>	<b>134</b>
Control Task RT	Multivitamin	958	135	933	97
	Placebo	928	113	887	93
Working Memory %	Multivitamin	62	9	62	11
	Placebo	63	10	64	12
Control Task %	Multivitamin	96	4	97	3
	Placebo	95	5	94	13

Bold font indicates significant main effect of time

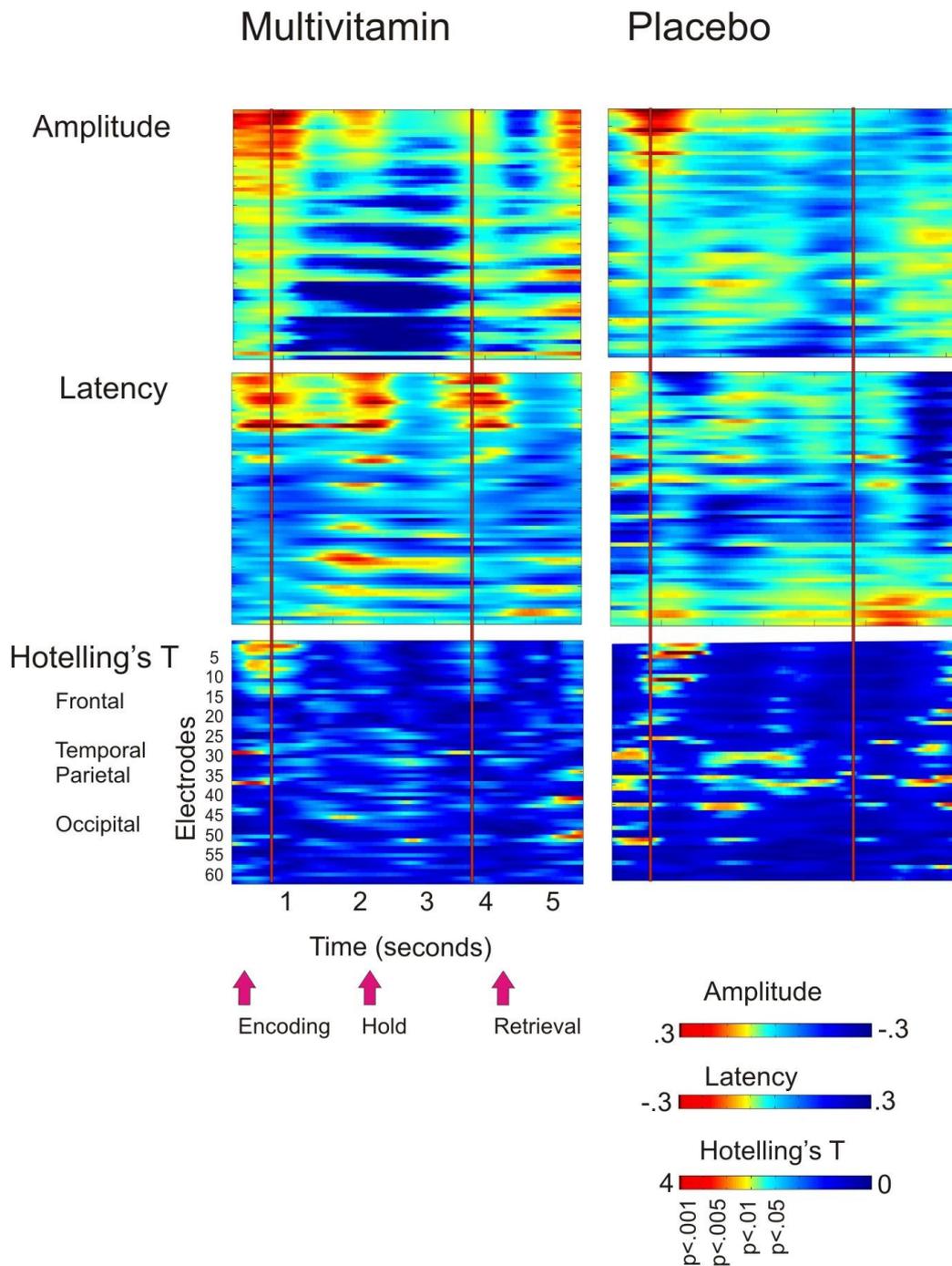
Multivitamin N =22, Placebo N = 19, RT = reaction time, % = percentage correct

### 7.3.2 SSVEP results

#### *SSVEP baseline spatial working memory DRT effects*

Figure 7.1 shows the SSVEP differences between the spatial working memory DRT and the control task at baseline. For both treatment groups, amplitude differences were segregated according to the encoding and hold components of the spatial working memory task. As represented by the data, the pattern of amplitude was similar for both the multivitamin and placebo groups, indicating a reduction in amplitude at frontal sites during encoding, and an increase during the hold. Both groups also demonstrated a latency increase across the hold period and a posterior latency reduction during retrieval. Latency was decreased frontally for the multivitamin group across most of the task. Differences between the baseline DRT and control task reached statistical significance at frontal sites during encoding and temporal parietal sites during retrieval for both treatment groups. The placebo group also demonstrated statistical differences at some temporal and parietal sites across the entire task duration. These results indicate that relative to the baseline control task, the multivitamin and placebo

treatments were associated with similar patterns of SSVEP at baseline. The effects for both groups resemble the pattern of SSVEP demonstrated across all participants in Chapter 6 (see Figure 6.1).



**Figure 7.1** Cluster plots displaying SSVEP amplitude, latency and Hotelling's T for the spatial working memory DRT at baseline for the multivitamin and placebo groups.

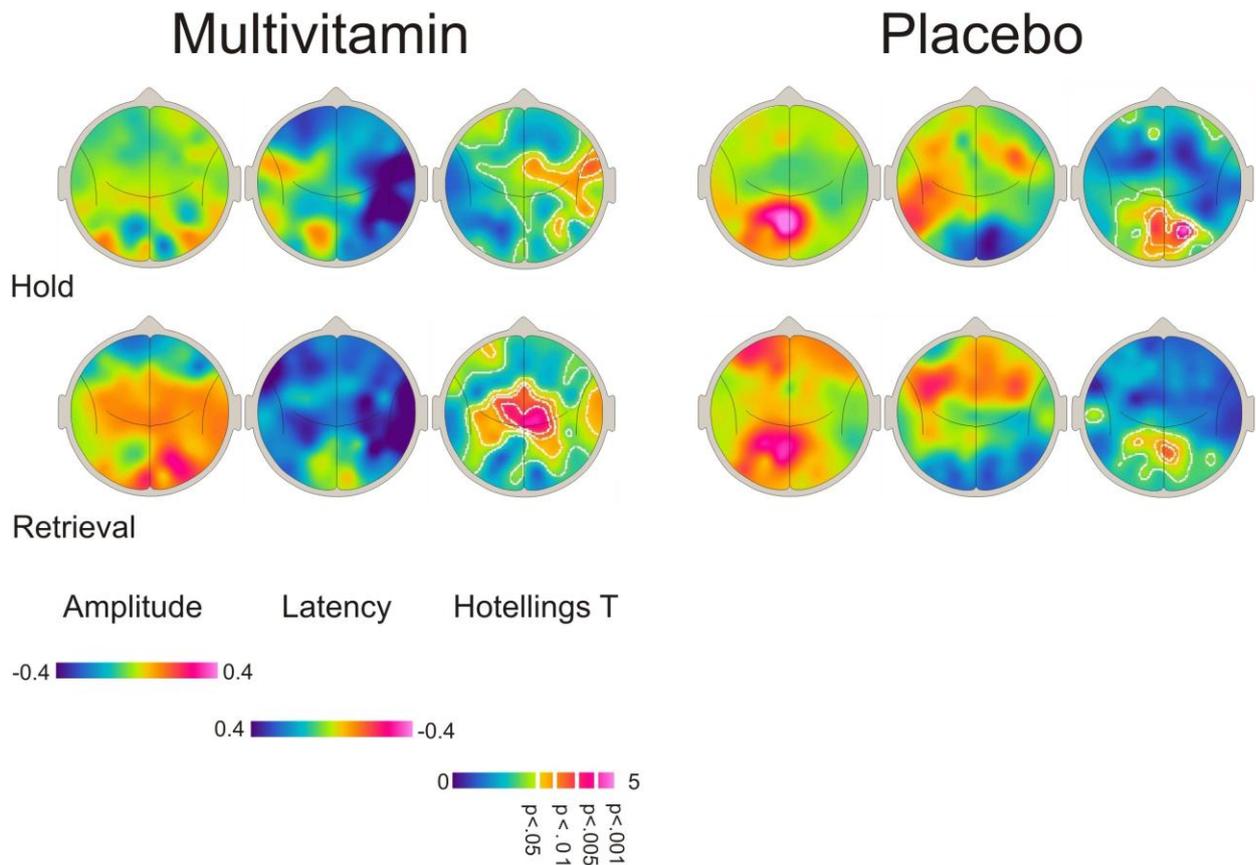
Time is presented on the  $x$  axis and electrodes on the  $y$  axis. Electrodes are approximately separated into frontal (electrodes 0 – 20), temporal parietal (electrodes 21 – 52), and occipital (electrodes 53 – 63) locations. Time points shown in the cluster plots represent the beginning of encoding, through to the end of the response of the DRT. Cluster plots show amplitude at the top and latency in the centre. Warmer colours represent amplitude or latency reductions relative to the control task, and cooler colours indicate amplitude or latency increases. Corresponding Hotelling's T statistics are shown at the bottom. Warmer colours represent greater statistical significance. Vertical lines separate sub-processes of encoding, hold and retrieval.

*SSVEP treatment effects*

Figure 7.2 presents the post-treatment working memory SSVEP as compared to the baseline working memory task SSVEP for the hold period and retrieval. Differences and Hotelling's T maps are presented separately for the multivitamin and placebo groups.

*Hold:* For the multivitamin group, difference maps reveal that there was an increase in latency across prefrontal sites, with a maximal latency increase observed over right temporal parietal regions. A more complex pattern of amplitude was presented, with both amplitude increases and decreases displayed across the scalp. Statistical significance was reached at a single right frontal electrode, associated with an amplitude and latency increase. For the placebo group, there was a posterior increase in latency, with a frontal latency decrease largest at left sites. A parietal amplitude decrease was also observed. The pattern of amplitude decreases and latency increases at occipital parietal sites was statistically significant.

*Retrieval:* For the multivitamin treatment, when the post-treatment working memory task was compared to the baseline working memory task, amplitude was decreased at all regions except prefrontal sites and latency was increased over all sites other than midline occipital electrodes. Latency increases were largest at right temporal and left frontal sites. The corresponding Hotelling's T map shows that amplitude reductions and latency increases were statistically significant at central, midline sites. For the placebo treatment, amplitude was reduced across most regions and latency increased at parietal occipital sites only. This pattern of activity reached statistical significance at a single parietal electrode only.



**Figure 7.2** Topographical maps presenting the post-treatment SSVEP relative to the baseline SSVEP during the hold period and retrieval.

Amplitude is shown on the left, latency in the centre and Hotelling's T on the right. At the top, warmer colours indicate amplitude decreases and latency decreases for the spatial working memory task at post-treatment relative to the same task at baseline during the hold period. Conversely, cooler colours indicate amplitude and latency increases for the post-treatment working memory task relative to the same task at baseline. On the bottom, maps present the same data for the retrieval period of the task. Contours correspond to  $p$  values of  $p < .05$ ,  $.01$ ,  $.005$ , and  $.001$ .

### *SSVEP analysis of variance*

In order to calculate the statistical effect of the multivitamin treatment on the SSVEP when compared to placebo, SSVEP data was entered into a 2 time (baseline, post-treatment) x 2 treatment (multivitamin, placebo) x 3 electrode (FZ, CZ, PZ) repeated measures ANOVA. A separate ANOVA was used for amplitude and latency. Preliminary assumption testing revealed no serious violations.

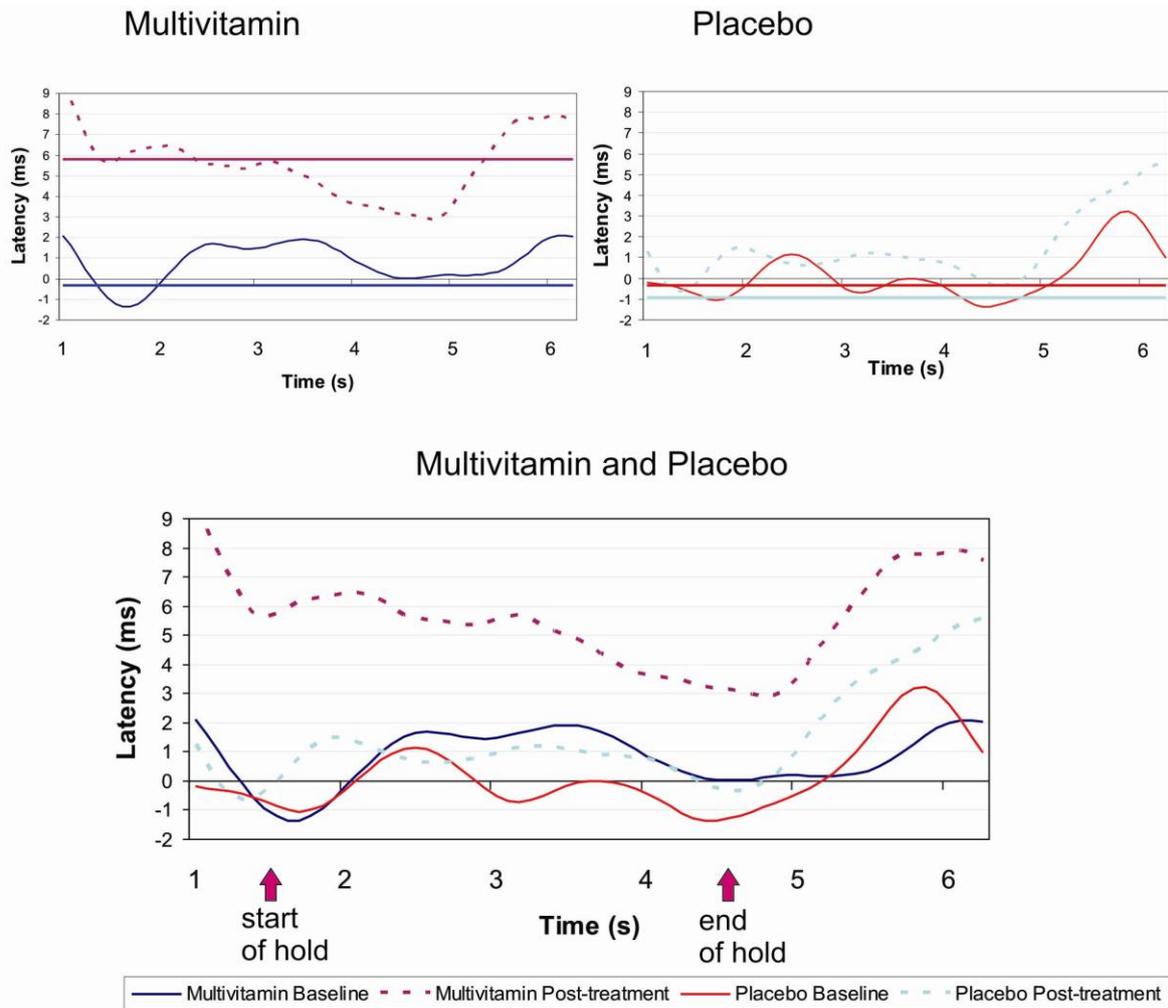
*Encoding:* During the encoding phase of the working memory task there were no significant time x treatment interactions for either latency or amplitude.

*Hold:* There was no significant time x treatment interactions identified for SSVEP latency or amplitude during the hold component of the working memory task.

*Retrieval:* The ANOVA analysis revealed that during retrieval there was a significant time x treatment interaction for SSVEP latency ( $F(1,39) = 7.99, p = .007, \eta^2 = .17$ ), indicating there was a significant increase in SSVEP latency following multivitamin supplementation. There were no significant time x treatment effects for SSVEP amplitude.

#### *SSVEP time series data*

As the multivitamin treatment effect for SSVEP latency, identified from the Hotelling's T and ANOVA analysis, was an increase in latency, and not the hypothesized latency decrease, a post hoc examination of time series data was undertaken to inspect the temporal characteristics of this latency effect. SSVEP latency data was presented for a right temporal electrode, as the latency increase appeared to be maximal across right temporal regions for the multivitamin group. Figure 7.3 displays the time series data averaged across subjects for both the baseline and post-treatment working memory tasks. The SSVEP mean latency across the corresponding time period of the control task, is also shown for baseline and post-treatment assessments. Data are presented separately for both groups. For the multivitamin treatment, time series data indicate that there was an overall increase in SSVEP latency at post-treatment, and there was a similar shift in the overall latency for the control task. In contrast, the time series data indicates that the same shift in latency did not occur for the placebo group. When displayed on the same axis, the latency increase from baseline to post-treatment was much larger for the multivitamin treatment than the placebo.



**Figure 7.3** Time series SSVEP latency data for the baseline and post-treatment working memory tasks.

Data are shown for at a right temporal electrode site, where SSVEP latency increases were maximal for the multivitamin group. Data for the multivitamin group is shown top left and the placebo group is shown on the right. The solid line represents the baseline working memory SSVEP latency, and the broken line displays the post-treatment SSVEP latency. Straight lines in the corresponding colour represent the average of the control task at baseline and post-treatment. The bottom figure displays the multivitamin and placebo SSVEP latency data on the same axis.

#### 7.4 Discussion

This experiment was conducted as the first trial into the effects of chronic multivitamin supplementation on brain activity. In this investigation, the SSVEP associated with the performance of a spatial working memory DRT was examined in elderly women at baseline,

and following 16 weeks supplementation with either a combined multivitamin, mineral and herbal formula, or a placebo. The major finding of the current study was that chronic multivitamin supplementation significantly increased SSVEP latency during working memory retrieval. There were no treatment effects of the multivitamin identified for SSVEP amplitude.

SSVEP latency is suggested to be indicative of neural information processing speed (Kemp et al., 2002). A reduction of the SSVEP latency has been interpreted as increased post-synaptic excitation (Silberstein et al., 2000), whilst latency increase has been proposed to reference decreased excitation, or an increase in post-synaptic inhibitory processes (Silberstein et al., 2000). In the current study, 16 weeks multivitamin supplementation was found to increase SSVEP latency. Whilst this result suggests that multivitamin supplementation is capable of modulating brain electrical activity, it does not support the hypothesis that multivitamin supplementation would be associated with a decrease in SSVEP latency, corresponding to faster processing speed in the brain. Instead, it appears that during spatial working memory activation, the multivitamin treatment increased inhibitory neural processes.

Behavioural results obtained from the spatial DRT revealed that for the multivitamin and placebo conditions, there was a reduction in spatial working memory response time, indicating that for both treatment groups, cognitive performance was improved. Whilst there was a significant SSVEP latency increase associated with the multivitamin treatment, a smaller, less extensive pattern of SSVEP latency increase was also identified in the topographical maps for the placebo. When combined with the behavioural improvements, it appears that the observed latency increase may index enhanced neural processes related to spatial working memory. However this interpretation is not consistent with the findings of the previous experiment, which demonstrated that faster spatial working memory response time was associated with larger SSVEP latency decreases, with this pattern of shorter SSVEP latency previously equated to faster neural transmission speed (Silberstein et al., 2000). Instead, these discordant findings potentially indicate that improvements to cognitive performance may occur via alterations in neural function, mediated by actions other than increased processing speed in the brain.

The finding from this study, which demonstrated that the multivitamin treatment did not improve neural processing speed, differs from the results of prior studies examining the neuro-regulatory effects of putative cognitive enhancing nutraceuticals. In these trials, increases in neural processing speed, as indexed by reduced P300 ERP latency, were identified after treatment with *Ginkgo biloba* or panax ginseng (Semlitsch et al., 1995; Kennedy et al., 2003; Page et al., 2005; Dimpfel et al., 2006). In the current study, the multivitamin formula under investigation contained a range of purported cognitive enhancing herbal extracts including *Ginkgo biloba*. Cognitive enhancing mechanisms of *Ginkgo biloba* are thought to involve the cholinergic neurotransmitter system (Di Renzo, 2000; Kennedy & Scholey, 2006), although when combined with a range of vitamin, mineral and herbal extracts, as in the case of the multivitamin, alternate neurotransmitter systems or aspects of brain function may also be influenced.

In the current study, multivitamin supplementation was associated with an increase in SSVEP latency during working memory retrieval. Increased SSVEP latency has been suggested to reference post-synaptic inhibition of cortical pyramidal neurons (Silberstein et al., 2000; Kemp et al., 2002; Kemp et al., 2004), indicating that chronic multivitamin supplementation may influence inhibitory neural processes. The ability to inhibit brain activity from task-irrelevant input has been described as a feature of intelligent cognitive performance (Doppelmayr et al., 2005), and may relate to the neural efficiency hypothesis introduced in Chapter 6. Specifically, this theory proposes that during cognitive performance, higher ability individuals require less brain activation than those with lower ability, and are therefore capable of recruiting neural resources in a more efficient manner (Neubauer et al., 1995; Neubauer & Fink, 2009). Evidence for this premise has been obtained from several prior studies of the SSVEP, indicating that increased inhibitory processes, as indexed by elongated SSVEP latency, may be important for the performance of cognitive tasks (Van Rooy et al., 2001; Song, 2005). In the study conducted by Van Rooy et al. (2001), a qualitative comparison between individuals with high and low WAIS IQ demonstrated that during performance of a spatial working memory task, those with a higher IQ displayed larger frontal latency increases. In a more comprehensive investigation of intelligence, Song (2005) concluded that frontal inhibitory processes were particularly relevant for the performance of the Raven Progressive Matrices. Again, once participants were divided into high and low

WAIS IQ groups, larger SSVEP latency increases, indicative of frontal inhibitory processes, were observed for the higher IQ group during performance of this task.

Post-synaptic inhibition of pyramidal neurons has been shown to be largely mediated by GABAergic interneurons (Koós & Tepper, 1999).  $\gamma$ -amino-butyric acid (GABA) is the major inhibitory neurotransmitter in the brain (Owens & Kriegstein, 2002) and at a cellular level, has been implicated in the working memory processes of spatial selectivity and preservation of memory storage against distracters (Compte et al., 2000; Rao, Williams & Goldman-Rakic, 2000; Brunel & Wang, 2001). In the current study, latency increases after multivitamin supplementation were maximal across right temporal regions, and surrounding frontal and parietal electrode sites. Previously, increased frontal and temporal SSVEP latency increases during the hold period of a spatial *n*-back task were interpreted as reflecting the efficiency of online maintenance through inhibition of adjacent neurons (Ellis et al., 2006). Examination of time series data in the present study indicated that for the multivitamin treatment, latency increases were not specific to the hold period. Instead, there was an overall increase in the post-treatment SSVEP latency across the entire task, when compared to baseline, and there was a similar shift in the overall latency for the control task. These findings are indicative of a modal shift in latency which may not be specific to any task components or working memory sub-processes. Subsequently, it may be that there is an alteration in the overall efficiency of working memory, mediated by an increase in inhibitory processes in neural regions relevant to spatial working memory. When compared to the placebo group, latency was increased across occipital and parietal regions only, and there was no modal shift in latency at temporal regions, indicating that this effect may be specific to the multivitamin treatment.

It is conceivable that the increased SSVEP latency associated with chronic multivitamin supplementation may represent greater efficiency of the brain via the inhibition of processes irrelevant to successful task performance. However, this interpretation diverges from the previous experimental chapter results which revealed that speed of neural processing may be central to performance of the same spatial working memory DRT. In this investigation, larger SSVEP latency reductions, rather than increases, were correlated with faster responses on the

DRT. It is conceivable that the approach adopted to measure cognitive performance in the previous chapter may have contributed to the discrepancy between the findings reported in Chapter 6 and the current experiment. In the previous chapter, working memory response time on the spatial DRT was correlated with SSVEP latency, indicating that faster responders demonstrated larger SSVEP latency reductions. As SSVEP latency has been suggested to index neural transmission speed in the brain (Silberstein et al., 2000; Kemp et al., 2002), it is possible that by correlating SSVEP latency with response time on the same cognitive task, both behavioural and neural parameters were measuring the same underlying construct of processing speed. In support of this premise, when SSVEP latency was correlated with a composite measure which also accounted for performance accuracy, the association with SSVEP latency was no longer apparent. In contrast, prior studies which have identified the opposite relationship between SSVEP latency and superior cognitive performance, have used IQ tasks rather than response time measures to differentiate cognitive ability (Van Rooy et al., 2001; Song, 2005). IQ instruments, such as the WAIS, provide an indication of ability across a range of higher order processes including crystallised intelligence, fluid intelligence, verbal ability and executive function (Davis, Pierson & Finch, 2011). These functions may rely more intrinsically on inhibitory processes in the brain than simple measures of reaction time. Therefore, it is possible that using a response time measure to index cognitive performance could have precluded the observation of inhibitory processes which may have been identified using a more comprehensive, higher order cognitive assessment instrument.

The identification of a an increase in SSVEP latency for the multivitamin group, across right temporal and surrounding cortical regions in the topographical maps, may be consistent with improved efficiency of the temporal and parietal lobes. Whilst the specific neural generators can only be speculated, as the SSVEP possesses relatively low spatial precision, this pattern of activity could potentially reflect alterations in medial temporal lobe activity as the hippocampus and parahippocampal cortex have also been suggested to be important for spatial memory (Lynch, 2004; Squire, Stark & Clark, 2004).

Thus far, the discussion has focused on the SSVEP latency, as the results demonstrated that chronic multivitamin supplementation increased this component of the SSVEP. In contrast,

there were no treatment effects identified for SSVEP amplitude. Instead, both treatment groups demonstrated a reduction in SSVEP amplitude at post-treatment. The absence of a statistical treatment effect indicates that the amplitude reductions may not be due to multivitamin supplementation, but may rather be the result of extraneous influences such as practice effects. In the current study, it is possible that the larger posterior amplitude reductions, observed following the intervention period for both groups, may reflect increased cortical activation as a consequence of repeated exposure to the same spatial working memory DRT. Similarly, practice-related increases in cortical activation have been observed in regions relevant to working memory storage such as the temporal and parietal lobes (Kelly & Garavan, 2005). Such practice related increases in activation have been accompanied by a decrease in frontal activation thought to reflect disengagement of executive control and attentional processes (Petersen et al., 1998).

In the previous chapter of this thesis, better spatial working memory performance, as measured by a combined accuracy/response time measure, was correlated with larger SSVEP amplitude reductions at prefrontal and left temporal regions during retrieval. Such SSVEP amplitude decreases have been equated to event related desynchronisation (ERD) in the upper alpha frequencies (Silberstein, 1995; Kemp et al., 2004). Specifically, ERD refers to a power decrease in the EEG, related to an increase in cortical activation (Pfurtscheller & Lopes Da Silva, 1999). Alpha amplitude has been shown to decrease with increased mental effort (Dujardin et al., 1993; Pfurtscheller & Lopes Da Silva, 1999), particularly over frontal regions (Stipacek et al., 2003). Analogously larger SSVEP amplitude reductions have been observed at high task demands for working memory and recognition memory (Pipingas & Silberstein, 1995; Ellis et al., 2006). An alternate explanation to that of practice effects, may be that the larger amplitude decreases observed at post-treatment for both the multivitamin and placebo groups represent an increase in cognitive effort from baseline to post-treatment.

#### **7.4.1 Limitations and future directions**

Behavioural measures of performance indicated that at post-treatment, spatial working memory response time was reduced for both the multivitamin and placebo conditions, indicating that behavioural improvements were not specific to the multivitamin treatment.

These findings differ from the results presented in Chapter 5, which demonstrated a reduction in spatial working memory response time associated with multivitamin supplementation only. Despite the use of alternate forms of the spatial working memory DRT at baseline and post-treatment, improved performance for both treatment groups may be due to the effects of practice on these measures. Several important differences between the two spatial working memory tasks may have contributed to the absence of a multivitamin treatment effect on the spatial DRT in the present experiment. Spatial working memory represents a cognitive domain which has been shown to be preferentially improved by nutraceutical intervention (Pipingas et al., 2008; Ryan et al., 2008), however there is some suggestion that the specific working memory measure may be an important determinant of whether treatment effects are detected (MacReady et al., 2011). The spatial working memory task included in Chapter 5 has demonstrated high sensitivity to age-related cognitive decline (Pipingas et al., 2010) and thus may also exhibit a greater responsiveness to nutraceutical effects. The spatial working memory DRT utilized in the current experiment was designed to activate the neural processes and brain regions associated with spatial working memory and may not necessarily be suitable to detect behavioural improvements following nutraceutical intervention.

In the current study, latency increases related to the multivitamin treatment, as displayed by topographical maps, appeared to be most prominent across right temporal and surrounding regions. However, the Hotelling's T statistic and topographical maps allow only a qualitative examination of differences in brain activity between the multivitamin and placebo groups. This represents a current limitation of the SST technique, and such indirect between-group comparisons have been raised as a major concern in the statistical analyses carried out in other trials in the field of neuroimaging (Nieuwenhuis et al., 2011). Whilst ANOVA analysis of the SSVEP enabled a direct comparison of the multivitamin and placebo groups, overcoming this limitation, this analysis was carried out at with limited spatio-temporal resolution, (i.e. less electrodes and averaged time periods) when compared to the SSVEP demonstrated in the topographical maps.

#### **7.4.2 Summary and conclusions**

The results of this experiment revealed that chronic multivitamin supplementation was capable of modulating brain electrical activity in older women. Specifically, the multivitamin treatment was demonstrated to increase SSVEP latency beyond effects observed for the placebo group, suggestive of increased inhibitory processes in the brain. These effects appeared to be prominent over right temporal and surrounding frontal and parietal regions. There were no effects of the multivitamin treatment observed for SSVEP amplitude. When coupled with the behavioural improvements to memory identified in Chapter 5, and the reductions to response time identified on the spatial working memory DRT for both treatment groups in the current experiment, these findings indicate that the pattern of SSVEP latency increase displayed by the multivitamin group may reflect an increase in neural efficiency, related to the treatment. Potential mechanisms, via which the multivitamin may have exerted influence on brain electrical activity, will be discussed in the subsequent chapter of this thesis.

## **Chapter 8 General discussion**

This thesis reported the findings from a 16 week, randomised, double-blind, placebo-controlled, clinical trial which investigated the effects of a multivitamin supplement on neurocognition in elderly women. The discussion will commence with a summary of the major findings of the experimental chapters included in this thesis. The suitability of computerised cognitive tests and the steady state visually evoked potential (SSVEP) measure of brain electrical activity to detect nutraceutical effects will then be discussed. Potential mechanisms by which the multivitamin may have exerted effects on cognition and brain activity will be proposed. Health benefits versus risks of multivitamin supplementation will also be considered. Future directions and implications of this thesis will precede the conclusion.

### **8.1 Summary of key findings**

There were two aims of the first investigation presented in Chapter 5. The first aim was to investigate the effects of chronic multivitamin supplementation on cognitive performance in the elderly. The second purpose of this experiment was to examine the potential mechanisms of cognitive improvement due to chronic multivitamin supplementation. Cognition was measured using an age-sensitive battery of computerised memory and attention tests, and a verbal recall instrument. It was hypothesised that chronic treatment with a combined multivitamin and herbal supplement would enhance the cognitive domains most vulnerable to age-related decline.

The results demonstrated that the multivitamin treatment significantly improved speed of spatial working memory response and provided small improvements to response time on a composite measure comprised of all the memory sub-tests of the computerised battery. However, the benefits to the composite measure were not supported by a statistical effect. There were no observed treatment effects for measures of attention or verbal memory. These findings are consistent with the identification of multivitamin treatment effects on

computerised measures of fluid intelligence in younger (Haskell et al., 2010; Kennedy et al., 2010) and elderly participant samples (Harris et al., 2011). These results also provide some evidence for the suggestion that following nutraceutical intervention, the cognitive processes most vulnerable to age, such as working memory, demonstrate the greatest improvements in the elderly (Ryan et al., 2008; Pipingas et al., 2010).

In relation to the potential mechanisms of cognitive enhancement, the results of the first investigation indicated that multivitamin supplementation significantly increased levels of vitamins B<sub>6</sub> and B<sub>12</sub>. There was also a trend for vitamin E to be increased and homocysteine to be reduced. Levels of vitamin B<sub>12</sub> and vitamin E were also found to correlate with cognitive performance at baseline, indicating that these vitamins may regulate cognitive function. There were no benefits of multivitamin supplementation identified for biochemical markers of inflammation, oxidative stress, or cardiovascular health parameters of cholesterol, blood pressure or arterial stiffness. Based on the results of this experiment, it was suggested that improvements to cognition may be due to the synergistic effects of the vitamin, mineral and herbal components on the function of the brain.

A considerable number of the vitamin and herbal components of the multivitamin under investigation are proposed to have neuro-regulatory effects (Kennedy et al., 2003; Mattson & Shea, 2003; Ramassamy, 2006; Huskisson et al., 2007; Gómez-Pinilla, 2008). Prior to this randomised controlled trial, no studies had examined the effects of a multivitamin, containing a combination of vitamin and herbal extracts, on brain activity. Subsequently, the second and third experiments of this thesis investigated the effects of chronic multivitamin supplementation on the SSVEP measure of brain activity, associated with the performance of a spatial working memory delayed response task (DRT). The aims of these experiments were to establish the SSVEP as a sensitive measure of the cognitive enhancing effects of multivitamin supplementation, and to examine the influence of chronic multivitamin supplementation on brain activity.

To establish whether treatment with a purported cognitive enhancing multivitamin was capable of improving the neural processes which subserve working memory performance as measured by steady state topography (SST), it was necessary to first examine the neural

underpinnings of the spatial working memory DRT in the elderly. The results of Chapter 6 revealed that the SSVEP was a sensitive measure of working memory sub-processes in an elderly sample. SSVEP amplitude and latency were found to be decreased over frontal regions during working memory encoding, and amplitude was increased over posterior brain regions during the hold period of the DRT task. These findings correspond to the pattern of SSVEP displayed by younger adults during working memory (Silberstein et al., 2001; Ellis et al., 2006). During the hold period of the DRT, SSVEP amplitude increases appeared to be attenuated. This reduction in neural processes is also consistent with previous findings that in the elderly, the SSVEP related to holding information „online“ is attenuated during the hold period of a working memory DRT (Macpherson et al., 2009).

In terms of identifying the SSVEP correlates of performance in the elderly, the results demonstrated that superior spatial working memory performance, as measured by faster response time, was related to greater latency reductions at frontal and prefrontal sites across the task duration. This result suggests that faster performers demonstrated an increased rate of neural processing, possibly corresponding to greater neural excitation at frontal cortical sites (Silberstein et al., 2000). In this thesis, better spatial working memory performance was also associated with larger amplitude reductions during retrieval, suggestive of an increase in cortical activation, at prefrontal, frontal and temporal sites.

Findings that the SSVEP was differentiated according to the sub-processes of the spatial working memory DRT indicate the SSVEP is a useful measure of working memory in the elderly. The identification of a relationship between performance on the DRT, and SSVEP amplitude and latency, demonstrates the SSVEP is sensitive to subtle inter-individual differences in working memory performance. On the basis of these observations, it was suggested the SSVEP may be responsive to the potentially cognitive enhancing and nutraceutical effects of multivitamin supplementation in the elderly.

The purpose of Chapter 7 was to investigate the effects of chronic multivitamin supplementation on the SSVEP. As the neural substrates of spatial working memory have been demonstrated to decline with age (Reuter-Lorenz et al., 2000; McEvoy et al., 2001;

Rypma et al., 2001), it was anticipated that the neural processes which subserve working memory performance may be enhanced following nutraceutical intervention. A proposed pattern of neural enhancement was based on the observation, in the previous chapter, that faster working memory responses on the spatial DRT, indicative of superior performance, were associated with larger SSVEP amplitude and latency reductions. Specifically, it was hypothesized that during working memory hold and retrieval, greater SSVEP amplitude and latency reductions, suggestive of increased cortical activation and processing speed respectively, would be observed across frontal, temporal and parietal brain regions after chronic multivitamin supplementation.

The results of the final investigation revealed that multivitamin supplementation significantly increased SSVEP latency during retrieval. Whilst this finding suggests that multivitamin supplementation is capable of modulating brain electrical activity, it did not support the hypothesis that multivitamin supplementation would be associated with a decrease in SSVEP latency, corresponding to faster processing speed in the brain. This result differed from past trials which have identified increases in neural processing speed, indexed by brain electrical activity, after treatment with reputed cognitive enhancing nutraceuticals (Semlitsch et al., 1995; Kennedy et al., 2003; Page et al., 2005; Dimpfel et al., 2006). Increased SSVEP latency has been suggested to reference post-synaptic inhibition of cortical pyramidal neurons (Silberstein et al., 2000; Kemp et al., 2002; Kemp et al., 2004), indicating that chronic multivitamin supplementation may influence inhibitory neural processes, rather than increase neural transmission speed in the brain. Topographical cortical maps presented for the multivitamin group indicated latency increases were largest across right temporal and surrounding cortical electrodes, regions known to be relevant to spatial working memory (Zimmer, 2008). There were no effects of the multivitamin on SSVEP amplitude, indicating that the treatment did not specifically influence levels of cortical activation, relating to holding information „online“ or retrieval processes in the brain.

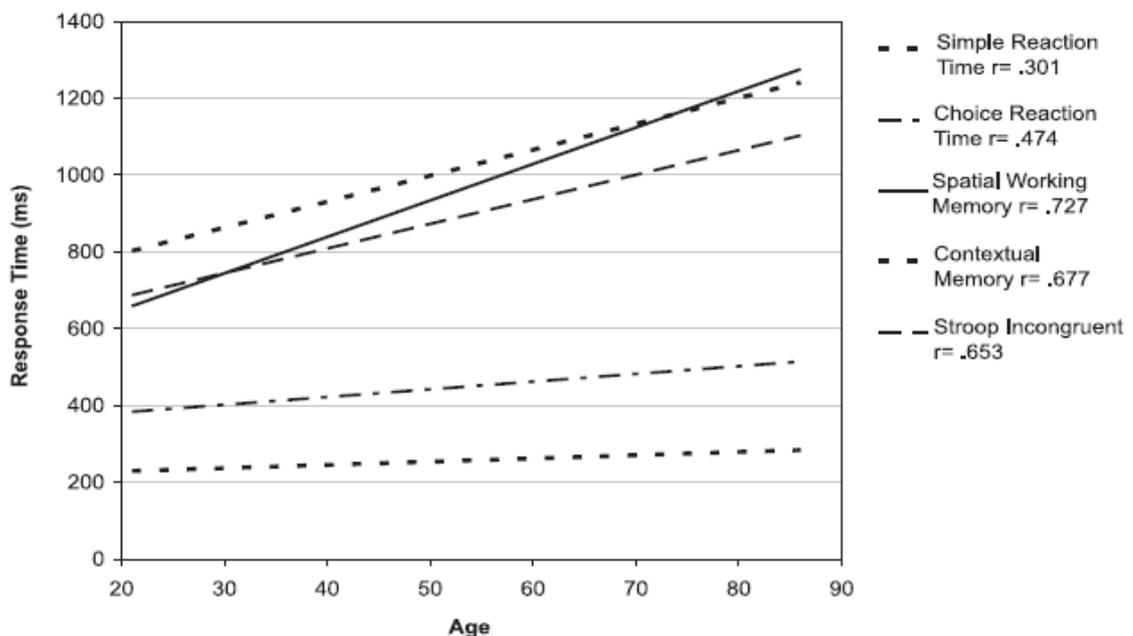
Combined with the behavioural improvements to memory identified in the first experiment, and the reductions to response time identified on the spatial working memory DRT for both treatment groups in the final experiment, the pattern of SSVEP latency increase displayed by

the multivitamin group was suggested to reflect greater neural efficiency via the inhibition of processes irrelevant to successful working memory performance. The ability to inhibit brain activity from task irrelevant input has been described as a feature of intelligent cognitive performance (Doppelmayr et al., 2005), and may relate to the neural efficiency hypothesis. This theory proposes that during cognitive performance, higher ability individuals require less brain activation than those with lower ability, and are therefore capable of recruiting neural resources in a more efficient manner (Neubauer et al., 1995; Neubauer & Fink, 2009). Consistent with this premise, prior studies of the SSVEP have also indicated that increased inhibitory processes, as indexed by elongated SSVEP latency, may be particularly important for intelligent behaviour (Van Rooy et al., 2001; Song, 2005). Findings from this thesis may suggest that the multivitamin supplementation was capable of improving cognition by increasing neural efficiency in the brain.

## **8.2 Utility of computerised cognitive assessments and the SSVEP measure of brain activity to detect nutraceutical effects**

The current thesis examined the neurocognitive effects of chronic multivitamin supplementation in an elderly sample, using a range of cognitive assessments and the SSVEP measure of brain electrical activity. The results of this study revealed that multivitamin supplementation was capable of improving memory performance, in terms of working memory response time, signifying that the multivitamin exerted cognitive enhancing effects. Comparable to prior trials (Haskell et al., 2010; Kennedy et al., 2010; Harris et al., 2011), cognitive enhancing effects of multivitamin supplementation were identified on computerised measures of fluid intelligence. Interestingly, small improvements to response time were observed for measures of memory only, and there were no benefits to computerised measures of attention and processing speed. Memory processes are especially vulnerable to the effects of age-related decline (Park et al., 1996; Ronnlund et al., 2005), and it is plausible that processes which deteriorate with age may demonstrate greater capacity for amelioration than cognitive functions with greater resistance to the ageing process. The observation that working memory was preferentially improved after multivitamin supplementation provides evidence for this premise.

In the current study, treatment with the multivitamin improved speed of spatial working memory response by 57ms. Pipingas et al. (2010) have observed a linear relationship between age and speed of working memory on this same measure, whereby response time increased, on average, by almost 10ms for each additional year of age (see Figure 8.1). In this study the observed reduction in response time of 57ms following multivitamin supplementation can be equated to a reversal of approximately 6 years of cognitive decline on this measure. Improvements to spatial working memory performance have also been identified in older adults in trials investigating chronic treatment with flavonoid containing nutraceuticals (Pipingas et al., 2008; Ryan et al., 2008). Consequently, it is recommended future studies also include a computerised measure of spatial working memory when assessing the potential cognitive effects of nutraceuticals in the elderly.



**Figure 8.1** Relationship between response time for SUCCAB cognitive tasks and age. Taken from Pipingas et al. (2010).

Benefits to cognition were not observed on all cognitive measures included in this investigation. The absence of an improvement to verbal processes after multivitamin supplementation is also consistent with several previous trials (Wolters et al., 2005; McNeill

et al., 2007). Findings from the current study, which utilised a range of cognitive measures, indicate that verbal memory measures may be less sensitive to the cognitive enhancing effects of multivitamin supplementation than speeded, computerised memory tasks. Whilst improvements to verbal memory have been observed in the elderly following chronic supplementation with a combined antioxidant and herbal formula (Summers et al., 2010), verbal processes tend to be less vulnerable to age-related decline (Christensen, 2001), and may not demonstrate the same scope for improvement by nutraceutical intervention. The selection of appropriate instruments to measure nutritionally-derived cognitive improvements has gained attention as an important consideration in the design of RCTs (Benton et al., 2005; MacReady et al., 2011). Evidence from the present study suggests that age-sensitive, computerised measures of fluid intelligence, such as the Swinburne University Computerised Cognitive Assessment Battery (SUCCAB) memory tasks (Pipingas et al., 2010), are suitable for the detection of cognitive enhancing effects of multivitamin supplementation in an elderly sample. Future studies may benefit from the inclusion of a larger sample size in order to strengthen the likelihood of identifying cognitive effects of nutraceuticals on these measures.

In this thesis, improvements to behavioural measures of memory and particularly working memory were relatively small and may not have provided convincing evidence that multivitamin supplementation is capable of modifying cognition when considered in isolation. However the identification of a treatment effect of multivitamin supplementation on the SSVEP measure of brain activity strengthens the argument that there is a genuine effect of multivitamins on neurocognition in this study. The current thesis not only represents the first trial to examine the effects of multivitamin supplementation on the SSVEP, but also contributes to the literature by presenting the findings of the first study of any nature to investigate the effects of multivitamin supplementation on brain activity.

In order to detect and interpret nutraceutical effects on measures of brain activity, it may be necessary to use a cognitive activation task based on a well understood paradigm. In this study, a spatial working memory DRT was used for this purpose. The neural correlates of working memory have been investigated in numerous studies and aspects of working memory pertaining to task difficulty (Silberstein et al., 2001), temporal dynamics of sub-processes

(Ellis et al., 2006), intelligence (Van Rooy et al., 2001) and ageing (Macpherson et al., 2009) have been examined utilising the SST methodology. In this thesis, the SSVEP was demonstrated to be sensitive to spatial working memory sub-processes in the elderly and to correlate with performance on the spatial DRT, indicating this measure of brain activity was suitable to detect variations in cognitive performance related to spatial working memory. On the basis of these features, it was suggested that the SSVEP associated with a working memory activation task, may have the potential to detect changes in neural activity which accompany the cognitive enhancing effects of multivitamin supplementation. Findings of a significant multivitamin treatment effect on SSVEP latency provided confirmation that the SSVEP was receptive to nutraceutical effects on brain electrical activity. Similar to behavioural testing, the SSVEP also appears to be vulnerable to practice effects due to repeated exposure to the same cognitive task. Specifically, topographical maps demonstrated a decrease in SSVEP amplitude from baseline to post-treatment for both the placebo and multivitamin groups. It may be beneficial, therefore, for a future investigation to examine the test-retest reliability of this measure of brain activity.

Several important findings of this clinical trial may not have been identified without the utilisation of a measure of brain electrical activity. The SSVEP possesses the ability to capture and monitor the timing of cognitive improvements, and enables the inspection of specific cognitive sub-processes (Silberstein et al., 1990; Silberstein et al., 2000; Ellis et al., 2006). In this study it was possible to examine the SSVEP time series data, with a high level of temporal precision, at a right temporal electrode where the latency increase appeared to be maximal (see Figure 7.3). Data from this electrode indicated that multivitamin-related latency increases were not specific to any single component of the working memory DRT. This finding is suggestive of a general alteration in neural efficiency which was not related to particular sub-processes of working memory hold or retrieval.

Behavioural improvements identified in the spatial working memory DRT demonstrated that there was a reduction in speed of response for both multivitamin and placebo groups at post-treatment, and that memory response time was improved on the SUCCAB working memory measure for the multivitamin group only. On the basis of improvements to response time alone, it may have been concluded that multivitamin supplementation was capable of

increasing neural transmission speed in the brain. However the examination of brain electrical activity revealed that SSVEP latency was increased rather than decreased, suggestive of greater inhibitory processes, and not an increase in processing speed in the brain. The identified effect of multivitamin supplementation on inhibitory processes in the brain may enable a more precise estimation of mechanisms underlying the cognitive enhancements. Whilst it cannot be confirmed that cognitive improvements in the SUCCAB were also related to an increase in inhibitory processes in the brain, it may be presumed that a similar neural mechanism was related to all observed memory enhancements in this study.

It has been proposed that SSVEP latency can provide unique insights into excitatory and inhibitory processes in the brain (Silberstein et al., 2000; Kemp et al., 2004). This is an advantage over metabolic imaging methodologies, such as positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), as it is not always clear whether changes in regional cerebral blood flow (rCBF) or blood oxygen level dependant (BOLD) signal reflect either excitatory or inhibitory processes (Arthurs & Boniface, 2002; Gray et al., 2003). In this study, SSVEP latency increases, indicative of an increase in inhibitory processes appeared to be largest across right temporal and surrounding frontal and parietal regions. The involvement of these regions in spatial working memory, as well as other memory processes has been established in this thesis and elsewhere (Wager & Smith, 2003; Zimmer, 2008), and improved neural efficiency of these regions may have contributed to the observed cognitive effects. Electrophysiological techniques such as SST are not without limitations and do not enable a precise estimation of the location of the brain regions responsible for such effects.

Findings from this study indicate that behavioural and brain electrical activity measures both provide unique contributions to the understanding of nutraceutical effects on neurocognition. The SSVEP measure of brain electrical activity associated with a spatial working memory DRT demonstrated sensitivity to the effects of multivitamin supplementation and may serve as a useful measure of the neurocognitive effects of nutraceuticals in future RCTs.

### **8.3 Potential mechanisms of neurocognitive improvement following multivitamin supplementation**

The following section will discuss potential mechanisms of the multivitamin which may have mediated the cognitive regulatory effects observed in this study. Putative actions will be discussed with reference to relevant cardiovascular, biochemical, cognitive and brain electrical activity effects of multivitamin supplementation, identified in this body of research. Before commencing, it may be important to note that it is outside the scope of this thesis to isolate a single component of the multivitamin which may be responsible for the cognitive effects. Without a full understanding of which components have crossed the blood brain barrier, and which ingredients may have influenced other physiological systems, mechanisms can only be speculated. Whilst findings of the current study indicated that the multivitamin increased levels of vitamin B<sub>6</sub>, B<sub>12</sub>, and E, and reduced homocysteine, correlations conducted in Chapter 5, indicated alterations in these biochemical parameters were not related to the cognitive improvements in memory response time. These results may indicate that increases to levels of a single vitamin, or a decrease in homocysteine alone, were not responsible for the observed neurocognitive benefits. Instead, mechanisms of neurocognitive enhancements may have occurred either as a result of synergistic effects of the vitamin, mineral and herbal components, or via the actions of components on the multivitamin which were not measured as a part of this investigation.

#### **8.3.1 Cardiovascular contribution to cognitive improvements**

There may be two predominant pathways by which multivitamin supplementation exerted effects on neurocognition in the elderly participants. The first route is via direct actions on the brain. The second route is via indirect influence on the cardiovascular system, although results from this study suggest this to be unlikely. A range of cardiovascular parameters including blood pressure, cholesterol, the high sensitivity C-reactive protein (hsCRP) marker of inflammation and arterial stiffness were measured in this clinical trial, and were not shown to benefit from 16 weeks multivitamin supplementation. In the elderly, these cardiovascular measures represent risk factors for cognitive decline (Wang et al., 2002; Elias et al., 2004; Hanon et al., 2005; Knecht et al., 2009). White matter lesions in the brain have been associated with hypertension (Longstreth Jr et al., 1996; Firbank et al., 2007; Kuller et al.,

2010), elevated cholesterol (Breteler et al., 1994), inflammation (Fornage et al., 2008) and arterial stiffness (Kim et al., 2011), and there is evidence to indicate that white matter damage may exert detrimental effects on cognition in older adults (Ylikoski et al., 1993; Rabbitt et al., 2007). Thus the relationship between poor cardiovascular health and cognitive function in the elderly may be partially due to the formation of white matter lesions in the brain.

Whilst it is unlikely that multivitamin supplementation over a 16 week period would be capable of reversing white matter damage brought about by cardiovascular pathology, other cardiovascular actions of the multivitamin may have been anticipated to influence cognition. For example, antioxidants, such as vitamin C (Diaz et al., 1997; Taddei et al., 1998) and flavonoid constituents of herbal extracts (Achike & Kwan, 2003; Vita, 2005), may increase the availability of nitric oxide. This compound is a major mediator of endothelium-dependent vasodilation, and possesses anti-inflammatory and antithrombotic properties (Landmesser et al., 2004). In turn, benefits to endothelial function may be associated with increased cerebral blood flow, enhancing the delivery of oxygen and glucose to the brain (Ghosh & Scheepens, 2009). Endothelial function can be inferred from measures of blood pressure, arterial stiffness and the plasma glycoprotein marker of fibrinogen (Wilkinson et al., 2002; Landmesser et al., 2004). The absence of any beneficial effects of the multivitamin on these indices, suggests that cognitive improvements were unrelated to endothelial function and corresponding increases to cerebral blood flow. Transcranial doppler ultrasound provides a measure of blood flow velocity in major arteries to the brain, and has been used previously to investigate alterations in cerebral blood flow following polyphenol consumption (Sorond et al., 2008). The inclusion of such methodology may be useful in future trials of multivitamins to further exclude the possibility of a cardiovascular contribution to cognitive enhancements.

A further cardiovascular variable of interest which was not examined in this trial was heart rate variability (HRV), a measure of cardiac autonomic modulation of heart rate. HRV is under tonic inhibitory control by parasympathetic influences via the vagus (Thayer et al., 2009). Together with a higher heart rate, lower HRV has been linked to higher cardiovascular disease risk in patients with depression (Taylor, 2010). In the elderly, HRV has been associated with all cause mortality, and may be a better predictor than other more commonly used measures of cardiovascular risk (Tsuji et al., 1994). In a ten week study investigating the

effects of omega 3 fatty acid in depressed patients with coronary heart disease, benefits were observed to a measure of HRV, indicating that omega 3 may have slowed deterioration in cardiac autonomic function (Carney et al., 2010). Interestingly, HRV has also been related to both the activity of the prefrontal cortex (PFC) and the executive functions subserved by this brain region (Thayer et al., 2009). In the current study, HRV may have provided a measure of cardiovascular function, potentially sensitive to the effects of the multivitamin. Due to the association between HRV and inhibitory processes in the brain, the inclusion of HRV may also have aided in the interpretation of the effects of the multivitamin on SSVEP latency, indicative of increased inhibitory processes. As a consequence, HRV may be a useful variable to examine in future trials.

### **8.3.2 Possible effects of the multivitamin on neurotransmitter production**

In the current study, 16 weeks multivitamin supplementation was found to increase levels of vitamins B<sub>6</sub> and B<sub>12</sub>. Along with folate, vitamin B<sub>12</sub> is required for the methylation of homocysteine to methionine, and vitamin B<sub>6</sub> is necessary for the metabolism of homocysteine to cysteine. In turn, methionine is essential for the synthesis of sadenosylmethionine (SAM), a vital component of one-carbon metabolism (Mattson & Shea, 2003), and is a methyl donor for neurotransmitters, proteins, phospholipids, DNA, and myelin (Selhub et al., 2000). As addressed in previous chapters of this thesis, members of the vitamin B complex, including B<sub>6</sub>, B<sub>12</sub> and folate, are required for the synthesis of neurotransmitters including adrenaline, serotonin, dopamine,  $\gamma$ -amino-butyric acid (GABA) and tyramine (Huskisson et al., 2007). In this study, it is possible that multivitamin-related increases in levels of B vitamins may have enhanced production of sadenosylmethionine (SAM), and consequently myelin, neurotransmitters, and membrane phospholipids in the brain (Rosenberg & Miller, 1992; Selhub, 2002). By increasing these nutrients through dietary supplementation, there may be a beneficial effect on the function and integrity of the nervous system, and ultimately cognitive performance.

Multivitamin supplementation was found to reduce concentrations of homocysteine in this study. Lowering of homocysteine may also result in alterations to levels of SAM and lead to comparable effects of B vitamin supplementation. When elevated, homocysteine can restrict

SAM, promoting hypomethylation and leading to lowered neurotransmitter production in the brain (Ho et al., 2002; Selhub, 2002). Given the 16 week duration of the clinical trial, an increase in myelin and neurotransmitter production represents a plausible mechanism via which cognitive improvements may have occurred after multivitamin supplementation.

Vitamin C has also been postulated to exert a range of functions in the brain and nervous system (Barabás et al., 1995; Harrison & May, 2009). This antioxidant is known to interact synergistically with the B vitamins and is found in high concentrations in the brain (Huskisson et al., 2007). Vitamin C is required for the transformation of dopamine into noradrenalin (Bourre, 2006), and the function of this vitamin has been suggested to extend to neuromodulation of dopamine, regulation of acetylcholine and catecholamine release, and glutamate and GABA-mediated neurotransmission (Rebec & Pierce, 1994; Harrison & May, 2009). It is possible that increasing levels of vitamin C through multivitamin supplementation may have contributed to increases in neurotransmitter production, and consequently cognitive function. However, vitamin C measures were unable to be included in the analysis of this trial, and therefore it is not possible to determine whether multivitamin supplementation was in fact capable of increasing circulating levels of vitamin C in this sample of elderly women.

If generalized improvement to the nervous system, resulting from an increase in neurotransmitter production, was to account for the improvements in memory performance in this study, similar effects would be anticipated across all speeded cognitive measures. This was not the case as there were no effects of the multivitamin treatment on measures of basic reaction time or attention. On the other hand, the identification of memory, but not attentional improvements, may also have occurred as a consequence of differences in cognitive task difficulty. Response time data, included in Chapter 5, indicated that the cognitive battery memory sub-tests have been more difficult than the attention measures, and therefore more responsive to nutraceutical effects. One possibility is that specific ingredients or synergistic effects of the multivitamin influenced the overall efficiency of neural processes relevant to general cognition function, yet treatment effects were only overtly demonstrated in the individual tasks most receptive to nutraceutical actions. The inclusion of an SST attentional task, enabling examination of the effects of the multivitamin on the neural correlates of attention, may be required in future trials to confirm or reject this prospect. Alternatively, it

may be that the actions of the vitamin, mineral or herbal components of the multivitamin, preferentially benefitted specific brain regions or neurotransmitter systems relevant to memory performance.

### **8.3.3 Suggested hippocampal mechanisms of multivitamin supplementation**

Considering that the cognitive improvements identified in this study were observed for memory processes only, hippocampal effects of the multivitamin may represent an appealing mechanism of cognitive enhancement. The hippocampus is a region implicated in learning and memory (Squire et al., 2004) and has a well established role in long term potentiation (LTP), the basic cellular mechanism thought to be involved in memory. LTP refers to an enhancement in synaptic strength that follows high-frequency electrical stimulation in the hippocampus or neocortex (Bliss & Collingridge, 1993; Bear & Malenka, 1994).

The hippocampus has been shown to decline in size with advancing age in healthy individuals (Zhang et al., 2010), and hippocampal atrophy is especially prominent in those with Alzheimer's disease (AD) (Jack Jr et al., 1997; Jack Jr et al., 1998). In the ageing brain, deficits in the induction and maintenance of LTP have been observed (DeToledo-Morrell, Geinisman & Morrell, 1988), and these deficits have been proposed to contribute to cognitive decline in the elderly (Rosenzweig & Barnes, 2003). The hippocampus is also a known site of adult neurogenesis, a process involving the generation of neurons throughout life (Eriksson et al., 1998). The potential of the hippocampus to regenerate suggests that this region may be amenable to the effects of intervention with nutraceutical substances.

Pertinent to the findings of improved spatial working memory following multivitamin supplementation in the current study, the hippocampus and parahippocampal cortex have also been suggested to be important for spatial memory (Lynch, 2004; Squire et al., 2004). The hippocampus receives sensory input including visual, spatial and auditory information from the entorhinal cortex. Specifically, the CA3 region is thought to possess the ability to integrate incoming information from the visual cortex regarding the presence of an object, with information from the parietal cortex, concerning object location (Rendeiro et al., 2009). This region of the hippocampus has also been demonstrated to be preferentially involved in

short term memory, but not necessarily longer term memory processes (Lee & Kesner, 2003). In the current study effects of the multivitamin were identified for spatial working memory, both in terms of behavioural and brain electrical activity measures. Interestingly, multivitamin supplementation may have increased inhibitory neural processes, as measured by increased SSVEP latency, across the right temporal cortices. Whilst the specific neural generators can only be speculated, as the SSVEP possess relatively low spatial precision, this pattern of activity could potentially reflect alterations in medial temporal lobe activity.

The suggestion that the memory enhancements observed in this study may be due to effects on hippocampal function is strengthened by evidence that several components of the multivitamin have previously demonstrated memory enhancing qualities, as well as modifications to hippocampal function. Several studies in samples including elderly subjects, have demonstrated an association between elevated homocysteine and atrophy of the hippocampus (Williams et al., 2002; Den Heijer et al., 2003; Firbank et al., 2010). Findings from a recently published trial revealed that in elderly with mild cognitive impairment (MCI), two years combined supplementation with folate, vitamin B<sub>6</sub> and B<sub>12</sub>, concurrently lowered levels of homocysteine and slowed the rate of brain atrophy, when compared to a placebo (Smith et al., 2010). Whilst these effects were not specific to hippocampus, findings from the study conducted by Smith et al. (2010) provide important evidence that lowering homocysteine, via B vitamin supplementation, may exert protective effects on the brain.

In this study there was a trend for multivitamin supplementation to increase levels of vitamin E. Due to the role of vitamin E as a powerful antioxidant, it may be anticipated to have contributed to a reduction in oxidative stress following multivitamin supplementation. As no such improvements to oxidative stress, measured by protein carbonyls, were identified in this study, these results suggest that any cognitive effects attributable to vitamin E were not due to alterations in oxidative stress. Putative effects of fat soluble antioxidants on the brain (Sen & Khanna, 2010), and particularly the hippocampus have been proposed (Cecchini et al., 2003). In rodents, vitamin E has been demonstrated to reduce neuronal death in the hippocampus (Hara et al., 1990) and vitamin E deficiency has been shown to increase hippocampal neuropathology (Fukui et al., 2005). Pertinent to cognitive functioning, other investigations have indicated that vitamin E may be an exogenous factor involved in regulation of some

stages of adult hippocampal neurogenesis (Cecchini et al., 2003), and this vitamin may facilitate LTP in neurons (Xie & Sastry, 1993). Roles of vitamin E in hippocampal neural processes may also indicate a capacity for modifying memory processes in the brain.

Selected memory improvements of the multivitamin may have been due to the botanicals *Ginkgo biloba* or *Bacopa monierra* which have demonstrated cognitive enhancing effects in humans, especially in the domains of learning and memory (Stough et al., 2001b; Mix & Crews, 2002; Calabrese et al., 2008). Actions of these herbals have been identified in the hippocampus, including an increase in antioxidant activity (Bhattacharya et al., 2000) and decrease in oxidative stress (Bastianetto, Zheng & Quirion, 2000; Jyoti & Sharma, 2006). In addition to neuroprotective functions, *Ginkgo biloba* has demonstrated the ability to increase hippocampal LTP both *in vitro* (Williams et al., 2004) and *in vivo* (Wang et al., 2006).

Other plant derived flavonoid components of the multivitamin may also have influenced hippocampal function and subsequently contributed to cognitive improvements. Herbal ingredients of the multivitamin known to possess flavonoid constituents include *Ginkgo biloba*, grape seed, bilberry, cranberry and hawthorn fruit (Häkkinen et al., 1999; Bastianetto et al., 2000; Zhang et al., 2001; Kar et al., 2006). Selected flavonoid extracts have previously been demonstrated to improve non-verbal working memory, the same cognitive domain affected by the multivitamin in the present study (Pipingas et al., 2008; Ryan et al., 2008). The impact of flavonoids on the functioning of the hippocampus has been proposed as a mechanism via which such cognitive improvements may occur (Rendeiro et al., 2009). Based on the results of rodent investigations, specific mechanisms in the hippocampus have been proposed, including reduced cell death by increasing intracellular glutathione, reduced reactive oxygen species and prevention of calcium influx which can be toxic to neurons (Ishige, Schubert & Sagara, 2001). It has also been suggested that flavonoids and their metabolites interact with neuronal signalling pathways to promote LTP and synaptic plasticity, and that these actions contribute to the beneficial effects of flavonoids on human cognitive performance (Spencer, 2008). In addition, the effects of flavonoids on memory may also be mediated by peripheral and cerebral vascular actions, which contribute to growth of new neurons in the hippocampus (Spencer, 2009).

Finally, other components of the multivitamin including vitamin D and zinc have been implicated in hippocampal function. The steroid hormone vitamin D has been suggested to be important for cognitive function (Buell & Dawson-Hughes, 2008). Vitamin D receptors have been identified in the rat hippocampus (Langub et al., 2001) and the presence of vitamin D has demonstrated a protective effect on hippocampal cells (Obradovic et al., 2006). Zinc is an important mineral for brain function (Takeda, 2001). It operates as a modulatory neurotransmitter released from hippocampal mossy fiber synapses (Vogt et al., 2000) and has been implicated in LTP (Lorca et al., 2011). Whilst there were no biochemical measures of these micronutrients included in this trial, it is possible that these multivitamin components may have contributed to the identified neurocognitive effects.

In summary, there is evidence from the literature that numerous components of the multivitamin, previously implicated in human cognitive function, also impart effects on the hippocampus, a brain region important for memory processes. The identification of possible memory specific effects of the multivitamin may suggest that cognitive benefits could be due to either the effects of individual constituents or combined components on the hippocampus.

#### **8.3.4 Putative cholinergic actions of the multivitamin**

The identification of memory specific effects of multivitamin supplementation in the current study may also implicate the role of the cholinergic neurotransmitter system. Cholinergic neurons project from the basal forebrain complex to the cerebral cortex and hippocampus and have been shown to be important for human cognitive function (Everitt & Robbins, 1997). Pharmacological studies have demonstrated that acetylcholine antagonists impair memory, and augmentation of cholinergic function is capable of enhancing memory (Gold, 2003). In AD, memory specific degenerative changes, including cell loss and down regulation of choline acetyltransferase occur in the cholinergic neurons of the basal forebrain complex (Schliebs & Arendt, 2006). This impairment to cholinergic function is believed to contribute to the marked memory dysfunction in AD (Gallagher & Colombo, 1995).

Several components of the multivitamin have been investigated for cholinergic enhancing properties. Actions of *Ginkgo biloba* have been proposed to involve direct effects on the cholinergic neurotransmitter system (Di Renzo, 2000). In a comparative study of the influence of extracts of *Ginkgo biloba* and *Bacopa monniera* extracts in rodents, the greatest inhibitory effect on acetylcholinesterase was observed for *Ginkgo* and not the latter (Das et al., 2002). However, a recent trial demonstrated that *Bacopa monniera* reduced the effects of scopolamine-induced amnesia, indicating that the memory enhancing effects of *Bacopa* extract may also operate via the cholinergic system (Anand et al., 2011). Due to the inclusion of these herbals in the multivitamin investigated in this thesis, it is possible that some of the neurocognitive effects were due to modulation of the cholinergic neurotransmitter system.

In the present study, improvements to the neural correlates of spatial working memory were identified following multivitamin supplementation. Specifically, there was an increase in SSVEP latency, indicative of an increase in inhibitory processes in the brain. As increased speed of spatial working memory response was also identified, these findings were interpreted as an increase in neural efficiency, possibly corresponding to an enhanced ability to inhibit task-irrelevant content. A series of PET experiments conducted by Furey and colleagues has revealed a similar pattern of neural activity during working memory for faces, following administration of Physostigmine, an acetylcholinesterase inhibitor that increases the duration of action of acetylcholine at the synapse. In the first of these trials, Physostigmine was found to improve response time and increase working memory efficiency by reducing activation in the right prefrontal cortex (Furey et al., 1997). In a subsequent study, cholinergic modulation increased activity in visual processing regions and decreased activity in regions relevant to working memory, such as the prefrontal cortex and temporal regions encompassing the hippocampus (Furey et al., 2000). This increase in neural efficiency was suggested to be mediated by effects of Physostigmine on the septohippocampal cholinergic system, operating to enhance the inhibitory effect of acetylcholine on the hippocampus.

Interactions between acetylcholine and other neurotransmitters have been suggested to be important for learning and memory (Decker & McGaugh, 1991). Acetylcholine interacts with

both glutamate, the major excitatory neurotransmitter in the brain, and GABA, the main inhibitory neurotransmitter (Owens & Kriegstein, 2002; Reis et al., 2009). In the current study, the multivitamin treatment-associated increase in inhibitory neural processes may implicate the GABA neurotransmitter system in neural activity alterations. Cholinergic innervations of GABA neurons have been identified in the septohippocampal region (Wu et al., 2000), and although not fully understood, septohippocampal acetylcholine and GABA interactions have been proposed to be important for memory (Parent & Baxter, 2004).

Taken together, the identification of memory improvements following multivitamin supplementation may be suggestive of an involvement of the cholinergic neurotransmitter in neurocognitive alterations. There is evidence linking several herbal components of the multivitamin to the cholinergic neurotransmitter system. Further support for this premise may be inferred from the observation that the multivitamin may have increased inhibitory neural processes, a pattern of activity also demonstrated by substances which enhance cholinergic function.

#### **8.4 Health benefits versus risks of multivitamin supplementation**

The focus of the current study was to investigate the neurocognitive effects of 16 weeks multivitamin supplementation in the elderly. As a range of health measures were also examined, it appears pertinent to address the potential health risks versus benefits of multivitamin supplementation. Certain herbal preparations and botanicals have been associated with hepatotoxic effects (Stickel et al., 2000), therefore it was important to assess the safety of a multivitamin containing numerous herbal extracts not regularly consumed as a part of the normal diet. Despite undergoing screening for allergies prior to entry to the trial, one participant, allocated to the multivitamin group, experienced a possible reaction and this was attributed to a previously unknown allergy. More generally, there were no effects of the multivitamin on any of the liver or kidney function tests, confirming that the vitamin supplement is safe for general consumption.

The current study did not identify many of the health benefits, particularly to the cardiovascular system, which have been observed in other investigations into the effects of

multivitamin supplementation (Salonen et al., 1991; Church et al., 2003; Arnaud et al., 2007; Li et al., 2010; Shargorodsky et al., 2010). Characteristics of the participants, such as age and health status at baseline, combined with the specific multivitamin formulation may influence the likelihood of identifying improvements to biochemical and cardiovascular parameters following vitamin intervention. Consequently, it remains important to investigate both the cognitive effects, and biochemical and cardiovascular mechanisms of the same multivitamin product, and not to infer mechanisms from separate investigations based on different multivitamin formulations and subject groups.

In terms of measurable health benefits of the multivitamin, an important outcome of this study was the identification of a reduction in homocysteine levels of 12% following multivitamin supplementation. Reductions of a similar magnitude in concentrations of homocysteine were observed in a previous trial conducted by our group, using a comparable multivitamin supplement (Harris et al., 2011), indicating that this is a replicable finding. At baseline, levels of homocysteine were 14 $\mu$ mol/L in the multivitamin group. In patients with vascular disease, lowering of homocysteine, even in individuals with concentrations below 14 $\mu$ mol/L, has been demonstrated to significantly slow the rate of disease progression (Hackam, Peterson & Spence, 2000). In women it has been suggested that levels above 10.4  $\mu$ mol/l may be indicative of elevated homocysteine (Selhub et al., 1999), leading to an increased risk of cardiovascular pathology (Selhub, 2006). Elevated homocysteine (Williams et al., 2002; Den Heijer et al., 2003; Firbank et al., 2010) and low levels of vitamin B12 (De Lau et al., 2009) have also been associated with neuropathology. Consequently, lowering of homocysteine via daily multivitamin supplementation may contribute to a lower risk of both cardiovascular and neural pathology.

Although not a focus of the current study, other trials have investigated the ability of multivitamin supplements to enhance aspects of mood, stress and general wellbeing. In studies which have assessed the effects of chronic multivitamin supplementation, beneficial mood effects have been reported in young adults (Benton, Haller & Fordy, 1995; Carroll et al., 2000) and in samples comprised of young through to middle aged individuals (Kennedy et al., 2010). Low levels of B vitamins and elevated homocysteine have been associated with

depression (Tiemeier et al., 2002), and folate depletion in depressed individuals has been linked to impaired methylation and monoamine neurotransmitter metabolism (Bottiglieri, 1996; Bottiglieri et al., 2000). Beneficial mood effects of multivitamin supplements may, in part, be due to the actions of B vitamins on these biological pathways.

In addition to potential benefits of multivitamin supplementation, there have also been reports of possible health risks. In 2010 a paper published in the *American Journal of Clinical Nutrition* indicated that multivitamin use was associated with an increased risk of breast cancer (Larsson et al., 2010). In this population-based cohort study of over 35,000 Swedish women, multivitamin use at baseline was associated with a 19% increased risk of breast cancer almost 10 years later. Considerable concern was expressed in the international media regarding the potential link between multivitamin use and breast cancer. In this Swedish trial, the higher cancer risk was potentially attributed to the folic acid included in multivitamin supplements. Conversely, the findings from another study have suggested that the folate included in multivitamins lowered breast cancer risk among women who consumed moderate amounts of alcohol each day (Zhang et al., 1999). The folate in multivitamins has also been suggested to lower the risk of colorectal cancer risk in moderate to heavy alcohol consumers (Jacobs et al., 2001), and comparable observations of lowered colon cancer risk have been identified irrespective of alcohol use (Giovannucci et al., 1998; Zhang et al., 2006). Currently there is no clear evidence to suggest that multivitamins increase cancer risk, and any possible health risks of multivitamin use should also be considered in light of potential benefits.

### **8.5 Limitations, implications and applicability of findings**

A number of limitations of this trial were addressed in the experimental chapters. Many of these caveats were related to the specific cognitive instruments used in this thesis, and the approaches cognitive performance and brain electrical activity measurement. There are also several issues pertaining to the participant sample and multivitamin treatment which must be addressed.

An issue worthy of consideration is that of experiment-wise error. In studies where many hypotheses are tested, there is a greater chance of making a Type 1 error (Shaffer, 1995). Within the individual experimental chapters of this thesis, statistical corrections have been applied to reduce the problem of multiplicity. However, statistical corrections were not extended to account for the number of hypotheses investigated across the entire thesis. For this reason, results of this thesis with borderline significance may need to be interpreted with some caution.

In terms of the sample, participants in this clinical trial were elderly women with complaints of subjective memory impairment. On the basis of their age and subjective reports of memory loss, this sample was classified as being at risk of cognitive decline. Despite attempts to include a healthy participant sample free from objective cognitive impairment or dementia, it is possible that some individuals may have been in a premorbid stage of AD.

The inclusion of a female only sample may limit the applicability of these results to a male population. However, a recent study by our research group identified memory benefits after eight weeks multivitamin supplementation on the same computerised cognitive battery, in a similar sized sample of elderly men, also at risk of cognitive decline due to their age and lifestyle factors (Harris et al., 2011). When considered together, these findings indicate that both genders at risk of cognitive decline may experience similar benefits of multivitamin supplementation to behavioural cognitive measures. A replication of this study may be required to ascertain health and brain activity effects of multivitamin use in males.

Due to the age range of the participants investigated in this study, it may be inappropriate to apply the findings of this study to younger participant groups. The multivitamin under investigation was designed for use by adults aged 50 years or above, and the focus of this trial was to identify cognitive improvements in the domains most vulnerable to age-related cognitive decline. The elderly often have lower dietary nutrient intake and absorption than younger adults (Baik & Russell, 1999) and are therefore likely to experience greater advantages from supplementation than those with adequate vitamin intake. Risk factors for cognitive decline, such as levels of oxidative stress and homocysteine, also increase over the lifespan, and contribute to cardiovascular and neural pathology in the elderly (Joosten et al.,

1996; Diaz et al., 1997; Floyd & Hensley, 2002; De Koning et al., 2003; Den Heijer et al., 2003; Kregel & Zhang, 2007; Reynolds et al., 2007). Combined, these factors could contribute to a below optimal level of cognitive function, which may be rectified with the appropriate dietary intervention.

This study included past vitamin supplementers and current fish oil supplementers. Whilst it would have been ideal to obtain a sample naive to dietary vitamin supplementation, the use of multivitamin supplements is common amongst seniors (Rock, 2007; Goh et al., 2009). Rather than completely excluding multivitamin users, a wash out period was applied prior to enrolment into the trial. Equivalent numbers of participants in each of the multivitamin and placebo groups reported supplementing their diet with fish oil. However, due to a growing body of evidence which suggests that the essential fatty acids found in fish oil supplements may be important for cognitive function in the elderly (Kalmijn, 2000; Uauy & Dangour, 2006; Yurko-Mauro et al., 2010), future studies should exclude users of fish oil or omega three supplements.

Finally, the vitamin components of a multivitamin may be derived from a natural, synthetic or partially synthetic form. There is evidence to suggest that some natural analogues of vitamins including vitamin E, are absorbed, transported and retained more efficiently in the body than synthetic forms (Burton et al., 1998; Zingg & Azzi, 2004). The use of a multivitamin supplement to increase dietary intake of vitamins and nutrients may therefore be less effective than maintaining a diet high in fruit and vegetable content. Implementation of a Mediterranean diet consisting of increased fruit and vegetable intake over a period of three months has been demonstrated to improve nutrient levels in healthy individuals (Polidori et al., 2009). Small cognitive benefits to spatial working memory have also been observed after a ten day Mediterranean diet intervention (McMillan et al., 2011). Although more difficult to implement and maintain, it may be anticipated that complete dietary modifications may be associated with greater cognitive benefits than multivitamin supplements.

## 8.6 Conclusions and future directions

This study aimed to investigate the neurocognitive effects of 16 weeks multivitamin supplementation in a group of elderly women, at risk of cognitive decline. Specifically, the effects of chronic multivitamin supplementation on cognitive performance and the SSVEP measure of brain electrical activity were investigated. A further objective of this trial was to examine the biochemical and cardiovascular mechanisms of multivitamin supplementation, which may have contributed to any neurocognitive benefits.

Prior studies examining the influence of multivitamin supplementation on cognition in non-demented, community-dwelling elderly have yielded inconsistent results. The selection of cognitive outcome measures used in these trials may have contributed to heterogeneity. In this study, consistent with the hypothesis that treatment with the multivitamin would preferentially improve the cognitive processes most vulnerable to the effects of ageing, multivitamin supplementation improved speed of response on a spatial working memory task. Response time on this task was improved by 57ms, an increase in speed which may be equated to a reversal of approximately 6 years of cognitive decline on this measure of spatial working memory. To maximise the likelihood of capturing cognitive enhancements in older adults, it is suggested that future studies include age-sensitive measures when examining the effects of multivitamin supplementation.

Findings from this investigation indicated that in the elderly participants, the multivitamin imparted effects on both behavioural and brain activity measures of neurocognitive performance. This is the first study to have demonstrated an effect of multivitamin supplementation on brain electrical activity. These findings indicate the SSVEP is sensitive to the nutraceutical effects of chronic multivitamin supplementation and may also serve as a useful measure of the neurocognitive effects of other nutraceuticals.

In this study, the multivitamin treatment increased SSVEP latency during retrieval, potentially reflecting increased inhibitory processes in the brain. The ability to inhibit brain activity from task irrelevant input has been described as a feature of efficient neural processing, and consequently, superior cognitive performance (Neubauer et al., 1995; Song,

2005; Neubauer & Fink, 2009). The results indicate that multivitamin supplementation may influence cognition by enhancing neural efficiency.

This study provides some insights into the mechanisms through which chronic multivitamin supplementation may enhance neurocognition. There was no evidence that the observed cognitive improvements were mediated by cardiovascular mechanisms. Instead, it is plausible that there were more direct effects of components of the multivitamin on aspects of neural function such as neurotransmitter production, hippocampal or cholinergic function. Future studies should endeavour to further elucidate these mechanisms of cognitive enhancement.

In the foreseeable future, population ageing will continue to be a global issue. Following replication of these findings in a larger sample, longer duration trials should examine the potential for multivitamin supplementation to slow the rate of cognitive decline and progression of neuropathology in the elderly.

## Chapter 9 References

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## Appendices

### Appendix A Publications relevant to this thesis

Literature reviewed in Chapter 3 has been included in a book chapter entitled „Micronutrients and neurocognition in the elderly“.

The cognitive and biochemical results presented in Chapter 5 have been accepted for publication in *Psychopharmacology*.

Preliminary SSVEP data from Chapter 6 was published as an abstract in *Clinical EEG and Neuroscience*, following presentation at the 19th Australasian Psychophysiology Society Conference in Australia.

Results from Chapter 7 have been presented at the International Evidence-Based Complimentary Medicine Conference in Australia, and the 1st Australasian Cognitive Neurosciences Conference in Australia. Findings from this thesis were also presented at the 15th World Congress of Psychophysiology in Hungary, with the abstract published in *The International Journal of Psychophysiology*.

The SSVEP results from Chapter 7 are currently undergoing review in *Physiology & Behavior*.

*Publications arising from this thesis*

Macpherson, H, (2011). Micronutrients and neurocognition in the elderly. In: Stough, C. & Scholey, A. (Eds). (2011). *Advances in Natural Medicines, Nutraceuticals and Neurocognition*. Taylor and Francis, New York

*Abstracts for accepted manuscripts*

Macpherson, H., Ellis, K., & Pipingas, A. (2011). Memory improvements in elderly women following 16 weeks treatment with a combined multivitamin, mineral and herbal supplement: A randomized controlled trial. *Psychopharmacology*, In Press

Abstract:

*Rationale:* There is potential for multivitamin supplementation to improve cognition in the elderly. This randomized, double-blind, placebo controlled trial was conducted to investigate the effects of 16 weeks multivitamin supplementation (Swisse Women's 50+ Ultivite ®) on cognition in elderly women.

*Methods:* Participants in this study were 56 community dwelling, elderly women, with subjective complaints of memory loss. Cognition was assessed using a computerised battery of memory and attention tasks designed to be sensitive to age-related declines to fluid intelligence, and a measure of verbal recall. Biochemical measures of selected nutrients, homocysteine, markers of inflammation, oxidative stress, and blood safety parameters were also collected. All cognitive and haematological parameters were assessed at baseline and 16 weeks post-treatment.

*Results:* The multivitamin treatment was found to improve speed of response on a composite measure of the computerised memory subtests and on a measure of spatial working memory. Concurrently, the multivitamin increased blood levels of vitamins B6, B12, and E, and there was a trend for homocysteine to be reduced. There were no hepatotoxic effects of the multivitamin formula indicating this supplement was safe for everyday usage in the elderly.

*Conclusion:* These findings indicate supplementation with a combined multivitamin, mineral and herbal formula may benefit memory performance in elderly at risk of cognitive decline.

*Abstracts currently undergoing peer review*

Macpherson, H., Silberstein, R. & Pipingas, A. Neurocognitive effects of multivitamin supplementation on the steady state visually evoked potential (SSVEP) measure of brain activity in elderly women. Submitted to *Physiology & Behavior*.

**Objective:** Growing evidence suggests that dietary supplementation with selected micronutrients and nutraceuticals may have the potential to improve cognition in older adults. Fewer studies have investigated the effects of these substances on brain activity. **Methods:** This study was a randomised, double-blind, placebo-controlled trial, conducted to explore the effects of 16 weeks supplementation with a combined multivitamin, mineral and herbal formula on the steady state visually evoked potential (SSVEP) measure of brain electrical activity. Participants were elderly women aged between 64 and 79 years, with subjective memory complaints. Baseline and post-treatment SSVEP data was obtained for 22 participants in the multivitamin group and 19 in the placebo group. A spatial working memory delayed response task (DRT) was performed during the recording of the SSVEP.

**Results:** Behavioural performance on the DRT was not improved by the multivitamin and there no multivitamin-related effects on SSVEP amplitude. The results revealed that when compared to placebo, multivitamin supplementation delayed SSVEP latency during retrieval, interpreted as an increase in inhibitory neural processes.

**Conclusion:** These findings indicate that in the elderly, multivitamin supplementation may influence cognition by enhancing neural efficiency.

*Abstracts for conference presentations related to this thesis*

Macpherson, H., Pipingas A., & Ellis, K. (2011). Can taking a daily multivitamin improve cognitive performance in the elderly? Evidence from a steady state visually evoked potential investigation of memory in elderly women. *International Journal of Psychophysiology*, 77(3), 261. Abstract from the 15<sup>th</sup> World Congress of Psychophysiology of the International Organization of Psychophysiology, Budapest, Hungary, September 1-4, 2010.

## Abstract:

*Background:* The concept of healthy cognitive ageing is gaining importance as rapid population ageing is taking place in the western world. As a consequence there is a growing interest in the potential for nutritional interventions to improve cognitive function in the elderly. Many vitamins such as the B vitamins and antioxidants are essential for normal neurophysiological function and a relationship between these vitamins and cognition in the elderly has been established in population studies. However, results from randomised controlled trials have been less promising, possibly due to methodological inconsistencies. To date, no studies have used electrophysiological measures to assess cognitive changes following multivitamin supplementation.

*Study Design, Aims and Hypotheses:* The present study utilized the steady state visually evoked potential (SSVEP) elicited by a task irrelevant 13 Hz light flicker to investigate the effects of multivitamin supplementation on working memory. The trial was designed as a 16 week, double blind, placebo controlled investigation into the effects of daily multivitamin supplementation on a battery of computerised cognitive tasks, verbal recall measures, and the SSVEP associated with performance of a spatial working memory (SWM) task. It was hypothesised that multivitamin supplementation would improve speed of response on computerised memory and information processing tasks and that effects of the multivitamin would be observed on the SSVEP.

*Method:* Participants were 51 elderly women, free from dementia and aged between 64 and 82 years. At baseline, participants completed the Swinburne University Computerised Cognitive Assessment Battery (SUCCAB) and the logical memory sub-test from the Wechsler Memory Scale. The SSVEP was elicited by a 13Hz light flicker superimposed on the visual field and was recorded from 64 electrode channels during the performance of a

SWM delayed response task and a control task. The same procedure using alternate forms of the cognitive tests was repeated at the 16 week post-treatment assessment.

*Results:* The results indicated that the 16 week multivitamin treatment significantly improved response time for spatial working memory and a composite memory measure from the SUCCAB. No effects of the multivitamin were identified for the information processing measures from the SUCCAB or for verbal memory. Following multivitamin supplementation, SSVEP amplitude during the SWM task was significantly reduced and latency was increased at right frontal, central and temporal sites across the task duration. In contrast, these effects were smaller, and less extensive in the placebo group, reaching significance only at occipital sites. As better working memory task performance across all participants at baseline was associated with larger frontal latency increases, this pattern of activity in those who received the multivitamin may represent an improvement in memory related neural processes. Behaviourally, there was a greater reduction in response time on the SWM for the multivitamin treatment than the placebo, however this effect did not reach statistical significance.

*Conclusion:* 16 weeks supplementation with a multivitamin was shown to improve measures of memory and the SSVEP appears to be a sensitive measure of these nutraceutical effects.

Macpherson, H., Pipingas A., Ellis, K., & Silberstein, R. (2010). The effects of multivitamins on cognition and the steady state visually evoked potential (SSVEP) in the elderly. Conference proceedings from the The Australian Cognitive Neuroscience Conference, Melbourne, Australia, 26-29 November, 2010.

Abstract:

Evidence from population studies indicate dietary intake, or blood levels of the B vitamins and antioxidant vitamins may be related to cognitive function in the elderly. Despite these findings, results from randomised controlled trials into the effects of individual vitamins or multivitamins have not consistently demonstrated cognitive benefits in the elderly using standardised neuropsychological or cognitive assessments. An alternate approach may be to examine brain electrical activity measures such as the steady state visually evoked potential (SSVEP). The current study was a 16 week, double blind, placebo controlled investigation into the effects of daily multivitamin supplementation on cognition and the SSVEP associated with performance of a spatial working memory (SWM) task. It was hypothesised that multivitamin supplementation would improve behavioural measures of cognition and that the SSVEP would be sensitive to the effects of the multivitamin. Participants were 51 elderly women, with subjective memory complaints aged between 64 and 82 years. At baseline, participants completed a range of computerised cognitive tests and the logical memory sub-test from the Weschler Memory Scale. Alternate forms of the cognitive tests were used at post-treatment assessment. The SSVEP was elicited by a 13Hz light flicker during the performance of a SWM delayed response task and a control task. The results revealed that multivitamin supplementation significantly improved memory response time on cognitive measures. At post-treatment, there was a decrease in SSVEP amplitude and increase in latency at right frontal, central and temporal sites during SWM response when compared to baseline. Smaller, less extensive effects were seen in the placebo group. In conclusion the SSVEP may be a useful addition when assessing the potential cognitive enhancing effects of vitamins or other nutraceuticals.

Macpherson, H., Pipingas, A., & Ellis, K., (2010). Working Memory in Women at Risk of Cognitive Decline and Dementia, *Clinical EEG and Neuroscience*, 41(2), 103-118. Abstract from the 19<sup>th</sup> Annual Conference of the Australasian Society for Psychophysiology, Newcastle, Australia, November 28-30, 2009.

Abstract:

Across the lifespan, the ageing process is associated with ongoing alterations to the brain and nervous system. The consequences of these neurophysiological changes are apparent during old age, when deleterious cognitive changes occur across a range of functions, particularly those which rely on working memory. This study aimed to investigate the steady state visually evoked potential (SSVEP) associated with performance of a spatial working memory task in 53 community dwelling women aged 64-82 years, who reported subjective memory complaints. Specifically, it was hypothesised that risk factors for cognitive decline such as advancing age, ApoE status and overall memory performance would exert effects on the neural correlates of working memory. The results of this study provided support for this prediction and will be discussed within a compensatory framework of ageing.

Macpherson, H., Pipingas A., & Silberstein, R. (2009). Changes in brain electrical activity (EEG) following 16 weeks of multivitamin supplementation: A double blind placebo controlled study. Conference proceedings from the International Evidence-Based Complimentary Medicine Conference, Armidale, Australia, March 13-15, 2009.

Abstract:

Growing evidence suggests that dietary supplementation with selected micronutrients and nutraceuticals may have the potential to improve cognition in older adults. The results from recent clinical trials have not consistently identified improvements in cognition in healthy elderly following multivitamin supplementation. This clinical trial aimed to investigate the effects of the Swisse Women's 50+ multivitamin supplement on memory and brain function in elderly women (aged 64-81 years). Participants were randomly allocated to receive the multivitamin treatment or a placebo. At baseline, 64 channels of EEG were recorded whilst participants completed a computerised short term memory task. An alternate version of this task was repeated after 16 weeks supplementation with either the multivitamin or the placebo. It was hypothesised there would be changes in brain activity in the treatment group, suggestive of more efficient brain activity, which would not be evident in the placebo group. Preliminary results from this study indicate that EEG may be a useful technique to further assess the effects of multivitamin supplementation on cognition.

*Other manuscripts by the author currently under review*

Grima, N., Pase, M., Macpherson, H., & Pipingas, A. (2011). The effects of multivitamins of cognitive performance: A systematic review and Meta-Analysis. Manuscript submitted for publication.

**Abstract:**

*Objective.* To determine if multivitamins can be used efficaciously to improve cognitive abilities

*Design.* Systematic review and meta-analysis of randomised controlled trials

*Data Sources.* Medline (PubMed), The Cochrane Library, SCOPUS and PsychINFO were searched independently until April 2011 by 2 researchers. Forward and backward searches were performed on all included studies

*Study selection.* Randomized, placebo-controlled, double-blind clinical trials reporting on the chronic effects ( $\geq 1$  month) of oral multivitamin supplementation on any valid cognitive outcomes. Studies must have been performed in cognitively intact adult samples. The quality of each trial was objectively assessed using an augmented Jadad scale.

*Data extraction and synthesis.* Cognitive outcomes were extracted and grouped into cognitive domains by one author and verified by another. Data was analysed using a random effects model with effect sizes calculated using a Standard Mean Difference expressed as Hedges  $g$ .

*Results.* Ten trials were included in analysis with a total of 3,200 participants. Qualitative analysis revealed a bias towards testing memory performance with other cognitive domains such as reasoning and mental speed less well studied. Meta-analysis indicated that multivitamins were effective in improving number facility ( $g = 0.34$  (95% CI: 0.01–0.67),  $p < 0.05$ ) and memory abilities ( $g = 0.23$  (95% CI: 0.05–0.40,  $p < .01$ ). The beneficial effect of multivitamins on memory was limited to immediate free recall ( $g = .24$  (95% CI: 0.06–0.43,  $p < .01$ ) with no enhancement in delayed free recall ( $g = 0.10$ ; 95% CI: -0.23–0.43,  $p = .56$ ). or visual memory ( $g = 0.29$ ; 95% CI: -0.22 –0.81,  $p = .26$ ). There was no evidence of publication bias. The only evidence of heterogeneity was in the overall memory analysis, which was overcome when memory was analysed according to its more specific components. There was no significant effect of multivitamins on any other cognitive domains.

*Conclusions.* Chronic multivitamin supplementation provides modest enhancement to immediate free recall memory and number facility with no evidence of enhancement to any other cognitive abilities.

Harris, E., Kirk, J., Vitetta, L., Macpherson, H., Sali, A., & Pipingas, A. (2011). Effects of a multivitamin, mineral and herbal supplement on cognition and blood biomarkers in a group of older males: A randomised, placebo controlled trial. Manuscript submitted for publication.

Abstract:

Cognitive change occurs across the lifespan, with various mental functions including memory susceptible to decline in old age. Growing evidence suggests that nutritional and vitamin status may be related to cognitive function and decline in the elderly. The current study was an eight-week, placebo controlled, double blind investigation into the effects of a multivitamin, mineral and herbal supplement (Swisse Men's Ultivite®) on cognitive performance in elderly men. Participants were 51 individuals aged between 50 and 74 years, at risk of cognitive decline due to lifestyle and health factors. Cognitive performance was assessed at baseline and post-treatment using a computerised battery of cognitive tasks, enabling the measurement of a range of attentional and memory processes. Blood measures of vitamin B<sub>12</sub>, folate and homocysteine were collected prior to, and after supplementation. The results of this study revealed that contextual recognition memory performance was significantly improved following multivitamin supplementation. Levels of vitamin B<sub>12</sub> and folate were significantly increased with a concomitant decrease in homocysteine, indicating that relatively short term supplementation with a multivitamin can benefit these risk factors for cardiovascular disease and cognitive decline. Findings from this study indicate that daily multivitamin supplementation may improve episodic memory in older men at risk of cognitive decline.

## Appendix B ethics declaration

To: Dr Andrew Pipingas/Ms Helen MacPherson, FLSS  
Dear Andrew and Helen

### **SUHREC Project 0708/063 The effects of multivitamin supplementation on cognition and brain function in memory impaired older adults**

Dr A Pipingas FLSS Ms Helen Macpherson  
Approved Duration: 19/02/2008 To 01/11/2009

I refer to the ethical review of the above project protocols by and on behalf of Swinburne's Human Research Ethics Committee (SUHREC).

I acknowledge notification of appropriate insurance coverage acceptable to Swinburne as emailed on 19 February 2008. With your previous revisions/clarification to date having been found satisfactory, I am pleased to advise that approval for the project to proceed takes effect in line with standard on-going ethics clearance conditions here outlined.

- All human research activity undertaken under Swinburne auspices must conform to Swinburne and external regulatory standards, including the *National Statement on Ethical Conduct in Human Research* and with respect to secure data use, retention and disposal.
- The named Swinburne Chief Investigator/Supervisor remains responsible for any personnel appointed to or associated with the project being made aware of ethics clearance conditions, including research and consent procedures or instruments approved. Any change in chief investigator/supervisor requires timely notification and SUHREC endorsement.
- The above project has been approved as submitted for ethical review by or on behalf of SUHREC. Amendments to approved procedures or instruments ordinarily require prior ethical appraisal/clearance. SUHREC must be notified immediately or as soon as possible thereafter of (a) any serious or unexpected adverse effects on participants and any redress measures; (b) proposed changes in protocols; and (c) unforeseen events which might affect continued ethical acceptability of the project.
- At a minimum, an annual report on the progress of the project is required as well as at the conclusion (or abandonment) of the project.
- A duly authorised external or internal audit of the project may be undertaken at any time.

Please contact me if you have any queries about on-going ethics clearance or if you need a signed ethics clearance certificate. The SUHREC project number should be quoted in communication.

Best wishes for the project.

Yours sincerely

Keith Wilkins  
Secretary, SUHREC

Ethics Declaration: The author would like to state that all ethics conditions pertaining to the ethics clearance were properly met, and all annual reports have been submitted.



Informed Consent

The effects of multivitamin supplementation on cognition and brain function in memory impaired older women

Principal Investigator : Dr Andrew Pipingas<sup>1</sup>
Chief Investigators : Ms Helen Macpherson<sup>1</sup>
Prof Richard Silberstein<sup>1</sup>
Dr Kathryn Ellis<sup>2</sup>

<sup>1</sup>Swinburne University
<sup>2</sup>University of Melbourne

I \_\_\_\_\_
(Name of Participant)

- have read and have understood the information provided in the Form of Disclosure and agree to participate in the study. Any questions I have asked have also been answered to my satisfaction

My agreement is based on the understanding that

- My consent to participate in this study is given freely
Any personal information that I provide will remain confidential
I understand that I am free to withdraw from the study at any time
I agree that research data collected for the study may be published or provided to other researchers on the condition that anonymity is preserved and that I cannot be identified
I agree that study investigators will inform me if tests reveal a possible memory or cognitive impairment. Similarly, study investigators can inform me if tests reveal that I am within "normal" limits of cognitive function.
I do not have epilepsy or a history of epilepsy.

Please tick the box if you agree for us to contact your GP if tests reveal a possible memory or cognitive impairment

Name of Participant.....

Signature.....Date .....

Name of researcher.....

Signature.....Date .....



Consent for APOE Testing

The effects of multivitamin supplementation on  
 cognition and brain function in memory impaired  
 older women

Principal Investigator : Dr Andrew Pipingas (Swinburne University of Technology)

Chief Investigators : Ms Helen Macpherson (Swinburne University of Technology)

Prof Richard Silberstein (Swinburne University of Technology)

Dr Kathryn Ellis (University of Melbourne)

I \_\_\_\_\_

(Name of Participant)

- have read and have understood the information provided in the Form of Disclosure and Alzheimer’s Disease Genetic Fact Sheet. Any questions I have asked have also been answered to my satisfaction

My agreement is based on the understanding that

- I understand that the APOE blood test is optional. If I DO NOT consent to have the APOE blood test this will in no way affect my relationship with the researchers involved in this project or with Swinburne University.
- I understand that the results of the APOE test will remain confidential and that the researchers will not disclose the results of this test to me.
- I understand that if I wish to obtain access to the results of the APOE test, the release of these results will be done in consultation with my General Practitioner. In this situation I agree to have my APOE results sent to my GP.

Name of Participant.....

Signature.....Date .....

Name of researcher.....

## Mood Data

### *DASS21*

The Depression, Anxiety and Stress Scale (DASS21) (Lovibond and Lovibond 1995) is a short questionnaire comprising three sub-scales: depression, anxiety and stress. The DASS is relevant for both clinical and non-clinical populations. There are 21 items encompassing a range of affect related symptoms, including physical symptoms and mood symptoms. Responses are made on a 4-point scale ranging from 0 to 3, with a possible range of scores from 0 to 63. Higher scores indicate more symptoms. A score of zero does not indicate positive mood, rather a lack of symptoms associated with dysphoric mood. Questions relate to feelings “over the past week”. In this trial the DASS was completed at baseline and post-treatment.

### *Depression, Anxiety and Stress Scale Scores at baseline and post treatment*

DASS Score	Treatment Group	n	Baseline		Post Treatment	
			Mean	SD	Mean	SD
Total	Multivitamin	25	9.1	4.7	9.1	7.5
	Placebo	25	5.6	6.2	7.1	6.8
Depression	Multivitamin	25	2.5	2.1	2.4	1.9
	Placebo	25	1.9	2.2	2.3	2.9
Anxiety	Multivitamin	25	2.3	1.9	2.3	2.4
	Placebo	25	1.4	2.5	1.9	2.2
Stress	Multivitamin	25	4.2	3.0	4.3	4.6
	Placebo	25	2.2	2.6	2.9	3.0

### *Mood results*

One way ANOVA revealed at baseline there was a trend for the multivitamin group to demonstrate a higher total DASS score than the placebo group ( $F(1,49)= 4.06, p =.05$ ). There were no group differences on the individual subscales at baseline.

Univariate analysis of co-variance (ANCOVA) was used to examine the effect of treatment group on change in DASS score, with the baseline DASS score included as a covariate.

There were no significant effects of treatment group on total DASS score or any of the subscales.