Effects of a Broad-spectrum Micronutrient Supplement Versus B Vitamins and Vitamin C on Anxiety and Memory in PVG/c hooded rats

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Acknowledgements

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Figure 6: *Mean ± S.E.M of the mean number corner squares occupied for treatment group.*

∗Significantly different (p<.05) from water control group.

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∗Significantly different (p<.05) from water control group.

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∗Significantly different (p<.05) from water control group for that particular sex.

^a Groups with superscripts in common significantly different (P<.05) for that particular sex.

Abbreviations

AD	Alzheimer's Disease
ADHD	Attention-Deficit/Hyperactivity Disorder
ANOVA	Analysis of Variance
°C	Degrees Celsius
CNS	Central Nervous System
CRF	Corticotropin-releasing Factor
dPFC	Dorsal Prefrontal Cortex
EMP+	EMPowerplus
F	Female
GABA	gamma-Aminobutyric acid
GAD	Generalised Anxiety Disorder
g	Grams
HPA axis	Hypothalamic-pituitary-adrenal axis
HPC	Hippocampus
Hyc	Homocysteine
IU	International Unit
M	Male
MCI	Mild Cognitive Impairment
mcg	Micrograms
mg	Milligrams
ml	Millilitres
mm	Millimetres
ms	Milliseconds
N	Number
NMDA	N-methyl-D-aspartate

OCD	Obsessive-Compulsive Disorder
PND	Post Natal Day
POMS	Profile of Mood States
PTSD	Post Traumatic Stress Disorder
RCT	Randomised Controlled Trial
RDA	Recommended Daily Allowance
ROS	Reactive Oxygen Species
S.E.M.	Standard Error of the Mean
SSRI	Selective-serotonin Reuptake Inhibitor
SNRI	Serotonin-noradrenalin Reuptake Inhibitor
TCA	Tricyclic Antidepressant
TX	Treatment
vPFC	Ventral Prefrontal Cortex
5-HTP	5-Hydroxytryptophan

Abstract

Numerous studies have assessed single nutrient and multiple nutrient supplements in the treatment of psychiatric symptoms. Recently there has been growing evidence suggesting that treatment with broad-spectrum micronutrient supplements (Rucklidge et al., 2012), B vitamin combinations (Stough et al., 2011) and vitamin C (Hughes et al., 2011) may have benefits for emotional and cognitive functioning. Whether a combination of nutrients acting synergistically is more effective than the administration of a B vitamin complex or vitamin C alone is yet to be determined. The present study was designed to address this question. To investigate this, 40 male and 40 female rats were administered a broad-spectrum micronutrient supplement (Berocca), a B vitamin complex supplement, or vitamin C alone in their drinking water, for seven weeks. A control group was provided unadulterated water. Animals were then examined through several behavioural measures of anxiety and one of short-term memory. The results revealed a number of significant behavioural differences between treatment groups, with the Y maze showing the greatest number of significant treatment effects. The Berocca group had a significantly higher percentage of entries into and occupancy of the changed arm, as well as total entries of both arms (ambulation) than all other treatment groups. There were also significant sex x treatment interactions in change arm entries and occupancy, with both measures increasing with Berocca supplementation in males, and increasing with both Berocca and B vitamin supplementation in females. This suggests that different treatments affected each sex differently. Overall rats receiving broad-spectrum supplementation (Berocca) showed significantly less emotionality and significantly improved short-term spatial memory when compared to other treatment groups.

<u>**1.0 Introduction:**</u>

1.1 General Overview

Anxiety symptoms and disorders represent an ever increasing burden on healthcare resources creating a huge strain in terms of social and economic costs (Kessler, 2007; Nutt et al., 2007). Currently, anxiety disorders are the most prevalent psychiatric illnesses in Europe and the USA (Alonso & Lepine, 2007; Kessler et at., 2005; Kessler, 2007; Nutt et al., 2007). Epidemiological studies (Alonso et al., 2004; Kessler & Merikangas, 2004) show anxiety disorders have the highest lifetime prevalence estimates of 13.6-28.8% and the earliest age of onset (11 years) of psychiatric disorders (Kessler et al., 2005; Kessler et al., 2005; Nutt et al., 2007).

Anxiety has been defined as a future oriented negative mood state associated with preparation for possible upcoming negative events, characterised by bodily symptoms of physical tension and apprehension about the future (Barlow, 2002). Symptoms of anxiety can be mild and transient and may not cause functional impairment in social and occupational areas. However for many people symptoms can create a severe and chronic impact on functioning across many areas (Baldwin et al., 2005; Cryan & Sweeney, 2011) significantly affecting an individuals ability to carry out normal life activities (Pilkington et al., 2006).

1.1.1 Causes of anxiety

The pathophysiology behind anxiety disorders is complex and still relatively unknown. There is no simple one dimensional cause. Instead it is thought to be associated with biological (inherited anxious traits), psychological (belief the world or situations are dangerous) and social contributors (Barlow & Durand, 2012) integrating to create a triple vulnerability (Barlow, 2002; Barlow & Durand, 2012). Under pressure a given stressor could activate

biological and psychological tendencies to feel out of control. Once this cycle begins it feeds on itself (Barlow & Durand, 2012).

From a neurobiological perspective, anxiety disorders are characterised by a variety of neuroendocrine, neurochemical and neuroanatomical disruptions and may also be influenced by oxidative stress (Bouayed et al., 2009). Neuroanatomically, a prefrontal-amygdala circuit appears to mediate the basic mechanisms involved in human anxiety such as selective attention to threats, interpretation of stimuli, the acquisition and extinction of conditioned fear (Canteras et al., 2010), the formation and retrieval of emotional and fear based memories (Martin et al., 2009). Dysfunction in this circuitry may contribute to the generation of anxiety disorders (Canteras et al., 2010; Bishop, 2007). Several pieces of evidence indicate these brain regions are similar in rodents and humans (Canteras et al., 2010).

These circuits are under the modulation of neurotransmitters and neuroendocrine hormones (Martin et al., 2009). The neurochemistry of anxiety has been dominated by focus on GABAergic and monoaminergic neurotransmission, based on evidence from effective anxiolytics that interact with these systems (Durant, Christmas & Nutt, 2010). The effects of anxiolytic drugs that interact with GABAergic systems and GABAergic abnormalities associated with anxiety in brain imaging studies (Durant, Christmas & Nutt, 2010), suggests increased activity in emotional processing brain regions may be a due to decreased inhibitory signalling by gamma-aminobutyric acid (GABA), or increased excitatory neurotransmission through glutamate (Martin et al., 2009). Antidepressant drugs that act on monoamine systems have implicated a role for serotonin, norepinephrine, and dopamine in the pathogenesis of mood and anxiety disorders (Martin et al., 2009).

Neuropeptides may also play a role (Martin et al., 2009). One important neuropeptide is corticotropin-releasing factor (CRF) which is released via the hypothalamic-pituitary-adrenal (HPA) axis in response to a threatening stimulus, resulting in the production of

glucocorticoids. Prolonged stress can cause dysfunction in the HPA axis and amygdala, leading to exposure to high levels of corticosteroids comprising the hippocampus (HPC). This also creates an increase in the activity of ventral prefrontal cortex and amygdala (involved in fear based memories) and decreases activity in the dorsal prefrontal cortex and nucleus accumbens which mediate an increased sensitivity to aversive events and decreased response to rewards respectively (Wilner et al., 2013).

Oxidative stress has been implicated in anxiety. Reactive oxygen species (ROS) affect a number of physiological functions. When ROS exceed the oxidative capacity of an organism (oxidative stress) they induce oxidative damage on cellular components. The brain is highly vulnerable to oxidative stress due to its high oxygen consumption, modest antioxidant defenses, and lipid-rich constitution (Ng et al., 2008; Halliwell, 2006). In the presence of high oxidative stress the lipid rich constitution of the brain favours lipid peroxidation, which results in a decrease in membrane fluidity and damage to membrane proteins, inactivating receptors, enzymes and ion channels. As a result oxidative stress can alter neurotransmission, neuronal function and overall brain activity (Delattre et al., 2005; Lebel, 1991; Cardozo et al., 1999). Recently a link between oxidative stress and certain anxiety disorders has been established in humans (Kuloglu et al., 2002, 2002a). There has also been links between oxidative and anxiety related phenotypes (Hovatta et al., 2005) and anxiety-related behavior (Bouyed et al., 2007).

1.1.2 The effects of anxiety on cognition

Patients suffering from anxiety disorders frequently present with other cormorbid illnesses including psychiatric disorders such as depression (Kessler et al., 2005) and medical conditions (Harter et al., 2003). Of importance to the present study is the association between stress and anxiety and cognitive functioning, particularly memory. Human and animal studies have indicated anxiety and stress are associated with poorer cognitive functioning (Pittenger,

2013**).** Chronic and uncontrollable stress has numerous effects on plasticity-associated processes throughout the brain in rodent models (Pittenger & Duman, 2008, Lupien et al., 2009; Radley & Morrison, 2005). These include dendritic atrophy, and inhibition of long-term potentiation (LTP) in the HPC and frontal cortex (Sapolsky, 2000; Kim & Diamond, 2002; Duman, 2004; Dranovsky & Hen, 2006; Lui & Aghajanian, 2008; Radley et al., 2006; Goldwater et al., 2009; Pittenger, 2013).

Several studies have found structural brain abnormalities in people with anxiety disorders (Mataix-Cols & van den Heuvel., 2006; Phan et al., 2009; Szezko et al., 2005; van den Heuvel et al., 2005), that could be linked to neuropsychological deficits (Casteneda et al., 2011). Research suggests brain structures subserving episodic memory functioning are affected in anxiety disorders (Araksinen et al., 2005). In a population-based study, impairments in verbal episodic memory and executive functioning in anxiety disorders in general were found, with the nature of the dysfunction depending on subtype (Araksinen et al., 2005).

In terms of specific anxiety disorders, research based on cognitive function of OCD has demonstrated episodic memory impairments for both verbal (Dison, 1995; Savage et al., 1996, 1999) and non-verbal information (Savage et al., 2000; Zitterl et al., 2001) and performance deficits in executive functioning (Mertinot et al., 1990; Parcell et al., 1998a,b; Veale et al., 1996; Head et al., 1989) and visual memory (Casteneda et al., 2011). Deficits have also been found in PTSD in verbal and visual memory and attention (Brewin et al., 2007; Golier & Yehud, 2002; Johnsen & Asbjornsen, 2008; Horner & Hamner, 2002). More information is needed for other anxiety disorders (Casteneda et al., 2011).

1.1.3 Current Pharmacological treatments for Anxiety

Over the last half century pharmacological treatments for anxiety disorders have become more tolerable, available and numerous (Farach et al., 2012). However despite these advances, between one third and one half of patients treated with current medications do not achieve sustained remission from anxiety **(**Pollack et al., 2008). To date, the main psychopharmacological treatments for anxiety are benzodiazepines and antidepressant (SSRIs, SNRIs, TCAs) medications.

Selective-serotonin reuptake inhibtiors (SSRIs) and to a growing degree Serotonin and norepinephrine reuptake inhibitors (SNRIs), are considered the first line pharmacological treatment for anxiety disorders (Ravindran & Stein, 2010) except for specific phobia. They are thought to work by blocking the reuptake of serotonin and norepinephrine, respectively, thereby increasing levels of these neurotransmitters at the synapse (Farach et al., 2012). This is then thought to start a cascade of downstream effects on other neurotransmitters, intracellular second messangers, and immediate early genes (rapidly activated) producing long term chemical changes in the brain (Kirshman & Nestler, 2008). This includes influences on intracellular neurotrophic factors, neurogenesis in the hippocampus, and the HPA stress response system (Wilner et al., 2013).

SSRIs have been touted as efficacious for a range of psychiatric disorders. They have been reported to have relatively fewer side effects and a low potential for abuse compared to other pharamcological treatments. They are also safer and more tolerable than older TCA antidepressants (Ravindran & Stein, 2010)**.** However, there are a number of drawbacks to SSRIs. To start with, the efficacy has been questioned as they have consistently shown only moderate response rates with around 30-40% of patients not responding to treatment (Wilner et al., 2013). Remission rates when treatment has been stopped are also high at around 40%

(Tollefson & Hoffman, 1994; Thase et al., 2001). There are serious questions about how much, and in whom, the placebo effect contributes to antidepressant response.

SSRIs and SNRIS usually take 2-6 weeks to produce a partial response with full benefits often not seen for another 4-6 weeks (Montgomery et al., 2002) and in some cases 10-12 weeks or longer (Tedeschini et al., 2011). They also come with a number of side effects. Approximately 30-50% of patients experience mild and transient side effects including nausea, diarrhea, headache, insomnia, jitteriness or restlessness, potentially increasing anxiety (Farach et al., 2012). Sexual side effects such as diminished interest, performance, and satisfaction occur in 30-50% of patients (Farach et al., 2012). Finally, they metabolically interact with and change blood levels of other drugs and are associated with a withdrawal syndrome that can mimic anxiety when stopped (Farach et al., 2012). Additionally, they have been associated with increased suicidal ideation, promoting the USA FDA's 'black box' warning. The lack of efficacy and serious side effects can make compliance with treatment difficult (Farach et al., 2012).

Benzodiazepines were initially considered first-line treatments for anxiety because of their tolerability and equal efficacy to tricyclic antidepressants (TCAs), but became second-line options when it became clear that SSRIs were both more tolerable and efficacious. Currently, benzodiazepines are primarily used for individuals who have had suboptimal responses to antidepressants (Farach et al., 2012). Benzodiazepines bind to a specific receptor site on the GABA–A complex and facilitate GABA inhibitory effects by acting on a chloride ion channel (Davidson, 1989). They are used for their potent, short-term effects and to help reduce anxiety during the initial weeks of an antidepressant when anxiolytic effects have yet to occur (Goddard et al., 2001). The potency of these medications are appealing to the patient but not always desirable, as they can reinforce pill taking, serve as a safety signal that undermines self-efficacy, and become incorporated into the conditioned fear response (Westra, et al.,

2002). This is exacerbated when benzodiazepines are taken on an as-needed basis. As-needed use links pill taking to rapid reduction in anxiety, powerfully reinforcing avoidance for anxiety-provoking situations and encouraging long-term reliance on the drug. This may be one reason why benzodiazepines have been associated with reduced response to cognitive behavioral therapy (Watanabe et al., 2007). Chronic benzodiazepine use is associated with physiological dependence, cognitive (Barker, 2004) and psychomotor impairment, and health problems (Pelissolo et al., 2007). Withdrawal from benzodiazepines can result in adverse reactions including rebound anxiety, paranoia, insomnia and depression. The effects of benzodiazepine withdrawal can ultimately worsen anxiety and potentially cause depression (Farach et al., 2012). Despite the advantages of benzodiazepines over previous drugs, their long-term use is hampered by dependence liability, tolerance, and cognitive and other behavioural side effects (Cryan & Sweeny, 2011).

The large number of unpleasant side effects and discontinuation effects associated with current pharmacological treatment and more specifically the addictive nature of benzodiazepines and inefficacy of antidepressant treatments has led to a search for alternatives. The hope being to reduce psychiatric symptoms with fewer side effects and risks associated with long-term use, as well as a treatment that is able to produce a response rate as good as or better than the current treatments. One area that has been gaining traction/seen an increase in investigation recently is the use of micronutrient (vitamins and minerals) supplementation in mental health.

Vitamin-based products are the most widely used food supplements throughout the developed world (Kennedy & Haskell, 2011). There is a wealth of evidence from epidemiological studies that clearly suggests a relationship between micronutrients and psychological functioning (Kennedy et al., 2010). This is not surprising as the central nervous system (CNS) is dependent on a wide range of micronutrients for optimal functioning

(Kennedy et al., 2010; Rucklidge & Kaplan, 2013). A number of studies have assessed single and multiple micronutrient supplements in the treatment of psychiatric symptoms with recent evidence suggesting that treatment with broad-spectrum micronutrient supplements (Rucklidge et al., 2012), B vitamin combinations (Stough et al., 2011) and vitamin C (Hughes et al., 2011) may have benefits for emotional and cognitive functioning. These effects are likely due to correction of micronutrient deficiencies, which may lead to the expression psychiatric symptoms, by broad-spectrum micronutrients and B-vitamins (Rucklidge & Kaplan, 2013), and the potent antioxidant properties of vitamin C (Cantuti-Castelvetri et al., 2000; Harrison et al., 2009a,b). Whether a combination of nutrients acting synergistically is more effective than the administration of a B vitamin complex or vitamin C alone is yet to be determined. The present study is designed to address this question by determining whether a broad-spectrum micronutrient supplement (Berocca, a combination of B vitamins and vitamin C) has greater benefits than a single nutrient supplement (Vitamin C or B vitamin complex) in decreasing anxiety-related behaviour and improving short-term memory in healthy rats.

1.2 Micronutrient formulas

The mixed results that have been reported in relation to single vitamins and minerals (Kaplan et al., 2007) and their effects on mental health have lead researchers to start investigating the effects of broad spectrum micronutrient formulas (multi-ingredient) may have on mental health. This recent shift in research focus has been controversial given the scientific method requires that only one independent variable, such as a single micronutrient, should be manipulated during an investigation of its effects on dependent variables to reduce the number of confounding factors (Kaplan & Leung, 2011). Research involving a number of independent variables is thrown out as having too many confounds and therefore interfering with the interpretation of results. Following this, designing studies with a multi-ingredient formula, as an intervention has required a shift in thinking in scientific and academic fields (Kaplan & Leung, 2011).

The growing body of research in this area has investigated the effects of micronutrient formulas in relation to a number of mental health areas including mood and anxiety disorders (Kaplan et al., 2002; Kaplan et al., 2004), ADHD (Rucklidge et al., 2011b) and OCD (Rucklidge, 2009). Of relevance to the present study is research showing that supplementation with Vitamin C alone (Hughes et al., 2011; Brody et al., 2002; Mazloom et al., 2013; Harrison et al., 2009a,b), B-vitamins alone and in combination (Stough et al., 2011; Abraham et al., 2010; Morales et al., 2010; Lewis et al., 2013), and a broad-spectrum micronutrient supplement (particularly Berocca) (Carroll et al., 2000; Kennedy et al., 2010; Cheng et al., 2004; Halliwell & Kolb, 2003) may be effective in reducing stress and anxiety, and improving memory in animals and humans.

The recommended dose of Berocca of one tablet per day has been shown to have therapeutic benefits in reducing stress and anxiety in humans (Carroll et al., 2000; Kennedy et

al., 2010). However both B vitamins and vitamin C have been shown to produce beneficial effects on stress or anxiety symptoms at doses higher than in the Berocca formulation (Stough et al., 2011; Hughes et al., 2011). This would suggest that a higher dose of two tablets per day of Berocca could produce a greater anxiolytic effect. Comparing single micronutrients to a broad-spectrum micronutrient supplement at a high dose is useful in determining appropriate supplementation and dosage for stress and anxiety. It may also help to determine further avenues of research in this area, such as whether deficiencies in a number of micronutrients or a single micronutrient contribute to anxiety related behaviour. A control group was also included in the present study to provide a comparison group of animals not receiving a supplementation as well as comparisons between treatment groups.

1.3 Assessing anxiety-like behaviour in rats

Anxiety is highly conserved during evolution as a vital emotion. Its interaction with cognitive parameters regulates behaviour in humans and animals (Ohl, 2005). It allows an individual to avoid a perceived threat or dangerous situation. The assessment of anxiety-related behaviour in rodent models is based on the assumption that anxiety in rodents is comparable to anxiety in humans (Ohl, 2005). However it cannot be proved that rodents experience anxiety in the same way as human beings (Ohl, 2005). Here the definition between anxiety and fear becomes very important. In humans anxiety is defined as a future oriented mood state associated with preparation for possible upcoming negative events (Barlow, 2002); and fear as an alarm response to present or imminent danger (real or perceived) (Craske et al., 2009). This is comparable to the animal predatory imminence continuum (Fanselow & Lester, 1988), whereby anxiety corresponds to an animal's state during a potential predatory attack and fear corresponds to an animal's state during predator contact (Craske et al., 2009). Models where a rodent is placed into a situation they perceive as potentially threatening have allowed the determination of distinct behavioural and physiological patterns that undisputedly indicate anxiety (Ohl, 2005). From these an association between anxiety in humans and rodents may be assumed (Ohl, 2005).

1.3.1 Validity in Animal Models of Anxiety

McKinney and Bunney (1969) proposed that an ideal animal model for any human clinical condition must fulfill three criteria. First, to satisfy predictive validity pharmacological treatments known to be effective in humans should induce comparable effects in the animal model. Therefore for anxiety the model should respond to anxiolytics such as benzodiazepines with reduced anxiety (Ohl, 2005). Second, to satisfy face validity, the responses or symptoms observed in humans should be the same in the animal model. For

anxiety the animal must display defensive or avoidant behaviour when exposed to a threatening stimulus (Ohl, 2005). Third, to satisfy construct validity, the underlying rationale should be the same in both humans and animal models. The underlying mechanisms of anxiety and its psychological causes must be identical (Ohl, 2005).

Due to the heterogeneous forms of pathological anxiety and subjective reports of symptoms in humans, meeting all three validity criteria is difficult for an animal model of anxiety. However aspects, such as the physiological and behavioural responses to aversive stimuli, are similar in humans and animals. This means animal models are useful as behavioural tests to screen for potential anxiolytic properties of new drugs and as tools to investigate specific pathogenic aspects of primary symptoms of anxiety disorders (Ohl, 2005).

1.3.2 Behavioural expressions and the approach avoidance conflict:

The main current behavioural tests of unconditioned anxiety in rodents focus on creating an approach-avoidance conflict in the animal (Encanner, 2014). They play on the conflicting drives of the animal to explore a novel environment and avoid any potential treat. This conflict moderates species-specific behavioral expressions of anxiety. It is well known that rodents tend to avoid the unprotected area of a novel environment upon entering it. Rodents typically explore the areas of new environments adjacent to the walls while avoiding an open area (Prut & Belzung, 2003). The aversive nature of an open area can be modified using differing light levels. A brightly lit area is thought to be more aversive for a rodent than a dim area and therefore produces more noticeable avoidance behaviour. The elevation of an open area is also thought to increase aversive behaviour (Ohl, 2005).

Exploratory behaviours encompass a wide range of behavioural patterns such as walking, rearing, climbing, sniffing and manipulating objects (Cryan & Sweeney, 2011; Ohl,

2005). It is hypothesized that explorative behaviours are inhibited by anxiety and therefore a reduction in the above behaviours would indicate an increase in anxiety. Cognitive dysfunctions in rats have also been suggested to be interrelated to anxiety-related behavior (Ohl, 2003).

The current study used four empirically supported behaviour tests. Three were of unconditioned anxiety-like behaviour; the emergence latency (light-dark box) in an open field, the open field test and an elevated plus maze. The responsiveness to brightness in a Y-maze test which is used to examine spatial working memory, has also been shown to be a useful index of anxiety (Aitchison & Hughes, 2006). Research to date has shown that known anxiolytic agents such as benzodiazepines, busipirone, paroxetine, propanolol and fluoxetine have produced a reduction in anxiety-like behaviour in rats using the above animal models (Cryan & Sweeney, 2011).

The emergence latency (light-dark box), open field and elevated plus maze tests play on the conflicting drives of avoidance an exploration of a perceived threatening stimulus (i.e. an open space or lit area) (Crawley, 1985; Hascoët & Bourin, 2003; Montgomery, 1955; Salum et al., 2003). When a rat is confronted with a novel environment its behaviour is determined by the conflict between its drive to explore the unknown area and its motivation to avoid potential danger (Ohl, 2003), which is assumed to represent anxiety (Ennaceur, 2014). The open field and elevated plus maze also assess exploratory behaviour (rearing, walking and grooming) as further indicators of anxiety (Carli et al., 1989; Lamprea et al., 2008; Escorihuela et al., 1999; Lepicard et al., 2001; Rodgers & Dalvi, 1997; Espejo, 1997; Hannigan & Isaacson, 1981; Kalueff & Tuohimaa, 2004; Kametani, 1988; Torres, 1961; Nin et al., 2012).

The Y-maze is a well known test of spatial working memory in rats (Kumar et al., 2013), that is based on the tendency of rodents to explore novel environments (Dellu et al.,

2000; Ma et al., 2007). Responsiveness to brightness change in a Y-maze has been shown to be a useful index of curiosity motivated spatial working/short term memory (Hughes & Maginnity, 2007; Hughes & Neeson, 2003; Hughes, 2004). The Y-maze can also be used to assess anxiety-like behaviour. Activity in the Y-maze is inversely related to anxiety (Archer, 1973). It is thought that higher levels of anxiety result in fewer changed arm entries and occupation (Aitchison & Hughes, 2006; Aitken, 1974; Hughes, 1987; Misslin & Ropartz, 1981).

1.4 Animal studies with micronutrients

To date minimal research has focused on the effects of broad spectrum micronutrient supplementation on anxiety-like behaviour in rats. This makes it difficult to predict the types of behavioural changes that could be exhibited following supplementation. There is slightly more research assessing the effects of individual nutrients on anxiety-like behaviour in rats which may provide a guideline for the types and size of behavioural effects expected. More specifically, vitamin C has been well researched with a number of studies reporting anxiolytic properties. Although there is little research into B vitamin complexes, individual B vitamins have shown similar anxiolytic effects.

1.4.1. Broad-spectrum micronutrient supplementation:

The majority of research into broad-spectrum micronutrient supplementation has focused on human trials. However some animal research has been conducted. The results from these studies suggest broad-spectrum micronutrient supplementation may be effective in reducing anxiety (Naismith Thomass, 2013) as well as mitigating the effects of stress-induced injury and traumatic brain injury on anxiety, memory and neuroanatomical outcomes (Cheng et al., 2004; Halliwell & Kolb, 2003; Halliwell, 2003; Hasan et al., 2013).

Naismith-Thomass (2013) investigated the effects of the broad-spectrum micronutrient formula EMPowerplus (EMP+) on anxiety-like behaviour in rats and the dose related anxiolytic effects. Rats received a diet of 0%, 1.25%, 2.5%, or 5% EMP+ for 141 days. Animals were tested on behavioural measures of anxiety-like behaviour and memory at mid (PND 136-138) and late adulthood (PND 186-188). The highest dose (5%) diet group occupied centre squares most and corners the least in an OFT compared to all other groups, suggesting a higher dose of EMP+ is associated with the greatest reduction in anxiety related behaviour. However this study found no significant differences between any treatment and

control groups in another test of anxiety (light-dark box) and a measure of short-term spatial memory (responosiveness to brightness in a Y-maze). Furthermore animals were found to display more anxiety-like behaviour at late adulthood regardless of receiving a supplemented diet or not. It is important to note that this study was interrupted by the September 4th, 2010 Canterbury earthquake, which may have compromised the results.

Cheng et al., (2004) investigated the protective effects of a broad-spectrum micronutrient supplement on stress-induced injury established by electric foot shock in rats. Rats were fed small, medium and large doses of the micronutrient supplement for 6 weeks. Compared to control, rats that consumed the micronutrient supplement exhibited less anxiety-like behaviour in an OFT, and had lower levels of serum cortisol, epinephrine and norepinephrine. A broad-spectrum micronutrient supplement was beneficial in improving stress-adapting ability and attenuating stress induced injuries in rats. A further study found supplementation with a broad spectrum of micronutrients reduced oxidative changes and DNA damage associated with chronic stress in mice (Hasan et al., 2013).

Halliwell and Kolb (2003) and Halliwell (2003) examined the effects of EMP+ on recovery from early brain injury. Rats lesioned at PND3 in the frontal cortex show drastic permanent reductions in cortical thickness and functional performance, including decrements on tasks involving motor skill and spatial learning and memory. When tested as adults those administered EMP+ in their food throughout their lives showed an increase in brain weight, cortical thickness and restoration of functioning in behavioural tasks as well as enhanced new dendritic growth and increased spine density. Rats with perinatal post parietal lesions, when fed EMP+ showed similar anatomical recovery and restoration of cognitive and motor capacity assessed by OFT, water maze, and tray reaching tests.

Halliwell et al., (n.d) investigated the effects of chronic stress during pregnancy and/or a prefrontal injury in rats on behavioural and anatomical outcomes, as well as the effects of

micronutrient supplementation following chronic stress and/or PF injury. It was found that the micronutrient treatment improved performance on a number of behavioural measures including skilled reaching, latencies in a MWM and exploratory behaviour in the EPM. These studies indicate there are some benefits of broad-spectrum micronutrient supplementation for cognition and emotionality in both normal and lesioned animals.

1.4.2 B Vitamins:

Although little if any research has investigated the effects of a B vitamin complex in animals, a number of studies have focused on individual B vitamins. This research has shown benefits of acute and chronic supplementation with high doses of specific individual B vitamins in anxiety (B6) (Abraham et al., 2010) and memory (B3 and B9) (Morales et al., 2010; Carletti et al., 2012; Matte et al., 2007) suggesting probable neuroprotective effects as well as influences on neurotransmitter systems as the mechanisms behind these effects.

Anxiety as well as alterations in serotonergic function in the hippocampus (HPC) are thought to be associated with diabetes mellitus. Abraham et al., (2010) found anxiolytic effects of 15 days of supplementation with a high dose of pyridoxine (B6) (100mg/Kg of bodyweight) in combination with insulin and aegle marmelose (plant extract) in an elevated plus maze (EPM) in diabetic rats compared to control non diabetic rats. They also found a normalisation of serotonin concentration and gene expression in the HPC to near control. This suggests pyridoxine in combination with insulin and aegle marmelose has a role in normalising diabetes-related stress and anxiety through HPC serotonergic function.

Morales et al., (2010) investigated the neuroprotective effect of acute doses (100mg/Kg of bodyweight) of nicotinamide (B3), administered 24, 48 and 72 hours after birth, against perinatal asphyxia in the HPC on behaviour at 30 and 90 days of age in rats. Nicotinamide prevented apopotosis in the HPC, working memory deficits in a novel object

recognition test and anxiety in an elevated plus maze in perinatal asphyxia compared to saline control. The authors proposed nicotinamide may protect against oxidative stress, ischemic injury and inflamation. These mechanisms may help prevent anxiety-related behaviour and memory impairment following perinatal asphyxia in rats.

A Further study (Carletti et al., 2012) investigated the effects of injecting folic acid (B9) once a day for 23 days, at a dose of .011 μmol/g of bodyweight, on Wistar rats that underwent hypoxia-ischemia (HI) at PND7. Folic acid prevented anxiogenic effects in an OFT and impairment of aversive memory in an inhibitory avoidance test of HI rats. This suggests potential neuroprotective and anxiolytic effects of folic acid following ischemic injury.

Another recent study looked at the effects of folate after induced chronic hyperhomocysteinemia (elevated homocysteine) in rats (Matte et al., 2007). Increase in homocysteine (Hyc) from a B vitamin deficient diet is hypothesised to contribute to cognitive decline. Hyperhomocysteinemia impaired reference memory for platform location in a water maze compared to controls. Treatment with folate at a dose of .011 μmol/g of bodyweight, for 22 days, prevented these impairments. It was suggested these effects were possibly due to its antioxidant effects of folate.

These studies suggest some anxiolytic and neuroprotective benefits for certain B vitamins in animal models. Possible mechanisms include effects on neurotransmitter function, oxidative stress, and inflammation. However research in this area is limited, and to date no research has looked at a B vitamin complex in animals.

1.4.3 Vitamin C

The anxiolytic and cognitive benefits of vitamin C have been well studied in animals. It has been implicated in anxiety and psychosocial stress-related behaviour (Hughes et al., 2011). These effects have been exhibited in poultry (Jones et al., 1996; Satterlee et al., 1994),

rats (Hughes et al., 2011), and humans (Brody et al., 2002; Mazloom et al., 2013). A number of studies have shown pre-treatment with vitamin C affects indices of fear in poultry, such as attenuated tonic immobility and decreased neophobia in Japanese quail (Jones et al., 1996) and broiler chickens (Satterlee et al., 1994). It has also been shown to reduce emergence latency in a light-dark test in Japanese quail (Jones et al., 1999).

Vitamin C has been reported to reduce time spent by mice in dark versus aversive light compartment (De Angelis & Furlan, 1995). DeAngelis and Furlan (1995) tested vitamin C and oxiracetam on scopolamine-induced amnesia in habituation tests in aged mice using a light-dark aversion test. Vitamin C alone (at doses of 62.5, 125, 250 mg/kg) or in combination with oxiracetam significantly reduced time spent in the dark and prevented significant alteration in learning patterns induced by acute scopolamine, compared to control. This suggests both anxiolytic and cognitive benefits.

Exposure to vitamin C (75-100 mg/kg) for 80 days has been found to markedly reduced anxiety in rats (Hughes et al., 2011). This was shown in increases in open field ambulation and occupancy of the four centre squares and decreased occupancy of corner squares, as well as decreased acoustic startle response. Further studies have shown benefits in zebra fish. Puty et al., (2014) treated zebra fish with ascorbic acid (a form or vitamer of vitamin C) before methyl mercury (a neurotoxin that causes learning deficits and memory impairments and anxiety-like behaviour) exposure. Pre-treatment with vitamin C (2mg/kg) blocked significant anxiogenic effects of methyl mercury in a light dark preference model, as well as preventing decreases in extracellular serotonin found in controls.

One study has investigated the effects of vitamin C supplementation on depression-like behaviour and ROS in rats following stress, using a model of chronic unpredictable stress (CUS) (Moretti et al., 2012). Just seven days of supplementation with vitamin C (10mg/kg) or fluoxetine (SSRI) prevented CUS-caused depressive-like behaviour in a tail suspension and

splash test. Vitamin C and fluoxetine also prevented oxidative damage found in controls. These studies provide evidence that vitamin C has been shown to reduce anxiety-like behaviour in the animal models used in the current study. It has also been as effective as current mainstream treatment (SSRIs) in preventing depression-like behaviour.

Vitamin C may also slow age-related degeneration of the CNS and associated cognitive decline especially in the presence of neurodegenerative diseases (Cantuti-Castelvetri et al., 2000; Martin et al., 2002; Morris et al., 1998, 2002). Evidence from animal research shows improvements in learning and memory following vitamin C treatment (Arzi et al., 2004; Harrison et al., 2009a,b; Hasanein & Shahain 2010; Hughes et al., 2011). Using the passive avoidance method (learning and memory) to assess cognitive function, Arzi et al. (2004), found chronic vitamin C treatment (300mg/kg) significantly improved cognitive function compared to control in aged mice. Hasanein and Shahain (2010) also found improvements in learning and memory in diabetic mice after chronic vitamin C (50 mg/kg) administration. Harrison et al. (2009a), showed acute administration of vitamin C (125 mg/kg) in APP/PSENI mice (mouse model of Alzheimer's disease) improved performance in tests of learning and memory. In another study Harrison et al. (2009b) found acute vitamin C (125 mg/kg) administration partially attenuated scopolamine-induced cognitive deficits in Morris water maze performance in young mice.

 Parle and Dhingra (2003) used exteroceptive (EPM, passive avoidance tests) and interoceptive (scopolamine, diazepam, aging) tests in mice to assess the effects of vitamin C (60 and 120 mg/kg) on anxiety-like behaviour and cognition. Vitamin C improved learning and memory of aged mice in the passive avoidance test and provided protection for young animals from scopolamine and diazepam induced memory impairment. Mhaidat et al (2015) found vitamin C (150 and 500 mg/kg) prevented oxidative stress in the HPC and impairments in short and long-term memory in a radial arm water maze following chronic sleep

deprivation. Finally, An et al (2015) found administration of vitamins C (300mg/kg) and E for 7 days to Wistar rats that had been administered melamine, significantly ameliorated cognitive deficits induced by melamine. This was assessed using a Morris water maze. Both vitamins also prevented oxidative damage to the HPC and ameliorated HPC apoptosis.

This research suggests benefits of acute and chronic vitamin C supplementation, at high and lower doses, on learning and memory in animal models. The anxiolytic and cognitive benefits found in these studies are likely due to vitamin Cs powerful antioxidant action.

1.5 Human studies with micronutrients

1.5.1 Broad-spectrum micronutrient supplementation:

Over the last decade a number of well-conducted trials have investigated the impact of broad spectrum micronutrient supplementation on stress, anxiety, and cognition, reporting benefits in both healthy and stressed individuals (Rucklidge & Kaplan, 2013). A number of studies have focused on the effects of the broad-spectrum micronutrient supplement EMPower plus (EMP+). In a randomised controlled trial Rucklidge et al., (2012) showed supplementation with Berocca (one tablet per day) and varying doses (low, medium and high) of CNE^{TM} (an identical formula to EMP+) reduced psychological symptoms of stress and anxiety in adults experiencing heightened anxiety/stress 2-3 months after an earthquake compared to control. In an open label trial, Rucklidge et al., (2011) reported that 8 weeks of treatment with EMP+ produced significant improvements in mood, anxiety and stress in adults with both ADHD and severe mood dysregulation. Positive effects of EMP+ treatments have also been reported in OCD with children (Kaplan et al., 2002; Kaplan et al., 2004; Rucklidge, 2009, Frazier, et al., 2009, 2012; Rodway et al., 2012) and adults (Rucklidge, Taylor & Whitehead, 2011; Kaplan et al., 2001).

Three double blind, placebo controlled trials have looked at the effects of Berocca supplementation on stress and anxiety. Carrol et al., (2000) showed 28 days of Berocca supplementation (one tablet per day) significantly reduced anxiety and perceived stress compared to placebo in healthy men. Investigating psychometric measures of stress in patients with high stress levels, Schlebusch et al., (2000) found a significant degree of improvement with Berocca (one tablet per day) treatment compared to placebo. Kennedy et al., (2010) found Berocca (one tablet per day) supplementation lead to improved ratings of stress, mental health and vigour, as well as improved cognitive performance during intense mental

processing compared to placebo. These results suggest broad-spectrum micronutrient supplementation may be effective in reducing anxiety and stress, and show some potential for improving cognitive performance in healthy and stressed individuals.

The extent to which broad-spectrum micronutrient supplementation improves cognition is uncertain (Pipingas et al., 2014), with several studies demonstrating no impact on cognitive performance (Cockle et al., 2000; Wolters et al., 2005), while more recent studies suggest potential benefits (Harris et al., 2012; Macpherson et al., 2012; Haskell et al, 2010). A recent meta-analysis (Grima et al, 2012) of ten trials showed one month of multivitamin supplementation produces some improvements in immediate recall, but no other cognitive domains. Another meta-analysis (Eilander et al., 2010) of 12 randomised controlled trials in healthy children (ages 5–16) found no significant effects on cognitive processing speed, working memory and long-term memory. However, four trials included in the analysis showed benefits in academic performance in school-age children (Magner et al., 2008; Schoenthaler et al., 1991; Vazir et al., 2006; Wang et al 2003).

A number of recent randomised placebo controlled trials have provided evidence that broad-spectrum micronutrient supplementation may yield some benefits in cognitive performance in adults and elderly populations. In a placebo controlled, double blind investigation, Harris et al., (2012) found 8 weeks of a multivitamin, mineral and herbal supplement (one tablet per day of Swisse Men's Ultivite) significantly improved episodic memory in middle-aged to elderly men compared to placebo. Macpherson et al., (2012) conducted a further randomised, double blind, placebo controlled trial to investigate the effects of a multivitamin, mineral and herbal supplement (one tablet per day of Swisse Women's 50+ Ultivite) on elderly women (aged between 64-82 years). They showed that 16 weeks of supplementation improved the speed of spatial working-memory compared to placebo. However they found no improvements with supplementation on measures of attention or

verbal memory compared to placebo. In a placebo controlled, double blind, randomised trial Haskell et al. (2010), investigated the effects of a multivitamin/mineral supplement (Supradyn, one tablet per day) on measures of cognitive function and fatigue in healthy females (aged 25-50 years). They found improvements on a computerised multitasking framework consisting of memory search, mathematical, stroop and numerical processing tasks after 9 weeks of supplementation compared to placebo. Therefore, although the evidence is inconsistent some results suggest broad-spectrum micronutrient supplementation may benefit cognition.

*1.5.2 B Vitamin Complex Supplementation***:**

As well as studies that specifically investigated Berocca (mentioned above), two studies have shown supplementation with a B vitamin complex reduces stress or anxiety in humans. In a randomised double-blind placebo-controlled trial, Stough et al. (2011) investigated the efficacy of 3 months administration of a B vitamin complex on mood and psychological strain associated with chronic work stress. The B vitamin complex group reported significantly lower personal strain and a reduction in confusion and depressed/dejected mood as compared with the placebo group, suggesting a decrease in the experience of workplace stress. Another randomised double-blind placebo-controlled trial (Lewis et al, 2013) evaluated the efficacy of a B vitamin complex for improving depressive and anxiety symptoms in adults diagnosed with a depressive disorder. The B vitamin complex group showed significant and more continuous improvements in depressive and anxiety symptoms, compared to placebo.

A number of cross-sectional studies have investigated B vitamin deficiency effects on mental health. Sanchez et al (2009) found an association between low blood B vitamin levels or high serum homocysteine and higher prevalence of depressive symptoms. This study was a cross-sectional analysis of 9670 participants using a validated semi quantitative food

questionnaire to ascertain vitamin intake. Similar results have been reported in a number of cross-sectional (Hvas et al., 2004; Ramos et al., 2004; Tolmunen et al., 2004; Sacholev et al., 2005) and case control studies (Lee et al., 1998; Tiemeir et al., 2002). Furthermore, a cross-sectional analysis of the West Australian Pregnancy Cohort (Raine) study (Herbison et al., 2012), found lower intakes of vitamins B1, B2, B3, B5, B6 and folate (B9) were associated with higher externalizing behavior (aggressive/delinquent) scores and reduced intake of B6 and folate were associated with higher internalizing (withdrawn/depressed) behaviour scores.

B vitamins play key roles at all levels of brain function (Kennedy & Haskell, 2011). It is therefore not surprising that a relationship exists between dietary consumption of B vitamins and cognitive performance. This has been investigated in cross-sectional and prospective studies of the elderly (Stough et al., 2011), revealing positive relationships between cognitive performance and B vitamin intake coupled with a negative relationship between cognitive performance and levels of plasma homocysteine (high in B vitamin deficiency, Del Parigi et al., 2006). B vitamin deficiency may be a casual factor in cognitive decline and dementia as a result of subsequent elevation in homocysteine levels (Kennedy & Haskell, 2011). Smith (2008) identified a total of 84 cross-sectional studies and 25 prospective studies that addressed this issue. Of these, 77 of the cross-sectional studies, involving more than 34 000 subjects, showed a negative relationship between cognitive deficits or dementia and the status of folate (B9), B6 or B12 and a positive relationship with homocysteine levels. However numerous reviews on the effects of B-vitamin supplementation in demented and cognitively intact cohorts have concluded that there is a lack of evidence to date for efficacy of these vitamins regarding cognitive function (Kennedy & Haskell, 2011).

A recent prospective longitudinal cohort study of 7030 postmenopausal women free of MCI/probable dementia, found folate intake below RDA at baseline was associated with increased risk of incidence of MCI/probable dementia after controlling for multiple

confounders (Agnew-Blias et al., 2014). However, there was no significant association with other B vitamins. Although the research into B vitamin supplementation is limited, the studies presented here provide some evidence that supplementation may benefit anxiety, mood and cognition.

1.5.3 Vitamin C Supplementation:

Animal research has implicated Vitamin C as an effective in agent in reducing anxiety and psychosocial stress-related behaviour (Hughes et al., 2011). These effects have also been shown in humans (Brody et al., 2002; Mazloom et al., 2013; Chui & Greenwood, 2008; Zhang et al., 2011). In a randomized double-blind placebo controlled trial with healthy young adults, Brody et al. (2002) found high dose vitamin C (3000mg/day) reduced blood pressure, subjective and state anxiety responses to acute psychological stress, and improved cortisol recovery after a stressor. Furthermore a randomized, placebo-controlled, single-blinded trial (Mazloom et al 2013) found vitamin C supplementation (1000mg/ day) significantly reduced anxiety in diabetic patients measured by a depression anxiety stress scale. These results suggest vitamin C may have a positive role in diminishing anxiety in humans. Zhang et al, 2011 examined in a double blind clinical trial the effects of vitamin C (500mg 2x /day) or vitamin D (1000IU 2x /day) on mood (POMs) in non-psychiatric hospitalised patients. Vitamin C therapy was associated with a 34% reduction in mood disturbances. These results suggest high dose vitamin C supplementation may be useful in reducing anxiety and improving mood.

In terms of effects on cognition and memory, one study, Chui and Greenwood (2008) found that consumption of 1000mg vitamin C and 800IU of vitamin E with a meal prevented meal-induced performance deficits on measures of delayed verbal recall (word list, paragraph recall) and working memory (digit span) in adults with type 2 diabetes mellitus. Memory

impairment is often observed in adults with type 2 diabetes mellitus with further acute deficits after meal ingestion.

Further research has investigated dietary intake and blood levels of vitamin C, and its effects on cognition and Alzheimer's Disease (AD). A number of large population studies have investigated associations between risk for AD and vitamin C intake. One study with data from the Chicago Healthy aging project found no dementia free participants aged over 65 developed AD over a follow up (mean of 4 years) when supplementing with vitamin C (Morris et al., 1998). However, other studies have found no beneficial effects of vitamin C intake on dementia (Gray et al., 2008; Luchsinger et al., 2003). The Honolulu Asia Aging Study (Masaki et al., 2000) found a more complex pattern (Harrison et al., 2014) with men aged 71-93 years. Higher vitamin C intake was associated with enhanced cognitive functioning in cognitively intact individuals and a lower likelihood of vascular dementia. However, there was no relationship between AD and vitamin C intake. The Rotterdam Scan Study (Engelhart et al., 2002) reported an 18% reduced relative risk for AD with higher vitamin C intake.

Higher blood vitamin C levels have been associated with improved cognitive function. Goodwin et al., (1983) showed higher plasma vitamin C was associated with improved verbal memory recall and mathematical calculations. Furthermore Gale et al. (1996) reported significantly increased risk for cognitive impairment in those with depleted plasma vitamin C or very low intake. Higher plasma vitamin C has also been associated with better free recall, recognition and vocal but not working memory in men over 65 (Perrig, Perrig & Strahelin, 1997). Although research into vitamin C supplementation in humans is limited, results from studies assessing dietary intake and blood levels suggest an association between vitamin C and cognitive performance.

1.6 Proposed mechanisms of action

1.6.1 Broad-spectrum micronutrient supplementation:

The rationale of broad-spectrum nutrient combinations for the treatment of psychiatric symptoms is based on human physiology. Many nutrients are required together in balance for optimal functioning. Dietary supplements with single nutrients may upset this balance and create deficiencies in other nutrients (Rucklidge and Kaplan, 2013). Mertz (1994) suggested single nutrients may create imbalances or deficiencies in other nutrients with any substantial change of one nutrient for extended periods affecting the requirement for other nutrients with which the first interacts. Single nutrient supplementation may be too simple and does not account for hundreds of interactions among nutrients (Mertz 1994).

Kaplan and Leung (2011) have proposed seven mechanisms by which micronutrients could influence mental function. They proposed broad spectrum micronutrient supplementation may help to correct in-born errors in metabolism, deficient methylation, alterations in gene expression, long latency effects (triage theory), down-regulation of micronutrient receptors, impaired growth and development of neurons and impaired mitochondrial functioning. These mechanisms are not mutually exclusive but may intertwine and overlap, working in conjunction with one another to alleviate mental illness.

Mental illness with a well-established genetic predisposition may be the result of inborn errors in metabolism that slow metabolic activity involving neurotransmitters in the brain. In-born errors in metabolism of key neurobiological pathways (i.e synthesis and uptake of neurotransmitters) may affect brain function because micronutrients are involved in many enzyme reactions, as cofactors, in the synthesis and metabolism of neurotransmitters. Deficiencies in these essential cofactors would lead to depletions in neurotransmitters essential

for optimal functioning and could impair ability to utilise nutrients, thereby contributing to psychiatric symptoms.

Micronutrient treatment may facilitate or correct that metabolic activity. Evidence shows at least a third of all known genetic mutations diminish binding affinity for a coenzyme by a known enzyme (Ames, 2004). Therefore suboptimal coenzyme (micronutrient) levels could decrease binding affinity and lower metabolic activity. Genetic diseases associated with this defect are in most cases treatable with micronutrients (Ames, Elson-Schwals & Silver, 2002).

Methylation (the addition of a methyl group [CH3] to a molecule) is an essential biological function involved in gene regulation, enzyme activation and the regulation of protein synthesis. DNA transcription and neurotransmitter synthesis are dependent on methylation processes. Micronutrients play a central role in methylation activity. Supplementation may enhance methylation of important brain enzymes, resulting in enhanced neurotransmitter synthesis (Kaplan & Leung, 2011).

There appears to be a strong genetic component for most mental disorders (Ames, 2004). However genetic effects on their own are not deterministic. Nutrition may be able to influence gene expression. This is supported by research in the fields of nutrigenomics and epigenetics, showing nutrient status is a strong modifier of genetic expression (Kaplan & Leung, 2011).

The triage hypothesis proposed by Ames (2004) posits that nature ensures our survival by rebalancing our metabolism when the availability of micronutrients is inadequate. Any available micronutrients are directed towards processes essential for immediate survival at the expense of long term health. This may be relevant to many individuals who do not present with mental illness until later in life. Mental disorders may reflect cumulative effects of the

body triaging suboptimal micronutrient levels that are critical for survival at the expense of the brain (Kaplan & Leung, 2011).

Impaired brain activity in the mentally ill may in some cases result from inherited deficiencies in protein regulation by micronutrient receptors. The basis of this theory comes from results of a recent study (Miler & Dulay, 2008) showing postmortem brain tissue from 12 patients with schizophrenia was significantly deficient in a particular protein critical for a niacin receptor. Though these results are specific to niacin, analogous processes related to other micronutrients may be unmasked in the future (Kaplan & Leung, 2011).

There is a large and growing body of literature showing reductions in brain tissue in association with poor mental health (Cotter et al., 2001; Keller et al., 2003; Smiley et al., 2009; Janssen et al., 2008). Studies on cortical thinning have demonstrated that changes are progressive (Keller et al., 2003). It may be that long-term experience of suboptimal nutrition, especially in an individual with a genetic predisposition, results in diminished capacity to maintain and initiate neuronal growth and development, producing a vulnerability to mental illness (Kaplan & Leung, 2011).

In a variety of neurological and psychiatric disorders the underlying pathophysiology has been demonstrated to induce mitochondrial dysfunction (Kato & Kato, 2000; Young, 2007; Gardener & Boles, 2005). Elevated levels of oxidative stress have also been implicated in neuronal damage in humans and in animal models of brain dysfunction (Wang et al., 2009). The primary treatment for mitochondrial disorders is micronutrients and carnitine (an amino acid). This suggests psychiatric disorders may be the result of cumulative deficiencies of micronutrients needed to deal with oxidative stress (Kaplan & Leung, 2011). Broad-spectrum micronutrient supplementation may correct these deficiencies and provide sufficient micronutrients to restore metabolic function, alleviating psychiatric symptoms (Rucklidge & Kaplan, 2013).

1.6.2 B Vitamin Supplementation:

The B vitamins play key roles at all levels of brain function (Kennedy & Haskell, 2011). They function as coenzymes and precursors to cofactors for many enzymatic processes involved in cellular metabolism (Kennedy & Haskell, 2011). They have essential involvement in every aspect of energy metabolism, including oxidative carboxylation reactions (B1), the Krebs cycle (B1, B5), redox reactions (B3), the metabolism of polysaccharides, lipids and proteins, and the mitochondrial respiratory chain (B2, B3) (Kennedy & Haskell, 2011).

Thiamine (B1) acts as a coenzyme for several metabolic pathways. In its triphosphate form, thiamine is somehow involved in nerve membrane function (Ball, 2004). It appears to control the functioning of voltage-gated ion channels by stabilizing the density of negative charges at the inner side of the nerve membrane (Fox & Duppel, 1975), potentially playing a role in the opening of chloride ion channels and influencing postsynaptic neuronal inhibition, by functioning as a membrane stabilizer (Bertendorff et al., 1990). Butterworth (1982) reported changes in the concentration or metabolism of monoamine neurotransmitters (NA, 5HT), GABA and acetylcholine in the brains of pyrithamin (an inhibitor of thiamine metabolism) treated animals. It is certain that thiamine modulates cognitive performance (Elsinger, 1997) especially in the elderly (Kanofsky, 1996). Deficiency in thiamine may also contribute to the development of AD (Glaso et al., 2004). Thiamine supplementation may act to reduce anxiety through its influences on neuronal inhibition, GABA and monoamine neurotransmitters, as well as providing neuroprotection to prevent cognitive impairments.

Riboflavin (B2) in its coenzyme forms (FMN and FAD) is important in key metabolic reactions involved in the conversion of folate (B9) and pyridoxine (B6) into their coenzyme forms. Both FAD and FMN function as coenzymes in a number of oxidoreductive processes including involvement in monoamine oxidase (breakdown of monoamines). They are necessary for reactions involving other vitamins. For example, pyridoxine phosphate oxidase

is an FMN dependent enzyme that converts pyridoxamine phosphate to pyridoxal phosphate, the primary B6 coenzyme. An FAD dependent hydroxylase is involved in the synthesis of tryptophan, a precursor to serotonin. FAD dependent enzymes are also involved in folate metabolism (Ball, 2004). Riboflavin also ensures the balance of B1 and B3 (Bourre, 2006). Its role appears to ensure there vitamins are transformed into appropriate cofactors.

Niacin (B3) plays a major role as a coenzyme in the transfer of electrons from metabolic intermediates in a number of pathways. Deficiency in niacin results in pellagra, a disease with neurological and neuropsychiatric symptoms including irritability, anxiety, and depression with dementia sometimes occurring in severe and chronic cases (Ball, 2004). It has been proposed to protect against oxidative stress, ischemic injury and inflammation (Zhang et al., 1995) and prevents apoptosis in the brain likely by stimulating neurogenesis (Morales et al., 2010). Niacin may play a role as an anxiolytic and neuroprotective agent by reducing oxidative stress in the brain and enhancing neurogenesis, which have both been linked to anxiety and cognitive impairments.

Pantothenic acid's (B5) biological activity is attributable to its incorporation into the molecular structures of coenzyme A and its acyl carrier protein. Deficiency in rodents' can result in necrosis and hemorrhage of the adrenal glands. This can impair adrenal endocrine functioning and create dysfunction in the HPA axis. In humans, clinical observations include irritability restlessness and insomnia as well as adrenocortical insufficiency (Ball, 2004). Pantothenic acid may benefit anxiety and stress by normalizing adrenocortical functioning.

Pyridoxine (B6) concentration in the brain is 100x greater than in the blood. It ensures the synthesis of chemical modulators to combat asthenia, irritability and depression. It acts as a coenzyme for amino acid decarboxylases in the synthesis of biogenic amines including GABA, dopamine and serotonin. Vitamin B6 is a cofactor for aromatic L-amino acid decarboxylase (AADC), an enzyme that catalyzes the conversion of L-DOPA to dopamine,

and hydroxytryptamine (5-HTP) to serotonin (Boadle-Biber, 1993). It plays a role in tryptophan metabolism and increases the production of serotonin (Galderon-Guzman et al., 2004). Vitamin B6 has also been shown to regulate the levels of 5-HTP (Calderón-Guzmán et al., 2004). There is evidence it plays a role in the modulation of steroid hormones including corticosteroids (Ball, 2004). These roles in monoamine neurotransmitter synthesis and in the modulation of stress hormones may explain potential anxiolytic effects of B6.

Folate (B9) plays a number of roles in the brain. It supplies the methyl group for the conversion of methionine to S-adenosylmethionine (SAMe), and therefore plays a role in the synthesis and integrity of DNA, and the methylation of proteins, phospholipids, and monoamine and catecholamine neurotransmitters (Kennedy & Haskell, 2011). Folate and vitamin B12 are required for processes involved in the synthesis and metabolism of monoamine neurotransmitters (Bottiglieri, 1996). Together these vitamins may provide benefits in anxiety by helping to normalize monoamine neurotransmitter synthesis and metabolism.

Folate plays an essential role in the synthesis of some of the nucleotides required for DNA synthesis. Folate deficiency may reduce methylation of cellular DNA and neurotransmitters (Gospe et al., 1995; Mazzerello et al., 1990) therefore impede neural function. Folate deficiency has been shown to have significant effects on DNA. Deficiency can cause chromosomal brakes, which result in loss of oligodendrocytes and demyelination. This in turn contributes to cognitive defects (Blount, 1997).

Neurological and neuropsychiatric changes associated with B12 deficiency include irritability, memory disturbances, mild depression and apathy. These changes reflect an inability to manufacture lipid content of myelin, which results in generalized demyelination of nerve tissue. B12 deficiency results in neural tissue deprived of a vital substrate, SAM, which

is required for myelination (Ball, 2004). Correcting B12 deficiency may help in the re-myelination of the CNS and return functioning to normal.

One hypothesised mechanism of the potentially beneficial effects of B-vitamins on cognitive function is their role in maintaining optimum homocysteine levels. Folate, B12 and B6 are implicated in the metabolism of homocysteine, which is produced as a by-product of methionine metabolism. Normally, homocysteine is converted back to methionine or used to create cysteine and other substances in the body. However, deficiencies in these vitamins may block this conversion leading to elevated homocysteine levels (Stough et al., 2011). Adequate levels of folate and B12 are also required for the re-methylation of homocysteine, while B6 plays a key role in this process as a coenzyme in the metabolism of homocysteine to cysteine (Mattson & Shea, 2003). Blocking of the conversion process (e.g. by deficiencies of folate and/or vitamins B6, B12) leads to elevated levels of homocysteine, which in turn may contribute to a range of neurodegenerative and psychiatric disorders (Reynolds, 2006). An association between stress and homocysteine levels also exists (Kang et al., 2005; Stoney, 1999). B-vitamin supplementation may therefore prove effective as a preventative strategy for stress reduction and cognitive decline by normalizing homocysteine levels.

1.6.3 Vitamin C Supplementation:

Vitamin C is transported into the brain against a steep concentration gradient, and accumulates at high concentrations in neuron-rich areas such as the hippocampus, cortex and cerebellum (Meffors et al., 1981; Mun et al., 2006). It has both antioxidant and nonantioxidant roles in the brain. In its role as a single electron donor, vitamin C is a powerful antioxidant, reducing oxygen, sulfur and nitrogen-oxygen radicals generated during normal cellular metabolism, and recycling other radicals to their previous forms (e.g. tocopheroxyl to a-tocopherol). It also contributes to the synthesis of tyrosine, carnitine, catecholamine

neurotransmitters and peptide hormones, (Padayatty et al., 2003) and plays roles in neural maturation, and as a neuromodulator of the activity of acetylcholine and the catecholamine neurotransmitters (Harrison & May, 2009)

The anxiolytic and cognitive benefits may be due to vitamin C's potent antioxidant properties (Cantuti-Castelvetri et al., 2000; Harrison et al., 2009a,b; Hughes et al., 2011). Vitamin C is a potent scavenger of free radicals. It plays an essential role in the CNS as a single electron donor, acting as a powerful antioxidant, reducing oxygen, sulfur and nitrogen-oxygen radicals generated during normal cell metabolism and recycling other radicals to their previous forms (Kennedy & Haskell, 2011). It is an effective scavenger of all aggressive ROS within the aqueous environments of the cytosol and extracellular fluids. It reacts with free radicals to produce ascorbyl radical and detoxified products through a range of single electron transfers (Ball, 2004), as well as supporting and regenerating cellular oxidative defence mechanisms (Harrison et al., 2014)

Therefore possible neuroprotective effects and cognitive benefits are likely the result of reduced oxidative stress-related neural damage (Martin et al., 2002). These antioxidant properties might also be responsible for the vitamin's anxiolytic effects (Hughes et al., 2011), as oxidative stress has been implicated in anxiety (Bouayed et al., 2009).

However, vitamin C's anxiolytic effects may not be a result of its antioxidant properties alone. Its concentration is higher in the brain than almost all other organs. It acts as a cofactor for dioxygenase enzymes in the synthesis of DA, NA, and 5HT. It is also vital for neuronal repair and new cell generation, and may play a direct role in the transcription and expression of hundreds of different genes (Harrison et al., 2014). Furthermore it modulates neurotransmitter systems in the brain by regulating receptor synthesis and facilitating the release of neurotransmitters (Ball, 2004; Harrison & May, 2009), including the inhibition of NMDA receptor activity (Majewska et al., 1990). This could lead to decreased anxiety since

NMDA antagonism has been shown to be anxiolytic in rats and mice in a similar fashion to benzodiazepines (Plaznik et al., 1994).

Vitamin C is involved in neural maturation enhancing differentiation of embryonic stem cells into neurons in culture (Lee et al., 2000). It is associated with increased expression of genes involved in neurogenesis, maturation and neurotransmission (Shin et al., 2003). It has been proposed to function as a neuromodulator of DA and glutamate-mediated neurotransmission (Rebec & Pierce, 1994; Grunewald, 1993), and in the regulation of both acetylcholine and catecholamine release from synaptic vesicles (Kuo et al., 1979). Furthermore it has been shown to attenuate levels of the stress-related hormone, cortisol, in an animal species (Satterlee et al., 1989) and humans (Brody et al., 2002).

The numerous effects of Vitamin C in the brain including preventing oxidative stress, neurotransmitter synthesis and modulation, neural maturation, and its effects on stress hormones and potentially neurogenesis suggest that vitamin C may be a potent anxiolytic with cognitive enhancing properties.

1.7 The Current study

The planned research was intended to determine whether a broad-spectrum micronutrient supplement (Berocca) has greater benefits than a single micronutrient supplement in decreasing anxiety-related behaviour and improving short-term memory in healthy rats. A number of studies have investigated the effects of single nutrient supplementation in psychiatric disorders finding only modest improvements (Rucklidge & Kaplan, 2013). Vitamin C alone has however been shown to significantly improve anxiety symptoms in rats (Hughes et al., 2011) and humans (Brody et al., 2002). B-vitamin combinations (Stough et al., 2011; Lewis et al., 2013) as well as the broad-spectrum micronutrient supplement Berocca (Carroll et al., 2000; Kennedy et al., 2010) have also been shown to be effective in reducing stress and anxiety in humans. Therefore it is important to determine whether these benefits can be attributed to a single vitamin or vitamin group, to assess which micronutrients should be considered in the treatment of anxiety.

The current study also investigated whether a higher dose of Berocca is more effective in reducing anxiety symptoms. The recommended dose of one tablet per day has been shown to be useful in reducing stress and anxiety in humans (Carroll et al., 2000; Kennedy et al., 2010). Both B vitamins (Stough et al., 2011) and vitamin C (Hughes et al., 2011) have been shown to produce beneficial effects on stress or anxiety symptoms at doses higher than in the Berocca formulation. This will be useful in recommending appropriate supplementation and dosage for stress and anxiety. It may also help to determine further avenues of research in this area, such as whether deficiencies in a number of micronutrients or a single micronutrient contribute to anxiety related behaviour. Rats received 8 weeks of supplementation with a broad-spectrum micronutrient supplement (Berocca), or a B vitamin complex supplement or vitamin C alone, and were examined through several behavioural measures of anxiety and one of short-term memory.

2.0 Aims and hypothesis:

Given the growing body of research investigating the effects of micronutrients on mental health in human populations, there is a need for animal research in the area. This will provide preclinical data, and may allow investigations into proposed mechanisms of action of micronutrient supplementation, as well as the use of techniques unavailable to human research. To date the main focus of micronutrient research has been on clinical human populations. Therefore research investigating the effects of micronutrient supplemenation on emotionality and cognition in animals is needed to guide future research in this area. The current study was a pilot study with two main aims:

1. To determine whether a broad-spectrum micronutrient supplement (Berocca) has greater benefits than a single nutrient supplement (Vitamin C) in decreasing anxiety-related behaviour and improving short-term memory in rats. Having a B vitamin complex group allowed investigation into whether there was an additive effect of B vitamins with Vitamin C. This was important as the Berocca formula is made of a combination of B vitamins and vitamin C.

2. To investigate whether a higher dose of Berocca, than has been previously used, is more effective in reducing anxiety symptoms. This will help to determine appropriate supplementation and dosage for stress and anxiety.

3.0 Method:

3.1 Subjects:

Subjects of the present study were 80 (40 male and 40 female) PVG/c hooded rats from the breeding colony in the Psychology Department, University of Canterbury, Christchurch, New Zealand. Animals came in two cohorts. The first cohort of forty of the rats (20 male and 20 female) were born on the 8/3/2014, the other forty (20 male, 20 Female) were born on the 24/3/2014. These rats were weaned on post-natal day (PND) 30 and housed in same-sexed pairs in plastic $560 \times 350 \times 215$-mm (length $\times$ width $\times$ height) cages. Each cage was divided in half by a 215-mm-high wire mesh partition that physically separated the two occupants but enabled them to see, smell and hear each other thereby minimizing any deleterious effects of social isolation. All rats were provided with their own drinking bottle and ad libitum food, and were kept in 12-h light: 12-h dark conditions with an ambient temperature of 20 ± 1 °C. All procedures were approved by the University of Canterbury Animal Ethics Committee.

Records of each rat's normal daily intake of water were kept for 10 days to enable estimation of the quantities of each vitamin that needed to be added to ensure that each rat in a specific group was given access to the same concentration. On the 3/10/2014 rats were randomly separated into four experimental groups, each made up of 10 males and 10 females, with a total of 20 animals in each group, shown in Table 1.

Table 1: Showing distribution of animals by sex within each treatment goup.

Sex	Control	Berocca	Vitamin C	B Vitamins
Males	10	10	10	10
Females	10	10	10	10

The number of rats of each sex in each treatment condition was sufficient to enable meaningful statistical calculation of the main effects of the vitamin treatment, and also calculation of sex x treatment interactions for each behavioural measure. The latter set of calculations is very important because of sex differences in responsiveness to various experimental manipulations.

Each group received a different vitamin treatment (B vitamins, vitamin C, Berocca or control), delivered in their drinking water. On the 3/10/2014 vitamin treatment began, with the start of treatment for each group being separated by a week. The first group (n=20) to begin treatment was the B vitamin group. The second group (n=20) was Vitamin C. The third (n=20) was Berocca and the fourth (n=20) was control. Animals were treated for seven weeks with behavioural testing taking place in the eighth week. Treatment was continued while rats were being tested. Measurements of the volume of fluid drunk were made twice a week when they were replaced with fresh solutions. This enabled calculation of approximate daily doses consumed during the 7 weeks of pre-test treatment and the time involved for behavioural testing.

3.2 Vitamin treatment procedure and rationale for micronutrient use and dosage:

Three different micronutrient treatments (B vitamin complex, Vitamin C and Berocca) and a control were used in the current study. The B vitamin complex and vitamin C were in powder form. The Berocca was an effervescent tablet. The B vitamin complex was made up in the lab from a combination of individual B vitamins to obtain the desired dose of each.

Records of each rat's normal daily intake of water were kept for 10 days prior to treatment to enable estimation of the quantities of each vitamin that needed to be added to ensure that each rat in a specific group was given access to the same concentration. The average fluid intake and bodyweights for male and female rats were used to calculate the appropriate dosages. All vitamins were administered through the animals' drinking water. This involved dissolving the following quantities of vitamins in the rats' drinking water to achieve the desired target doses. The Berocca was administered a dosage equivalent to two tablets a day for a human. The B vitamin complex group was administered a dosage of only the B vitamins in the Berocca formula equivalent to two tablets per day for a human. The Vitamin C group was administered a dose of 81.6 mg/kg/day which is equivalent to a human consuming 1000mg of Vitamin C per day via 2 tablets of Berocca i.e., 13.23mg/kg/day.the equivalent of 1000mg of Vitamin C per day for a human. The exact dose of each of each individual micronutrient administered to each treatment group is shown in Table 2. This table also shows the quantities of each micronutrient in the previously established therapeutic dose of Berocca (one tablet per day for a human) (Carroll et al., 2000; Kennedy et al., 2010). All control rats were provided with unadulterated water. The concentrations of male and female vitamin solutions were different because the females drank more water relative to their bodyweight (i.e., females had a weaker solution than males). The dose/kg for both sexes was the same.

Table 2: *Composition of micronutrient treatments comparing equivalent human therapeutic dose for Berocca (1 tablet per day), double therapeutic dose for Berocca (2 tablets per day), B vitamin complex and vitamin C.*

Micronutrient	Berocca Therapeutic dose (1 tablet/ day)	Berocca Treatment Group Double Therapeutic dose (2 tablets/day)	B vitamin Complex	Vitamin C
B1 (Thiamine)	15 mg	30 mg	30 mg	-
B2 (Riboflavin)	15 mg	30 mg	30 mg	-
B3 (Nicotinamide)	50 mg	100 mg	100 mg	-
B5 (Panthenoic acid)	23 mg	46 mg	46 mg	-
B6 (Pyridoxine)	10 mg	20 mg	20 mg	-
B12 (Cyanocobalamin)	10 μg	20 μg	20 μg	-
C (Ascorbic Acid)	500 mg	1000 mg	-	1000mg
H (Biotin)	150 μg	300 μg	-	-
B9 (Folic acid)	400 μg	800 μg	-	-
Calcium	100 mg	200 mg	-	-
Magnesium	100 mg	200 mg	-	-
Zinc	10 mg	20 mg	-	-

Measurements of the volume of fluid drunk were made twice a week when they were replaced with fresh solutions. This enabled calculation of approximate daily doses consumed during the 7 weeks of pre-test treatment plus the time involved for behavioural testing. Treatment was continued while the rats were being tested.

All rats were housed in pairs in large cages (as described in 3.1) and provided with their own food and water supply. Therefore, it was possible to measure the volume of fluid drunk by individual rats thereby enabling calculation of individual vitamin dose consumed.

The rationale for dosages relates to effective therapeutic doses previously found in animal and human studies. Recent research has investigated the benefits of single and broad-spectrum micronutrient supplementation in psychiatric disorders. Vitamin C alone has been shown to significantly improve anxiety symptoms in rats (Hughes et al., 2011) and humans (Brody et al., 2002). B-vitamin combinations (Stough et al., 2011; Lewis et al., 2013) as well as the broad-spectrum micronutrient supplement Berocca (Carroll et al., 2000; Kennedy et al., 2010) have also been shown to be effective in reducing stress and anxiety in humans. The recommended dose of one Berocca tablet per day has been shown to be useful in reducing stress and anxiety in humans (Carroll et al., 2000; Kennedy et al., 2010). Both B vitamins (Stough et al., 2011) and vitamin C (Hughes et al., 2011) have been shown to produce beneficial effects on stress or anxiety symptoms at doses higher than in the Berocca formulation.

3.3 General procedure and behavioural testing apparatus:

Once normal daily intake of water was determined, estimation of the quantities of each vitamin that needed to be added to ensure that each rat in a specific group was given access to the same concentration were be made. Rats were then treated with vitamins for 7 weeks. Observations of the rats' behaviour at the end of the treatment period involved four tests of anxiety-related behaviour i.e., emergence latency and open field activity, responsiveness to brightness change (Y-Maze), and elevated plus maze. The responsiveness to brightness change Y-Maze was also used to test short term memory by examining the time spent in a novel changed arm following an acquisition trial.

	B vitamin	Vitamin c	Berocca	Contol
8/3/2014	**Cohort 1 born**			
24/3/2014		**Cohort 2 born**		
12/9/2014-19/9/2014	Fluid (water) intake recorded for all rats 10 days. Initial bodyweight recorded for all rats. Dosage calculated for each treatment condition.			
3/10/2014	Tx begins			
10/10/2014		Tx begins		
17/10/2014			Tx begins	
24/10/2014				Tx begins
17/11/2014 -23/11/2014	Behavioural Testing			
24/11/2014 -30/11 2014		Behavioural Testing		
1/12/2014 -7/12/2014			Behavioural Testing	
8/12/2014 -14/12/2014				Behavioural Testing

Figure 1. *Timeline showing when each cohort began treatment and when each treatment group underwent behavioural testing.*

Tests were conducted every second day over a period of 8 days. This allowed a minimum of 48 hours between tests for each rat. Each day 15 rats from a specific treatment

group were tested. As each group began treatment at different times the order of group testing was: B-vitamins, vitamin C, Berocca, Control.

The tests were carried out in the same experimental room, which maintained a stable temperature of 22℃ ± 2℃ with humidity control of 48% ± 10%. All tests were carried out between 9.00 and 15.00 hours in the light phase of the rats' normal light/dark cycle. Over each eight-day testing period, each animal completed the emergence latency and open field tests once, the elevated-plus maze test once and the responsiveness to brightness in a Y-maze test once. The order of the tests was varied in a non-systematic way for individual rats. Each animal completed one test per day.

3.3.1 Emergence latency and open-field activity:

The emergence portion of this test is used to assess anxiety or fearfulness, whereas the open field portion enables assessment of activity and emotionality. The emergence latency acts as a light-dark preference box test and is based on the rats' innate avoidance of brightly illuminated open areas (Hascoët & Bourin, 2009; Sanchez, 1996). Higher emotionality, thus anxiety, is indicated by the tendency to avoid the light side (Hughes et al., 2004; Hascoët & Bourin, 2009). Once in the open field, location and behavior were measured to assess activity and emotionality, with higher anxiety indicated by less time in the centre squares and specific patterns of exploratory behavior (Cryan & Sweeny, 2011). The open field test is designed to elicit anxiety by separating the animal from its home cage and placing it in an inescapable novel environment (Prut & Belzung, 2002; Walsh & Cummings, 1976). The test was originally developed to measure emotionality in rats by Hall (1934) and now is one of the most widely used measures of anxiety-like behaviours on animal studies (Prut & Belzung, 2002). As well as being used to measure anxiety-like behaviours, it has also been used to

demonstrate the behaviourally stimulant and sedative properties of drugs in animals (Gould et al., 2009).

For the emergence latency test, each rat was placed in a small, darkened start box dark side for 1 minute. Then, a slide separating the dark box from a 600 x 600 x 250mm dimly lit open field was withdrawn to allow the rat free access to the field. The time taken for the rat to emerge into the open field fully (all four paws) was recorded using a hand held stopwatch. If the rat did not emerge from the dark side after five minutes the trial was terminated. Rats that did not emerge were excluded from emergence latency and open field data analyses.

In this study, the open field apparatus consisted of a wooden open field measuring 600 x 600 x 250mm which was placed on a table 700mm high. The floor was painted flat black and divided into a 4 x 4 grid of sixteen identical squares each measuring 150 x 150 mm. There were twelve peripheral squares and four central squares. The wooden walls were also painted black and were 250mm high. Garau et al. (2000) suggested that uncertainty (therefore anxiety) is increased by placing an animal in a novel environment and also by altering light levels. In this study, the light level was low (45 lux) and the testing area was unfamiliar to the animals producing low to moderate anxiety (File & Seth, 2003). An infrared camera was mounted on a single wooden arm 850mm above the open field and this was connected to a television monitor in the same experimental room which was used by the observer to monitor the animal's position and behaviour within the open field.

Once in the open field it was noted which square the rat was located in every three seconds, for 5 minutes with the slide between the field and the start box closed. It was also noted whether the rat was rearing up on its hind legs, walking or grooming itself. At the end of the trial the number of faecal boluses left in the apparatus were counted. This test and the behaviours that are observed are designed to provide a measure of general activity and emotional reactivity in animals (Hall, 1934a; Royce, 1977; Walsh & Cummings, 1976).

The total number of faecal boluses was also recorded as defecation increases when the animals are under stress. In this experiment, grooming was defined as the touching of front paws to mouth, paws to ears, mouth to sides, mouth to tail, mouth to feet, mouth to genitals, mouth to abdomen, feet to head/ears and feet to sides (Mayaho et al., 1995; Pleskacheva, 1995). The number of times the animal was located in a different square to where it had been 3 seconds prior was also calculated to indicate the number of transitions the animal made and to provide a measure of locomotor activity (ambulation). As it has been argued that animals prefer the outer squares to the exposed central squares (Prut et al., 2002), the total frequency of centre and corner square occupation was calculated. After each trial the dark box and open field were washed with Paraquat Blue 20% solution.

3.3.2 Elevated plus-maze test:

The elevated plus maze creates an approach-avoidance conflict by placing rats a novel environment that also stimulates a natural aversion for height and open spaces (Kumar et al., 2013; Salum et al., 2003). The open arms are considered to be more aversive than the closed arms (Cryan & Sweeney, 2011) and it is thought that a less anxious animal will explore open arms more. This has been shown with anxiolytic drug treatment increasing both entries into and time spent in open arms, while anxiogenic dugs do the opposite (Braun et al., 2011; Handley & Mithani, 1984; Hogg, 1996; Lister, 1987; Moser et al., 1990; Pellow et al., 1985).

In this study, a four-armed plus maze was used of which two arms were open (clear Perspex walls), and the other two were closed (wooden walls). The whole maze is elevated 1m above the floor. The light level was low (45 lux) and the testing arena was unfamiliar to the animals producing low to moderate anxiety (File & Seth, 2003). An infrared camera was mounted above the elevated plus maze and this was connected to a television monitor in the

same experimental room, which was used by the observer to monitor the animal's position and behaviour within the open field.

Individual rats were removed from their home cage and placed into the centre of the maze facing one of the closed arms. Every three seconds for 5 minutes the arm the rat was occupying was recorded. The number of entries into each arm was also recorded thereby enabling calculation of preferences for entering and occupying open versus closed arms. Exploratory behaviours rearing, walking and grooming were also recorded and it was noted in which arm these behaviours were exhibited. The primary indices of anxiety in the elevated plus maze are the percentage of entries and time spent in open arms (Salum et al., 2003). The total number of entries into both arms is a reliable measure of motor activity, since it is little affected by anxiety (Cruz et al., 1994; Salum et al., 2003). The maze was washed down and disinfected with 20% Paraquat Blue after each animal.

3.3.3 Responsiveness to brightness in a Y-maze:

This test was used to assess short-term spatial memory and curiosity. It also exploits the rats' natural tendency to explore novel areas. It is thought that a less anxious animal will enter the novel arm more often than the familiar arm, whereas a more anxious animal will show a preference for familiarity rather than novelty (Hughes, 2001; Hughes & Neeson, 2003; Samyai et al., 2000).

In this study, a wooden apparatus was placed on a table 700mm high which was illuminated overhead by low (45 lux) fluorescent lighting 1390mm above the maze. An infrared video camera was mounted 1390mm directly above the apparatus. This was used by the experimenter to monitor the rats' location within the maze. The arms of the maze were 450mm long with a 120˚ angle between them. The stem of the maze was 300mm long with a black compartment at the base, which was separated from the rest of the stem by a guillotine

slide. Both the stem and arms were 100mm wide and 140mm high. One arm contained a black painted metal insert while the other contained a white painted metal insert which occupied the width, height and 400mm length of each arm. A clear Perspex lid covered the entire apparatus.

Each animal experienced an acquisition and retention trial. Each animal was placed in the black compartment at the base of the stem and then released. It was allowed to roam freely within the maze for 5 minutes. This is known as the acquisition trial. During this trial one arm contained a black insert while the other arm contained a white insert. After 5 minutes the animal was returned immediately to its home cage where it had free access to food and water. The inserts were replaced with two clean black inserts and the entire apparatus was wiped down and disinfected with 20% Paraquat Blue solution. The rat was then allowed access to the maze again for a 3-min retention trial during which it was faced with two black arms, one of which had changed from white to black i.e., the novel arm. Every 3 seconds it was noted which arm the rat was occupying, and how often each was entered. It was therefore possible to subsequently calculate the percentage of entries into and time spent in the changed novel arm, as well as the total entries of both arms. This information enabled calculation of the rats' preferences for the changed versus unchanged arm (and thus curiosity/anxiety, and memory for which arm has changed).

During the acquisition trial, one half of the animals (n = 40) experienced the maze with the black insert in the left arm while the remaining half experienced it with the black insert in the right arm. It was randomly determined which side the black insert would be on for each animal. Within each treatment group ten rats (five male, five female) completed the maze with the black insert on the left and ten rats (five male, five female) completed it with the black insert on the right. The entire apparatus was wiped down and disinfected with 20% Paraquat Blue solution at the end of the retention trial.

3.4 Rationale for Behavioural apparatus:

The current study used four empirically supported behaviour tests. Three were of unconditioned anxiety-like behaviour; emergence latency (light-dark box) in an open field, the open field test and an elevated plus maze. The Y-maze was used to examine spatial working memory, and it has also been shown to be useful for assessing anxiety (Aitchison & Hughes, 2006).

The emergence latency (light-dark), open field and elevated plus maze tests play on the conflicting drives of avoidance an exploration of a perceived threatening stimulus (i.e. an open space or lit area) (Crawley, 1985; Hascoët & Bourin, 2003; Montgomery, 1955; Salum et al., 2003). When a rat is confronted with novelty, its behaviour is determined by this conflict between its drive to explore the unknown area and its motivation to avoid potential danger (Ohl, 2003). This approach-avoidance conflict is assumed to represent anxiety (Ennaceur, 2014).

The open field and elevated plus maze also assessed rearing and grooming behaviours inside the apparatus. Rearing is an exploratory behaviour (Carli et al., 1989; Espejo, 1997) and is used as an indicator of anxiety in the open field (Carli et al., 1989; Lamprea et al., 2008), and the elevated plus maze (Escorihuela et al., 1999; Lepicard et al., 2001; Rodgers & Dalvi, 1997). Reductions in rearing are thought to indicate heightened anxiety in the animal (Belzung & Griebel, 2001; Rodgers et al., 1997). Grooming is considered to be an adaptation to stressful situations and conflict (Espejo, 1997; Hannigan & Isaacson, 1981; Kalueff & Tuohimaa, 2004; Kametani, 1988; Torres, 1961). Stressors such as exposure to a novel environment, handling, and immersion in water, increase grooming in rats (Eguibar & Moyaho, 1997; Van Erp et al., 1994). Anxiolytic drugs have been shown to decrease grooming (Barros et al., 1994). Therefore an increase in grooming behaviour is thought to be a valuable tool for assessing heightened anxiety in animals (Nin et al., 2012).

The emergence latency test in the open field serves as a form of light-dark box test. In this test it is thought the light compartment (open field) is more aversive than the dark compartment (Cryan & Sweeney, 2011). Therefore the more anxious an animal is, the longer its latency to emerge from the dark compartment into the open field compared to an animal experiencing lower anxiety levels (Hascoët et al., 2000). Numerous studies have shown anxiolytic drug treatment increases the number of entries into the light compartment, the time spent in the light compartment, and reduces freezing behaviour (Crawley & Goodwin, 1980; De Angelis & Furlan, 2000; Hascoët et al., 2000; Hascoët & Bourin, 2003; Jones et al., 1988).

In the open field tests rodents have a preference for the periphery of the apparatus over central parts (Prut & Belzung, 2003). The avoidance of unprotected areas is used as an indicator of anxiety (Ohl, 2003). The more anxious an animal is, the more time it will spend in the corners of the apparatus and less time in the centre squares (Hall, 1934; Prut & Belzung, 2003). Rearing and grooming behaviours are also observed and it is thought that a more anxious animal will display less rearing and more grooming (Ohl, 2003; Prut & Belzung, 2003). An increase in defication is indicative of an increase in anxiety (emotional stress) (Hall, 1934; Wills et al., 1983). Therefore the number of fecal boluses left by each rat in the field was recorded.

The elevated plus maze is based on creating an approach-avoidance conflict between curiosity and exploration of a novel environment and rodents' natural aversion for height and open spaces (Kumar et al., 2013; Salum et al., 2003). The open arms are considered to be more aversive than the closed arms (Cryan & Sweeney, 2011). Therefore rats will typically enter the closed arms more frequently and spend more time in them compared to the open arms (Salum et al., 2003). A less anxious animal will show greater exploration of the open arms. This has been shown with anxiolytic drug treatment increasing both entries into and time spent in open arms, while anxiogenic drugs have the opposite effect (Braun et al., 2011; Handley & Mithani,

1984; Hogg, 1996; Lister, 1987; Moser et al., 1990; Pellow et al., 1985). The primary indices of anxiety in the elevated plus maze are the percentage of entries and time spent in open arms (Salum et al., 2003). The total number of entries into both arms is a reliable measure of motor activity, since it is little affected by anxiety (Cruz et al., 1994; Salum et al., 2003).

The Y-maze is a well-known test of spatial working memory in rats (Kumar et al., 2013), that is based on the tendency of rodents to explore novel environments (Dellu et al., 2000; Ma et al., 2007). Responsiveness to brightness change in a Y-maze has been shown to be a useful index of curiosity-motivated spatial working/short term memory (Hughes, 2004; Hughes & Maginnity, 2007; Hughes & Neeson, 2003). Following an acquisition trial the number of entries and time spent in the changed arm are used to indicate memory performance. Rats that make more entries into and spend more time in the changed (novel) arm are thought to have a better short term memory compared to those that spend more time in the unchanged arm. This is because the rats must remember the nature of both arms in order to identify the one that has been changed. If the animals recognise which arm has been changed they are more likely to make more entries and spend more time in it due to their tendency to approach and explore novel stimuli. The Y-maze can also be used to assess anxiety-like behaviour. Activity in the Y-maze is inversely related to anxiety (Archer, 1973). It is thought that higher levels of emotional reactivity (anxiety) result in fewer changed arm entries and occupation. Higher levels of anxiety result in the animal being less inclined to explore the novel environment. Therefore, they make fewer entries and spend less time in the changed arm because they have a preference for a familiar environment over a novel environment. This can occur with anxiogenic experiences in other situations (Aitchison & Hughes, 2006; Aitken, 1974; Hughes, 1987; Misslin & Ropartz, 1981).

4.0 Statistical Analysis:

The main focus of this study was to examine the effects of receiving micronutrients on animal emotionality and whether this effect was maintained over time while receiving micronutrients as part of a normal diet.

All raw data sets were analysed using the statistical program Statistical Package for the Social Sciences (SPSS) 19.0. Each measure was treated to a separate 4 (treatment group) x 2 (sex) factorial ANOVA. Fischer LSD post hoc tests were carried out when a main effect was significant ($p < .05$). Sex differences were included in the analyses due to sex differences in brain maturation and in brain maturation rates (Anderson, 2003). Given male and female brains develop differently, receiving micronutrients, as part of their diet would likely affect the two sexes differently over time. Differences in behaviour at each testing age were investigated to detect any additional changes after receiving micronutrients over an extended period of time.

5.0 Results

5.1 Fluid Intake, Dosage and Bodyweight

Each animal had their fluid intake recorded twice a week. Bodyweights were taken before and after treatment. This enabled the calculation of average dosage (mg/kg) for males and females in each treatment group. ANOVAs were used to assess the effects of micronutrient treatment, sex and any interactions on fluid intake, dosage consumed and bodyweight.

Table 3: ANOVA results for fluid intake (mL/100g/day) and final bodyweight for treatment group and sex.

Treatment group	F	Df	P
Fluid intake (mL/100g/day)	28.98	3,120	.0001
Final Bodyweight (g)	1.25	3,72	.298
Sex	**F**	**df**	**P**
Fluid intake (mL/100g/day)	3235.39	1,120	.0001
Final Bodyweight (g)	2788.97	1, 72	.0001

5.1.1 Fluid intake:

The mean ± S.E.M (mL/100g) of fluid intake per day for rats in B vitamin, vitamin C, Berocca and control groups respectively were 8.87 ± .59, 9.03 ± .72, 10.44 ± .72, 9.61 ± .72. There was a significant main effect of sex on fluid intake, see Table 3. Females (13.24 ± .09) drank significantly more than males (5.74 ± .15). There was a significant main effect of treatment on fluid intake, see Table 3. Fischer post hoc tests revealed that the Berocca group drank significantly more than the B vitamin, vitamin C and control groups (all $p < .0001$). The

control group also drank significantly more than B vitamins (p < .0001) and vitamin C (p < .01) groups. Therefore it is important to note that only the Berocca group drank significantly more fluid per day than the control group. There was a significant interaction between sex and vitamin group, shown in Figure 2. The males in the B vitamin group drank almost the same amount of fluid per day as the control group. However females in the B vitamin group drank significantly less fluid than the control group.
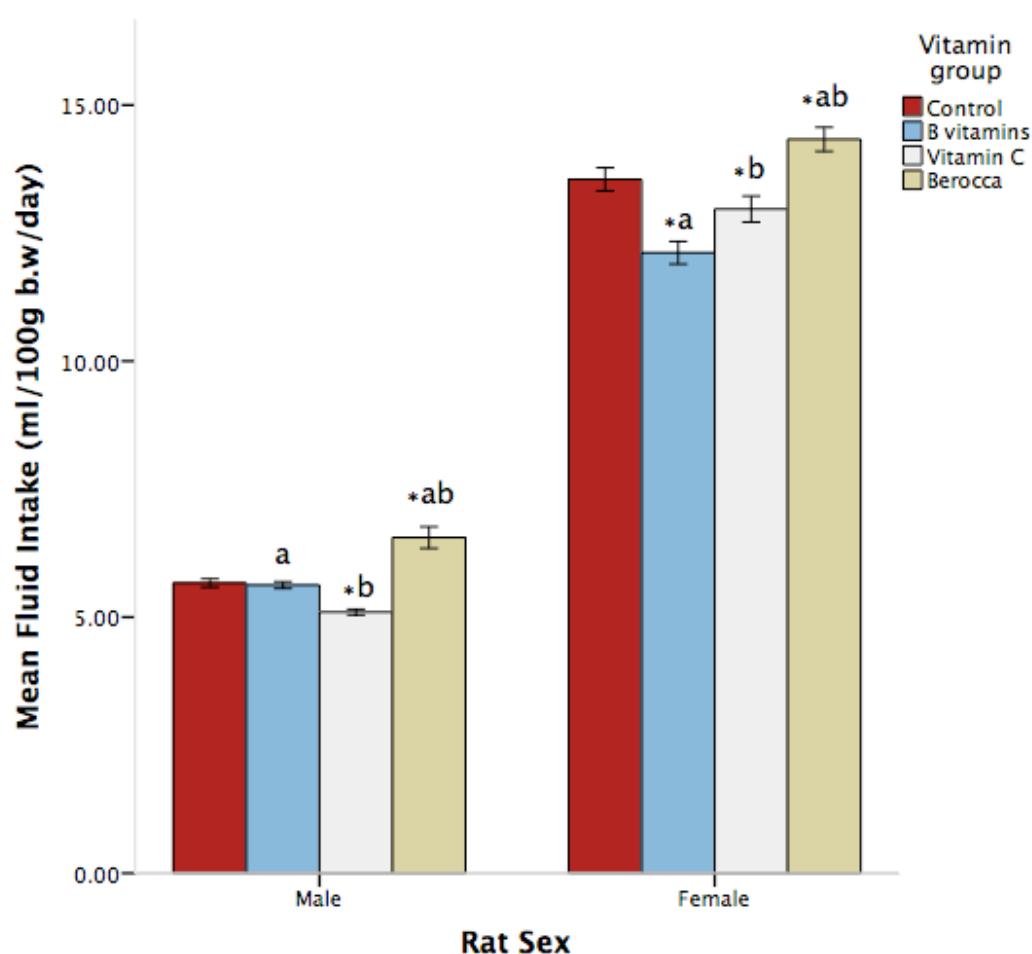
Figure 2: *Mean ± S.E.M fluid intake (ml/100mg b.w./day) for males and females separately following exposure to B vitamins, Vitamin C, Berocca, or unadulterated drinking water (Control).*

∗ Significantly different (p<.05) from water control group for that particular sex.

ab Groups with superscripts in common significantly different (P<.05) for that particular sex.

5.1.2 Dose:

The Berocca formula contains both vitamin C and all the B vitamins in the B vitamin formulation. The dosages of vitamin C and B vitamins Berocca was known prior to treatment. By measuring the dosage of Berocca it was possible to calculate the dosage of B vitamins and vitamin in Berocca that were consumed. The mean ± S.E.M dose per day (mg/Kg) of B vitamins alone, vitamin C alone, B vitamins in Berocca and vitamin C in Berocca, were respectively 18.92 ± .29, 85.23 ± 2.74, 25.98 ± .61, 114.95 ± 2.73. There was a significant main effect of sex on dose (see Table 4), with females consuming a significantly higher dose of vitamins than males. There was a significant main effect of treatment group on dose, see Table 4. Fischer LSD post hoc tests revealed that the Berocca group consumed significantly more B vitamins and vitamin C than the B vitamin and vitamin C group alone (all p < .0001). There was a significant interaction between sex and treatment group, shown in Figure 3. Females consumed a significantly higher dosage of vitamin C, vitamin C in Berocca, and B vitamins in Berocca, than males. However they did not consume a significantly higher dose of B vitamins alone, than males.

Table 4: *ANOVA results for dose (mg/kg/day) for treatment group and sex.*

Dose (mg/kg/day)	F	Df	P
Treatment group	1664.01	3,120	.0001
Sex	154.72	1,120	.0001
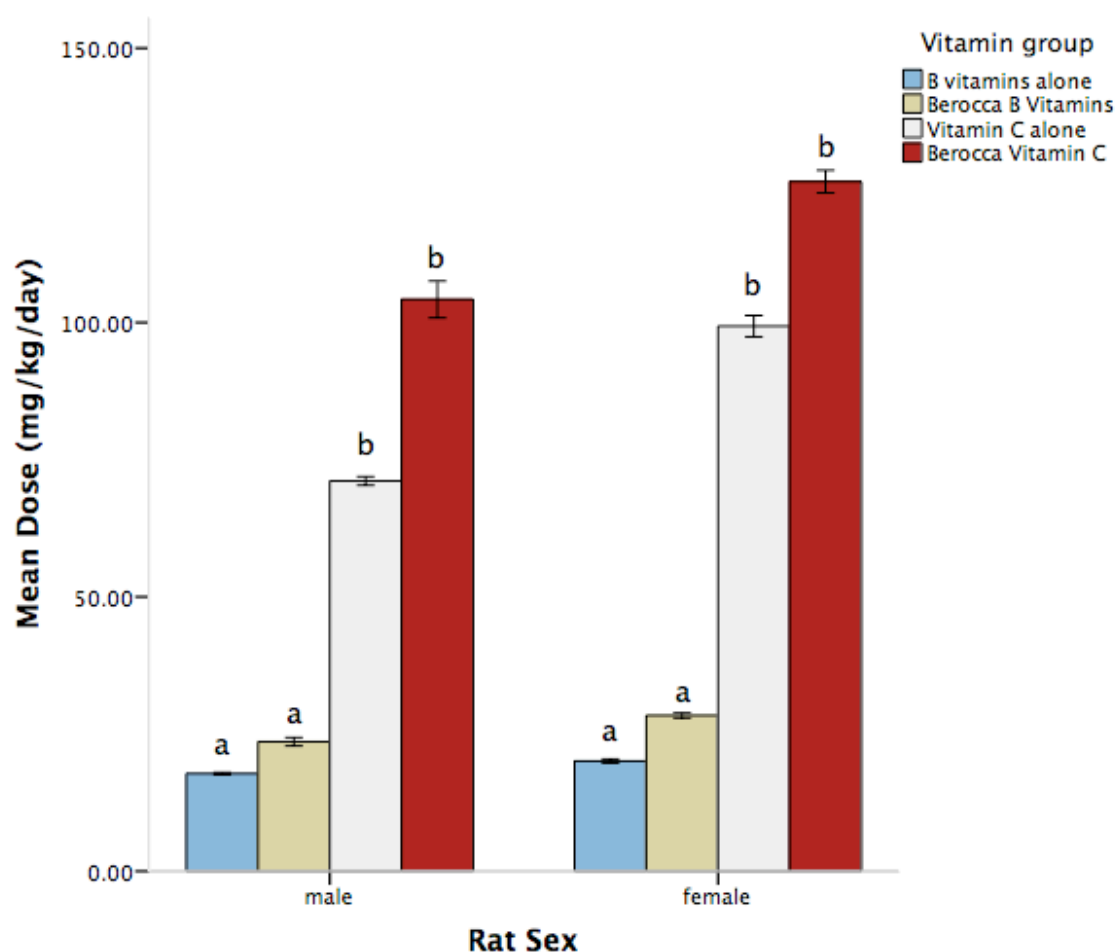
Figure 3: *Mean ± S.E.M dose (mg/kg b.w/day) for males and females separately following consumption of B vitamins alone, vitamin C alone, B vitamins in Berocca, and vitamin C in Berocca.*

[ab] *Groups with superscripts in common significantly different (P<.05) for that particular sex.*

5.1.3 Final Bodyweight and comparison with pre-treatment bodyweight:

Animals were weighed before and after treatment. The pre-treatment bodyweights were taken before the animals were divided into treatment groups and are therefore an average for all animals and not for each individual treatment group. The mean ± S.E.M final bodyweight (g) for rats in B vitamin, vitamin C, Berocca and control groups respectively were 311.75 ± 20.54, 314.50 ± 23.19, 305.80 ± 21.49, 314.05 ± 22.63. There was a significant main effect of sex on bodyweight with males (406.05 ± 3.01) being significantly heavier than

females (217 ± 2.11), see Table 3. The main effect of treatment on bodyweight was not significant nor was the interaction between sex and treatment, see Table 3.

The mean ± S.E.M pre-treatment bodyweights (g) for males and females were respectively 375.85 ± 2.84 and 204.10 ± 1.78. When compared with the post-treatment bodyweights males gained significantly more weight than females. The pre-treatment bodyweights were significant less than the final bodyweights for all treatment groups (control, B vitamins, vitamin C and Berocca) (all $p < .0001$), shown in Figure 4.
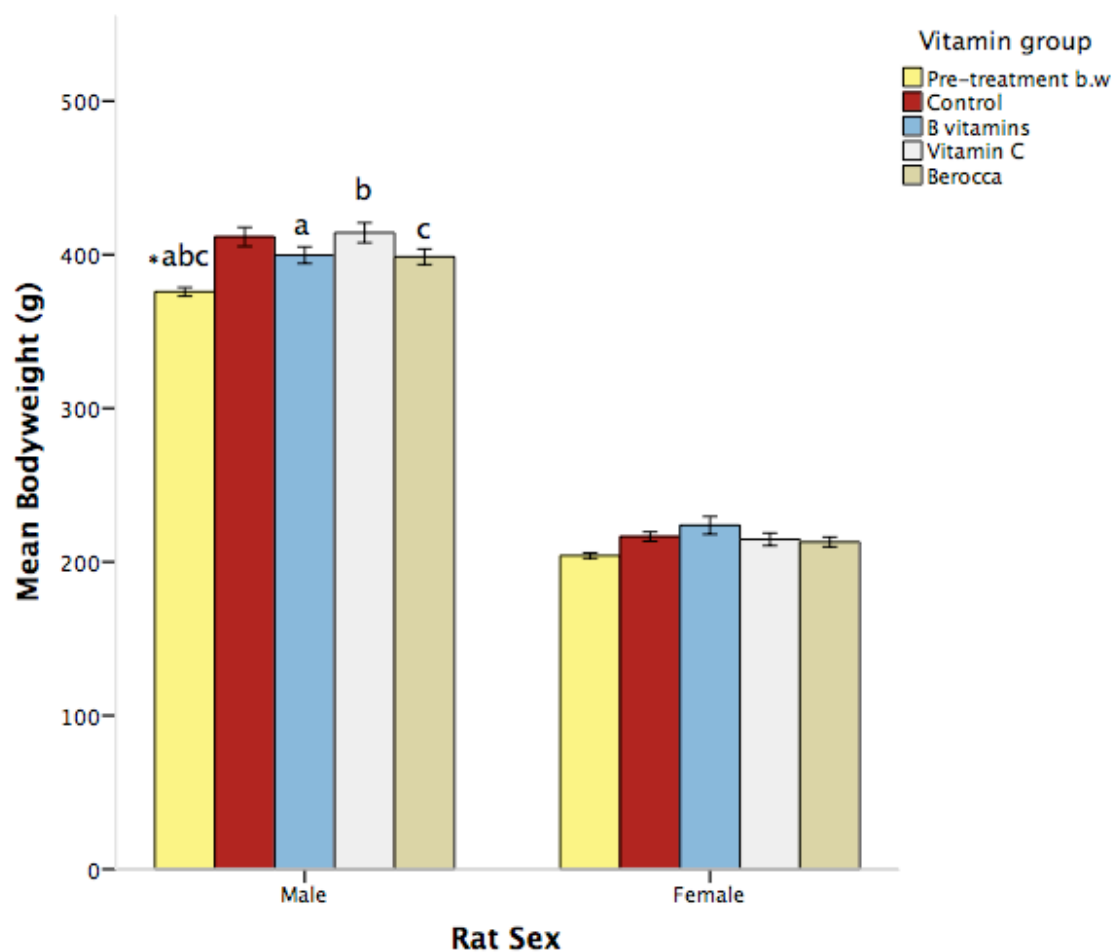
Figure 4: *Mean ± S.E.M final bodyweights for following treatment with unadulterated drinking water (control), B vitamins, Vitamin C, or Berocca, compared to pre-treatment bodyweight (pre-treatment b.w.), separately for male and female.*

∗ Significantly different (p<.05) from water control group for that particular sex.

[abc] Groups with superscripts in common significantly different (P<.05) for that particular sex.

5.2 Emergence Latency and Open Field Tests

The emergence latency and open field tests were conducted consecutively. Each animal was tested once. After the animal emerged for the darkened box into the open field measures were taken for the open-field portion of the test. ANOVAs were used to assess whether there was an effect due to micronutrient treatment group, sex, and micronutrient x sex interactions. For the emergence latency and open field tests 28 rats were excluded from the analysis because they failed emerge. Seven were from the B vitamin group (2 male, 5 female), six male rats from the vitamin C group, six rats from the Berocca group (3 male, 3 female) and nine from the control group (5 male, 4 female).

Table 5: Mean (± S.E.M) for responses in the open field test for control (n=11), B vitamins (n=13), vitamin C (n=14), Berocca (n=14) micronutrient treatment groups and for male (n=24) and female (n=28).

Treatment Group	Control	B	C	Berocca	F (3, 43)	P
Transitions	39.43 (3.41)	36.50 (2.47)	38.68 (3.42)	42.32 (2.22)	.73	.542
Center square occupancy	5.00 (1.01)	3.76 (.85)	4.55 (1.11)	5.88 (1.83)	.47	.706
Corner square occupancy	59.35 (3.28)	57.81 (3.34)	47.70 (4.38)	42.09 (3.48)	4.73	.006
Walking	66.67 (2.38)	50.28 (2.77)	60.90 (2.72)	68.17 (2.65)	9.69	.0001
Rearing	27.38 (2.26)	31.83 (3.36)	27.63 (3.03)	24.36 (2.24)	1.31	.285
Grooming	2.0 (1.02)	7.63 (1.87)	5.25 (2.28)	5.74 (1.89)	1.41	.252
Faecal boluses	1.00 (.55)	1.51 (.45)	1.73 (.44)	1.38 (.64)	.36	.785

Sex	Male	Female			F (1, 43)	P
Transitions	36.24 (1.66)	42.22 (2.17)			4.23	.046
Centre square occupancy	4.36 (1.01)	5.23 (.79)			.43	.518
Corner square occupancy	51.64 (3.30)	52.44 (2.60)			.133	.717
Walking	60.55 (2.79)	62.46 (1.81)			.52	.476
Rearing	29.65 (2.19)	25.95 (1.89)			1.79	.188
Grooming	3.77 (1.04)	6.54 (1.49)			2.04	.160
Faecal boluses	2.18 (.41)	.63 (.25)			.81	.495

5.2.1 Emergence latency:

There was a significant main effect of sex on time (seconds) to emerge (F (1,43) = 15.872, p < .001, partial η^2 = .270), with females (112.987 ± 12.73) taking significantly longer to emerge than males (49.16 ± 9.43). The mean ± S.E.M time to emerge (seconds) for rats in B vitamin, vitamin C, Berocca and control groups respectively were 125.40 ± 25.12, 66.93 ± 12.54, 65.63 ± 14.92, 66.33 ± 15.45. There was a significant main effect of vitamin group on emergence latency (F (3,43) = 3.5, p < .05, partial η^2 = .196). Fischer LSD post hoc tests revealed the B vitamin group took significantly longer to emerge than the Berocca group (p < 0.05), shown in Figure 5. The B vitamin group also took longer to emerge than the control and vitamin C groups but this was not statistically significant (p = .086 and .099 respectively). There was a non-significant interaction between sex and vitamin group on emergence latency (F (3,43) = .873, p = .463, partial η^2 = .057).
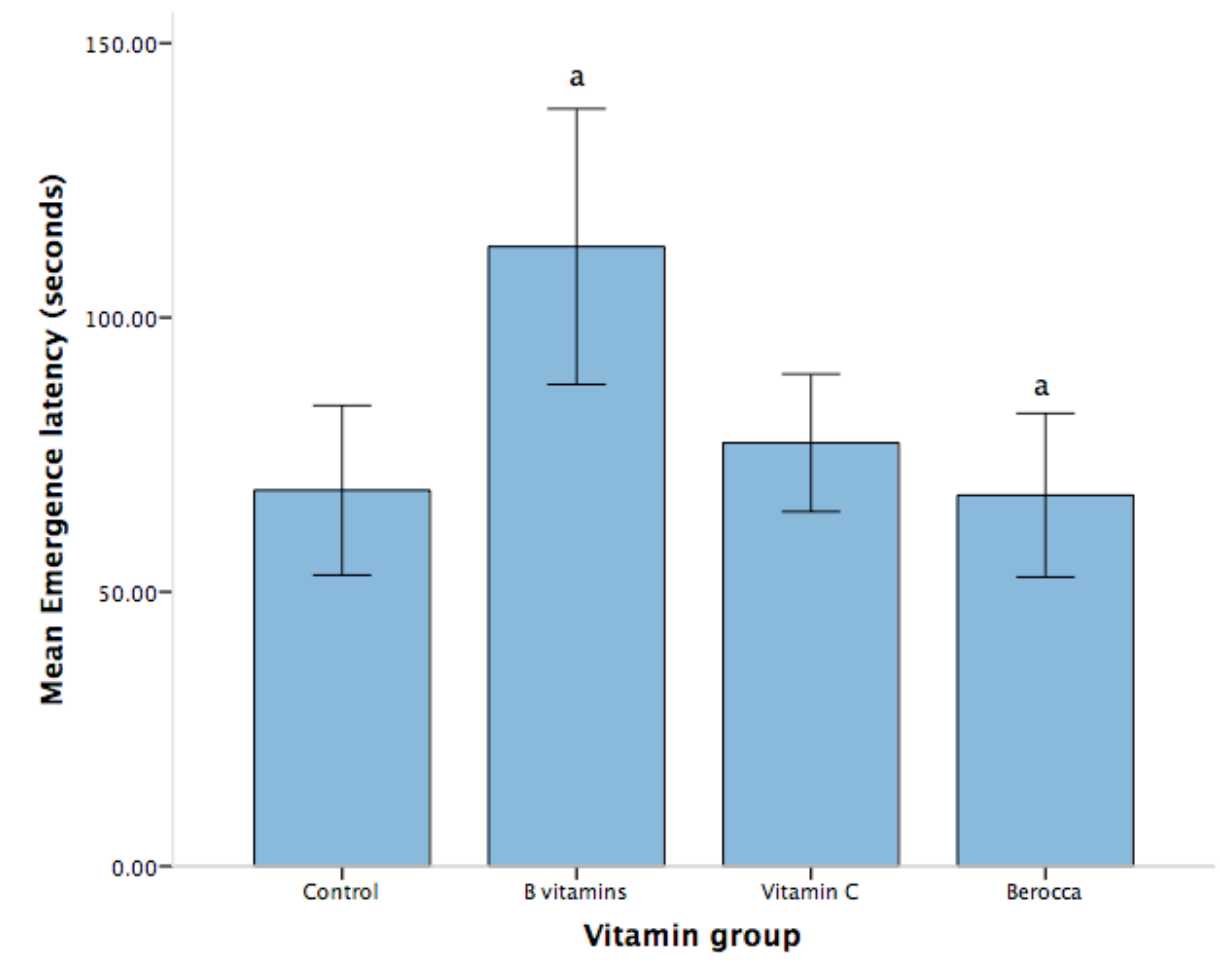
Figure 5: *Mean ± S.E.M of the mean emergence latency times (seconds) for treatment group.*
[a] Groups with superscripts in common are significantly different (P<.05).

5.2.2 Transitions:

There was a significant main effect of sex on the number of transitions made, with females making significantly more transitions than males, shown in Table 5. The main effect of treatment group on the number of transitions was not significant nor was the interaction between sex and treatment group, shown in Table 5.

5.2.3 Centre Square Occupancy:

The main effects of treatment group and sex on centre square occupancy were not significant. Nor was the interaction between sex and vitamin group, shown in Table 5.

5.2.4 Corner Square Occupancy:

There was a significant main effect of treatment group on corner square occupancy (see Table 5). Fischer LSD post hoc tests revealed that the Berocca group spent significantly less time in the corner squares than the B vitamin and control groups (both $p < .01$), shown in Figure 6. The vitamin C group also spent less time in corner squares than B vitamin and control groups but this was not statistically significant ($p = .093$ and $.088$ respectively). The main effect of sex on corner square occupancy was not significant, nor was the interaction between sex and treatment group (see Table 5).
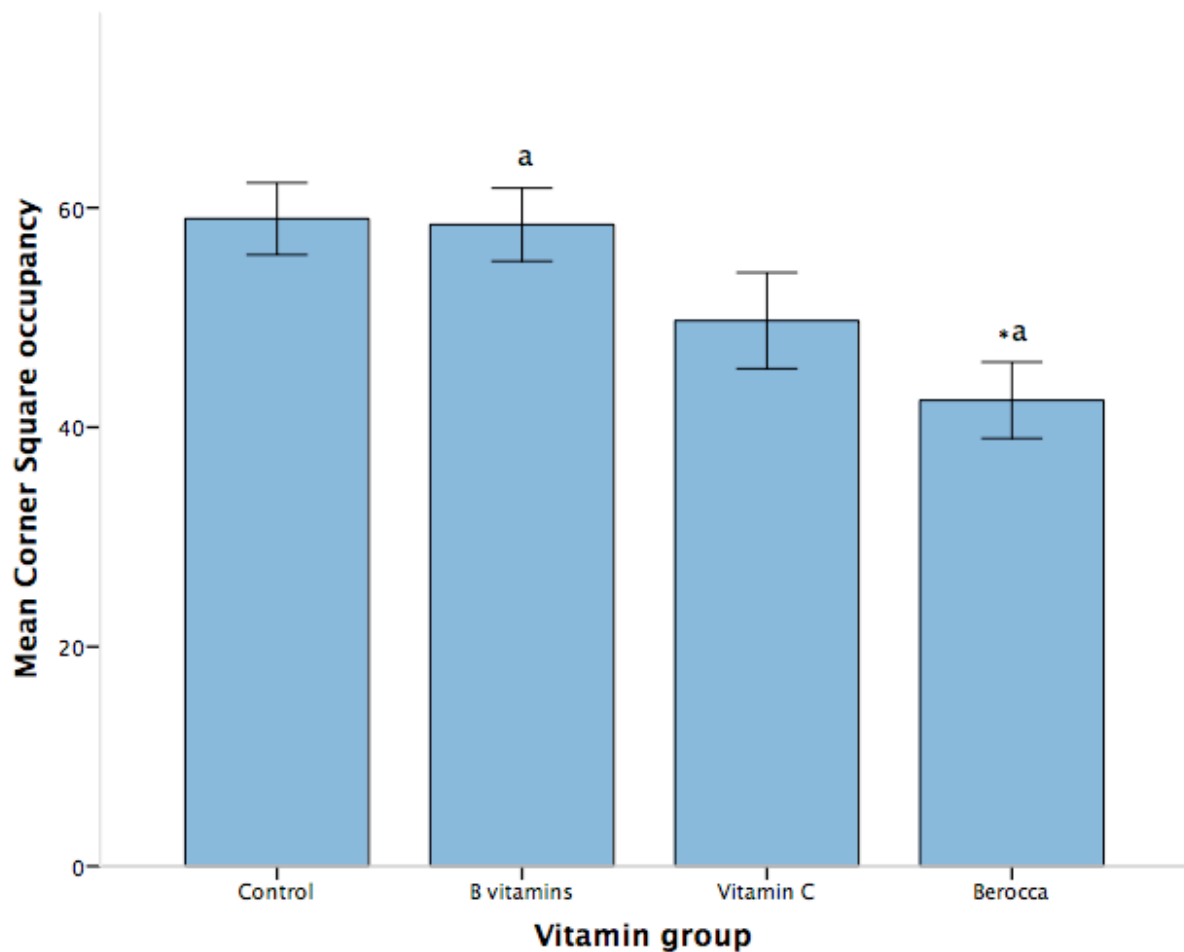
Figure 6: *Mean ± S.E.M of the mean number corner squares occupied for treatment group.*

∗*Significantly different (p<.05) from water control group.*

ᵃ *Groups with superscripts in common are significantly different (P<.05).*

5.2.5 Walking:

There was a significant main effect of vitamin group on time spent walking (see Table 5). Fischer LSD post hoc tests showed the B vitamin group spent significantly less time walking than all other groups (all $p < .001$), shown in Figure 7. The vitamin C group also spent less time walking than the Berocca group but this was not statistically significant ($p = .062$). The main effect of sex on time spent walking was not significant nor was the interaction between sex and treatment group (see Table 5).
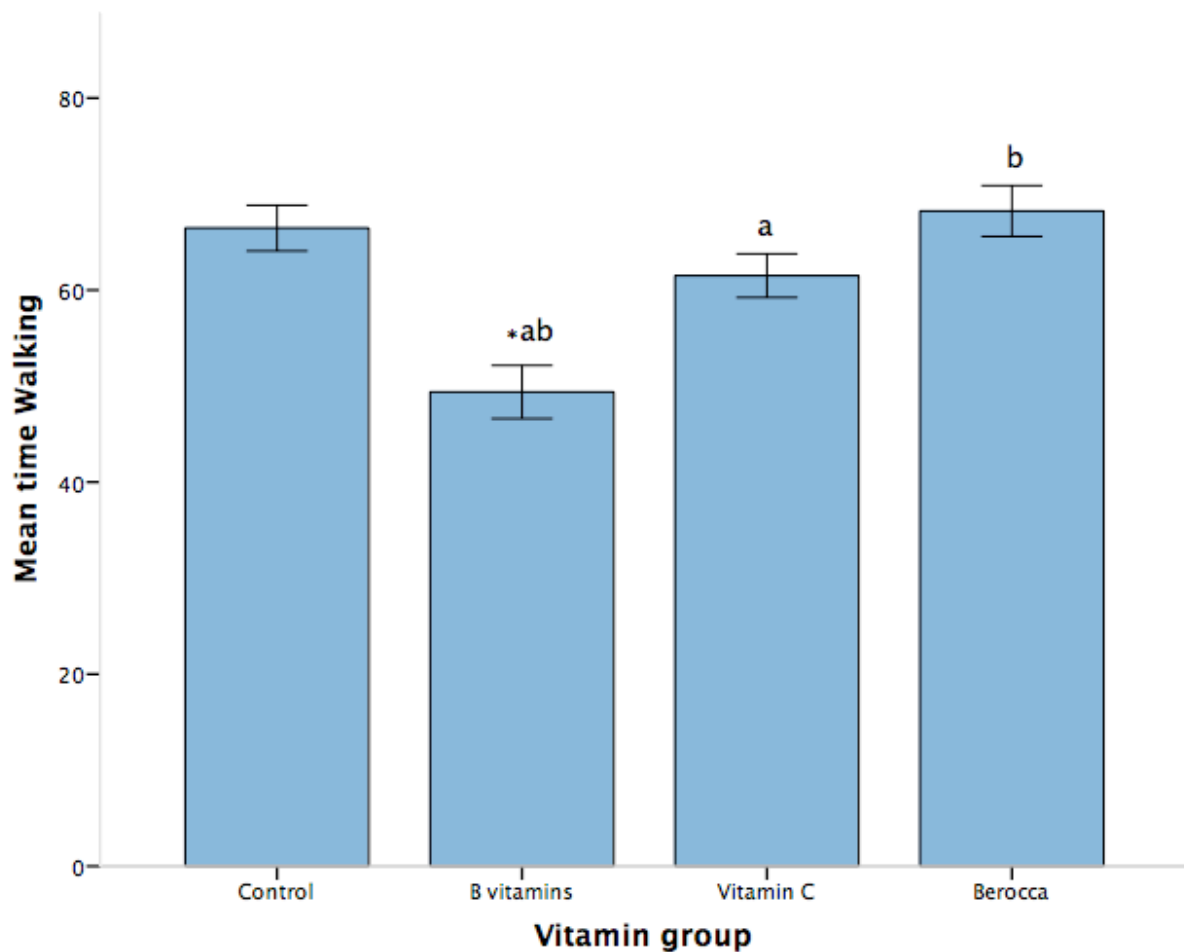
Figure 7: *Mean ± S.E.M of the mean time spent walking for treatment group.*

∗Significantly different (p<.05) from water control group.

ab Groups with superscripts in common are significantly different (P<.05).

5.2.6 Rearing:

The main effects for treatment group and sex on time spent rearing were not significant. Nor was the interaction between gender and treatment group (see Table 5).

5.2.7 Grooming:

The main effects of treatment group and sex on time spent grooming were not significant. Nor was the interaction between treatment group and sex (see Table 5).

5.2.8 Faecal Boluses:

There was a significant main effect of sex on the number of faecal boluses, with females leaving significantly fewer boluses than males (see Table 5). The main effect of treatment group on number of faecal boluses was not significant, nor was the interaction between sex and treatment group on number of faecal boluses (see Table 5).

5.3 Elevated plus maze:

In the elevated plus maze each animal was tested once. ANOVAs were used to assess whether there was an effect due to treatment group, sex differences, and treatment x sex interactions.

Table 6: Mean (± S.E.M) for responses in the elevated plus maze for control (n=20), B vitamins (n=20), vitamin C (n=20), Berocca (n=20) micronutrient treatment groups and for male (n=40) and female (n=40).

Treatment Group	Control	B	C	Berocca	F (3, 72)	P
% open arm entries	36.30 (3.34)	39.65 (4.76)	38.99 (3.72)	48.15 (3.37)	1.76	.163
% closed arm entries	63.69 (3.34)	60.69 (4.29)	61.01 (3.72)	52.59 (3.56)	1.62	.192
% open arm occupancy	36.30 (3.34)	39.65 (4.76)	38.99 (3.72)	48.15 (3.37)	1.76	.163
% closed arm occupancy	63.69 (3.34)	60.69 (4.29)	61.01 (3.72)	52.59 (3.56)	1.62	.192
Total entries both arms	77.15 (2.15)	77.55 (1.90)	76.00 (1.24)	75.70 (1.43)	.27	.847
Rearing open	11.80 (1.24)	11.55 (1.54)	10.15 (1.28)	15.05 (1.35)	2.26	.089
Rearing closed	19.05 (1.15)	18.30 (1.60)	17.70 (1.18)	19.45 (1.32)	.33	.805
Walking open	16.75 (1.59)	18.05 (1.91)	19.30 (1.91)	21.20 (1.45)	1.23	.304
Walking closed	28.30 (2.11)	23.10 (2.02)	23.90 (1.83)	19.15 (1.80)	3.89	.012
Sex	**Male**	**Female**			**F (1, 72)**	**P**
% open arm entries	38.63 (3.22)	42.89 (2.18)			1.21	.275
% closed arm entries	61.56 (3.06)	57.44 (2.22)			1.19	.278
% open arm occupancy	38.63 (3.22)	42.89 (2.18)			1.21	.275
% closed arm occupancy	61.56 (3.06)	57.44 (2.22)			1.19	.278
Total entries both arms	75.05 (1.47)	78.15 (.78)			3.28	.075
Rearing open	11.75 (1.12)	12.53 (.83)			.32	.576
Rearing closed	18.63 (.91)	18.63 (.96)			.00	1.0
Walking open	16.93 (1.29)	20.73 (1.10)			4.96	.029
Walking closed	23.05 (1.69)	24.18 (1.15)			.349	.556

5.3.1 Percentage of entries into open arms:

The main effects of sex and treatment group on the percentage of entries into open arms were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.2 Percentage of entries into closed arms:

The main effects of sex and treatment group on the percentage of entries into closed arms were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.3 Percentage open arm occupancy:

The main effects of sex and treatment group on the percentage of open arm occupancy were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.4 Percentage closed arm occupancy:

The main effects of sex and treatment group on the percentage of closed arm occupancy were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.5 Total entries both arms:

The main effects of sex and treatment group on the total number of entries into both arms were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.6 Rearing in open arms

The main effects of sex and treatment group on the time spent rearing in open arms were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.7 Rearing in closed arms:

The main effects of sex and treatment group on the time spent rearing in closed arms were not significant. Nor was the interaction between sex and treatment group (see Table 6).

5.3.8 Walking in open arms:

There was a significant main effect of sex on time spent walking in open arms, with females spending significantly more time than males walking in open arms. The main effect of treatment group on time spent walking in open arms was a not significant, nor was the interaction between sex and treatment group (see Table 6).

5.3.9 Walking in closed arms:

There was a significant main effect of treatment group on time spent walking in closed arms (see Table 6). Fischer LSD post hoc tests revealed the Berocca group spent significantly less time walking in closed arm then control ($p < .001$), shown in Figure 8. The Berocca group also spent less time walking in closed arms than vitamin C but this was not statistically significant ($p = .082$). The main effect of sex on time spent walking in closed arms was not significant, nor was the interaction between sex and treatment group (see Table 6).
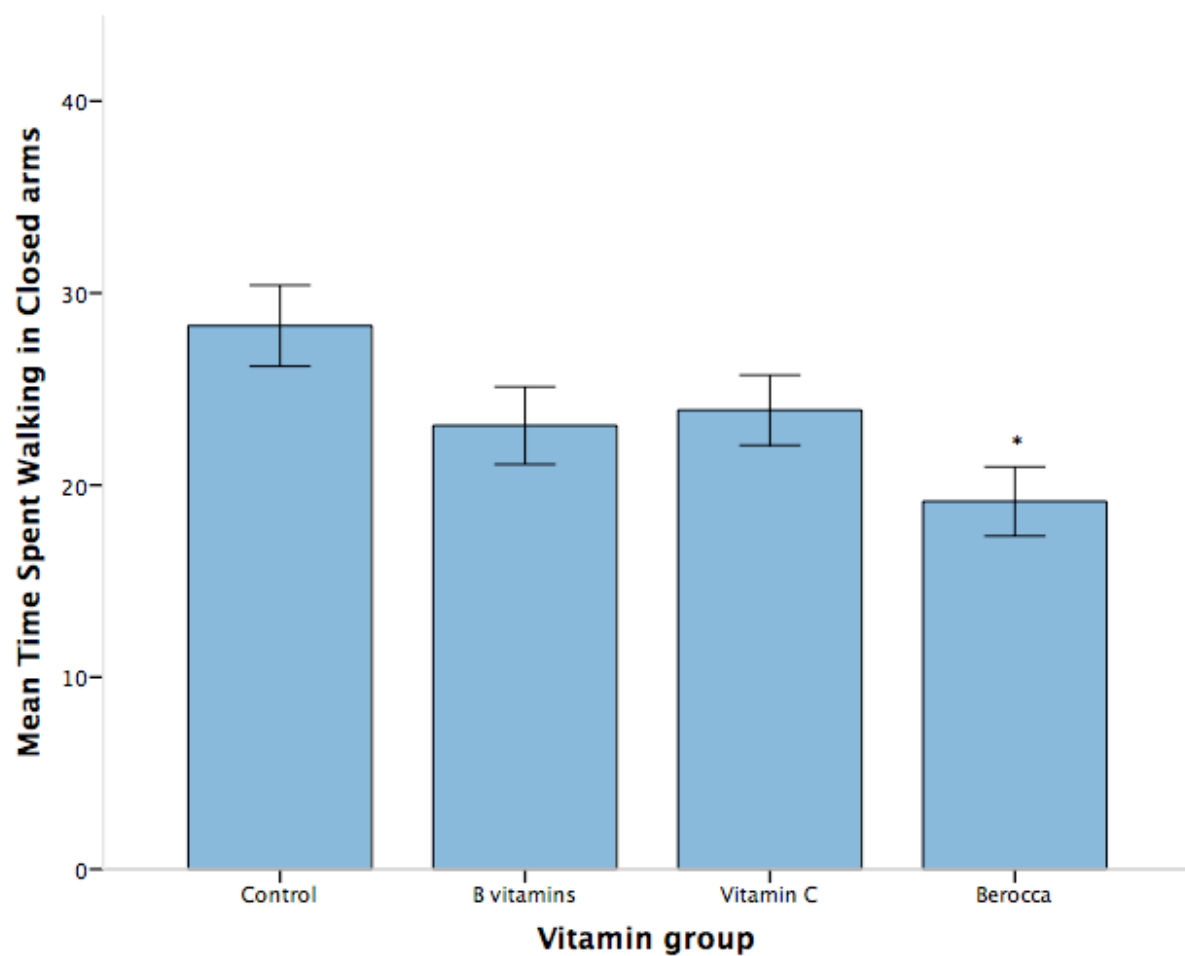
Figure 8: *Mean ± S.E.M of the mean time spent walking in closed arms for treatment group.*

∗ *Significantly different (p<.05) from water control group.*

5.4 Responsiveness to Brightness Change in a Y-maze:

In the Y-maze each animal was tested once. ANOVAs were used to assess whether there was an effect due to micronutrient treatment group, sex differences, and treatment x sex interactions.

Table 7: Mean (± S.E.M) for responses in responsiveness to brightness in Y-maze test (n=20), B vitamins (n=20), vitamin C (n=20), Berocca (n=20) micronutrient treatment groups and for male (n=40) and female (n=40).

Treatment Group	Control	B	C	Berocca	F (3, 72)	P
% change arm entries	47.16 (1.15)	47.66 (7.26)	48.31 (4.97)	62.21 (2.55)	2.91	.04
% change arm occupancy	37.71 (2.52)	50.63 (8.01)	58.82 (5.27)	66.29 (3.54)	5.96	.001
Total entries both arms	6.10 (.42)	3.30 (.64)	5.00 (.72)	5.75 (.45)	6.27	.001

Sex	Male	Female			F (1, 72)	P
% change arm entries	48.51 (4.25)	54.16 (2.08)			1.76	.189
% change arm occupancy	47.62 (4.79)	56.10 (2.88)			3.13	.081
Total entries both arms	3.85 (.43)	6.23 (.34)			22.78	.0001

5.4.1 Percentage Change arm Entries:

There was a significant main effect of treatment group on percentage of change arm entries (see Table 7). Fischer LSD post hoc tests revealed the Berocca group had significantly higher change arm entry percentage than all other groups (all $p < 0.05$). There was no significant difference in change arm entry percentage between all other groups. The main effect of sex on percentage of change arm entries was not significant (see Table 7).

There was a significant interaction between sex and treatment group on the percentage of change arm entries, shown in Figure 9. This effect indicated males and females may be affected differently by vitamin treatment. Specifically, percentage of change arm entries for males was increased with Berocca treatment but decreased by B vitamin treatment. However for females the response was different. Both Berocca and B vitamin treatment increased percentage of change arm entries compared to control, for females.
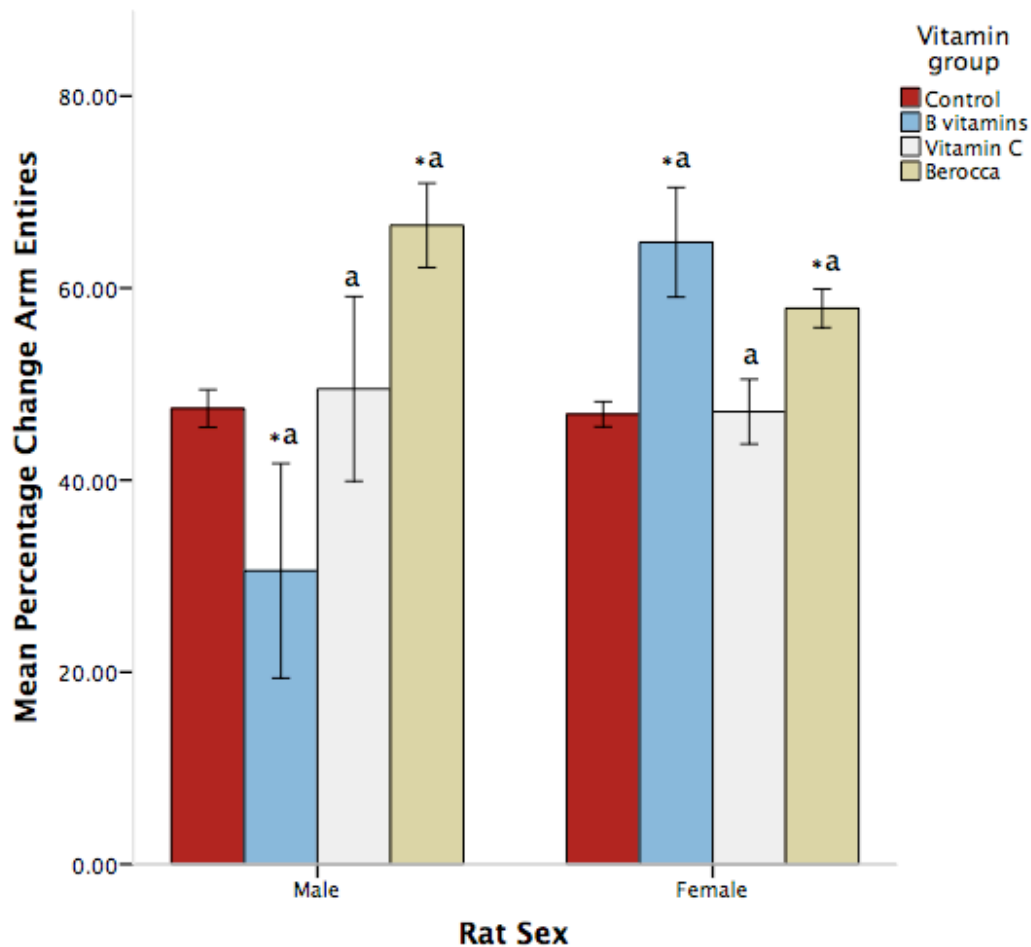
Figure 9: *Mean ± S.E.M percentage change arm entries for males and females separately following treatment with B vitamins, vitamin C, Berocca, or unadulterated drinking water (control).*

✲Significantly different (p<.05) from water control group for that particular sex.

[a] Groups with superscripts in common significantly different (P<.05) for that particular sex.

5.4.2 Percentage Change Arm Occupancy:

There was a significant main effect of treatment group on percentage change arm occupancy (see Table 7). Fischer LSD post hoc tests revealed that the Berocca group spent significantly more time in the change arm than the B vitamin and control groups (p < 0.05). Berocca also spent more time in the change arm than the vitamin C group, but this was not

statistically significant (p = 0.051). The main effect of sex on percentage change arm occupancy was not significant (see Table 7).

There was a significant interaction between sex and vitamin group on percentage change arm occupancy, shown in Figure 10. This effect indicates males and females may be affected differently by vitamin treatment. Specifically, percentage of change arm occupancy for males was increased with Berocca treatment but decreased by B vitamin treatment. However for females the response was different. Both Berocca and B vitamin treatment increased the percentage of change arm occupancy compared to control, for females.
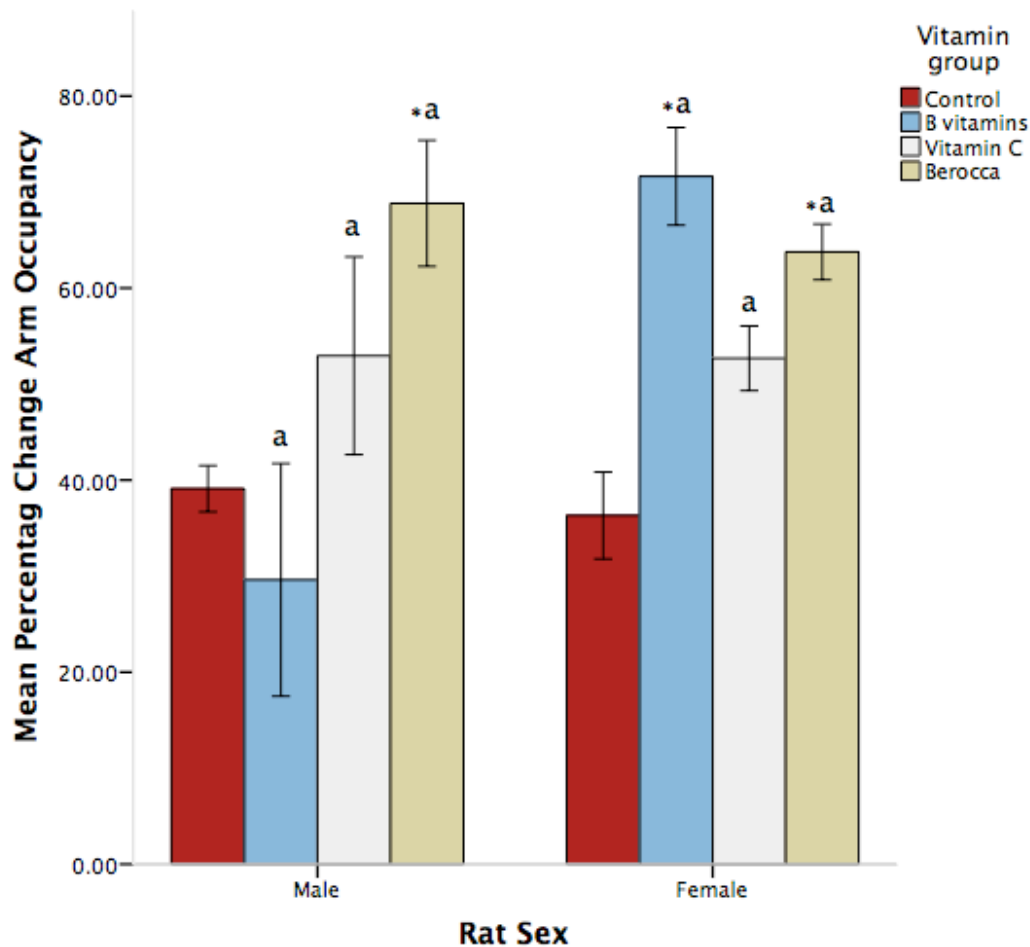
Figure 10: *Mean ± S.E.M percentage change arm occupancy for males and females separately following treatment with B vitamins, vitamin C, Berocca, or unadulterated drinking water (control).*

∗Significantly different (p<.05) from water control group for that particular sex.

[a] Groups with superscripts in common significantly different (P<.05) for that particular sex.

5.4.3 Total Entries into both arms

There was a significant main effect of vitamin group in the total number of entries into both arms (see Table 7). Fischer LSD post hoc tests revealed the B vitamin group made significantly fewer total entries into both arms than all other groups (all p < 0.05), shown in Figure 11. There was a significant main effect of sex on the total number of entries into both

arms, with females making more entries into both arms compared to males. The interaction between sex and vitamin group was not significant (see Table 7).
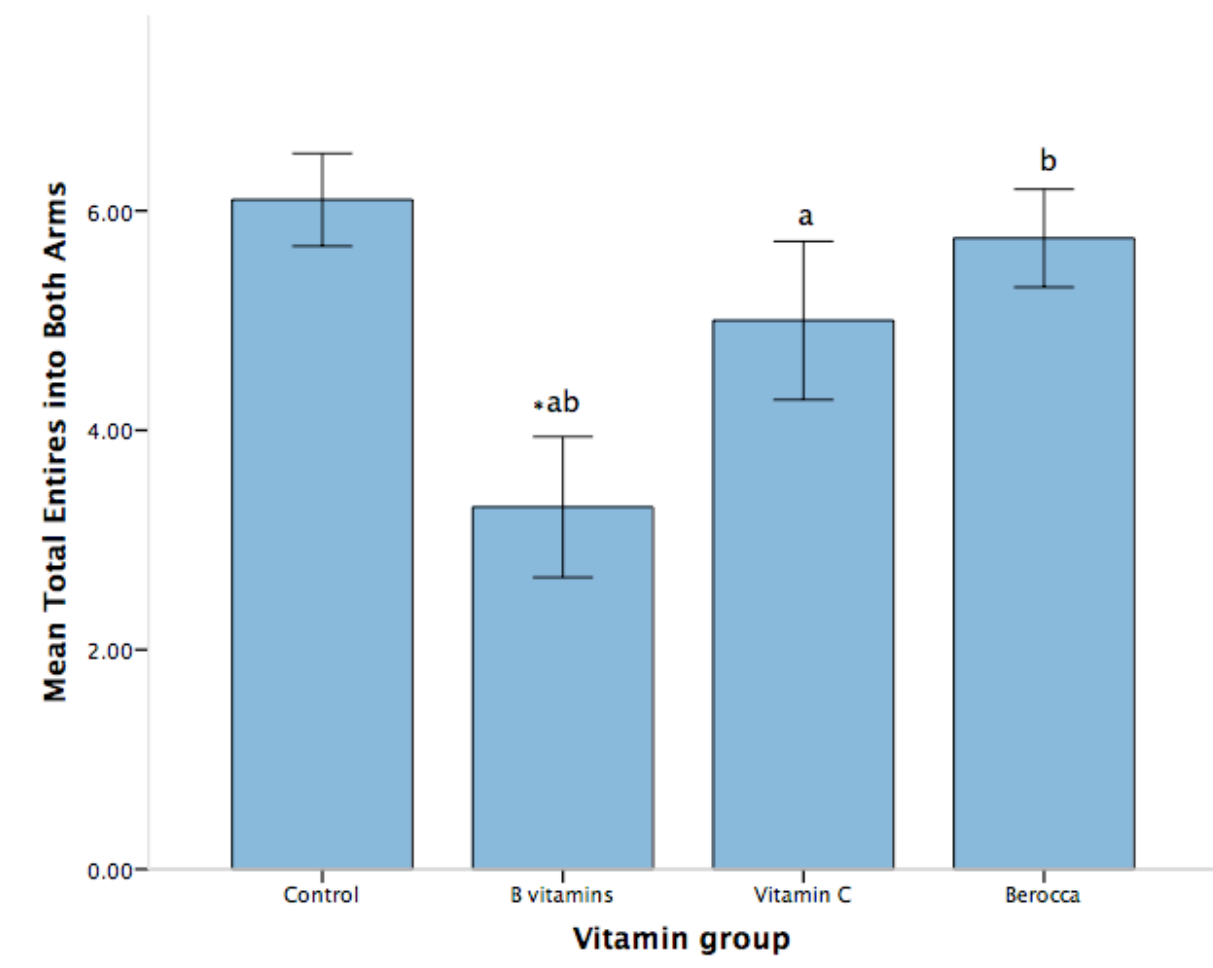
Figure 11: *Mean ± S.E.M total number of entries into both arms for treatment group.*

∗Significantly different (p<.05) from water control group.

ab Groups with superscripts in common significantly different (P<.05)

6.0 Discussion of results:

In the current study, 80 rats were divided into four treatment groups each with a different micronutrient treatment administered through their drinking water. Treatment groups consisted of a B vitamin group, a vitamin C group and a broad-spectrum micronutrient group (Berocca, B + C), and a control group (unadulterated water). Dosages used were determined to be at a therapeutic level, based on previous studies (Hughes et al., 2011), and above therapeutic levels to investigate whether a higher dose (B vitamins and Berocca) may have greater benefits or a negative effect. Each treatment group received vitamin treatment for seven weeks and remained on treatment during testing which occurred on the 8th week of treatment. Three behavioural tests of anxiety related behaviour (emergence latency, OFT, EPM) and one of short term spatial memory (responsiveness to brightness change in a Y Maze) were used to assess the effects of micronutrient supplementation on anxiety-like behaviour and memory. Male and female rats were used to allow for possible sex differences in the effects of vitamin treatment. Conclusions were then drawn from any significant results about the behavioural outcomes of different micronutrient supplements over a prolonged period (chronic supplementation).

6.1 Summary of results

The results revealed a number of significant behavioural differences between treatment groups. The rats receiving broad-spectrum supplementation (Berocca) showed significantly less emotionality and significantly improved short-term spatial memory when compared to other treatment groups. Sex differences were found quite often as well as interactions between sex and treatment group especially in the Y maze.

The B vitamin group was the slowest to emerge in the emergence latency test with Berocca being significantly faster, but not than other groups. Females also took longer to emerge than males. In the OFT the Berocca group spent significantly less time in the corner squares than B vitamin and control groups, suggesting lower anxiety levels than these groups. The Y maze revealed the greatest number of significant differences between treatment groups. The Berocca group had a significantly higher percentage of entries into and occupancy of the changed arm, as well as total entries of both arms (ambulation) than all other treatment groups. In the Y maze there was also a significant sex difference in ambulation, with females making more entries into both arms than males. There were significant sex x treatment interactions in change arm entries and occupancy suggesting different treatments affected each sex differently. The most notable interactions here were differences in the effects of B vitamin and Berocca supplementation on males and females. Specifically, the percentage of change arm entries and occupancy for males was increased with Berocca treatment but decreased by B vitamin treatment. However for females the response was different. Both Berocca and B vitamin treatment increased the percentage of change arm occupancy compared to control, for females. Nothing of major significance was found in the EPM.

These results provide evidence that a broad-spectrum of micronutrients may be more effective than a single micronutrient or vitamin group in reducing anxiety-related behaviour and improving memory in a normal rat population. The sex x treatment interactions also

suggested that certain micronutrient treatments may be more appropriate for different sexes. Also, it was noteworthy that the Berocca group consumed on average a significantly higher dose of both B and C vitamins than the two other treatment groups.

6.1.1 Dose and Fluid intake:

Fluid intake and doses were higher for females among all treatment groups. The Berocca group also drank significantly more than all other groups (possibly due to a better taste of the solution) and accordingly ingested a significantly higher dose of B vitamins than the B vitamin group, and a higher dose of vitamin C than the vitamin C group. This may have affected the behavioural results. It is possible that both the B and C groups' doses were at a sub-therapeutic level, which would explain the effects due to Berocca (a combination of B and C) but not the single vitamins. This may be true for vitamin C, as high doses of this vitamin have been shown to have an anxiolytic effect in rats (Hughes et al., 2011). These results make it difficult to determine exactly whether the anxiolytic effects were due to higher dose of a single vitamin group in the Berocca, or to a synergistic effect of the combination of B vitamins, vitamin C and minerals.

6.1.2 Treatment effects:

In the emergence latency into an open field test, the B vitamin group took significantly longer to emerge than the Berocca group, suggesting that broad-spectrum supplementation may have a greater anxiolytic effect in this measure of anxiety related behaviour than B vitamins alone. However there was no significant difference between the Berocca and control groups. The B vitamin group actually took longer to emerge than both vitamin C and control groups, but this was not statistically significant. It may be that high dose B vitamins alone could be eliciting an anxiogenic effect in this measure.

In the OFT the Berocca group spent significantly less time in the corner squares than both B vitamin and control groups, but not vitamin C. The vitamin C group also spent less time in the corner squares than the B vitamin and control groups but this was not statistically significant. This suggests that, compared to the control condition, Berocca had an anxiolytic action in the OFT. Given that this action was greater than vitamin C alone, it suggests the combination of B and C in Berocca may act synergistically so that B enhances any anxiolytic effects of vitamin C reported in previous studies (Hughes et al., 2011). It may also be possible the combination of minerals (magnesium and zinc) in Berocca influenced the anxiolytic actions of vitamin C or even somehow exerted an anxiolytic effect on its own.

The only significant treatment effect in the EPM was that Berocca-treated rats spent significantly less time walking in the closed arms than control subjects. This suggests either the Berocca treatment had a small anxiolytic effect in the EPM, or that Berocca-treated rats spent more time in the middle of the maze (i.e. not in any arm), or more time immobile in the closed arms, compared to control. There were no significant differences in rearing in closed arms or rearing and walking in open arms between any of the treatment groups. Therefore, it is unlikely that there was any anxiolytic effect of Berocca treatment in the EPM, and it is more likely that animals were either spending more time immobile or in the middle of the maze.

For responsiveness to brightness change in a Y-maze, the Berocca group made a significantly higher percentage of entries into the changed arm than all other groups. The Berocca group also showed a significantly higher percentage of changed-arm occupancy than either the control or B vitamin group. The Berocca group occupied the changed arm for longer periods of time than the vitamin C group but this difference just failed to reach statistical significance (p= .051). The Berocca group also made a significantly higher number of entries into both arms (ambulation) than all other groups. Low ambulation is thought to be indicative of increased anxiety. These results suggest that Berocca may have both decreased anxiety and

improved memory in the Y maze. Berocca supplementation may have improved encoding, consolidation or retrieval of information or decreased neophobia arising from heightened emotional reactivity (or "anxiety"), as can occur with anxiogenic experiences in other situations (Aitken, 1974; Hughes, 1987; Missilin & Ropartz, 1981) thereby making the animals more comfortable in the novel environment (Atchinson & Hughes, 2006). Given the higher levels of ambulation in the Berocca group, and as activity is inversely related to anxiety (Archer, 1973), decreased anxiety in the Berocca treated animals may be the best way of accounting for all the behavior observed in the Y-maze.

These anxiolytic effects may be due to synergistic action of B vitamins with vitamin C, or the additional mineral content of Berocca on its own or its enhancement of the effects of vitamins B and C. The Y maze was the last behavioural test for the animals so it may also be that the significant results of Berocca in this test were due to a longer treatment time i.e. the animals had reached a threshold for treatment effects that had not been met in the OFT and EPM.

6.1.3 Sex differences and interactions:

A number of sex differences and interactions were found in each of these tests. In the open field, females took significantly longer to emerge from the start box than males thus suggesting that they may be more anxious when exposed to open illuminated novel environment. However, once in the open field, they made significantly more transitions than males suggesting that (given lower ambulation is indicative of increased anxiety) they may have been less anxious than male rats. The number of faecal boluses left by females in the OFT was also significantly lower than males, which further supports the idea that females are less anxious than males once in the open field.

In the EPM females spent significantly more time than males walking in open arms. Given there were no significant differences in rearing it is possible that males spent more time immobile in the open arms suggesting they were more anxious than females in the open arms.

In the Y maze there was a sex difference in ambulation and also sex x treatment interactions in percentages of entries into and occupancy of the changed arm. There was a significant difference in ambulation between males and females, with females making significantly more entries into both arms than males. There were significant interactions between sex and treatment in percentages of changed-arm entries and occupancy. These results indicate that males and females were affected differently by the different micronutrient treatments. Specifically, the percentage of changed-arm entries and occupancy for males was increased with Berocca treatment but decreased by B vitamin treatment. However for females the response was different. Both Berocca and B vitamin treatment increased the percentage of change arm occupancy compared to control, for females. This indicates B vitamin treatment was more effective for females than males in terms of anxiolytic and memory enhancing effects. However the addition of more nutrients in Berocca resulted in the anxiolytic and memory enhancing effects being of greater benefit to males. It may be that the additional minerals and vitamin C in the Berocca formulation provides greater anxiolytic benefit for males.

7.0 General Discussion:

7.1 Methodological strengths:

The current study had several methodological strengths. The first was the use of rats. Rats develop quickly reaching adulthood in approximately 90 days (Anderson, 2003). Therefore research can be completed in a much shorter time period and the long-term behavioural effects of exposure can be examined faster than in human populations. Rats provide a number of advantages including overall control of experimental conditions, reducing confounding variables associated with humans such as non-compliance with treatment, missed appointments, prior history of medication use and illness, and no expectations about what the treatment might do. This ultimately limits a number of confounding variables that might be present in human populations. The use of rats allows us to ensure that all subjects are exposed to the same experiences (i.e. handling, living conditions) and that it is only the variables being manipulated that determine the results.

The use of a control group allowed investigation into whether the supplement was responsible for any behavioural effects in each treatment group. Having a control meant comparisons could be made between supplement groups and a group that had never taken the supplement.

The use of male and female rats allowed investigation into different effects of each supplement in each sex and this proved to be useful due to the sex differences and interactions that were found. This is important because the supplementation regimens here are intended for use by both sexes in a human population.

As mentioned in section 3.4 the validity and reliability of the behavioural measures adopted were high, thereby providing considerable confidence in the methodology.

The rats were provided in one cohort thus avoiding any problems with them arriving at different times. Consequently, all rats in each group began their treatment and were tested at the same time. This further limited the number of possible any confounding variables.

The use of different micronutrient formulas enabled the establishment of the most effective supplementation regimen in relation to the reduction of anxiety like behaviour and improvements in short term spatial memory in normal rat populations. Comparing B vitamins and vitamin C with Berocca (a combination of the two) which have all been shown to reduce anxiety in humans and rats (Carroll et al., 2000; Kennedy et al., 2010; Stough et al., 2011; Hughes et al., 2011) alongside the control animals allowed the assessment of whether a single nutrient or nutrient group is more effective than another in reducing anxiety or whether they act synergistically to have the greatest effects in reducing anxiety like behaviour in rats. Furthermore using doses of the B vitamins and Berocca that were higher than a therapeutic level (as determined by previous studies, Carroll et al., 2000; Kennedy et al., 2010) provided information regarding the effects of higher doses of micronutrient supplementation.

This study showed that receiving a high dose of a broad-spectrum micronutrient supplement gave the greatest reduction in anxiety-like behaviour and the most improvement in measures of short-term memory compared to single micronutrient supplementation. Effects of single micronutrients did not differ greatly from controls and in some cases rats in these groups even performed worse than controls. It has been suggested that higher doses may have possible toxic effects and therefore produce greater anxiety (De Oliveria et al., 2007). This was possibly the case with the B vitamins, as treated rats exhibited poorer performance on some measures of anxiety than controls. However the broad-spectrum supplementation resulted in significantly improved performance.

The administration of supplements in the drinking water was another strength. This allowed exact measurement of the appropriate dose for each supplement to be easily dissolved

into the rats' drinking water. It also made it very easy to calculate accurately how much each rat was drinking and subsequently the dose each rat was consuming. The manner in which rats were housed allowed social interaction but at the same time, because they each had their own drinking solution, individual doses could be calculated.

The presence of an observer during behavioural tests could conceivably have affected responding especially in the emergence latency and open field tests. To ensure this was kept to a minimum an external video camera was mounted above each apparatus and connected to a T.V. monitor in the experimental room where the observer was able to record movements without sitting in close proximity to either apparatus. The same testing room was also used for all tests and animals and the apparatus was oriented in the same location for every animal.

This study serves as a pilot for the behavioural effects of a broad spectrum versus single micronutrient supplement. No other research has been done in rats or humans in this specific area and consequentially little is known about the differences in behavioural effects between single and broad-spectrum micronutrients. The purpose of this study was to detect any differences between these micronutrient supplementation regimens and their long-term behavioural effects. As there is a lack of animal research in this area, the results should be viewed as preliminary and providing a starting point for future research.

7.2 Methodological Limitations:

There were a number of limitations to the current study. Firstly, the rats were not weighed during treatment (only before and after) so the average dose consumed each week could not be calculated. An average dose for each week of treatment would have been useful for determining exactly how much of each micronutrient each group was consuming, and how this was related to the behavioural results.

One possible limitation might be the fact that the animals were drawn from a normal rat population. Because the majority of human research in this area has focused on individuals with psychiatric disorders, perhaps the use of an animal model of anxiety using genetic manipulation to produce animals with higher anxiety traits would provide more insight into the effects of different micronutrients on an anxious population.

Rats in each experimental group were not tested on each measure before treatment. Therefore there is no baseline of behavioural results for each treatment group. It is possible, but unlikely, that the two cohorts were significantly different in measures of anxiety and memory before treatment due to individual characteristic and genetic differences. Establishing a baseline measure of anxiety and memory would have allowed for control of these individual differences. However this could also be viewed as a strength as rats did not have a chance to become habituated and therefore less anxious in the experimental environment.

There were two factors that need to be considered with the micronutrient administration. First the Berocca may have tasted better, given it contains artificial sweeteners, which were not in the other treatment groups. This would explain the higher dose ingested by the Berocca group. Another limitation is that Berocca contains minerals (calcium, magnesium, and zinc). These comprised one variable that was not controlled which may have influenced the results.

7.3 Implications

This was a pilot study designed to assess the effects of broad spectrum and single micronutrient supplementation on behaviour in normal rats. There is a lack of research into the effects of vitamins on animal behaviour. The results of this study are therefore important and provide additional knowledge to a body of research with mixed results across a variety of individual vitamins and minerals and their effects on a number of measures of animal and human behaviour. The current study highlights some important issues and provides a base for future research. It is important that animal research into micronutrient supplementation is continued as it adds to our understanding of this area. This will be significant in the future as the number of studies looking at the effects of micronutrients in the human population increases.

This research provides a valuable stepping-stone in animal micronutrient research. It has helped to identify gaps in the literature that need addressing, and has highlighted some important considerations that need attention when planning future research. It highlights a need to establish a complete understanding of the effects of micronutrients.

The most significant findings of the current study were the effects of the Berocca treatment on measures of anxiety and memory in the responsiveness to brightness change in a Y maze test. Berocca treatment resulted in a significant reduction in anxiety and improvements in short term spatial memory compared with all other treatment groups and controls. In the Y maze there were also significant sex differences in ambulation, and a sex treatment interaction in the percentage of change arm entry and occupancy, suggesting different treatments affected each sex differently. Specifically, the percentage of changed-arm entries and occupancy for males was increased with Berocca treatment but decreased by B vitamin treatment. However for females the response was different. Both Berocca and B vitamin treatment increased the percentage of change arm occupancy compared to control, for

females. This indicates B vitamin treatment was more effective for females than males in terms of anxiolytic and memory enhancing effects. However the addition of more nutrients in Berocca resulted in the anxiolytic and memory enhancing effects being of greater benefit to males. It may be that addition minerals and vitamin C in the Berocca formulation provides greater anxiolytic benefit for males.

These results provide evidence that a broad-spectrum of micronutrients may be more effective than a single micronutrient or vitamin group in reducing anxiety-related behaviour and improving short-term memory in a normal rat population. The sex-treatment interactions also suggest that certain micronutrient treatments may be more appropriate for different sexes. Also noteworthy is that the Berocca group consumed on average a significantly higher dosage of both B vitamins and vitamin C than the two other treatment groups. This may explain some of the effects, as the B vitamin and vitamin C supplementation may have been below therapeutic levels.

Any potential biochemical mechanisms are unable to be identified from these results, given that the study was focused on behavioural effects rather than mechanisms of action. The study results do however suggest that the broad-spectrum rationale mentioned in section 1.6.1 may be important to take into consideration with the effects of vitamins on anxiety-like behaviour and short-term spatial memory in rats. It is important to note that the broad-spectrum group did consume higher doses of B vitamins and vitamin C that the single vitamin groups. This may implicate the usage of higher doses of these vitamins alone in future studies.

Overall the results from the current study suggest that a high dose of a broad spectrum micronutrient supplement is more effective in reducing anxiety and improving memory compared to single micronutrients or single micronutrient groups (B vitamins). However future research needs to be conducted to replicate these results before any firm conclusions are

made about the efficacy of a broad-spectrum micronutrient supplement over a single

micronutrient supplement.

8.0 Future directions:

This was the first study comparing the effects of broad-spectrum versus a single micronutrient supplement on anxiety like behaviour and short-term memory in rats. Future research in this area is essential. Firstly future research should aim to address the limitations of the current study. From here the focus should be further animal studies to see if these results are able to be replicated, and to provide more evidence to support these findings.

Establishing a baseline for behaviour may be useful in the future to help determine more accurately whether behaviour was influenced by the intervention or through individual differences between different animal cohorts. This would involve running the behavioural tests prior to the intervention then comparing the results with behavioural tests following intervention. Future research may also benefit from the use of animals that have been bred to express anxious traits. There are a number of genetic models of anxiety in rodents (Cryan & Sweeny, 2011) that could be used. This would provide a better analogue for human anxiety and improve the face and construct validity of future studies.

Addressing the taste of the vitamin solution would also be useful. This could be done either by removing the artificial sweeteners from the Berocca and making it up in the laboratory, or adding sweeteners to the B vitamin and vitamin C mixtures. The former would be a better option to limit any confounding effects of artificial sweeteners. Making the broad-spectrum supplement in the laboratory may also be useful, as it would allow the exclusion of the minerals in Berocca, thereby further reducing confounding variables.

Future research should endeavour to establish the effects of each individual vitamin and mineral in Berocca. This would mean testing each B vitamin alone as opposed to in a complex. While this would be difficult it could enable a clearer understanding of whether all the micronutrients in Berocca are needed together or not. This would give a more in-depth and

confident understanding of how micronutrients affect behaviour. It may add or detract to the rationale behind broad-spectrum supplementation, that nutrients are required together in balance.

Another area that would be useful to investigate is the length of treatment. Both longer and shorter treatment periods would provide a better idea as to a minimum effective period for optimal effect.

Once these issues have been adequately addressed in animals, research should then focus on the effects of broad-spectrum micronutrient supplementation compared to single micronutrient supplementation for anxiety in humans. The use of randomised placebo controlled trials, here may be helpful in determining if these effects are replicable in humans. This also gives a further option of examining a variety of different single micronutrients alone and comparing them to broad-spectrum supplementation.

There is a need for future research to investigate the biochemical mechanisms of micronutrient action. This could involve in-vitro investigations into specific effects of single micronutrients on brain tissues, including neurotransmitter pathways and potential antioxidant effects. In-vivo studies could test the same parameters with individual micronutrients and a broad-spectrum supplement to compare and elucidate any synergistic interactions between micronutrients. This may provide support for the mechanisms of action proposed by Kaplan and Leung (2011).

There have been recent investigations into potential mechanisms of micronutrient supplementation. Du et al (2015) have investigated the role of nutrients in protecting mitochondrial functioning and neurotransmitter signalling with implications in the treatment of depression, PTSD and suicidal behaviour. More research of this kind is needed to establish a more concrete idea of the mechanisms involved in the beneficial effects of micronutrient supplementation on mental illness.

The growing fields of nutrigenetics and neutrogenomics may help offer insight into micronutrient mechanisms of action. These fields allow the development of a more targeted approach to micronutrient supplementation by mapping out genetic information. This would provide some insight into any genetic mutations that may cause in-born metabolic errors and micronutrient deficiencies, which can then be targeted with supplementations. The field of epigenetics offers promise, as does the use of omic genetic technologies. These areas include pharmacogenomics (how genes affect responses to drugs), proteomics (the study of proteins expressed by the genome) and metabolomics (chemical processes involving metabolites). Technologies in these areas could help to provide answers on pharmacodynamics properties, toxicity and safety effects, and the clinical efficacy of micronutrient supplementation.

9.0 Conclusions:

The most significant finding in the current study was that supplementation with a broad-spectrum (Berocca) micronutrient resulted in less emotionality and improved short-term spatial memory when compared to other treatment groups. Sex differences occurred, as did interactions between sex and micronutrient treatment group. The main outcome of the study is to favour the view that a broad-spectrum micronutrient supplement may be more effective in reducing anxiety and enhancing short-term spatial memory, than a single micronutrient, at least in a normal rat population. The sex x treatment interactions also suggested that certain micronutrients have different effects on anxiety and memory depending on the sex of the animal. These interactions suggested that B vitamin treatment was more effective for females than males in terms of anxiolytic and memory enhancing effects. However the addition of more nutrients in Berocca resulted in the anxiolytic and memory enhancing effects being of greater benefit to males. It may be that addition minerals and vitamin C in the Berocca formulation provided a greater anxiolytic benefit for males.

This was a pilot study and has highlighted a number of limitations that can be addressed in future research. The current study provides evidence that micronutrient supplementation does indeed affect behavioural responses in animals. Further research with randomized controlled designs in both animals and humans will help establish a greater understanding of these effects. Research into the mechanisms of action in the brain and body of micronutrients, alone and in combination will help to develop this understanding further. Together this knowledge may help provide insights into how micronutrient supplementation can be integrated into clinical practice.

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Appendix A

AEC Ref: 2014/06R

13 June 2014

Andrew Henderson

Department of Psychology

UNIVERSITY OF CANTERBURY

Dear Andrew

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "Effects of a broad-spectrum micro-nutrient supplement versus B vitamins and vitamin C in anxiety and memory".

Approval has been granted: (a) for the use of 80 Rattus norvegicus(b) for your research project to be undertaken from 11 June 2014 to 31 December 2014. If you require

an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MPI statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MPI Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, whichever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

Associate Professor Jim Briskie

Chair

University of Canterbury Animal Ethics Committee