NEUROTROPHIC BIOMARKER CHANGE AFTER PHYSICAL ACTIVITY AND MINDFULNESS INTERVENTIONS.

A thesis submitted for the degree of Master of Science at Federation University, Ballarat, Australia.

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ATTRIBUTION OF COLLABORATIVE CONTRIBUTION.

The Wellness project was established by Prof. Britt Klein at Faculty of Health & Centre for Biopsychosocial eHealth Research and Innovation, Federation University (Ballarat) and staff and students, who advertised, recruited and organised attendance on site for assessments and blood collection. The original study question and hypothesis involved in analysis of neurotrophin mRNA change in association with mood was established by the candidate in association with Prof. Fadi Charchar (Faculty of Science and Technology, Federation University, Ballarat)

Blood collection was carried out by the candidate on the Ballarat campus along with sample preparation and stabilisation prior to the RNA extraction.

Priscilla Prestes and the candidate extracted the RNA and Priscilla subsequently prepared the cDNA and processed the raw data output from the qPCR analysis in the FOST Genetics lab (Ballarat). The candidate completed all gene expression assays, raw data analyses and statistical analyses.

Except where explicit reference is made in the text of the thesis, this thesis contains no material published elsewhere, nor work relied upon or used without due acknowledgement in the main text or references listed. This thesis does not include work with copyright provisions or requiring copyright approvals.

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CONTENTS

ABSTRACT

BACKGROUND AND AIM AND HYPOTHESIS: BDNF, FGF2 and NGF are neurotrophins associated with neuroplasticity, nervous system development and psychiatric disorder in the literature. BDNF in particular is suggested as a useful biomarker of mood disorder. Both mindfulness and physical activity are shown to improve mood, reduce stress and are widely used as part of a multi-component treatment approach, reducing distressing symptoms of mood and affect disorders. The utility of protein level as a biomarker has been controversial in the literature following issues concerning the assessment of peripheral levels as a proxy for central levels. The principal aim of this study was to investigate the gene expression of three neurotrophins BDNF, FGF2 and NGF as potential biomarkers of mood disorder, at an early stage of these disorders, which are now widely recognised as having pathogenesis related to dysregulation in the neuro-immuno-endocrine axis. In addition, the study will explore the effect of both physical activity and mindfulness on neurotrophin expression clarifying the associations between the success of these interventions seen in the literature and their effect on the change of neurotrophin expression. Current literature reports increased levels of BDNF protein both centrally and peripherally following mood disorder treatment and participation in both physical exercise and mindfulness activities. Based on similarity of structure and function amongst the three neurotrophins, this thesis will hypothesize an increase in *BDNF* and potentially *FGF2* and *NGF* mRNA following participation in the two interventional modules designed to improve wellbeing in clinical and non-clinical communities.

METHOD: In this independent measures design, 28 non-clinical volunteers were randomly allocated to an 8 week intervention, comprising digital health tracking modules and participation in an unstructured increase in Physical Activity or Mindfulness program, to

assess the effect of these interventions on levels of mRNA expression. RTqPCR was used to compare relative mRNA abundance in peripheral blood at baseline and 8 week time interval. The control group were allocated to a waitlist for the period of the 8 week study, followed by access to the program of their choice. Change in emotional state was measured using the DASS.

RESULT: *BDNF* expression is shown significantly increased (p 0.01, n=5) in the Physical Activity group, and significantly decreased in the Mindfulness group (p 0.01, n=11). *FGF2* and waitlisted controls showed no significant change. In the case of *NGF* no expression was seen in human peripheral blood either before or after the intervention. DASS scores revealed a significant decrease in negative affect in the Mindfulness group $p = 0.03$.

CONCLUSION: This study revealed a significant positive association between physical activity and *BDNF* mRNA, although no significant reduction in distressing mood symptoms was shown. This was potentially due to the small group size. Mindfulness was significantly associated with decreasing negative affect, despite an unexpected decrease in *BDNF* mRNA consistent with pathophysiology of depression, likely related to neuro-immuno-endocrine axis disturbance as suggested in the published literature. It is suggested decreasing mRNA levels reflect lower numbers of immune activated leucocytes present in the blood following mood improvement, albeit not verified in the study. This study suggests even in a small non-clinical sample there may be potential benefits to well-being by increasing levels of physical activity or becoming mindful, and that *BDNF* has potential as a biomarker of emotional state.

1. INTRODUCTION

Anxiety and depressive disorders remain the most prevalent psychiatric disorders worldwide resulting in pronounced distress and reduced quality of life for those affected. Those motivated to seek treatment are sometimes unable to find long term relief using pharmacological avenues and even if relief occurs it can be a period of weeks before there is an appreciable improvement. Depressive disorders are ranked as the second leading cause of disability worldwide (Ferrari et al., 2013), and anxiety estimated to be the sixth leading cause of disability globally (Baxter, Vos, Scott, Ferrari, & Whiteford, 2014). Conceptually stress fails to achieve consensus in respect to an all-encompassing definition and as a result often becomes difficult to measure. Good stress (eustress) and distress, all can cause emotional, physical, mental strain and tension. However its contribution, either as causation or an exacerbating factor to depression and anxiety is without doubt. As a result it impacts on health and economic statistics either individually or as part of the disorder spectrum.

Failure to resolve mood and anxiety disorders can result in complex comorbidities in both an individual's mental and physical wellbeing. Without lifelong reliance on medication, or in the case of treatment resistance, relapse is likely, and feelings of hopelessness and desperation may result in self-harm or suicide. A recent study by Thibodeau et al. (2013) reported suicidal ideation and attempts are independently linked to anxiety disorders beyond the associations with comorbid depression (Thibodeau, Welch, Sareen, & Asmundson, 2013) making anxiety a potential risk factor of suicide along with depression.

Depression and anxiety are widely recognised to be co-morbid. Gorman reported that 85% of patients with depression also exhibit anxiety symptoms and conversely 90% of those suffering anxiety, exhibit symptoms of depression (Gorman, 1996). Indeed DSM – IV and the current version of ICD 10 both include classifications recognising atypical depression and mixed anxiety-depressive disorders, underlining this strong comorbidity (*Diagnostic and statistical manual of mental disorders (DSM-IV)*, 1994; WHO, 1993). It has been

controversially withdrawn from DSM 5, due to questions around sub-threshold versus threshold presentations and diagnostic stability over time (Möller et al., 2016). Nevertheless, comorbidity is widely accepted in clinical practice, and indeed anxiety and stress presentations provide clear neurobiological mechanisms for the progressive nature of depressive disorders.

Conceptually stress is a widely used descriptive that most of us can relate to. The daily dynamics of a busy life, with conflicting demands of relationships, finances, time pressure, and work-life balance may explain why we feel stress. But stress is also and perhaps more accurately, a function of disturbed internal homeostasis and the systemic load imposed to return the system to equilibrium. Early work by Wheatley (1997), as shown in Figure 1, describes an interaction that is still relevant today though much expanded and incorporating a multi-disciplinary approach to the interactions (Wheatley, 1997).

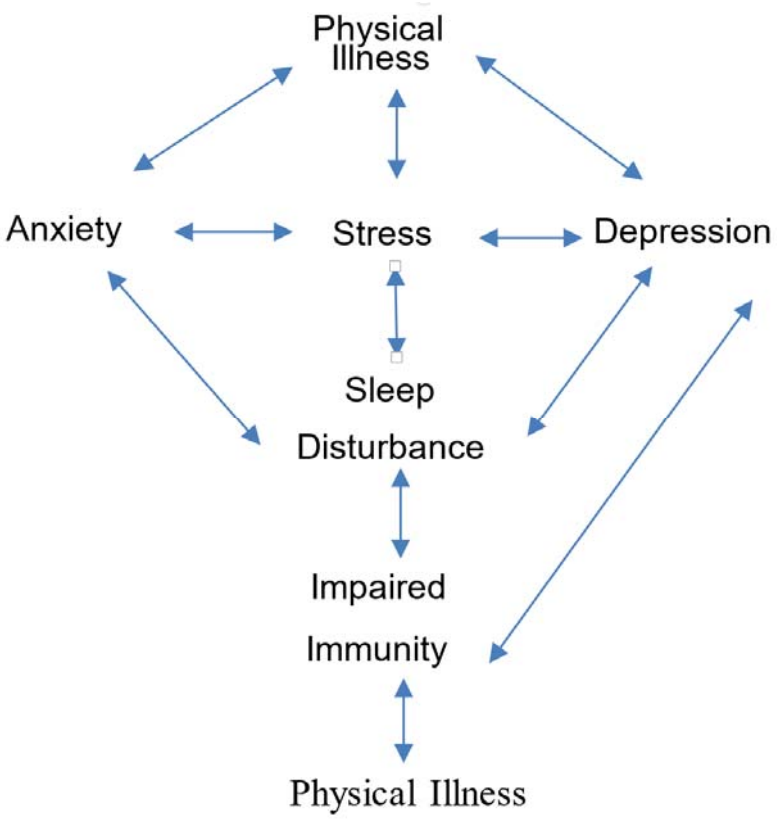


Figure 1 Physiological Interactions

Both internal and external stresses may result in emotion and mood disturbance including fear, worry, tension, anger and sadness, which may then cause hypothalamic-pituitary-adrenal-axis (HPA-axis) hormone disturbance and inflammatory reactivity. Both have become widely recognised as important theories in the development of mood and anxiety disorders and although not a new concept, contemporary theory is beginning to more clearly understand the effects of mood and emotion on the nervous control of the physiology. Neuro-visceral integration and the pathways of signal and communication between the nervous system, immune system and the endocrine system has necessitated a new interdisciplinary approach. Psychoneuroendocrinoimmunology or PNEI describes the construct of interacting systems, which on the one hand appear to add levels of complexity to the investigation of psychological disorder, may also offer the potential to reveal new biomarkers for these disorders and with each interacting system and process, opportunities for successful treatment.

For many reasons some individuals experiencing anxiety, depression or stress disorders are either averse to seeking treatment, or require support in addition to their pharmacological or psychotherapeutic treatment plan. Digital health programs offer an opportunity to make available online intervention modules, combining valuable tools and resources. Easily integrated into daily routine, they may be offered in a single or multiple-module format, as a beneficial strategy to improve recovery. Digital health intervention can be undertaken in the home environment at a pace at which each individual can integrate, reducing dependence on overburdened health services, and encouraging proactive approach to recovery. Reliable and credible intervention information may be developed by mental health specialists on proven strategies to reduce particular symptoms and recognise the signals that should prompt action. Not only allowing timely and often immediate intervention of acute phases but if employed lifelong may reduce relapse rates. .

In the clinical setting, mindfulness and physical activity have been shown to offer symptom relief, with some early success (Babyak et al., 2000; Kabat-Zinn, Lipworth, & Burney, 1985). Both lend themselves well to a digital format although compliance monitoring relies on self-report measures. In mood disorder treatment programs, they may be helpful as an adjunct in association with pharmacological treatment, or as stand- alone self-help strategies in the pursuit of individual wellness. Although seemingly polar opposites, one requiring physical exertion, the other relaxation, with cognitive and cerebral effort, their efficacy is showing great potential in treatment and prevention of a wide range of disorders. This diametric opposition suggests simple theories of endorphin release following exercise or reduction in stress levels after meditation alone are not adequate to explain the positive outcomes.

Research is progressing to determine the neurochemical and neurophysiological mechanisms that mediate their success. Recently the capacity of the brain to demonstrate neuroplastic change (the ability of the brain to reorganise and remap by forming new neural connections), in association with the expression of neurotrophin Brain Derived Neurotrophic Factor (BDNF) and the subsequent improvement in mood, has emerged as a promising avenue to explore in remediation. In addition it has become widely investigated as a potential biomarker in the origin and progression of mood disorder.

Studies implicate dysregulated levels of brain derived neurotrophic factor (BDNF) nerve growth factor (NGF) and fibroblast growth factor 2 (FGF2), along with changes in neuroplasticity and brain volume, in the development and progression of many psychological disorders (Berry, Bindocci, & Alleva, 2012; Warner-Schmidt & Duman, 2006) (Turner, Watson, & Akil, 2012). In particular these growth factors have become important in pathogenesis of depression and anxiety. Restoration of depleted levels and volumetric

increase in neural tissue are reported in association with resolution of symptoms (Pittenger & Duman, 2008). Moreover, there has been much evidence presented to suggest interventions such as physical activity (PA) and mindfulness also mediate changes in brain volume and neurogenesis, (Cahn, Goodman, Peterson, Maturi, & Mills, 2017; Farmer et al., 2004; Håkansson et al., 2017; Hölzel et al., 2010; Kronenberg et al., 2003) and despite inconsistencies in results, this is thought to be mediated by increased *BDNF* gene expression.

2. DEPRESSION

Recent research has highlighted the development and progression of depression as a disorder involving dysregulation of multiple physiologic, molecular and neural systems. Theories on pathogenesis of depressive disorders have been many and varied and not necessarily mutually exclusive. Dysregulated expression of *BDNF* is still widely considered to be a potential causative factor in tissue volume deficits seen across many clinical studies, and improved neural tissue density and synapse efficiency seen following effective pharmacological intervention. The failure of pharmacological treatments to prevent relapse in a subset of clinical cases, has indicated multi-system involvement and has prompted research to consider a more system-wide approach. Recent insights into depression have advanced the contemporary theory that components of the immune system, which mediate inflammation and endocrine system (HPA-axis) are intimately involved in both the initiation and perpetuation of depression. Complex interactions between psychological stressors, physiological stress (allostatic load), inflammatory reactivity, oxidative and nitrosative stress, along with neural, genetic and epigenetic factors, that modulate these interaction are becoming well established mediators of depressive mood, and will present opportunities for treatment intervention and identification of new and earlier biomarkers. Current research is effectively integrating theories of anxiety, stress and depression in to a model interrelating

the neuroendocrine and immune system with its autonomic innervation facilitating crosstalk communication between central and peripheral compartments, to potentially explain the development of mood disorder its trajectory and comorbidities.

The question which is still perhaps outstanding is which of these systems begins the process. Slavich and Irwin (2014) present cumulative evidence that anxiety precedes depression with neuro-inflammatory sensitisation as a prequel (Slavich & Irwin, 2014). Experiences of adversity which create stress and anxiety activate the innate immune system, and immune cells such as monocytes, macrophages and other non-immune cells release cytokines (pro and anti-inflammatory) to regulate the process. Although inflammation is a highly conserved evolutionary and necessary process, which protects against physical injury and pathogen invasion, it is widely believed that perceived threat of impending danger, social threat, and adverse conditions often associated with fast-paced modern lifestyles also activate this pathway creating anxiety, and stress (see fig 1). Persistent activation of the HPA-axis and desensitization of the feedback control result in systemic glucocorticoid-mediated damage. Neuro chemical receptors allow this information to be transmitted bi-directionally between periphery and the brain facilitating processes which attenuate the potential problem, but chronic activations of these systems reduce efficacy and result in damage and pathology. The following sections will explore in a little more detail depression, stress and anxiety and the pathways through which they are interconnected.

Diagnosis of depression according to DSM V requires five of nine potential symptoms to be present consistently for a two week period and must include a depressed mood (feeling sad, hopeless or empty) or anhedonia (loss of interest in activities normally found pleasurable). Three or four or more of the following, weight loss, difficulty sleeping, psychomotor agitation or retardation, fatigue, feelings of worthlessness, thoughts of death or suicidal ideation and

difficulty concentrating also being characteristically present (*Diagnostic and statistical manual of mental disorders : DSM-5*, 2013). However, recent reports from a British journal have evidenced 8.4 -9.9% of people in a community sample experience non-specific and sub-syndromal symptoms which do not necessarily fit the conventional categorical diagnosis. In these cases symptoms typically ebb and flow and cluster along a continuum (Ayuso-Mateos, Nuevo, Verdes, Naidoo, & Chatterji, 2010), impacting significantly physiologically, psychosocially and on wellbeing. These individuals represent a sizable sector of the community that either go undiagnosed or simply do not present to health care practitioners for consultation. Nevertheless they often undertake the search for practical self-help strategies to minimise their symptoms.

3. ANXIETY

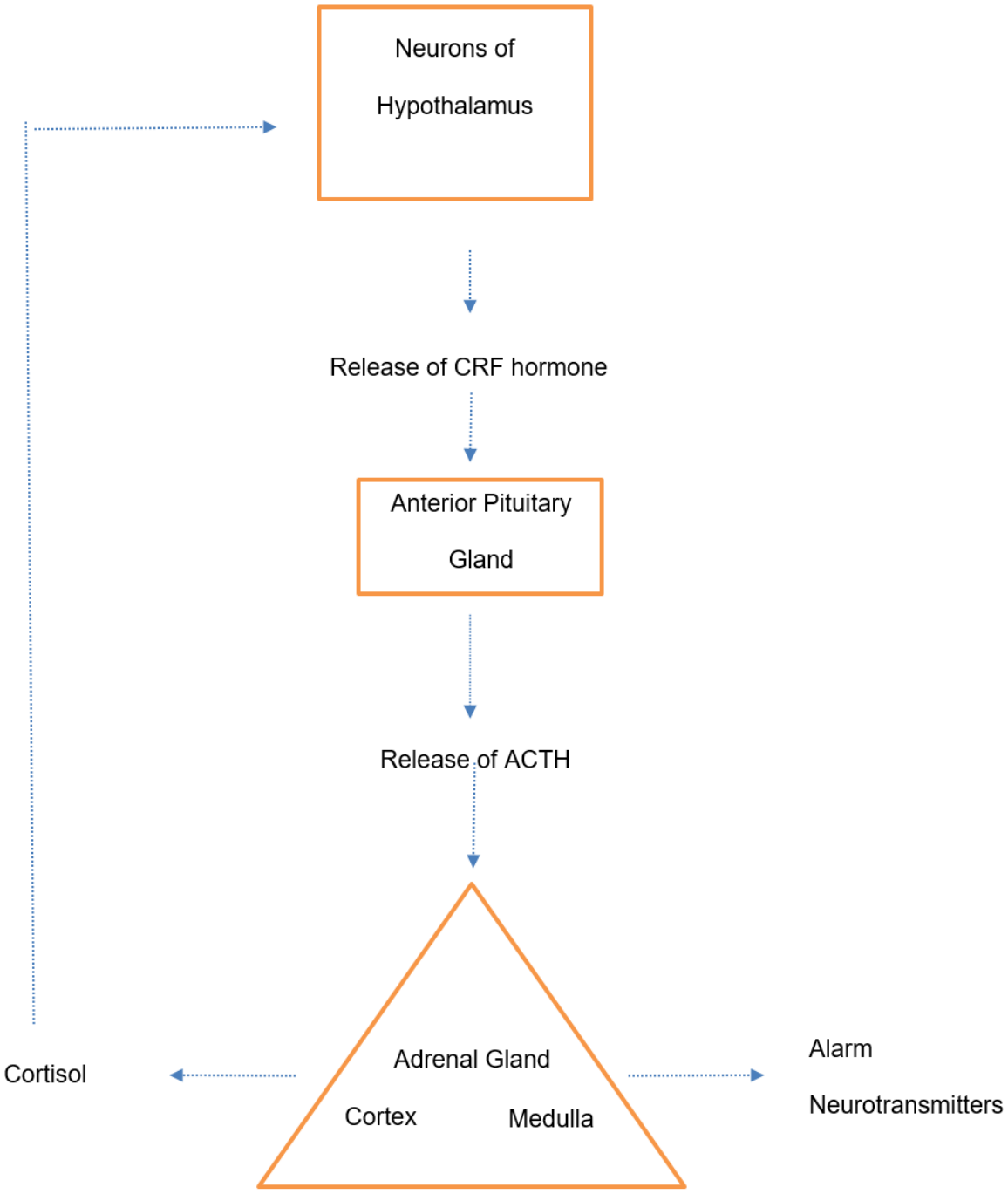
Anxiety is a normal part of the human experience described as a feeling of unease directed towards an impending event or situation about which we may be apprehensive. Most frequently arising from non-specific stimuli, and giving rise to an increase in arousal and vigilance (Dias, Banerjee, Goodman, & Ressler, 2013). In normal circumstances it is short lived and dissipates following the event, however if the individuals perception of threat persists, anxiety can become a prolonged, future directed chronic state of fear, worry and dread, which becomes disruptive to normal physiologic and psychological function. An individual's perception of social and environmental threat, real or imagined, can induce anxiety. Interpersonal conflict, fear of rejection or exclusion, paranoid feelings, adverse environmental conditions within the family, at the home or workplace are all commonplace potential anxiety provoking situations. Individual responses vary, but if not resolved, may trigger systemic stress, and progression to depression. Furthermore, early life stress predisposes development of a susceptible anxiety phenotype (Nugent, Tyrka, Carpenter, & Price, 2011) by way of epigenetic changes to genes shaping developing regions of the brain

and circuitry involved in behaviour (McEwen, 2010; Roth & Sweatt, 2011) and depression by way of epigenetic regulation of the *BDNF* gene (Seo et al., 2016), and other genes important across the spectrum of mood regulation (Boulle et al., 2012; Tsankova, Renthal, Kumar, & Nestler, 2007). Epigenetic modification of gene expression, especially following early life adversity has become an important mechanism resulting in phenotypes vulnerable to stress, anxiety and depression.

4. STRESS

Stress is a word often used to describe feeling overburdened by conflicting requirements of daily life. But more accurately in a physiological sense, any condition internal or external which disrupts homeostasis creates stress, and the body mediates the effect of stress through allostasis, a process of physiological change aimed at returning the body to homeostatic condition. However allostasis may come at a cost known as allostatic load (AL). AL is the result of allostatic mediators such as glucocorticosteroids, inflammatory cytokines, and activations of autonomic nervous system (ANS) and hypothalamic-pituitary-adrenal axis (HPA-axis), and central nervous system (CNS) neurotransmitters (McEwen & Wingfield, 2003) creating systemic stress on the body. When allostatic load becomes persistent pathology results.

Stress provides an added risk dimension to the anxiety and mood disorders particularly when prolonged, creating a vicious cycle of anxiety, fight or flight responses, HPA-axis disturbance, and eventually resulting in damage (Figure 2.)



Neurons of the hypothalamus release corticotrophin releasing factor (CRF), this hormone stimulates anterior pituitary to release adrenocorticotrophic hormone (ACTH) which in turn stimulates the adrenal gland to produce alarm catecholamine neurotransmitters (norepinephrine and epinephrine and serotonin) from the medulla. The adrenal cortex releases stress hormone cortisol. At critical blood concentrations of cortisol, a feedback mechanism signals hypothalamus to reduce CRF secretion, which reduces adrenal production of cortisol and returns system to homeostasis. Following normal exposure to periodic stress, the system experiences brief episodes of alarm, but repeated and prolonged exposure may result in habituation. Protracted HPA-axis activation beyond normal limits reduces efficacy of feedback and produces sustained high levels of stress hormone cortisol.

Figure 2 HPA-axis and Stress

Tightly regulated coordination of the HPA-axis by the CNS and the endocrine system is maintained to reduce prolonged exposure to glucocorticoid (GC) activity and damage, but in depression, disrupted serotonin, dopamine and norepinephrine transmission also impair regulatory feedback. Concomitant overactivity of the sympathetic NS contributes to immune activation and resulting release of inflammatory cytokines.

There is substantial body of evidence that psychological stress can trigger increases in inflammatory activity in the absence of any invading pathogen or physical injury, via the above pathway. The action of chemical messengers released by macrophages neutrophils dendritic cells and other non-immune related cells, and subsequent neuroimmune crosstalk are thought to mediate profound changes to behaviour, including symptoms of depression such as anhedonia, fatigue, psychomotor retardation, withdrawal (Slavich & Irwin, 2014) and sickness behaviour (Dantzer, 2009).

Maes et al. (2011) suggests severity of depression is positively associated with cytokine-mediated activation of inflammation and oxidative and nitrosative (O&NS) stress circuits which may be triggered by several factors including monocyte activation, psychological stress and strenuous exercise (Maes, Kubera, Obuchowiczwa, Goehler, & Brzeszcz, 2011). O&NS refers to an imbalance in the production and elimination of reactive oxygen and nitrogen species and antioxidant systems of the body, resulting in damage or alteration to molecular and cellular components, immune pathways, gene regulatory pathways, and cellular apoptosis. Inflammatory cells generate a variety of reactive oxidative and nitrogen species along with destructive proteases which damage tissue and homeostatic mechanisms

5. INTEGRATING INFLAMMATION AND BEHAVIOUR

The neuro-immune axis connects CNS with peripheral immune system and regulates homeostatic and inflammatory function. Under normal circumstances there is a circadian rhythm of norepinephrine (NE) release which activates sympathetic βadrenergic receptors on nerve endings in the bone marrow neural niches, resulting in release of hematopoietic cells in to the blood stream when the body is at rest. In this way adequate numbers of blood cells are made available to sustain normal physiological requirements. When activated, cells of the immune system produce cytokines (signalling molecules by which the immune system informs the brain about the inflammatory status of the periphery), which dramatically increase the proliferation rate and recruitment of immune competent cells from the hematopoietic pool. This pathway is highly relevant in psychosocial stress which has been shown to increase proliferation of leucocytes, and when stress is chronic, monocytosis and neutrophilia are also shown (Heidt et al., 2014). Some depressive disorders have shown increased pro-inflammatory markers, (Frommberger et al., 1997; Maes et al., 1995), and an associated positive relationship between severity of illness and white blood cell count (Maes et al., 1992) . Research is beginning to understand how mood and affect disorders might influence immune responses in the periphery, and vice-versa. But whether dysregulated mood produces disturbance in inflammation or inflammation results in mood disorder remains to be determined.

CNS circuits communicate with the periphery bi-directionally. The vagus nerve (tenth cranial nerve) has long been associated with models of neurovisceral integration due to its innervation of many of the visceral structures including the spleen, heart, liver and digestive organs and it transmits sensory information from the periphery to its brain stem terminus and

back to peripheral targets. Cytokine receptors found amid the nerve fibres, signal and regulate the neuro immune reflex through afferent (sensory) and efferent (motor) pathways. These bidirectional circuits extend to areas of the brain intimately involved with depression, stress and anxiety, in particular the locus coeruleus (stress and panic) the rostral ventromedial medulla (nociception) and the hypothalamus (HPA axis modulation) via the nucleus tractus solitarius (NTS) of the brain stem. Vagus nerve terminations lie in the NTS, the junction of these two structures form the dorsal vagal complex (Pavlov & Tracey, 2004) and are in involved in regulation of autonomic state, vagal tone (an indicator of stress resilience), emotion and social behaviour according to the polyvagal theory (Porges, 2009).

Cytokine signalling to the brain occurs by a variety of routes. Firstly bi-directionally along the vagus nerve via receptors on vagal nerve afferents, usually considered the pro-inflammatory route (Goehler et al., 2000), and the anti-inflammatory route via vagal efferents. Secondly, directly into the brain across the blood brain barrier (BBB) by active transport mechanisms, or structures of the brain where BBB is lacking. Thirdly via a cellular route, peripheral immune cells may enter the CNS with the aid of chemoattractant mediators or again through compromised areas of BBB (Corsi-Zuelli et al., 2017) and finally through a recently discovered lymphatic pathway (Louveau et al., 2015)

Cytokines translate environmental cues into molecular signals, and the majority of brain cell populations are susceptible to regulation by these cytokine signals (Bilbo & Schwarz, 2012). These molecules have autocrine and paracrine function locally but can also act at a distance when released into the bloodstream, signalling to the brain, with neuromodulatory effects. Cytokine receptors are found throughout the CNS and are of particular relevance on microglia, vascular endothelia, monocytes and neurons (Bilbo & Schwarz, 2012; Hopkins & Rothwell, 1995).

Microglia make up 5-20% of total brain cells (Sousa, Biber, & Michelucci, 2017) and carry out surveillance of their immediate environment, sensitive to inflammatory signals from trauma and stress. On detection of signal stimulus microglia become activated, modify their gene expression and facilitate release of cytokines which recruit leucocytes to CNS. In addition early nervous system and vasculature development situate nerve and blood vessel pathways close together expediting cytokine signalling between the endothelial cells lining the blood vessels, neurons, glial cells and interaction between cellular and neural components of the peripheral and central compartments. This multi-system pathway is becoming central to the theory of mood and depressive disorders providing a mechanism to understand how the different anatomical circuits of the brain influence mood and behaviour and in turn communicate signal to the periphery resulting in systemic responses involving neuroimmune and endocrine systems.

6. WELLNESS

Wellness is a term popularised by the commercial health and nutrition industries and directs towards maximising or at the very least optimising the wellbeing of the individual. Wellness as a concept refers to diverse interconnected aspects of social, physical, and mental dimensions which encompasses not just health and vitality, but freedom of choice, optimistic attitude, awareness and a directive to self-actualise amongst others.

In the current therapeutic physical and mental health environment, encouragement of a self-directed, proactive engagement of self-improvement strategies is encouraged, increasing self-regulation, self-awareness and less reliance on medical, psychological and pharmacological intervention. These practices are widely believed to improve wellbeing, and

the rapidly increasing opportunities to access strategies aimed at increasing wellbeing offered by popular media, health care providers and online agencies attests to this.

Defining wellbeing has proved difficult, hampered by complexity and broad reaching concepts which have been difficult to apply universally and even more difficult to measure. Furthermore, a tendency to use wellness and wellbeing interchangeably in the absence of an operational definition has complicated the issue. But Dodge et al (2012) have offered a definition and a measure, which hinges on an individual's capacity to manage the dynamic flux between challenges an individual faces and their resources to meet the challenges (Dodge, Daly, Huyton, & Sanders, 2012). Not so much a constant state of happiness with an absence of distress, but an innate sense and confidence of one's own capacity to engage resources to manage the stresses and strains of life, restoring balance and homeostasis.

The benefits of improving wellbeing are wide reaching, including greater work productivity, income levels and satisfaction, more successful marriages, more positive social interaction and social support networks, increased vitality and physical health improvement, decreased stress related illness, less pain, strengthened immunity and improvements in mental health and behavioural indices with improved self-confidence, prosocial behaviour, creativity, and self-regulation (Lyubomirsky, King, & Diener, 2005).

7. MODALITIES OF SYMPTOM RELIEF

7.1. MINDFULNESS

From the psychological point of view, mindfulness and meditation both focus on training the mind to develop non-judgmental awareness of moment to moment thoughts, sensations, affective state and mental imagery in an attempt to improve attentional and emotional self-regulation. It may encompass specific mindfulness exercises, meditation practice, yoga, tai chi and other eastern philosophies. Meditation has arisen from Buddhist tradition but ethical concerns regarding secular, as opposed to religious context and whether to be explicit regarding the religious connotation during training have been put forward (Compson, 2017). Current mindfulness practice as used in mindfulness based interventions, distances itself from the esoteric, spiritual and religious inferences which may deter some individuals, and reduce acceptance of the practice, but uses meditation-based exercises to train individuals to experience mindfulness.

Mindfulness has been associated both in theory and evidence-based studies with psychological wellbeing, facilitating resilience to psychological distress and worry, including rumination, anger and tendency to either avoid or over-engage distressing emotion (Borders, Earleywine, & Jajodia, 2010), anxiety (Kabat-Zinn et al., 1992), and a broad range of physical distress and mental health issues including pain management, stress, depression and relapse rates (Grossman, Niemann, Schmidt, & Walach, 2004). Tang et al (2007) reported findings that even a short period of meditation (5 days of 20 mins a day) increased immunoreactivity, and produced significant decreases in stress, cortisol production and anxiety (Tang et al., 2007).

Two components which feature repeatedly in attempts to define mindfulness, are non-judgmental awareness of streams of internal and external stimuli as they arise (Baer, 2003) and self-regulation of one's attention or an ability to selectively switch attention from one experience to another (Brown & Ryan, 2003). As mindfulness grew in popularity, a definition for mindfulness became an imperative in order to accommodate comparisons and analysis among the large number of studies which attempted to explain its neurobiological mechanism and success. In 2004 Bishop et al., produced a consensus driven operational definition encompassing a two component model. The first involves self-regulation of attention, maintained on immediate experience, allowing a recognition of mental process in the present. This requires the capacity to maintain sustained attention, for example, on the breath, and the ability to switch attention at will, allowing the individual to accept interfering thoughts and feelings but return awareness back to breathing once the intrusive thought has arisen. The second requires adoption of a particular orientation of openness, curiosity and acceptance towards the present moment experience, thus fostering an attitude of receptiveness to whatever arises, and reducing cognitive and behavioural strategies aimed towards avoidance of experience (Bishop et al., 2004). Cardaciotto et al. (2008) caution care here in interpreting acceptance not as resignation or passivity, but rather as the capacity to examine the experience without judgement or self- criticism (Cardaciotto, Herbert, Forman, Moitra, & Farrow, 2008). Allowing introspection with self- acceptance and detachment, rather than avoidance, and in the knowledge that emotions are impermanent and need not be further elaborated. Mindfulness training is therefore proposed to result in enriched mental content and experience, a more accurate representation of reality, and ultimately, an ability to act more effectively with the additional internal and external information, without resort to default reaction/over-reaction (Grossman et al., 2004). The results should yield a more resilient approach to everyday stresses and strains and thus less physical and mental distress.

Neuroscience research reports areas of the brain engaged during mindfulness practice include those mediating;

Attention control – anterior cingulate cortex (ACC) and striatum

Emotional regulation – prefrontal region, limbic regions and striatum

Self-awareness – insula, medial prefrontal cortex (mPFC), posterior cingulate cortex (PCC), default mode network (DMN)

7.1.1. Attention control

Increased cortical thickness and white matter changes have been reported in Zen meditators (Grant, Courtemanche, Duerden, Duncan, & Rainville, 2010; Tang et al., 2010), and shown frequently linked to activation changes in the anterior cingulate cortex (ACC) (Grant et al., 2010; Holzel et al., 2007; Tang et al., 2010). This region is associated with conflict resolution (Botvinick, Cohen, & Carter, 2004; Van Veen, Cohen, Botvinick, Stenger, & Carter, 2001) and the ability to evaluate and choose to attend to salient information in the event of conflicting or incompatible incoming cues.

7.1.2. Emotional Regulation

Emotion regulation (ER) is a key factor in many mood and anxiety disorders. Mindfulness training aids in the diversion of attention deployment, allowing a decision on which emotion to attend to, and a change in default appraisals of feelings, usually resulting in negative self - judgement. Often suppression of negative emotion, avoidance and escape strategies, are adopted in an effort to control distress, this is therapeutically regarded as maladaptive, and results in increased sympathetic nervous system activity and potentially reduced immune

responses, with adverse health consequences. Although universally accepted therapeutically, techniques involving cognitive reappraisal of negative feelings and distress, may rebound, with attention brought back to aversive dysphoria, especially in those with an inability to control intensity and perpetuation of the negative experience (Farb, Anderson, & Segal, 2012). Mindfulness studies have convincingly reported positive effects, including reduced intensity and frequency of negative affect (Chambers, Lo, & Allen, 2008; Goldin & Gross, 2010). Goldin's study also reported improved anxiety, depressive symptoms, increased self-esteem, decreased amygdala activity and increased activity in attention networks, following a mindfulness based stress reduction program.

7.1.3. Self-referential processing and self-awareness.

Self-referential processing involves concepts of self-awareness and altered self-representation. Meditators have consistently scored higher on scales of self-acceptance and self-esteem (Thompson & Waltz, 2008) and (Koole, Govorun, Cheng, & Gallucci, 2009), while mindfulness has been reported to moderate the effect of poor self-esteem on depression (Michalak, Teismann, Heidenreich, Ströhle, & Vocks, 2011) stress, anxiety and depression (Cash & Whittingham, 2010), negative self-concept, poor mood regulation and low positive emotions (Jimenez, Niles, & Park, 2010). Self-referential processing involves activation of midline structures, proposed as components of the default mode network (DMN) exhibiting highest activity when the mind is at rest or wandering, monitoring the environment without conscious thinking, or envisioning the future. A relative deactivation of DMN across several types of meditation practice is shown associated with increased cognitive control over DMN function (Brewer et al., 2011).

Mindfulness training has been shown to disassociate the insula (an area associated with interpretation of body sensation as an indicator of serious illness, and anxiety) and mPFC which was strongly coupled in novices, this being interpreted as evidence that mindfulness mediates a more self- detached analysis of interoceptive awareness (Farb et al., 2007).

7.1.4. Mindfulness and Plasticity

A number of studies have looked at changes in tissue density and volume in regional areas of the brain following mindfulness training perhaps evidencing a role for neurotrophins in mediating the positive effects of mindfulness. Human studies have shown inconsistencies perhaps due to differing morphological and data measures used, use of clinical compared to non-clinical cohorts, and the divergent mindfulness based stress reduction (MBSR) programs used. Most reports cited in the literature focus on increased grey matter morphometry demonstrated by MRI across a number of areas of the brain involved in affective disorders. Trait anxious individuals frequently demonstrate increases in amygdala activation, an area implicated in detection of threatening and stressful stimuli, (Stein, Simmons, Feinstein, & Paulus, 2007), while trait mindful subjects demonstrate reduced resting state amygdala activity (Way, Creswell, Eisenberger, & Lieberman, 2010). In a MRI study by Hölzel et al (2010) a group of stressed but otherwise healthy individuals showed reductions in perceived stress linked to volumetric decreases in the amygdala, with less tissue density and activation of these circuits as stress levels reduced. This study however reported the volume change was significant only in the right basolateral region of the amygdala (Hölzel et al., 2010). Of interest in this study, is the fact that the basolateral regions of the amygdala is suggested to be important in receiving input from sensory modalities (detecting threat stimuli) and directing information flow to other subcortical and cortical structures. This information flow may facilitate stress and anxiety related physiological sequelae, perhaps explaining why mindful individuals are less reactive to

stress and less anxious. A later longitudinal study by Hölzel et al (2011) found increases in grey matter volume in posterior cingulate cortex (PCC), the cerebellum, temporo-parietal junction and left hippocampus after 8 weeks of mindfulness based stress reduction program. The hippocampus has shown critical relevance in psychopathologies especially anxiety and stress related disorders, with decreased volumes seen in anxiety (Gross & Hen, 2004; Kalisch et al., 2005) and depression (Campbell, Marriott, Nahmias, & MacQueen, 2004; MacQueen & Frodl, 2011) and contribution to the regulation of emotion (Davidson, Jackson, & Kalin, 2000; Hartley & Phelps, 2009; Immordino‐Yang & Singh, 2013)

Novice practitioners of mindfulness techniques may employ different emotional regulation circuits to long-term practitioners (Chiesa, Calati, & Serretti, 2011; Murakami et al., 2015; Xu et al., 2014) and as a result, show different areas modulated by neuroplasticity. Tang et al (2015) suggests novices trigger circuits activated during learning of a new skill and evidence distinct patterns of neural activation and morphology between expert and novice practitioners which may suggest neurotrophin mediated plasticity augments frequently activated circuits and rewires the brain following mindfulness and meditative training and practice (Tang, Holzel, & Posner, 2015).

The brain has been shown to be capable of adaption to its environment, either to facilitate meeting the demands of new experience, learning or repair following injury or dysfunction. Several studies now have shown neurotrophins associated with neuroplasticity and this thesis surmises that the increased grey matter volumes seen in many studies following meditative or mindfulness training are mediated by one or a combination of neurotrophins. There have however been comparatively few successful experimental protocols reported, demonstrating statistically significant increases in BDNF or indeed any of the neurotrophins

following mindfulness interventions with associated reductions in distressing symptoms of mood disorder.

7.2. PHYSICAL EXERCISE

The benefits of regular physical activity ranges widely across physical and mental health issues, including metabolic and inflammatory reactivity, cardiovascular health, mood and general wellbeing, and has been extensively reported over the last 20 years. Mechanisms by which these systems mediate their positive effects are still incompletely understood but the success of PA as a therapeutic intervention in the treatment of mood disorder has become well established. Amidst the large body of literature available on this topic, exercise and physical activity terms are often used interchangeably. Silverman and Deuster recommend the assumption that exercise is a planned and structured form of physical activity, scheduled regularly, while physical activity describes spontaneous ad hoc participation without regular schedule in some studies (Silverman & Deuster, 2014).

Potential mechanisms facilitating the positive effects on mental health, physiological function and counteraction of volumetric losses, seen following exercise regimens, are suggested by Cotman et al (2007) to be improved growth factor signalling, increased synaptic plasticity and strength, increased vascular function and, in addition, the reduction of tissue damage caused by inflammatory cascades and other associated peripheral risk factors which increase allostatic load (Cotman, Berchtold, & Christie, 2007).

Increased levels of BDNF following exercise have been widely reported (Kim et al., 2005; Szuhany, Bugatti, & Otto, 2015). Epigenetic mechanisms of chromatin remodelling, and

changes to methylation status of the *BDNF* gene, are the current favoured theories to explain the increases in *BDNF* gene expression following exercise. Ntanasis-Stathopoulos et al. (2014) review a number of important examples of epigenetic modifications, induced by mechanical stress in muscle, metabolic and inflammatory processes, along with changed expression of glucocorticoid receptors, following exercise, all known to be important in mood disorder development. This group suggesting physical exercise causes epigenetic modifications, which regulate the transcriptional mechanisms of several genes in the brain, and periphery, coordinating the adaptive behavioural responses to stressful events (Ntanasis-Stathopoulos, Tzanninis, Philippou, & Koutsilieris, 2013). A recent study by Sleiman et al. (2016) suggests endogenous β-hydroxybutyrate, a ketone which increases following exercise, may be responsible for the epigenetic modification of the *BDNF* gene by inhibiting histone deacetylases allowing transcription to occur (Koppel & Timmusk, 2013; Sleiman et al., 2016).

Cardio-respiratory and aerobic fitness has been shown in imaging studies associated with increases in grey and white matter volume in both PFC and hippocampus (Hayes, Hayes, Cadden, & Verfaellie, 2013). In healthy study cohorts, benefits are seen in increased cognitive flexibility (Masley, Roetzheim, & Gualtieri, 2009), improved learning (Winter et al., 2007), and executive function (Hötting & Röder, 2013). Interestingly the Hötting & Röder review highlights that neural networks with a high capacity for neuroplastic change are the first to show positive improvement following exercise. Running has been directly correlated with hippocampal neurogenesis (Kronenberg et al., 2003; Rhodes et al., 2003; van Praag, Christie, Sejnowski, & Gage, 1999), increased *BDNF* mRNA in hippocampal and caudal areas (Neeper, Gomez-Pinilla, Choi, & Cotman, 1996) and synaptic plasticity with increased *BDNF* gene expression (Farmer et al., 2004). A mouse study by Gömez-Pinilla et al shows increased expression of *FGF2* mRNA in the hippocampus has been shown between day 2 and 4 in a study of variable voluntary wheel running, suggesting exercise also modulates

FGF2 expression, and that there are potentially other growth factors contributing to the positive effects of PA (Gómez-Pinilla, Dao, & So, 1997). Further work by Gomez-Pinilla et al showed *FGF2* mRNA increased in animals subject to a physical exercise task combined with a spatial memory task. The effect of *FGF2* increase was augmented when the animals had to perform both tasks together compared to physical activity alone and were associated with increased density of astrocytes in the hippocampus (Gómez-Pinilla, So, & Kesslak, 1998).

Results may differ depending on intensity and duration of activity. Indeed, many studies have shown a dose dependent relationship. In a large cross-sectional US study using self-report data, individuals exercising for less than 2 hours a week and more than 7 hours a week, had poorer mental health assessed on a measure of psychological distress, compared to those managing to do 5-7 hours a week. The relationship was curvilinear and held across gender, age and other metrics of physical health (Kim et al., 2012). A 2006 meta-analysis evidenced intensity of physical activity shows dose dependent association with all-cause mortality, metabolic disorders, cardiovascular disease, diabetes and other chronic diseases as well as anxiety and depression, such that those with the highest levels of physical activity and fitness are at the lowest risk of premature death (Warburton, Nicol, & Bredin, 2006). Mechanisms proposed to facilitate such wide-reaching improved mental and physical health include improved autonomic tone, sympathovagal balance, reduced systemic inflammation and inflammatory mediators and enhanced endothelial function (Pavlov & Tracey, 2012; Tomfohr, Martin, & Miller, 2008)

7.2.1. Exercise Stress and HPA connection

Regular exercise has been reported in association with lower sympathetic nervous system activity and HPA axis reactivity (Crews & Landers, 1987; Rimmele et al., 2007) both important mediators of the stress response and mood disorder. Voluntary exercise in a preclinical group altered the release of CRF from hypothalamus and ACTH from the anterior pituitary, evidencing exercise may effect change in mechanisms involved in both responses (Droste et al., 2003). However the physiological sequelae of PA often appears discrepant, both activating stress and HPA responses and yet effectively decreasing it. A study by Luger et al (1987) reported acute exercise activates HPA axis in a dose dependent manner, along with both the sympathetic nervous system and immune system. Both intensity and duration determining magnitude of the stress response. While low intensity activated minimally in acute conditions, in conditioned individuals (training regularly), markedly reduced HPA axis activation was recorded (Luger et al., 1987). The stress response system is an adaptive system which allows the body to prepare for fight or flight during challenge, once the challenge abates, this system restores to homeostasis. In the early phase, release of excitatory catecholamines and glucocorticoids stimulate energy mobilisation, cardiovascular responses and cognitive arousal, vigilance and attention, and a subsequent raise in inflammatory response. However prolonged exposure to glucocorticoids and its downstream inflammatory effect produce detrimental effects if not attenuated in a timely fashion. To plausibly account for the discrepancy between exercise-induced systemic stress with HPA reactivity, and favourable change in physical and mental health status, exercise is proposed to buffer against stress-related dysregulated mental and physical condition, in a complex interaction, which may both optimise and blunt neuroendocrine and physiological response. Central to this proposal is a physiological toughening, through which regular exposure to exercise increases tolerance to stress and reduces emotional, physiological and metabolic reactivity. Proponents of this theory suggest that by allowing appropriate recovery time between exercise stress loading, lower basal rate of catecholamine activity with increased

sensitivity and delayed pituitary-adrenal-cortical responses to challenge, not only improves performance and tolerance to stressors, but improves emotional stability and immune system response (Dienstbier, 1991). In order to explain the seeming counter-intuitive finding that endurance training which progressively increases work load but seemingly does not produce the damaging effects of excess GC exposure, adaptions to the tissue sensitivity of cortisol is proposed. This potential adaption mechanism of the HPA axis seen in studies of endurance training, is compared by Duclos (1998) to acute stress conditions, where excessive release of cortisol, necessary to mobilize energy resources, may produce reduced pituitary sensitivity to cortisol, without reduced adrenal ACTH sensitivity, and a consequent reduction in potential glucocorticoid damage. Duclos suggests this reduced sensitivity results in the blunted activation of HPA axis seen in trained individuals (Duclos et al., 1998) and this may contribute to reduced emotional and physiological disturbance and thus increased positive mood (Strasser & Fuchs, 2015).

8. SIGNIFICANCE OF THE NEUROTROPHINS

The capacity of the nervous system to reorganise its structure and function to meet demand or repair damage is known as neuroplasticity. This refers to the capacity of the brain and nervous tissue to rewire and remap. It accomplishes this by adapting the strength of its synaptic connections and increasing or decreasing the density of cells according to demand. Thus frequently firing connections are strengthened, while the less frequent ones are rewired or lost, in effect, use it or lose it. These changes take place not only throughout early development but constantly throughout life as we learn new skills and in response to damage or environmental stimulus. Mediating these changes are neurotrophins, nervous system growth factors which co-ordinate growth, survival, differentiation, maturation and apoptosis of neurons according to a regulated program in embryonic development. The same factors continue to influence neuronal function into and throughout adulthood,

although their influence in adulthood is tightly restricted, and activated by demand in specific neural populations and by necessity in specific physiological neural states (Knüsel & Hefti, 1993).

Nerve growth factor (NGF) was the first to be characterised by Levi-Montalcini and Hamburger in the 50's. Numerous more growth factors regulating nervous tissue have since been described. They are divided into distinct families, based on structural similarity which influence genesis, differentiation, maturation and survival of subsets of neurons and tissues, and actions range from regulation of central and peripheral NS to neuroendocrine, cytokine and immune system interactions. (Levi-Montalcini, Dal Toso, della Valle, Skaper, & Leon, 1995). Both NGF and BDNF belong to the neurotrophin family of growth factors and are strongly related in structure and function. Studies have revealed similar patterns of expression, and in some instances, each has been shown to regulate expression of the other (Apfel et al., 1996; Krüttgen, Möller, Heymach, & Shooter, 1998)

During vertebrate development up to 80% of neurons die as the nervous system develops, ensuring adequate numbers of neurons establish appropriate innervation densities in a competitive environment (Northcutt, 1989). This is thought to be tightly regulated by the innervation target cell population itself.

Stress, especially when chronic with high levels of GC exposure, decreases neurotrophic factor expression, neurogenesis, signalling and gliogenesis (Banasr & Duman, 2007), particularly in brain regions associated with mood disorder and anxiety. Neurotrophin effects have been widely reported in association with changes in cortical volumes, mood and brain function. Hippocampal and PFC volume losses have been widely associated with anxiety

responses and mood disorders. Indeed hippocampal neurogenesis is becoming a potential pharmacological target for treatment of many mental health issues (DeCarolis & Eisch, 2010).

8.1. BDNF

Brain derived neurotrophic factor protein (BDNF) is widely reported as the most abundant neurotrophic factor in the brain and has been the most extensively studied and characterised. It is a member of the neurotrophin family along with NGF and neurotrophins 3 (NT3) and 4 (NT4) to which it is structurally related. The neurotrophin family have a central role across developmental, proliferation, plasticity and survival of subpopulations of neurons in the peripheral and central nervous system. The critical importance of the neurotrophin family has led to widespread interest in their dysregulation, in particular BDNF expression and function, as a mediator of psychiatric disorder. It is found widely expressed throughout areas of the brain especially in the frontal cortex, hippocampus, amygdala, thalamus, brain stem, pons and cerebellum (Murer et al., 1999). There is widespread evidence linking decreased levels of BDNF and its genetic variants to anxiety and depressive behaviour (Duman & Monteggia, 2006; Hashimoto, 2007; Martinowich, Manji, & Lu, 2007).

Throughout embryogenesis, post-natal development and later life, BDNF regulates neuronal development, differentiation, axonal and dendritic growth, neuronal pathfinding, survival and apoptosis. Moreover it plays an important role in formation of synaptic connections required for formation of memory and learning throughout. Synaptogenesis allows the brain to modify in response to experience and environmental stimulus. The number of synapses and strength of their connections is mediated by active function, or practice of an activity, although the mechanism by which this occurs is as yet still under investigation. This synaptic plasticity allowing variation in the number and strength of synapse connectivity, facilitating

behavioural interaction between organism and environment, crucial in learning and survival. But it is widely thought its function in neuronal growth and repair maintains brain volume in the healthy brain, and its reduced level, regarded as the potential cause of brain volume losses seen in psychiatric disease.

8.1.1. Biosynthesis and cellular sorting

All the neurotrophins are synthesized as precursor proteins called pro-neurotrophins. However pro-BDNF has been shown to have activity independent of the mature form, signalling through a low affinity receptor p75 and thought to be involved in neuron apoptosis (Woo, 2005) while mature BDNF signals via binding high affinity TrkB receptor resulting in the activation of three distinct signal transduction pathways, see Figure 3. Each pathway confers unique functionality, the phospholipase Cγ pathway mediating release of intracellular calcium stores with subsequent ion channel influence on synapse function. The phosphotidyl-inositol 3-kinase pathway involved in cell survival and the mitogen-activated protein kinase pathway in cell growth and differentiation. All three pathways converge downstream on the cellular transcription factor cAMP response element binding protein (CREB) (Autry & Monteggia, 2012) which can increase or decrease transcription of the BDNF gene.

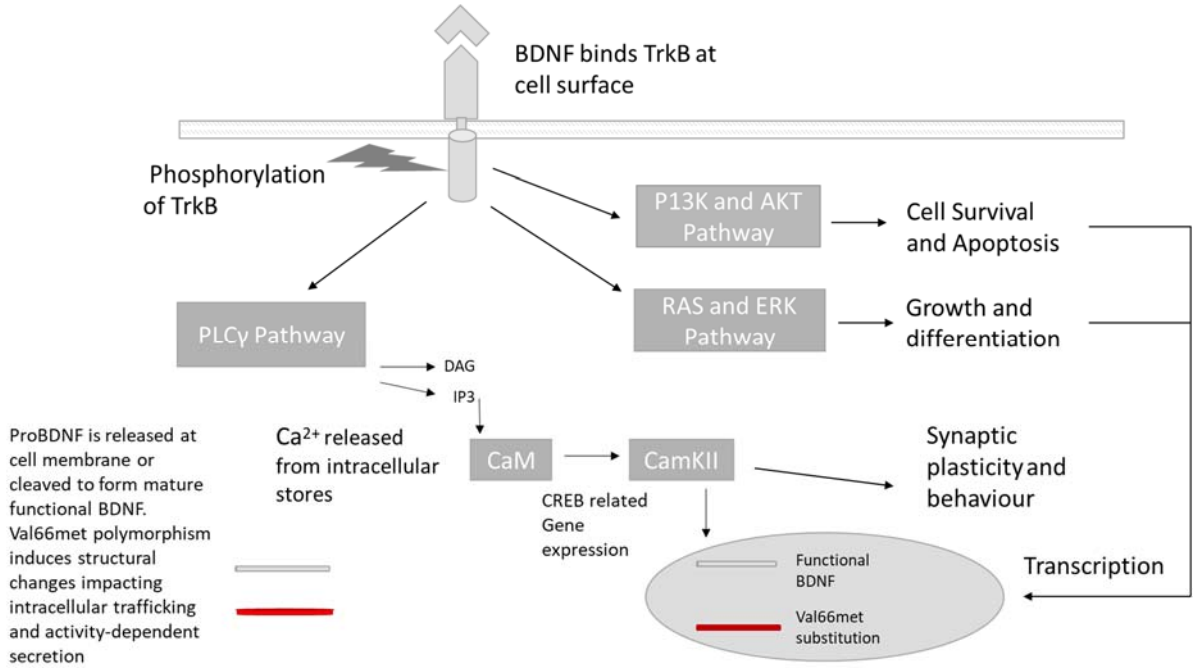


Figure 3 . BDNF Regulatory Signal Transduction Pathway

TrkB binding at cell surface mediates three distinct signal transduction pathways which result in regulation of *BDNF* gene expression and behaviour. P13K- phosphoinositide 3-kinase enzyme, AKT- protein kinase B, RAS- membrane associated guanine nucleotide binding protein activated by binding of growth factors, receptor tyrosine kinases and T cell receptors, ERK extracellular signal-regulated kinase, PLCγ- phospholipase C gamma, DAG- diacylglycerol, IP3- inositol triphosphate, CaM- calmodulin (calcium binding messenger) CamKII- calmodulin-dependent protein kinase.

BDNF precursor protein (prepro BDNF 32 kDa) is cleaved into pro-BDNF (28kDa) and further cleaved extracellularly by enzyme action of tissue plasminogen activator upon its release in an activity dependent manner. Significantly, the full length precursor protein becomes folded facilitating correct sorting and trafficking in to vesicles and subsequently directed into the regulated pathway essential for correct processing and activity dependent secretion of BDNF. However, the spatial and temporal transport and secretion of BDNF is an important regulatory issue which remains to be fully understood.

The final mature form is a smaller 13.5kDa protein (Du, 2014). Some groups claim the mature form is small enough to cross the BBB and suggest this allows peripheral levels to be used as a proxy for central levels (Gass & Hellweg, 2010; Pan, Banks, Fasold, Bluth, & Kastin, 1998b). As such, it has been suggested as a potential biomarker for these disorders. There is however considerable disagreement regarding transport across BBB particularly amongst groups investigating BDNF as a pharmacological treatment, claiming effective transport across BBB is negligible (Zhang & Pardridge, 2001), requires a delivery system due to impermeability of BBB to BDNF (Pilakka-Kanthikeel, Atluri, Sagar, Saxena, & Nair, 2013), requirement for lysosomal transport vectors to deliver BDNF to target structure as therapeutic agent (Xing, Wen, Li, & Xia, 2016) and findings of no significant association between peripheral BDNF and central levels (Piepmeier & Etnier, 2015). The detection of BDNF protein in peripheral blood provides a convenient method of measuring BDNF levels, but since it is expressed on a variety of different cell types in both central and peripheral compartments, and its passage across BBB is controversial, the utility of protein levels measured from peripheral samples as a proxy for central levels remains in doubt.

8.1.2. Gene Expression

The *BDNF* gene is located on chromosome 11, between band p13 and 14 (Hanson, Seawright, & van Heyningen, 1992) extends over 70kb and reportedly contains 11 exons and 9 functional promotors (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007b), which regulate a highly complex, and as yet still poorly understood, pattern of site specific and cell specific expression (Aid, Kazantseva, Piirsoo, Palm, & Timmusk, 2007; Chiaruttini, Sonego, Baj, Simonato, & Tongiorgi, 2008). Of these, only one is reported to be a coding exon (IX) which codes the pro-BDNF and contains two polyadenylation sites at the 3´untranslated

region of the coding exon. These promotors and adenylation sites reportedly give rise to 34 different spicing transcripts (Aid et al., 2007). Pruunslid et al (2007) also report the two polyadenylation sites in coding exon produce either a short or long 3´-UTR which are expressed in the neuron soma and dendrites respectively. Differential mRNA transcripts are also reported to be found localized in distinct cellular and subcellular locations (Aliaga, Mendoza, & Tapia-Arancibia, 2009; Baj et al., 2013). Expression of transcripts containing exon I has been shown high in the testis, brain specific transcripts include those containing exons II, III, IV and V while transcripts containing exons V, VI and IX are expressed widely in the periphery and in blood (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007a).

BDNF transcription regulation is highly complex, each exon is regulated by its own unique promotor, in an activity dependent fashion and with specific temporal and spatial sequence. Both *BDNF* and *NGF* have been shown to increase in an activity dependent manner consistent with its role to facilitate synaptic plasticity and neural activity in rat hippocampus (Zafra, Hengerer, Leibrock, Thoenen, & Lindholm, 1990). It seems likely distinct mRNA splice variants differing either in the 5´ or 3´ extremities in conjunction with differential targeting of *BDNF* gene promotors by diverse stimuli, mediates temporo-spatial regulation of gene expression. In effect, multiple transcripts may be obtained from the *BDNF* gene, with differential subcellular locations and temporal profile which are regulated in response to neuronal activity

BDNF has been strongly associated in the literature with depression, mood and anxiety disorders, human studies frequently showing lowered levels of BDNF protein and reduced hippocampal volume in depressed individuals (Campbell et al., 2004). Lower levels of BDNF protein and mRNA expression have been associated with depression, while restoration of BDNF mRNA and protein levels in hippocampal and cortical regions are seen following

antidepressant treatment (Lee & Kim, 2010; Molendijk et al., 2011). Epigenetic modifications of the *BDNF* gene are also strongly associated with mood disorder in the literature. In episodes of early life stress, increased methylation of the *BDNF* gene reduces expression and is rescued by treatment with a methyl inhibitor (Roth, 2009), epigenetic reprogramming of *BDNF* occurs both spatially and temporally, following exposure to environmental stimuli, including stress exposure and exercise, producing expression change (Karpova, 2014). Associations with a valine/methionine substitution in the *BDNF* gene at position 66 (*Val66Met*) reducing its expression are also widely reported in humans (Bueller et al., 2006; Frodl et al., 2007; Hajek, Kopecek, & Höschl, 2012) leading to suggestions that this common *BDNF* gene polymorphism may be the causative factor in depression and lowered hippocampal volumes seen in mood and anxiety disorders. The met allele confers abnormal intracellular packaging and secretion of BDNF (Egan, 2003) and a study of clinically depressed patients showed those with two copies of the met allele displayed abnormal adrenocorticotrophic and cortisol responses (Schule et al., 2006).

8.2. **NGF**

8.2.1. Biosynthesis and Genetics

Since its early discovery as a target-mediated regulator of neuronal growth in mammals, NGF has been extensively studied in tissues of the peripheral and central nervous system, playing an important role in the regulation of the sensory and sympathetic nervous system, and the cholinergic function of the CNS.

NGF has been mapped to chromosome 1p 13.2 on the reverse strand. The amino acid and mRNA sequence has been characterized and found to be highly conserved, sharing

significant homology between species (Götz & Schartl, 1994).The NGF molecule has structure composed of 3 sub units α, β and γ in a 2:1:2 ratio and together forms a 140kDa protein. The neuronal growth factor function relates specifically to the 26.5 kDa homodimer β sub-unit. When translated it encodes a 241 amino acid prepro-protein which, following translocation and enzyme processing, results in an elongated monomer which, only biologically active in a homodimeric form, consisting of two parallel monomers. (Wiesmann & De Vos, 2001).

NGF binds preferentially to TrkA in the same way as BDNF binds TrkB although it will bind the pan neurotrophin receptor p75-NTR with limited affinity. Its biological activity is dependent, also much like BDNF, on binding at cell surface receptor (transmembrane tryrosine kinase) and subsequent phosphorylation, triggering cell signalling cascades of the RAS, P13-kinase, PLC-γ1 pathways and their downstream effectors (Reichardt, 2006).

While of critical importance during early development of the nervous system it seems in adulthood there is less dependence on constitutive expression of NGF for neuron survival once maturity is reached. Rather, NGF facilitates maintenance of differentiation state and phenotype (Sofroniew, Howe, & Mobley, 2001a). In adult life the amount of NGF synthesis is correlated with density of innervation suggesting tight control by target tissue. Levi-Montalcini suggesting only limited amounts of NGF are released by target organs to effect proliferation, differentiation and survival of developing peripheral autonomic and sensory neurons (Levi-Montalcini, 1987), seemingly only that which is needed is synthesized.

8.2.2. NGF Function.

NGF also has a function in neural repair in the periphery following injury. In normal healthy nerve tissue NGF is expressed minimally, but following injury, the normally low level of expression by nerve cells is increased in activated Schwann cells along with its receptors, mediating increase in density of Schwann cells distally, to accommodate regenerating axon. NGF has both a cell proliferative role and also a tropic function (Frostick, Yin, & Kemp, 1998) in guiding outgrowth of axon. It has been shown to have function in angiogenesis and microvascular remodelling especially in inflamed tissues (McDonald, 2001) and in vitro and in vivo in endothelial cells (Cantarella et al., 2002). Analysis of functional candidate genes for peripheral neuropathy and loss of pain perception have revealed point mutations in the *NGF* gene coding region, which has revealed a separation in the functional effects of nervous system development and cognitive development (Einarsdottir et al., 2004)

In human muscle tissue pathology such as muscular dystrophy, regenerating muscle fibres, myofibroblasts and mast cells consistently express NGF protein while healthy subjects show no immunoreactivity or NGF protein expression (Capsoni, Ruberti, Di Daniel, & Cattaneo, 2000). More recently, NGF has been shown active in the immune system and neuroendocrine system and it is likely this neurotrophin has a more broad functionality than originally thought. However it seemingly is active in disease state or repair of damage rather than healthy tissue.

NGF protein is not normally expressed highly in venous circulation but expression has been shown substantially increased following immune challenge, in particular allergic and inflammatory reaction. Elevated levels of circulating NGF protein have been found in asthma, rhinitis and atopic eczema (Nockher & Renz, 2006). Messenger RNA and protein

were found in freshly isolated eosinophils from peripheral blood with the capability to induce a secretory response on activation, to produce inflammatory mediators. (Solomon et al., 1998). However 99% of fully formed eosinophils reside in mucosal tissue ready to be mobilized to periphery in the case of immune challenge (Kita, 2011). Mast cells, eosinophils and lymphocytes (Bonini, Rasi, Bracci-Laudiero, Procoli, & Aloe, 2003) are shown to store and synthesize NGF, and monocytes are prompted to differentiate to macrophage phenotype by NGF evidencing a role as a immunoregulatory cytokine (Ehrhard, Ganter, Stalder, Bauer, & Otten, 1993). It is shown to have a role in pain perception following injury and pathology (Dyck et al., 1997; Mantyh, Koltzenburg, Mendell, Tive, & Shelton, 2011) (Seidel, Wise, & Lane, 2013). Overexpression studies have revealed sensory hyperinnnervation in genetically modified animals, resulting in increased sensory and sympathetic nerve fibres in airways (Hoyle et al., 1998) and self-perpetuating pain sensation (Sofroniew, Howe, & Mobley, 2001b). However the expression of NGF in human blood and indeed many of the tissues in which it is expressed is highly programmed, coordinated and regulated by necessity its continuous expression resulting in disadvantageous consequence.

8.2.3. NGF in Psychological disorders.

Its contribution to psychological disorder and anxiety, centres on its role in the stress response. It has been shown linked to inter-male agonistic behaviour in mice with circulating NGF protein levels highly correlated with number of exposures to aggressive behaviours (Aloe, Alleva, Bohm, & Levi-Montalcini, 1986), male to male aggressive behaviour in mice following social isolation (Aloe, Alleva, & De Simone, 1990), psychosocial stress in male mice, NGF levels being recorded higher in subordinate males compared to dominants of the hierarchy in animal models (Axelrod & Reisine, 1984) and recently in humans confronted with a romantic conflict situation, (Laurent, Laurent, & Granger, 2013). Novice soldiers under psychological stress before and after their first parachute jump recorded increased blood NGF levels followed by elevated cortisol and ACTH, correspondent to pre jump stress (Aloe

et al., 1994b), leading to suggestions that NGF levels may represent a stress-response system concomitant with the HPA- axis and thus a potential biomarker for human stress. Several lines of evidence are now pointing to the role of hypothalamic sources of NGF involved in neuroendocrine function thereby influencing behaviour. One potential mechanism for the role of NGF exerting influence over physiology is its effect on the adrenal gland. Exogenous NGF administration is shown to increase adrenal weight and volume in a dose dependent fashion (Bigi, Maestripieri, Aloe, & Alleva, 1992) with the potential to influence fight or flight response. Alternatively, behaviour might regulate NGF expression, inter-male fighting in mice shown to result in a marked increase in *NGF* mRNA production in hypothalamus evident at 30 minutes post fight and peaking at 3 hour mark. Increased mRNA was associated with increased NGF protein in hypothalamic region, with levels declining 6-12 hours post (Spillantini et al., 1989). Interestingly, a study by Scaccianoce et al. (2000) showed differential effects of different stressors on activation of the HPA-axis on hippocampal and basal forebrain NGF in male rats. These variations in NGF concentrations seen in young animals were no longer present in aged animals, indicating a complex relationship between stress and NGF which varies with type of stressor and age and remains to be resolved, but the author suggest differences may be due to degree of involvement of neurohormone or neurotransmitter for each stressor (Scaccianoce, Lombardo, & Angelucci, 2000).

Anxiety, tremor and hyper-excitability linked to alcohol or heroin withdrawal were shown associated with higher levels of NGF (Aloe et al., 1996), and epigenetically down regulated by putative methylation of CpG sites in the promotor region between day 7 to 14 into a withdrawal program. Concomitant decrease in plasma NGF levels were reported (Heberlein et al., 2013) and with chronic stress and depressive state in caregivers (Hadjiconstantinou et al., 2001).

NGF has remained along with BDNF at the forefront of research into neurodegenerative, and psychological disorder research. Both neurotrophins showing structural and functional similarity with altered levels of both frequently associated across many studies with psychological disorder. Both participating in plasticity, repair and differentiation, either singly or in concert. Restoration of normal levels are frequently seen following treatment or resolution of disorder

8.3. **FGF2.**

8.3.1. Biosynthesis and Genetics.

Fibroblast growth factor 2 (FGF2) belongs to the 22 member FGF polypeptide growth factor family with pleiotropic effect across different tissues of mesodermal and neuro-ectodermal origin. FGF2, along with other members of this family, act through binding tyrosine kinase receptors FGFRs (fibroblast growth factor receptors) which contain three immunoglobulin-like domains and a heparin-binding sequence (Lee, Johnson, Cousens, Fried, & Williams, 1989). Interaction with FGFRs activates intracellular second messenger cascades. Much like the other neurotrophins BDNF and NGF, its receptors signal through three main pathways, in this case, the phospholipase Cγ (PLCγ), mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) pathways (Turner et al., 2012). FGF2 similarity in function and regulation has led to its recognition by many as a neurotrophin.

FGF2 maps to chromosome 4 in humans in the region 4q25-27 on the forward (sense) DNA strand and is 70990base pairs in length. The gene contains 2 introns, 3 exons and a large

5´and 3´UTR with a variety of regulatory elements influencing polyadenylation, translation initiation and message stability. Its expression is regulated in response to cell density, neurotransmitters, hormones and 2nd messenger interaction. However its transcription is bidirectional, producing an antisense transcript, the function of this transcript is still under investigation but may potentially play a role in transcription regulation. The antisense strand encodes nudix hydrolase 6 (NUDT6) recognized as a member of the nudix-motif housekeeping enzyme family. Two regions of the 3´UTR are complementary to the 3´ end of the *NUDT6* transcript which permits sense-antisense pairing. This along with a destabilizing element located in-between the first and second polyadenylation site are implicated in *FGF2* mRNA stability. Transcription of these two genes shows an inverse relationship which has prompted theories that the antisense transcript regulates FGF2 expression , according to MacFarlane and Murphy (2010) repressing transcription with downstream effects on cell cycle progression and cell adhesion (MacFarlane & Murphy, 2010). In addition the sense – antisense relationship is highly conserved across species indicating an important functional relationship (Baguma-Nibasheka, MacFarlane, & Murphy, 2012). Transcript stability appears to vary with the length of the UTR at the 3´end. The shortest transcript is the most stable and produces an 18kDa protein which according to Delrieu et al (2000) is an efficiently secreted, cytosolic form while the higher molecular weight isoforms contain nuclear targeting sequences localizing them to the nucleus. The low molecular weight 18kDa form is the form isolated from blood and biological fluids, and interacts with specific transmembrane receptor FGFR, while the higher molecular weight forms are FGFR-independent.

Regulation of FGF2 expression is likely to be as complex as BDNF and NGF indeed 5 different molecular isoforms have been characterized from a single mRNA. These isoforms result from alternative initiation from 5 different initiation codons and have different cellular destinations and functions (Bugler, Amalric, & Prats, 1991). Expression and localization of FGF2 isoforms is determined by cell type, environment, cell density, cell cycle phase and level of differentiation.

8.3.2. FGF2 in Psychological disorders

In embryonic development FGF2 serves a role in development of hippocampal and cerebral cortex volume and circuitry. It is a potent central nervous system growth factor and glial mitogen encouraging mitotic proliferation of glial cells but also functions in wound healing, angiogenesis, neurogenesis and differentiation of the central nervous system (CNS) in later life. It has been characterised as a neurotrophin and mice lacking in FGF2 have been shown to be defective in neuronal cell development (Dono, 2003; Dono, Texido, Dussel, Ehmke, & Zeller, 1998; Ortega, Ittmann, Tsang, Ehrlich, & Basilico, 1998). Its role in neural function and development has resulted in interest in FGF2 in neurodegenerative disorders and its implication in affective disorders.

The neurogenesis hypothesis of depression and affective disorders focusses on two key areas of the brain referred to as neurogenic niches, lining the wall of the lateral ventricle called the sub ventricular zone (SVZ) and the sub granular zone (SGZ) of the denate gyrus of the hippocampus. These regions are rich in neural stem cells. They are proliferative regions and provide the optimum environment to encourage and preserve the survival of neural stem cells. According to Merkle et al (2004) the neural stem cells of these regions are a sub population of astrocytes which differentiate from embryonic radial glia in early development, and continue throughout adulthood, to provide the progenitors of neurons and stem cells throughout life (Merkle, Tramontin, Garcia-Verdugo, & Alvarez-Buylla, 2004). FGF2 receptors are differentially expressed on the cells of the neurogenic niches and are believed responsible for the differentiation of neural cell populations (Y Galvez-Contreras, E Gonzalez-Castaneda, Luquin, & Gonzalez-Perez, 2012).These receptors begin to be expressed at day 12.5 (E12.5) in the CNS and are involved in neuronal differentiation in the

embryonic period and as such play a role in the neonatal development of the hippocampus and thus hippocampal volume. It is conceivable then that FGF2 is relevant in the development of hippocampal dysfunction in line with reports of FGF2 role in anxiety and depression

Early studies reported decreased expression of FGF2 in post-mortem brains of depressed individuals (Evans et al., 2004; Perez, Clinton, Turner, Watson, & Akil, 2009; Turner et al., 2012) and antidepressant use increasing FGF2 levels with a concomitant reduction of depressive symptoms in those diagnosed with depression (Mallei, Shi, & Mocchetti, 2002). By contrast though, a recent meta-analysis found significantly higher FGF2 proteins and its receptor RNAs in patients with diagnosed major depressive disorder (MDD) compared to healthy controls and propose this as a novel direction for exploration. However inconsistencies in the literature are highlighted by this group, citing paucity of available human data, and in their own analysis, cite small study numbers, failure to examine changes following treatment, and questions around correlation between central and peripheral levels are discussed as potential limitations amongst these studies (Wu, Tseng, Chen, Tu, & Lin, 2016). Turner et al (2008) suggested endogenous FGF2 had antidepressant effects following repeated FGF2 administration in rats undergoing forced swim and novelty-suppressed feeding models of depression. Not only did FGF2 administration act as an antidepressant but levels of FGF2 receptor 1 also increased, suggesting FGF2 can also promotes increases in its own receptor, augmenting its own effect (Turner, Calvo, Frost, Akil, & Watson, 2008)

Moreover increased levels of FGF2 may be achieved by epigenetic manipulation, using environmental enrichment to induce *FGF2* expression, and further implicate FGF2 as an integrator of genetic and environmental factors to modify anxiety phenotype (Perez et al.,

2009). Early life administration of FGF2 as a single injection in animals bred for susceptibility to anxiety 24 hours after birth, resulted in altered developmental trajectory of both the hippocampus, and cell density of the dentate gyrus, altered gene expression and a marked effect on later emotional regulation in anxiety-prone animals (Turner, Clinton, Thompson, Watson, & Akil, 2011). A knock-down model to reduce FGF2 in rat denate gyrus and CA3 region of the hippocampus produced anxiogenic response with no other behavioural alterations (Eren-Kocak, Turner, Watson, & Akil, 2011) and modulated allostatic stress load (Salmaso & Vaccarino, 2011). A later study by Salmaso et al reported increased HPA-axis activity and reduced glucocorticoid receptor expression with associated increased anxiety behaviour in FGF2 knockout mice. FGF2 administration was enough to exert an anxiolytic effect and this effect was reversed when the glucocorticoid receptor was blockaded, suggesting the receptor is necessary for the anxiolytic effect of FGF2 (Salmaso et al., 2016). Turner et al (2016) however cautions against misinterpreting the relationship between stress and anxiety. This group suggest the impact of FGF2 is conditional on whether the HPA axis is normally functioning or dysregulated, and whether the stress is acute or long term, along with the affective phenotype of the animal.

All three neurotrophins have shown robust connections to the development and maintenance of neural tissue. Their involvement in repair, differentiation remodelling and plasticity of neural circuits, and their associations with anxiety and depression in the literature identify, each one as a rational target for investigation in the pathways mediating the success of the two intervention models under investigation.

9. THE USE OF mRNA AS A BIOMARKER

While convenient to access from peripheral blood, in order to have clinical utility as a biomarker, protein levels should in some way reflect change which characterises disease state and be reliably measurable at an early or late stage of pathogenesis. In the case of mood disorder, multiple interrelated systems potentially dysregulated in depression, anxiety and stress, opens the opportunity for markers to be present in any of the physiological pathways associated with development of the disorder.

It is still a subject of debate in the literature whether BDNF protein crosses the BBB. Some groups claim BDNF arising in the brain is easily transported across the BBB to the peripheral compartment and can be used as proxy for central levels (Pan, Banks, Fasold, Bluth, & Kastin, 1998a; Suliman, Hemmings, & Seedat, 2013). However, pharmacological studies provide evidence to the contradictory, with peripheral introduction failing to reach central targets (Baseri et al., 2012; Lu, Nagappan, Guan, Nathan, & Wren, 2013; Pilakka-Kanthikeel et al., 2013).

Once neurotrophins are released to the blood stream their origins are unknown. Cells of the lung, heart, spleen, gastrointestinal tract and liver are all known to synthesise and release BDNF along with platelets, fibroblasts, smooth muscle cells, thymus and vascular endothelia. BDNF also has functional importance across a wide diversity of physiological systems, responding to food intake signals controlling obesity and weight regulation (Gómez-Pinilla, 2008; Morales-Marín et al., 2016), diabetes and insulin resistance (Krabbe et al., 2007) and chronic inflammatory skin diseases (Rossing et al., 2011) throwing doubt on correlation between peripheral and central levels as a function solely of mood disorder. A meta-analysis by Fernandes et al. (2014) was unable to differentiate between different types

of psychological disorder using BDNF protein levels, or determine whether low levels of BDNF was due to reduced release from brain tissue or lower levels released from peripheral sources (Fernandes, Berk, Turck, Steiner, & Gonçalves, 2014). In addition Molendijk et al. (2014) reported levels of BDNF protein did not correlate with severity of depression symptoms and as such is unlikely to be a useful clinical biomarker in the opinion of this group (Molendijk et al., 2014).

Analysis of mRNA levels of neurotrophin in place of protein, takes a different approach. Contemporary research on models of depression suggest it as a disorder with systemic inflammation and dysregulated endocrine responses as the mechanism of pathogenesis. Direct cytokine communication with the brain is facilitated via neuro-immuno-endocrine crosstalk, regulating production of BDNF by peripheral immune cells in response to dysregulated mood.

Analysis of *BDNF* mRNA levels and gene regulation dynamics in the immune system avoids the central-peripheral protein argument favouring a more direct biomarker of pathogenesis particularly for depressive illness. In a brief review by Gass and Hellweg (2010), contribution of BDNF protein from peripheral sources is discussed, along with a review of work by Cattaneo et al. (2010) showing both protein and mRNA levels of BDNF were reduced in serum and leucocytes respectively of depressed patients compared to healthy controls, levels improved following treatment, and correlated with clinical symptom improvement. Cattaneo and colleagues presented results which indicated BDNF protein synthesis, seen in serum levels, is associated with changes in *BDNF* mRNA levels in leucocytes. According to Gass and Hellweg, BDNF serum levels probably represent a composite, derived from multiple sources and may not necessarily correlate directly with central levels. Gass and Hellweg speculate the regulation of mRNA in leucocytes may more accurately reflect not

only temporal dynamics of central BDNF change but also of physiological inflammatory mechanisms associated with depressive illness and might therefore represent a valuable early biomarker of disorder. Notwithstanding, Cattaneo presents an argument that peripherally derived BDNF protein may still exert effect on brain function and behaviour, but by the simplest mechanism, this will ultimately depend on whether BDNF protein does indeed pass easily across the BBB (Cattaneo et al., 2010; Gass & Hellweg, 2010).

10. AIM

The current study investigated the comparative change in *BDNF* gene expression, and its potential as a biomarker of negative mood and affect, following an 8 week intervention program of increased physical activity or mindfulness. The gene expression of two other neurotrophins NGF and FGF2, with associations in the literature to neuroplastic change and psychological wellbeing, will be compared to establish whether these neurotrophins also have utility as biomarkers for mood and affect disorders. A non-clinical community sample of volunteers were randomly allocated to each intervention model, the control group were waitlisted. Gene expression was measured from peripheral blood using RTqPCR method, and change in mood measured using subscales of the DASS (Depression, Anxiety and Stress Scale).

11. HYPOTHESIS

In line with reported trends in both protein and mRNA studies, this study hypothesized an increase in *BDNF* gene expression and lowered symptoms of psychological distress following both mindfulness and physical activity interventions. NGF and FGF2 are also frequently associated with pathogenesis of mood disorders in the literature. They have similarity in structure and function to BDNF, and are involved in co-ordination of neurotrophic response. It is suggested they may represent additional novel biomarkers of mood and possibly anxiety disorders. Since the study group comprises a healthy non-clinical sample, any observed change is expected to be small. Increase in neurotrophin expression, in association with improved mood following interventions, not only suggests these neurotrophins are influential in the widely reported success of both physical activity and mindfulness in treating mood disorders, but will also be beneficial to the well-being of healthy individuals, and have utility as biomarkers in clinical assessment of these distressing disorders.

12. MATERIALS AND METHODS

This Study was approved by Federation University Human Research Ethics Committee with approval number A14-095. An overview of the study protocol is shown in Figure 4.

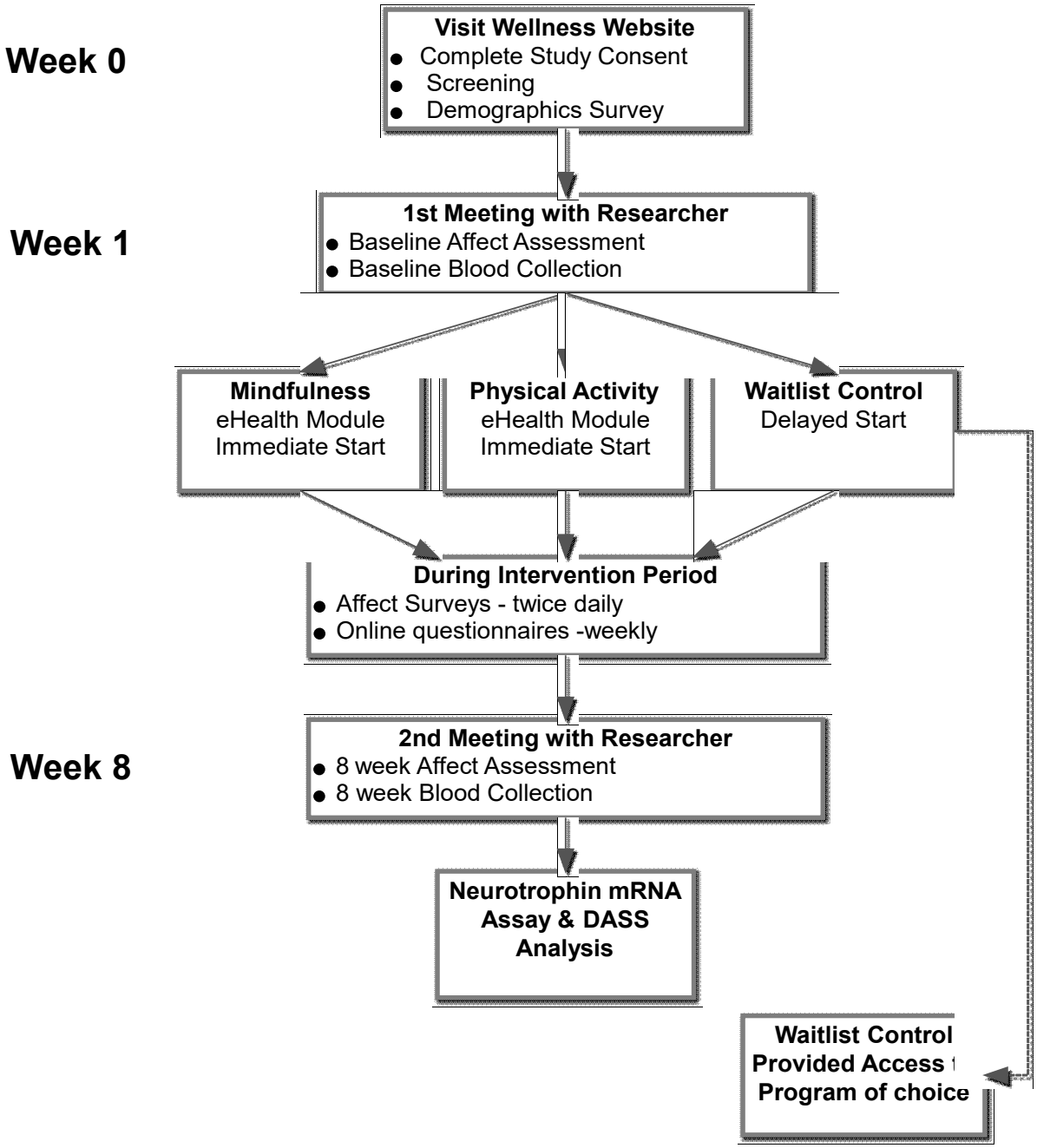


Figure 4 Overview of Study Protocol

12.1. PARTICIPANT SELECTION

Inclusion criteria; To be eligible each volunteer was required to have access to the internet, and internet-connected mobile phone, availability to attend two onsite assessment appointments and be 18 years of age or over.

Exclusion criteria; Potential participants were excluded if they had a documented life-threatening illness or injury, cardiovascular disease, a diagnosed mental illness or psychosis, had a debilitating physical mobility condition or were pregnant.

Recruitment procedure;

Participants were recruited as part of a larger randomised controlled trial conducted by Federation University to determine the effect of physical activity compared to mindfulness on a range of biopsychosocial assessments of wellness. The study was advertised by social media, and email, to attract participants from the general community and university staff and students. To register interest in participating in the study, people were asked to visit the university hosted Wellness website, specifically created for the study, complete an online form after reading the plain language information statement and consenting to it. Exclusion criteria were clearly stated on the website and participants advised of their ineligibility if any documented physical or mental impediment present. Participants were then contacted by a member of the research team to arrange a time discuss the study and set up a time for the pre-intervention assessment.

12.2. PRE-INTERVENTION ASSESSMENT

At a time mutually convenient, an onsite meeting was arranged for each participant with a researcher. Each participant was asked to give a non-mandatory blood sample (baseline pre-condition), and complete physical and computerised measurements to collect a range of biopsychosocial data. The researcher also explained and downloaded the Affective App (a mobile mood survey application) onto each participant's mobile phone. The Basis™

Wristwatch (Basis, San Francisco) was fitted (records the biometric data) which was part of a larger biopsychosocial data set recorded for a wider analysis and further studies. Those participants unwilling to give a blood sample or for whom the results were incomplete were eligible to be included in other analyses in the wider study. Following the initial meeting, participants were then asked to complete a battery of online questionnaires and once completed, they were notified of allocation to one of three intervention conditions, mindfulness, physical activity or waitlist (control).

12.3. INTERVENTION PERIOD

The immediate start group began their allocated intervention immediately following completion of their pre-intervention questionnaires, the waitlisted group (controls) did not receive access to their program for 8 weeks. However they were required to complete the same surveys and measures as the experimental groups. During the course of the study, all participants receive 5 daily SMS alerts to prompt completion of the daily Affect app Survey and every 4 days, an SMS alert to upload the data collected by the Basis watch to the server. Compliance with condition intervention was monitored through online questionnaire, and at assessment appointments. The Mindfulness and Increasing Physical Activity intervention websites also recorded each time participants logged in. Participants using less than two modules were prompted to complete a questionnaire about the barriers to completing the intervention which they may have encountered. All participants were offered access to modules for a further 3 months following completion of the study to encourage maintenance of activities. All data analysed was de-identified.

12.4. COMPONENTS OF THE INTERVENTIONS

12.4.1. Mindfulness

The Mindfulness digital health program consisted of 3 modules, designed to help the user become aware of their moment- to- moment experience. Each module took approximately 15 minutes to complete, and participants were recommended to complete one module per week. Participants were free to work through these at their own pace and were asked to complete five minutes of mindfulness practice activity each day.

Module one consisted of an introduction to mindfulness followed by an audio MP3 downloadable version of the introduction to mindfulness exercise which was used as the practice exercise throughout the intervention. Module two introduced the concepts of observing, guiding and non-judgemental awareness, and module three encouraged the user to complete five minutes of mindfulness daily

12.4.2. Physical activity

The physical activity intervention modules were designed to encourage participants to increase their current level of exercise in a way that is practical and achievable, facilitating ongoing maintenance and benefit at the end of the intervention period. Information on goal setting and behavioural change was outlined.

In the first module the benefits of physical activity, achievable goal setting and strategies for making change were presented, and participants were asked to set an activity goal for the next session. In module two this goal was reviewed and any barriers identified, along with any necessary strategy revision. Participants were required to set a new goal if necessary prior to the next session. In module three the new goal was reviewed, stages of change revisited, and if successful, encouragement to continue and extend current activity was offered, or suggestions of alternative strategies were made such as joining a class or a gym.

During the 8 week intervention participants are required to attend 2 onsite assessments. Pre-intervention as above and at 8 weeks (post-intervention). On arrival for each assessment, participants handed in a cortisol sample which they had completed at home, had their resting heart rate variability measured and completed computerised cognitive testing (for unrelated analysis). Each participant was asked a series of questions including any physical activity undertaken, dietary consumption that morning, stage of menstrual cycle where appropriate, and completed on-line self –report questionnaires which included the DASS (Depression Anxiety and Stress Scale) a 21 item screening scale for measuring negative affect beginning at pre-assessment, and subsequently at week 8 (see Appendix A). Blood was drawn according to professional best practice guidelines at pre-intervention and week 8 (post-intervention).

12.5. DASS MEASUREMENTS DURING INTERVENTION.

Depression, Anxiety, Stress Scales (DASS-21). As shown in Appendix A, the DASS-21 (Lovibond & Lovibond, 1995) is a short form of the DASS (containing 42 items) and contains three self-report scales, each with 7-items designed to measure the emotional states of depression, anxiety and stress. Respondents are asked to use a four-point severity/

frequency scale from 0 (did not apply to me at all) to 3 (applied to me very much, or most of the time) to rate the extent that they had experienced each emotion over the last week (e.g., “I found it difficult to relax”). High scores indicate high levels of disturbance. The DASS-21 wields adequate Cronbach’s alpha values for scales depression (α = .86), anxiety (α = .55) and stress (α = .87) subscales (Lovibond & Lovibond, 1995).

12.6. GENERAL LABORATORY PROTOCOLS

For the purposes of this project I collected, processed and undertook the technical aspects of the laboratory work myself at the university laboratory. The extraction and PCR was supervised by a laboratory technician. Blood was collected using Safety-Lok™ butterfly Blood Collection Set (Becton, Dickinson and Company, NJ. USA) 1 PAXgene Blood RNA Tube (PreAnalytix Qiagen/BD Switzerland) and 3 BD Vacutainer® EDTA tubes (Becton, Dickinson and Company, NJ, USA) for further unrelated analysis each time, including one discard EDTA tube (drawn first, in order to absorb potential volumetric shortfall due to air filled tubing).The EDTA tubes were immediately put on ice prior to transport to the laboratory. Since the PAXgene tubes maintain RNA stably for a period of days, tubes were kept at room temperature on the day of collection, for the enzyme degradation of cell components to complete prior to RNA extraction, and periodically transported to laboratory freezer during the course of the day.

On arrival at the lab both the PAXgene tube and the EDTA discard tube were frozen as whole blood overnight at -20^{O}C while the remaining 2 EDTA tubes were centrifuged at 2700rpm and the plasma combined into a sterile falcon tube, frozen at -20^{O}C overnight and transferred to -80^{O}C along with the corresponding discard whole blood tubes as soon as practicable. These tubes were maintained at -80^{O}C for further studies.

12.6.1. Gene Expression Assay

In order to measure and compare whether there was any difference between expression of our three genes before and after intervention, Quantitative Reverse Transcription Polymerase Chain Reaction (RTqPCR) assay was used in conjunction with Comparative Cycle Threshold (cT) method for calculations. Using this method a standard curve is not required permitting all the wells to be utilized for samples and reference gene, and a higher sample throughput per plate. This in turn minimizes dilution errors and pipetting errors across plates.

12.6.2. RNA extraction

All RNA work was undertaken in RNA handling designated work areas and kept in closed Eppendorf tubes to avoid contamination and degradation. The PAXgene Blood RNA tubes were thawed and maintained at room temperature for 2 hours to ensure complete lysis of cellular material according to the manufacturer's recommendations. The tubes were centrifuged at 3000g for 10 minutes to pellet nucleic acids and cell debris. The supernatant was discarded in 10% bleach solution. The pellet was resuspended in 4ml RNase free water, centrifuged at 3000g. The pellet was used for RNA purification using the Isolate II Biofluids RNA Kit (Bioline).

The nucleic acids were resuspended in the PAXgene tube using Lysis Buffer RX and vortexed for 15 seconds, then transferred to a sterile RNase free 1.5ml tube and kept on ice. Genomic DNA was removed using the Isolate II Genomic DNA Removal Column, and the flow through used to purify the RNA using the supplied RNA Binding Column. Wash buffer for RNA purification was prepared by adding 90mls of 100% ethanol to Buffer Concentrate W1. To optimize the performance of the RNA purification column, 100% ethanol was added to the flow through 60µl to every 100µl to produce an ethanolic lysate. Lysate was applied to the RNA column and the flow through discarded. In order to minimise any remaining

genomic DNA contaminants bound RNA was further purified using an optional DNase treatment supplied in the kit. DNase digest was prepared as directed using 15µl enzyme to 100µl Reaction Buffer DRB and the reaction incubated for 15mins at room temperature, followed by a column wash. Instances where the column appeared clogged, and lysate remained on the column after repeated centrifuging were resolved by gently pipetting remaining lysate on and off the column until homogenised.

RNA was eluted in Elution Buffer (kit supplied) and RNA yield quantified using NanoDrop 2000 (ND-2000) UV-Vis Spectrophotometer (Thermo Fisher Scientific, MA, USA) by absorbance at 260nm. Elution buffer was used as blank subtraction and purity was ascertained by the A260/280 and A260/ 230 ratios. RNA was stored at -80C.

12.6.3. Synthesis of cDNA.

This step is necessary firstly in order to store the samples over time. RNA is highly unstable and making a cDNA copy allows the RNA to be maintained stably at -80^{O}C long term and cDNA sequences of interest to be synthesized for each subsequent analysis. In addition most commercial kits are designed to amplify DNA strands not RNA, it is therefore necessary to synthesis a DNA copy of the target mRNA sequence for use in the PCR reaction.

Total cellular RNA purified from whole blood samples was converted to single stranded cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems ™ California from Thermo Scientific).

For each sample a reaction mix was prepared containing;

2.0µl 10X Reverse Transcriptase (RT) Buffer

0.8µl 25X dinucleotide triphosphate (dNTP) mix (100mM)

2.0µl 10X RT Random Primers

1.0µl Multiscribe Reverse Transcriptase

4.2µl Nuclease free H20

Each sample reaction mix was multiplied by total number of samples (64) and aliquoted to wells of the reaction plate.

The plate was sealed with an optical seal sheet, and spun to eliminate air bubbles and collate all reagents at the base of the well.

Plates were placed in the thermal cycler.

Thermal Cycler Program for synthesis of cDNA

The plate was programmed through 4 cycles and ran for approx. 135 mins

Cycle	1	2	3	4
Temp^{O}C	25	37	85	4
Time mins	10	120	5	∞

The resulting cDNA may be stored at -20^{O}C or diluted to desired working concentration for PCR.

The concentration of all but one of the samples was 10ng/µl, but due to a particularly low RNA yield, one of the samples had a final concentration of 5ng/µl. For consistency, all other samples were standardized at the lower concentration of 5ng/µl. 10µl of each RNA sample was made up to 20µl total volume with 10µl of 2x RT Mastermix providing a final concentration of 10ng/µl or 200ng RNA per sample.

12.6.4. Expression Assay RTqPCR

Quantitative Reverse Transcription polymerase chain reaction RTqPCR is the method of choice when the starting material is total cellular RNA. Although starting with mRNA transcript of interest may provide more sensitivity, total RNA is preferable to mRNA requiring fewer purification steps, ensuring a greater yield, and provides a closer relative quantification and standardization to normal cellular content. The RNA however must be transcribed into cDNA before PCR can proceed. Target mRNA was differentiated by the use of a commercial probe complementary to the target of interest.

Expression of the three genes was compared to an invariant endogenous reference gene *GAPDH*, which has consistently shown a reliable and stable rate of expression across a wide range of tissues previously in this lab. The use of a reference gene (housekeeping gene) ensures the accuracy of the expression profile of the target genes by minimizing sample to sample variation in RT qPCR efficiency, differences caused by sampling obtained over different time courses which may produce variations in RNA integrity, and run-run variations. In order to further minimize intra-run variations a 384 well plate was used to enable all 64 samples to be analysed for the 3 target genes, in the same run along with *GAPDH* and controls.

A TaqMan® Universal Master Mix II with UNG and TaqMan Gene Expression Assay kits (Applied Biosystems ™ California) were chosen for the gene expression assays. TaqMan gene expression assays are commercially available oligonucleotide kits of sequence specific primers and probes which are fluorescently labelled. Kits are designed to be sequence specific to the target sequence of interest. The fluorescent dye is attached at the 5´ end and a quencher at the 3´ end. Following attachment of the primer, TAQ polymerase extends the

primer sequence synthesizing a nascent strand, closely followed by exonuclease activity of the polymerase enzyme which degrades the probe. The removal of probe proximal to the quencher allows release of the fluorescence bound to each synthesized strand, detection of fluorescence resulting from the amplification cycles will be proportional to the number of copies of the target present at the end of the cycle sequence.

PCR analysis was performed on the VIIA™ 7 Thermal Cycler (Applied Biosystems) equipped with fluorescence detection and amplification analysis software. The change in fluorescence over time is used to determine the amount of target (amplicon) produced in each amplification cycle. Amplification occurs in two phases the exponential phase and the plateau phase. The reaction proceeds as the amplicons are exposed to a number of pre-programmed timed temperatures (cycles). During the exponential phase target amplicon doubles each cycle, but consumption of reaction components creates a rate limiting stage or plateau phase. Despite exponential amplification fluorescence is not detectable in the early cycles. Fluorescence is detected at the point at which amplicon levels cross the threshold. The threshold value represents background levels. The Cq (quantification cycle) is determined by the amount of target present in the initial sample at the start of the amplification cycles, the more target present, the fewer the number of cycles required to amplify the target to the threshold level thus the reaction will have an early Cq, if there is a low level of target present the reaction will have a late Cq or may not reach threshold during the programmed sequence. This might indicate degraded sample or absence of target nucleotide sequence. In the latter case corresponding to an absence of expression of the gene in question.

TaqMan gene expression assay kits chosen are shown in Table 1 are all designed to be sequence specific to the target of interest (do not detect homologs, map to multiple genes, or

detect off-target sequences) and produce short amplicons for a more efficient PCR reaction. Primer design included in the assays all spanned exons except the *BDNF* assay which mapped to within a single exon. This assay was chosen because it has the best coverage across a range of possible transcripts. However probes mapping within single exons increases the risk of amplification of genomic DNA (gDNA). Since every precaution was taken during the RNA purification protocol to remove any contaminating gDNA by treatment with DNase enzyme, the advantage of using a probe which detected multiple transcripts was considered to outweigh the risk.

Table 1 TaqMan Gene Expression Assay Kits

Gene	Analysis	Assay ID	Supplier
BDNF	TaqMan™	Hs02718943_s1	Life Technologies Australia Pty Ltd
NGF	TaqMan™	Hs01113193_m1	Life Technologies Australia Pty Ltd
FGF2	TaqMan™	Hs00266645_m1	Life Technologies Australia Pty Ltd
GAPDH	TaqMan™	Hs02758991	Life Technologies Australia Pty Ltd

BDNF – Brain Derived Neurotrophic Factor, *NGF* – Nerve Growth Factor, *FGF2* Fibroblast Growth Factor, *GAPDH* Glyceraldehyde-3-Phosphate Dehydrogenase.

12.6.5. Gene Expression Assay Protocol

All four gene assays (*BDNF, FGF2, NGF and GAPDH*) were able to be run on the same MicroAmp® Optical 384 Well Reaction Plate minimizing run to run error. Two positive

controls were included on the plate for each gene assay. Extra controls were set up on a separate plate due to lack of space on the gene analysis plate.

Gene assays were thawed vortexed and spun to mix thoroughly, then kept on ice during the preparation of reaction mixes.

Four reaction mixes were made in 1.5ml Eppendorf tubes one for each of our genes.

For each sample reaction volumes were as follows;

TaqMan™ Fast advance Master Mix 2X 2.5µl

TaqMan™ Gene Expression Assay 20X 0.25µl

Nuclease free H2O 0.25µl

cDNA 2µl at 5ng/µl concentration.

Total volume per well 5µl

Total volumes prepared were dependent on number of samples assayed and then multiplied by two for duplicate samples. The reaction mix was further extended by 10% to allow for potential shortfalls generated by multichannel pipetting.

Assay Controls.

Two technical replicates were included for each sample and showed a good level of consistency on thermal cycler output. PCR efficiency was monitored by the computer interface. Control samples for the purposes of the Wellness project included individuals which were waitlisted, i.e. not engaged in either of the intervention models during the assessment period, but were later allowed access to all information modules and intervention models at the termination of assessments.

Quality controls to monitor the validity of the assay method were No Template Controls (NTC), and the invariant *GAPDH*. The NTCs were able to be obtained from the remaining reaction mixes and prepared minus the cDNA, to monitor contamination and primer-dimer formation which could produce false positives.

The reactions were run on a Viia™7 Thermal Cycler (Applied Biosystems) and run cycle programmed.

Thermal Cycler Program for Gene Assay

Cycle	Temp ^{O}C	Time mins/secs
1x	50	2mins (hold)
1x	95	20secs
40x	95	1sec
	60	20secs

The *NGF* assay produced an unexpected amplification plot. While replicates were reasonably consistent the target crossed the threshold very early in the sequence of cycles and failed to show expected amplification of a gene which is being expressed.

In order to test whether the assay kit was at fault, failure due to technical error or *NGF* was simply not highly expressed in whole blood, the *NGF* assay was repeated using tissue homogenates from a tissue library stored in the lab. Tissues chosen were heart, kidney and brain and all had worked well in previous assays, showing high levels of *NGF* expression.

Assay plate was set up to include both *GAPDH* and *NGF* duplicates with a duplicates of a NTC.

Reaction volumes were mixed in 1.5ml Eppendorf tubes as follows;

TaqMan™ Master Mix 2x 2.5µl

TaqMan™ Gene Expression Assay 20x 0.25µl

Nuclease free H2O 0.25µl

cDNA 2.5µl of 5ng/µl (stored at 20ng/µl the stock was diluted 2.5µl in 7.5µl nuclease free H20. As before 3µl of reaction mix was pipetted in to each well corresponding to both *NGF* and *GAPDH* genes for each of the three tissues samples.

The analysis of this run showed normal and expected levels of expression of the *NGF* gene in the selected tissues eliminating the possibility that the assay kit was faulty. In order to establish no operator error in the original experimental assay run on the blood samples, the Wellness samples were re run by a laboratory research assistant. These results delivered exactly the same outcome, showing early Ct and no amplification, further suggesting no *NGF* expression in the Wellness blood samples.

12.6.6. Statistical Analysis

Paired t-test was used for the statistical analysis of both gene expression study and DASS sub-scales using GraphPad Prism® 7 statistical package.

13. RESULTS

The experimental intervention groups Mindfulness and PA were compared to a control (Waitlisted) group. Participants were randomly allocated to one of the three conditions in an independent measures design study. Participant demographics are shown in Table 2. The study recruited a sample of 28 adults aged between 19 and 57 years. Mean age of the mindfulness group was 33.08, *SD*=13.40, physical activity was 31.17, *SD*=13.20 and the waitlist 31.70, *SD*=13.60. It was observed that there was a large gender bias, the majority of participants were female (82.14%), males (7.85%). A large proportion of the participants ad

never smoked (64.28%) and 3 individuals had never had alcohol (10.71%). Participants working part-time (64.28%) and studying (57.15%) respectively were the most prevalent. Although it should be noted some participants answered in more than one response category e.g. they may have been studying and volunteering or working part-time.

Table 2 Demographics at Baseline.

Demographic		Total n (%)	Mindfulness (n=12)		PA (n=6)		Wait (n=10)	
			Mean (*SD*)	n (%)	Mean (*SD*)	n (%)	Mean (*SD*)	n (%)
Age			33.08 (13.40)		31.17 (13.20)		31.70 (13.60)	
Female		23 (82.14)		11 (91.67)		5 (83.33)		7 (70.00)
Male		5 (17.85)		1 (8.33)		1 (16.67)		3 (30.00)
Smoking	Never	18 (64.28)		10 (83.33)		5 (83.33)		3 (30.00)
	Past	7 (25.00)		1 (8.33)		1 (16.67)		5 (50.00)
	Intermittent	3 (10.71)		1 (8.33)		0		2 (20.00)
	Regularly	0		0		0		0
Alcohol	Never	3 (10.71)		1 (8.33)		1 (16.67)		1 (10.00))
	Monthly	8 (28.57)		7 (58.33)		0		1 (10.00))
	2-4 Monthly	11 (39.28)		4 (33.33)		3 (50.00)		4 (40.00))
	2-3 Weekly	4 (14.28)		0		2 (33.33)		2 (20.00)
	≥4 Weekly	2 (7.14)		0		0		2 (20.00)
Employed	F/T	7 (25.00)		2 (16.67)		0		5 (50.00)
	P/T	18 (64.28)		7 (58.33)		6 (100)		5 (50.00)
	Volunteer	5 (17.85)		1 (8.33)		1 (16.67)		3 (30.00)
	Studying	16 (57.14)		8 (66.67)		3 (50.00)		5 (50.00)

PA – Physical Activity, Wait - Waitlisted Control, F/T - Full Time, P/T – Part Time, *SD* – Standard Deviation.

13.1. DASS Analysis

The DASS assessment was administered online and measures the negative emotions associated with states of depression anxiety and stress (see appendix A). Analyses of paired pre and post intervention scores were done with GraphPad Prism ® using a paired T test. Table 3 summarises results of the statistical analysis. Data has been checked for normality and outliers and participants with incomplete data removed.

Depressive and anxiety symptom mean reduction was observed in both of the interventions yet only significantly for depression (p = 0.03), whereas the waitlist group (control) scores showed non-significant elevations on both depression and anxiety (p = 0.27 and 0.51 respectively). Stress scores similarly showed both non-significant increases and decreases in the intervention groups, and in the case of the waitlist the scores remained the same from baseline to the final analysis. Figure 5 and Figure 6 show a comparison of pre and post analyses on the DASS.

DASS analysis of the PA condition revealed no significant decrease in depression, anxiety or stress level. Comparing the means pre and post it can be seen there are small reductions in the depression and anxiety scores by week 8 with stress scores increasing slightly. However, given the study sample size was small, it may have been insufficiently powered to detect significance.

Table 3 DASS subscales means pre and post intervention compared with waitlist

		Depression					Anxiety					Stress				
Condition	n	Mean Pre	SEM Pre	Mean Post	SEM Post	*p*	Mean Pre	SEM Pre	Mean Post	SEM Post	*p*	Mean Pre	SEM Pre	Mean Post	SEM Post	*p*
Min	10	3.60	1.09	1.90	0.98	0.03	1.60	0.62	0.60	0.27	0.19	5.40	1.36	3.30	1.18	0.21
PA	5	3.60	2.29	1.80	1.80	0.14	1.60	0.68	1.40	0.68	0.62	5.60	3.01	6.40	3.33	0.74
Wait	7	3.00	1.70	4.00	1.53	0.27	2.43	0.43	3.14	1.20	0.51	8.14	2.61	8.14	2.30	>0.99

p is significant at the level ≤0.05, M = mean, SEM = Standard Error of the Mean. PA – Physical Activity, Wait – Waitlisted Control.

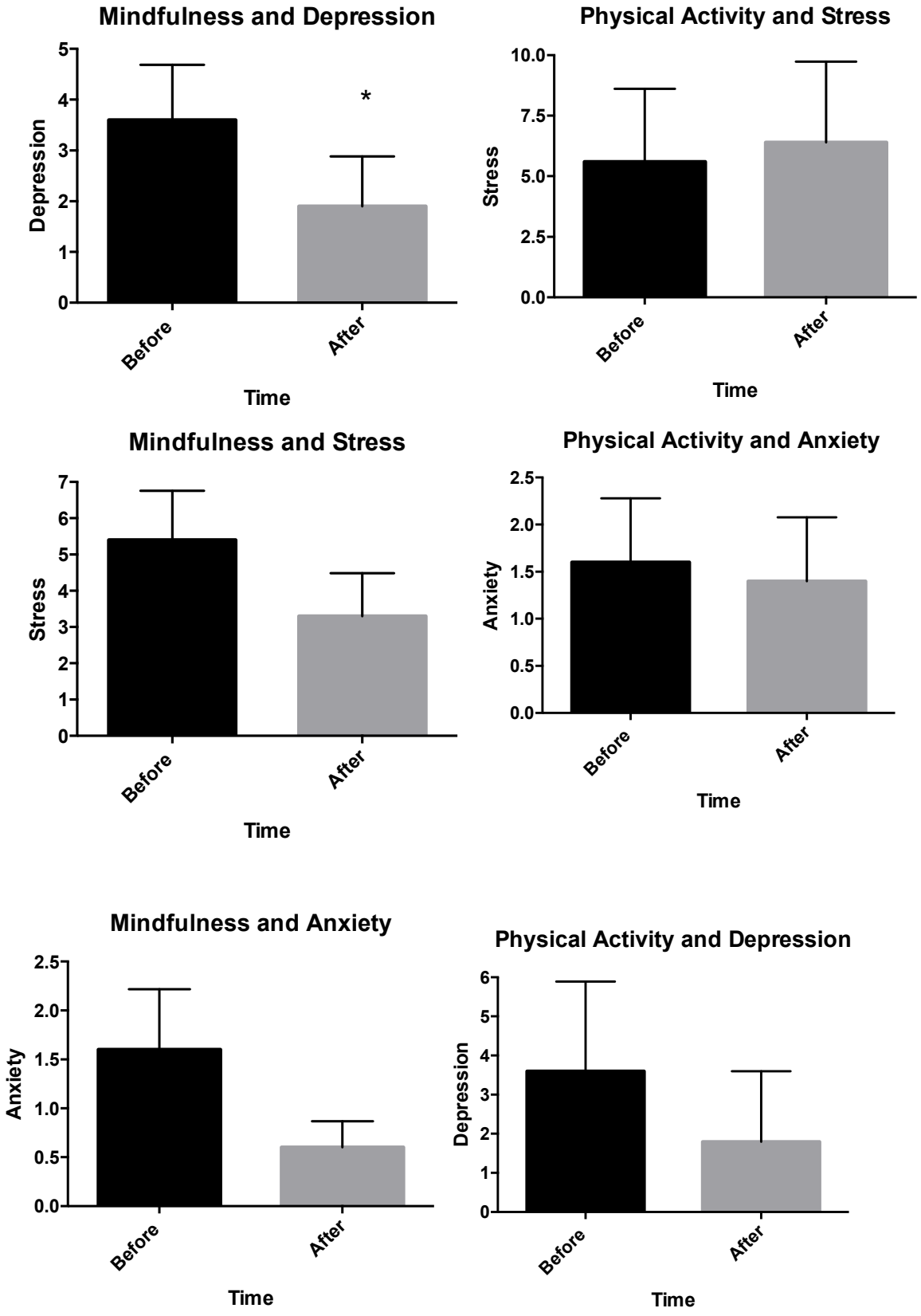


Figure 5 DASS Analysis of Depression and Anxiety with Interventions.

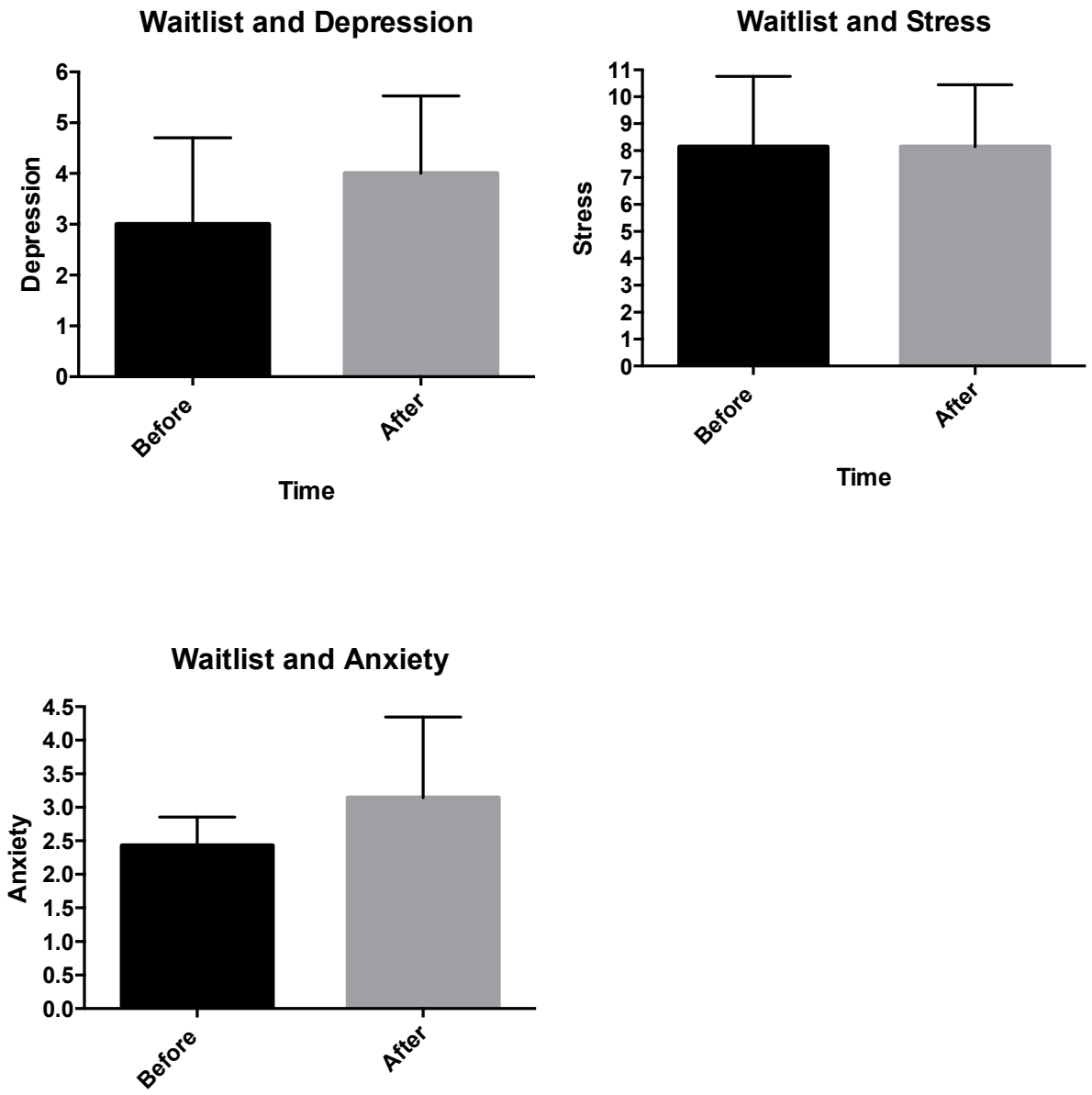


Figure 6 DASS Analysis of Stress with Interventions.

13.2. NEUROTROPHIN ANALYSIS

Table 4 summarises the analysis of change in neurotrophin levels across each intervention. The data has been checked for normality, outliers and participants with incomplete data removed. Examination of individual sample levels revealed one outlier with unusually high level of *BDNF* in the post intervention analysis. This case was a female smoker, deficient in vitamin B12 and vitamin D and taking oral contraceptives. This participant described herself as being in “very good” physical health, and “good” mental health although requiring B12 injections and taking oral contraceptives. While she did attend for her final blood collection, she did not finish the biometric upload of data or on-line questionnaires at week 8 and as a result data for this case was incomplete. Since it was not possible to determine any reason why the post score was unusually high, this score was excluded from the analysis. Removing this case resulted in a significant decrease in *BDNF* message abundance following the mindfulness intervention by the final assessment $p = 0.01$ (n=11) as shown in Table 4 and Figure 7.

Taken together with the significant reduction in depression scores on the DASS, the result is in line with a large body of influential research reporting the success of mindfulness practice in the reduction of psychological distress. However, the results of this study also reveals an association with reduced levels of *BDNF* and decreasing depression scores following 8 weeks of mindfulness practice.

Table 4 Abundance mRNA comparing pre and post means of the two interventions with waitlist across the three neurotrophins

	BDNF					*FGF2*				
Condition	n	Mean Pre	SEM	Mean Post	SEM	*p*	n	Mean Pre	SEM	Mean Post	SEM	*p*
Mindful	11	3.03	0.82	0.91	0.32	0.01	9	4.07	1.91	3.58	1.10	0.78
PA	5	0.44	0.16	2.28	0.51	0.01	0	0.00	0.00	0.00	0.00	0.00
Wait	10	1.42	0.73	0.65	0.41	0.19	9	2.31	0.49	4.15	0.91	0.11

Mindful – Mindfulness, PA – Physical Activity, Wait – Waitlisted Control, *BDNF* Brain Derived Neurotrophic Factor, *FGF2* Fibroblast Growth Factor 2.

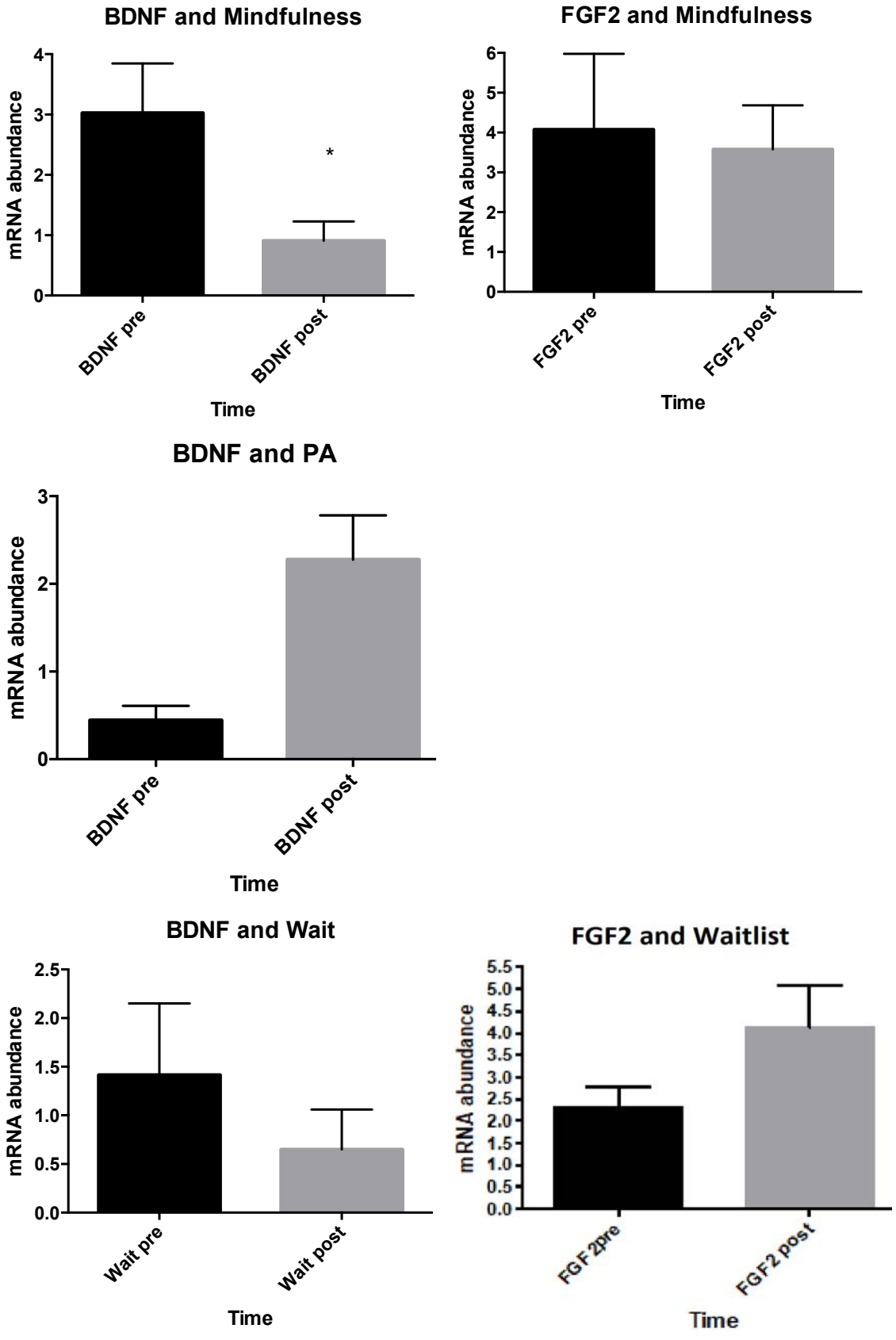


Figure 7 Neurotrophin Abundance with Interventions.

All cases considered, results for *BDNF* abundance in the physical activity group pre- to post-intervention, were not significant, although there was a general trend towards increased levels of *BDNF* following increased levels of physical activity. However, the analysis revealed one extreme outlier with an unusually high pre-intervention level. The outlier was a female reporting limited capacity for physical activity due to difficulty bending. She reported her physical health as “fair”, mental health as “good”, and reported no medication use, but did report vitamin B12 deficiency (treated with B12 injections) and anaemia. Although arthritis was not reported as a pre-existing condition, if spinal inflammation was present it may have artificially inflated her *BDNF* level at baseline (Forsgren, 2009). Removing this outlier from the analysis, mRNA abundance was shown significantly increased $p = 0.01$ (n = 5).

Both *BDNF* mRNA abundance and symptoms of depression, anxiety and stress on the DASS in the waitlist group, showed no significant difference, from pre to post condition. All available data was included and there were no obvious outliers. The PA condition was the smallest group in the study with participant attrition considerably affecting group size.

Data for *FGF2* abundance and mindfulness were incomplete, 4 of the 12 samples recording null on the thermal cycler data output. For the remaining data set, analysis showed no significant differences in expression pre-to-post intervention. Outliers were present but once removed had no appreciable effect on the significance value. Comparison of the means showed a slight decrease in *FGF2* following the mindfulness intervention see Figure 7.

FGF2 results in the group assigned to the physical activity intervention were also incomplete. Null results were recorded on the output from the thermal cycler for 4 of the 6 samples. When incomplete data is uploaded from the thermal cycler, the software analysis program records a zero and these samples have to be discarded. As a result, with only two data

points, a valid analysis was unable to be attempted for the difference between *FGF2* pre- and post-intervention in the PA condition.

FGF2 abundance in the waitlist group was missing 1 data pair, but no outliers present. The resulting analysis was not significant although comparison of the means showed a slight increase in *FGF2* by week 8.

13.3. Investigations into the Amplification Curve for NGF

The amplification curve for *NGF* was unexpected. Output from the thermal cycler showed very early accumulation of fluorescence crossing the threshold at a very early cycle number, and no exponential amplification of our target sequence. The gene analysis was repeated twice during the period of the original study and once again at a later date by another laboratory staff member, all outputs showing similar expression curve.

Outputs of this nature are usually attributable to one of the following causes. Potential contamination from genomic DNA (gDNA), in this case the samples were treated with DNase enzymes during RNA purification stage to minimize any potential genomic DNA contaminants. In addition the samples analysed for the *NGF* target expression were aliquoted from the same source samples, analysed for the other genes, and run on the same plate. The other targets of interest showed no evidence of gDNA contamination in their amplification curves.

Failure to amplify maybe due to a limiting of reagents, either due to degradation of reaction reagents, or errors in dilution of either reagents or sequences of interest. In order to eliminate this as a possibility the analysis was rerun several times including a run by another member of our group as a control. In all instances the resulting output showed the same profile and we can assume the analysis did not suffer dilution errors. The viability of *NGF* gene expression assay kit was subsequently tested against tissue samples known to

demonstrate normal levels of *NGF* expression with successful and normal exponential amplification of the target. This not only validated the kit reagents were not degraded but also evidenced the specificity of the probe and enzymes to the target of interest. Another potential source of failure which was a consideration since *NGF* probe was not commercially available off the shelf and had to be custom made especially for this analysis.

A further potential source of this kind of early fluorescence detection and failure of target sequence to amplify is formation of primer-dimers. The potential of one region of a primer, for example the 3´terminal end, having complementarity with itself, or another primer region, producing a secondary structure (artefact) by hybridizing to itself. When the original copy number of target gene is low or primer anneals with low specificity to the target, amplification of non-specific product and primer-dimer artefacts can amplify quickly using the high availability of nucleic acids in the reaction mix. Competition in the thermodynamic amplification phase between artefact and low availability of target reduces target yield reducing both sensitivity and linear range of the reaction time. The result will be early fluorescence detection, and lack of detection of target amplicon. Whilst primer-dimer formation is one potential explanation for the unexpected amplification curve, the salient point remains that the analysis showed no amplification of the target sequence and it seems most likely there is an absence of expression of *NGF* in all the blood samples in this study, having eliminated potential sources of experimental error.

14. DISCUSSION

Robust associations between pathophysiology and symptom relief in psychological disorders have suggested neurotrophins as potential biomarkers for mood disorders. BDNF protein has received most attention. However both FGF2 and NGF protein are similarly involved in plasticity, growth and differentiation of the nervous system, and have shown similar associations in the literature with mood disorder regulation. Nevertheless, protein as a biomarker remains controversial. This study measured change in gene expression of neurotrophins with potential to serve as more direct biomarkers of depressive mood, anxiety and stress pathophysiology and avoiding the controversial problem of peripheral protein levels as a proxy for central levels. The study hypothesised an increase in *BDNF* mRNA in line with the reported increases in BDNF protein following participation in physical activity and mindfulness in the literature. In addition a potential increase in *FGF2* and *NGF* was expected, in line with their role as growth factors in neural repair and proliferation.

14.1. DEPRESSION, ANXIETY AND STRESS ASSOCIATION WITH NEUROTROPHIN EXPRESSION AND MINDFULNESS

Results of the study showed a significant decrease in expression of *BDNF* mRNA following 8 weeks of mindfulness training. This was accompanied by a significant reduction in scores on the depression subscale of the DASS. This significant result suggests mindfulness reduces depressive symptoms and this may be associated with reducing levels of *BDNF*. Consistent with theories on inflammatory mechanism as a causal factor in mood disorder, decreasing levels of depressive symptoms, along with a reduction in activated leucocytes may produce lower abundance of *BDNF* mRNA, as the mindfulness training progresses.

Mindfulness has been associated consistently in the literature with improved emotional regulation and mood improvement, with accompanying modification of cognitive-affective processes (Goldin & Gross, 2010; Grossman et al., 2004). Such modification processes likely involve neuroplastic change and as such, BDNF is expected to be involved. However, data associating *BDNF* gene expression and mindfulness with improved mood are limited.

Although research in this area is nascent, a similar pilot study conducted in Thailand using medical students undertaking a four day mindfulness meditation program found similar, although in this case non-significant, downward trend of serum BDNF protein (Turakitwanakan, Mekseepralard, & Busarakumtragul, 2015). An analysis of cortisol level was also separately published from the same group of volunteer medical students, with results indicating a significant reduction in cortisol following the mindfulness program consistent with modulation of the neuroendocrine pathway. Their results on the stress (GHQ28) measure were non-significant but the four day intervention was short. Such a short period of practice was likely enough time for the temporal regulation of gene expression to take place, but perhaps not long enough for the brain to modify circuitry to achieve robust findings with regard to stable changes in mood.

Mindfulness has been widely shown to increase plasticity, and brain volumes in brain imaging and central expression studies (Hölzel et al., 2011; Kirk, Fatola, & Gonzalez, 2016; Tang et al., 2015). While a corresponding increase in BDNF would be anticipated following increases in brain volume and plasticity in the central compartment, it would not necessarily be representative of peripheral *BDNF* gene regulatory mechanisms involved in the peripheral physiology of mood disorder pathogenesis. Mounting evidence suggests mindfulness may work by reducing stress induced inflammation and endocrine dysregulation, with corresponding modulation of neural cross-talk reducing distressing mood

symptoms (Cahn et al., 2017; Kaliman et al., 2014). Neural modulation may facilitate disconnection of fear circuits in the amygdala, insula, hippocampus and other areas important in reducing worry and rumination, and augment emotional regulation pathways. In this model the decrease in depressive, anxious and stress related symptoms would be expected, concomitant with a reduction in endocrine dysregulation and inflammation and leucocyte mediated *BDNF* mRNA abundance.

The number of studies analysing the effect of mindfulness on BDNF is still small, however increased BDNF protein levels have been reported. Cahn's study (2017) compared a range of psychoneuroendocrine and inflammatory markers of wellness after a 3 month yoga and meditation retreat finding, BDNF protein in samples of whole blood significantly increased when comparing pre-to post- assessments (Cahn et al., 2017). Indices of anxiety and depression were significantly improved, but this group did concede a possible contribution from sustained physical postures (potentially comparable to PA) during the yoga component of the retreat which may have increased BDNF protein. In addition breathing techniques, and postures may have an effect on sympathovagal tone not seen in mindfulness alone. Also, If BDNF freely crosses the BBB, studies which have shown increased BDNF protein in peripheral circulation, following mindfulness interventions, may have detected protein from composite brain and peripheral sources (Gass & Hellweg, 2010) as suggested earlier. BDNF protein increases in the brain may be expected in association with neural remapping and neuroplastic change, consistent not only associated with learning of a new skill, but also mediating increased functional connectivity in regions involved in executive processing, mediating sustained attention and focus, emotional regulation and self-referential processing. Contributing to the increase in serum protein levels. While abundance of message in the periphery is more likely a result of specific cellular activation most likely in the neuroimmunoendocrine pathway.

14.2. Depression Anxiety and Stress association with Neurotrophin expression and Physical Activity

This study has shown a significant increase in abundance of *BDNF* mRNA in peripheral blood following 8 weeks of the increased physical activity intervention. Since this was the smallest group in the study, with participant attrition considerably affecting the size of the data set, the result needs to be interpreted with caution. There is a large body of influential research evidencing increased levels of both protein and mRNA in association with significant improvement to mood, following experimental PA conditions (Moylan et al., 2013; Neeper et al., 1996; Ströhle et al., 2010).These associations have replicated across samples of different age, gender, clinical and non-clinical populations alike (Pasco et al., 2011; Rebar et al., 2015; Strawbridge, Deleger, Roberts, & Kaplan, 2002). However, contrary to these reported associations in the literature, this study, did not produce any significant reduction in distressing mood symptoms on the DASS subscales.

Plausibly, the study method may have affected this unexpected result. Most of the studies reported used stringent laboratory managed exercise time and intensity conditions, with each participant undertaking exactly the same activities. This study was designed to assist those seeking to make lasting changes and lifestyle modifications, beneficial to future health and wellbeing. As such it was important to offer a program which could be managed and integrated easily at home. In order to facilitate compliance with the study protocol and encourage ongoing maintenance, participants were offered free choice in the physical activity they adopted. Emphasis was put on gentle introduction of increased activity along with strategies in setting and achieving goals. Protocols adopting laboratory monitored activities with moderate to high intensity levels have almost universally reported successful reduction of mood disorder symptoms, along with improved BDNF expression. Indeed in terms of mood disorder alleviation, there is substantial evidence that reduction of symptoms

is, dependent on intensity, type and time course of activity (Chang, Lu, Hu, Wu, & Hu, 2017), consistent with a dose-response relationship (Evangelista, Cacciata, Stromberg, & Dracup, 2017), with dose-dependence, intensity and whether exercise is defined as occupational (mandatory and often intense) or leisure time (Kim, Shin, Nam, Choi, & Kim, 2008) and length of time the exercise condition runs, especially with regard to alleviation of clinical depressive symptoms (Craft & Perna, 2004). The Kim et al. 2008 study emphasised the necessity to make comparisons between studies, based on equivalent measures of intensity and duration between experimental groups, indicating the strength of the association with symptom reduction fails in the event different types and intensity of activities are used comparatively, and type of activity is an important determinant of whether it produced psychological benefit. In this very small group of study participants, all likely accomplishing their goals at different rates with differing activities it is perhaps improbable, given the importance of duration and intensity above, that a significant change in mood would be recorded.

It is broadly accepted that exercise induces an increase in circulating leucocytes (Gabriel, Schwarz, Born, & Kindermann, 1992; McCarthy & Dale, 1988; Neves et al., 2015) and this could plausibly account for the increase in *BDNF* seen in this group. In healthy individuals this early inflammatory response is countered by mediators aimed to restrict the duration of inflammatory reaction and extent of damage. To further reveal any physiological effect from the increased numbers of circulating immune cells, an early study by Connolly et al. (2004) characterised the genomic response of peripheral blood mononuclear cells from healthy subjects exposed to a bout of exercise, finding a wide array of genes associated with inflammation, cellular repair, growth and stress mediators were up-regulated. Although in this study gene response was rapid and transient returning to baseline within 1-2 hours of recovery, an interesting rodent study by Burchtold et al (2005) suggested exercise primes a molecular memory for BDNF gene and protein induction, and following repeated bouts of

exercise, significant increases in BDNF were maintained, although incrementally reducing, during 7 days of subsequent inactivity (Berchtold, Chinn, Chou, Kesslak, & Cotman, 2005). Therefore, it is possible the participants in this study sustained a significantly increased *BDNF* level due to increased leucocyte population following repeated bouts of PA, but due to variations in intensity and duration of activity, it did not produce significant change in mood. The DASS depression subscale however did show a downward trend in 3 of the 5 mean scores available for analysis and a larger data set might have yielded results in line with reported trends.

It cannot be discounted *BDNF* mRNA expression increased as an inflammatory response to exercise induced strains, sprains, and repair of minor damage by mechanical agitation to muscle and nerve tissue, particularly in those participants dramatically increasing the intensity of their workload (without laboratory supervision) when commencing the study. Reactive oxygen species are formed during intense exercise in muscle. Oxidative and antioxidative dynamics have evidenced a capacity to epigenetically alter the methylation status of the *BDNF* gene (Lindholm et al., 2014; Nguyen, Duquette, Mamarbachi, & Thorin, 2016) and reasonably contribute to change in *BDNF* mRNA levels in the periphery in the exercise condition.

14.3. INTERVENTION *FGF2* AND *NGF* EXPRESSION.

In the literature, expression of both NGF and FGF2 have been shown increased in response to exercise in brain tissue (Gómez-Pinilla et al., 1997; Neeper et al., 1996), but there are thus far no reports in peripheral blood in association with PA or mindfulness. In this analysis, neither *FGF2* nor *NGF* showed any significant associations with change in mood symptoms on the DASS measure. FGF2 functions in angiogenesis, wound healing, and embryonic development, regulation, proliferation and differentiation of a wide variety of cells (Wilkie,

Morriss-Kay, Jones, & Heath, 1995). It has been associated with neurological and psychological disorder including decreased levels in post-mortem brains of depressed individuals (Evans et al., 2004) and utility as a potent anxiolytic (Graham & Richardson, 2011; Perez et al., 2009) and warranted investigation as a biomarker on the strength of these association. However results of this study do not suggest it has any utility as a biomarker for depression, anxiety or stress related mood disorders. Results from the *NGF* expression analysis showed this neurotrophin seemingly at sub-threshold levels, or absent in peripheral blood both at pre-condition and at post. NGF has been shown increased in peripheral blood following acute stress response in humans, undertaking an anxiety provoking situation such as a first parachute jump (Aloe et al., 1994a), increasing concomitantly with elevated cortisol and ACTH leading to suggestions of utility as a biomarker for stress and HPA-axis activation. In the absence of acute stress or aggression it is found elevated in peripheral blood in allergic reactions (Nockher & Renz, 2006). While leucocytes populations are capable of synthesis, most of the NGF mRNA in blood is found in eosinophils and these reside mainly in mucosal tissue until they are released into circulation following immune challenge (Kobayashi, Gleich, Butterfield, & Kita, 2002). However NGF is not reported highly expressed in peripheral circulation under normal circumstances, and indeed in this study.

15. STRENGTHS AND LIMITATIONS

15.1. STRENGTHS

This is the first study to directly compare expression change in three different neurotrophin genes *BDNF NGF and FGF2* with strong associations to dysregulated mood, following mindfulness and PA intervention conditions. The use of mRNA bypasses the current debate on the utility of protein as a biomarker, reflecting more directly neuroimmune and endocrine

models of mood disorder pathogenesis and physiology. In particular neural regulation of BDNF expression in response to physiological mechanisms is suggested to show more direct association with mood change and offers an earlier stage window on the status of disorder.

Participants were randomly assigned to their interventions in this between subjects design, ensuring any observed effect from the condition (PA or mindfulness) to be reasonably attributed to the activity, as opposed to pre-existing differences in individuals, experimental and control groups or carry over from the comparison condition which may be observed in repeated measure designs.

The study design emphasises the ease with which these strategies can be incorporated into daily life. Instead of the necessity to attend time consuming and expensive courses, retreats or punishing gym and personal trainer sessions, these strategies can be implemented in or around the home environment without inconvenience. The significant nature of the change in mood and *BDNF* expression should maximise the impact of this study, since it evidences even small amounts of easily manageable change to daily activities produces effective changes at the molecular level which facilitates wellness, hopefully promoting broad acceptance and engagement of exercise and mindfulness in the general community.

15.2. LIMITATIONS

RNA molecules are inherently unstable and prone to degradation by RNases. Their stability, in circulating blood, subject to clearance and degradation by a wide array of cellular lysis and bacterial enzymes. It is reasonable to question the longevity of transcripts found in

circulation. While the PAXgene system preserves the integrity of transcripts once extracted, low levels and sub-threshold levels may be due perhaps either to entire transcript degradation, or partial degradation of the message in vivo, or potentially during laboratory extraction and purification of RNA, with the subsequent failure of probe specificity.

Whilst every effort was made to achieve enough participant engagement throughout the course of the intervention, there was a large attrition rate. Frequently cited was the difficulty in wearing the Basis wristwatch 24 hours a day, particularly amongst female participants, and difficulty sleeping when wearing the watch. For some participants the frequency of prompts to complete the daily self-monitoring mobile survey were intrusive and inconvenient, and led to failure to complete the required elements. For one particular cohort, the final 8 week assessment coincided with their final university examination schedule resulting in some participants sending apologies, declining to attend. The attrition rate in particular had most impact on the PA group. In addition most respondents were female, resulting in only 5 male participants able to be included in analysis across all conditions.

This was a sex biased study. Literature reports several sex biases particularly in BDNF levels, gender effects and sex moderated BDNF levels are widely reported in the literature (Chan & Ye, 2017) in particular in PA studies (Szuhany et al., 2015) and serum and platelet levels (Lommatzsch et al., 2005) and due to such small numbers we are unable to account for any gender effect in our analysis.

Resources did not permit strict adherence to timing of blood sampling. Timing ranged from morning to late afternoon, diurnal effects were therefore not able to be accounted for in this analysis. Some studies have reported diurnal effects particularly in BDNF levels (Trajkovska

et al., 2007).In addition there are circadian rhythms in the production of leucocytes in the bone marrow which may be a factor when considering numbers of leucocytes and BDNF expression. Finally in terms of research method design, this was a between subject design each subject experiencing a different intervention. BDNF in particular has been shown to demonstrate large inter-individual differences and variations over extended time periods, with females showing higher levels than males (Trajkovska et al., 2007) making the necessity for large study numbers an imperative to eliminate noise and increase statistical power.

An accidental freezer malfunction resulted in a freeze thaw event which went unnoticed for an unknown period for some of the samples. These samples were included in the analysis due to small numbers of participants. While the PAXgene tube stabilizes mRNA for a period of 3-5 days at room temperature, a freeze thaw event of unknown duration may have had a detrimental effect degrading RNA content in some of the samples.

The sample was comprised of generally physically and mentally healthy individuals. Consistent with the purpose of the project, each participant was free to choose any physical activity convenient to their circumstance. The physical and mentally healthy status of the participants, and non-prescriptive PA model, suggested the experimental effects would be small, perhaps indistinguishable from inter-individual differences especially given the intensity and duration dependence seen in corresponding studies. In addition, most individuals were well educated about the value of regular exercise and its contribution to a healthy lifestyle, and were already actively engaged in some form of activity to maintain fitness. As a result effects on mood change may have been blunted in the PA group. However it was encouraging to find significant changes in *BDNF* levels in both intervention groups. Attesting to the robust association of *BDNF* with PA and mindfulness, and showing

both these activities as convincing strategies to boost wellbeing. Nonetheless this open-choice physical activity protocol may be considered both a limitation, making comparison amongst more rigorously prescriptive protocols difficult, and an advantage as it offers an easily attainable options to improve health and wellness in non-clinical and clinical populations alike.

16. CONCLUSION

This randomised control trial with between subject design assessed the effect of brief physical activity and mindfulness training on the level of expression of three neurotrophin genes *BDNF, NGF and FGF2* in a physically and mentally healthy community sample.

A brief overview of the results is presented in Figure 8.

The data has revealed a significant positive association between physical activity and BDNF mRNA, although no significant reduction in distressing mood symptoms was shown. This was potentially due the small group size. Mindfulness was significantly associated with decreasing negative affect, despite an unexpected decrease in BDNF mRNA, consistent with pathophysiology of depression, which is likely related to neuro-immune-endocrine axis disturbance, as suggested in the published literature. It is suggested that decreasing mRNA levels might reflect lower numbers of immune activated leucocytes present in the blood, following mood improvement; albeit this was not verified in this study. This study suggests that even in a small non-clinical sample there may be potential benefits to wellbeing by increasing levels of physical activity or the practice of mindfulness, and that BDNF has potential as a biomarker of emotional state. It will be useful to follow up this study with quantitative assessments to establish the sensitivity of BDNF as a biomarker, and analysis to determine whether changed levels correlate with severity of symptoms.

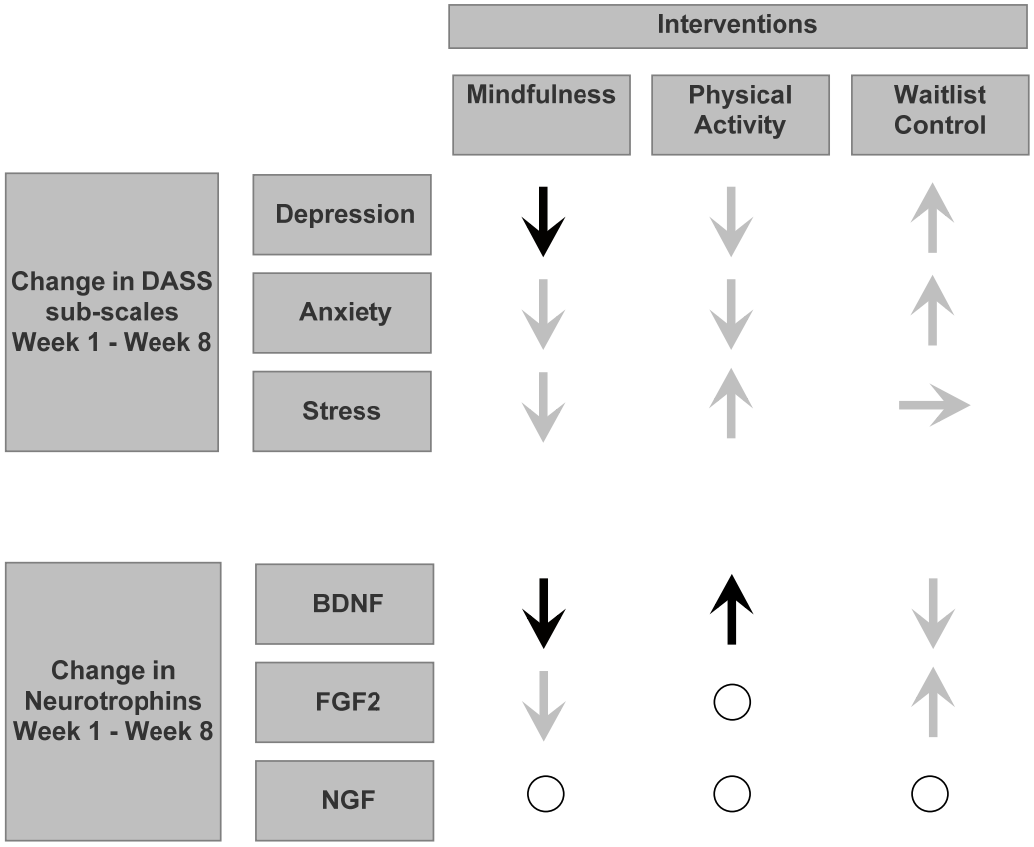


Figure 8 Overview of Results.

Significant results are shown with black arrows; grey arrows represent non-significant trend, and null results are indicated by a circle. DASS – Depression Anxiety and Stress Scale, *BDNF* – Brain Derived Neurotrophic Factor, *FGF2* – Fibroblast Growth Factor 2, *NGF* Nerve Growth Factor.

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Appendix A. The DASS 21

The DASS 21 is designed as a self-report scale to measure negative emotional states associated with depression, anxiety and stress (Lovibond & Lovibond, 1995) In this abridged form of the 42 item scale, there are 7 items measuring dysphoria, anhedonia, hopelessness, self-deprecation, and inertia, lack of interest and devaluation of life, to assess depression. Autonomic arousal, skeletal muscle effects and situational or subjective anxious experience, to assess respondent level of anxiety and nervous arousal, while irritability and impatience measures non-specific states of arousal associated with stress.

Respondents rate the severity and frequency of their feelings and experience on a 1-4 point scale. The scores are summed and coded. Results separate respondents on the basis of normal, moderate and severe dimensions of emotional disturbance, rather than a categorical diagnostic.

I found it hard to wind down	0	1	2	3
I was aware of dryness in my mouth	0	1	2	3
I couldn't seem to experience any positive experience at all	0	1	2	3
I experienced breathing difficulty, e.g. excessively rapid breathing/ breathlessness in the absence of physical activity	0	1	2	3
I found it difficult to work up the initiative to do things	0	1	2	3
I tended to over-react to things	0	1	2	3
I experienced trembling e.g. in the hands	0	1	2	3
I felt I was using a lot of nervous energy	0	1	2	3
I was worried about situations in which I might panic and make a fool of myself	0	1	2	3

I felt I had nothing to look forward to	0	1	2	3
I found myself getting agitated	0	1	2	3
I found it difficult to relax	0	1	2	3
I felt down-hearted and blue	0	1	2	3
I was intolerant of anything that kept me from getting on with what I was doing	0	1	2	3
I felt I was close to panic	0	1	2	3
I was unable to become enthusiastic about anything	0	1	2	3
I felt I wasn't worth much as a person	0	1	2	3
I felt that I was rather touchy	0	1	2	3
I was aware of the action of my heart in the absence of physical exertion e.g. sense of heart rate increase or heart missing a beat	0	1	2	3
I felt scared without any good reason	0	1	2	3
I felt life was meaningless	0	1	2	3

SCORING

0 = Does not apply to me/ never

1 = Applied to me occasionally/ some of the time

2 = Applied to me a considerable degree/ a good part of the time

3 = Applied to me most of the time

CODING

	Depression	Anxiety	Stress
Normal	0 - 4	0 - 3	0 - 7
Mild	5 - 6	4 - 5	8 - 9
Moderate	7 - 10	6 - 7	10 - 12
Severe	11 - 13	8 - 9	13 - 16
Extremely Severe	14 +	10 +	17 +