

**Neuroimaging Biomarkers in Generalised Anxiety Disorder, and
Associated Modulations Following an Anxiolytic Intervention**

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Abstract

Introduction and Aims

Generalised anxiety disorder (GAD) comprises a debilitating cluster of psychological and physiological symptoms that markedly impairs quality of life. GAD is characterised by hallmark cognitions of persistent worry and anticipatory anxiety. Evidence exists for dysregulation in excitatory/inhibitory neurobiological pathways in prefrontal and limbic brain regions, with the dorsal anterior cingulate cortex an area of particular interest. However, limited research exists assessing regional activations and the role of metabolites such as gamma-aminobutyric acid in these regions, nor modulations as a function of treatment. The aim of the thesis was to investigate the functional and metabolic features of this region, and to assess the role of neuroimaging biomarkers of anxiolytic treatment response.

Methods

Two investigations were conducted utilising structural features of the region of interest: task-based functional magnetic resonance blood oxygen level-dependant signal activation and GABA levels via magnetic resonance spectroscopy together with relevant psychometric and psychiatric measures. The first study was a cross-sectional investigation undertaken to compare neuroimaging biomarkers in 41 participants with GAD with 35 healthy control participants. The second study was an 8-week RCT sub-study involving 41 participants randomised to either daily 240mg of kavalactones *Piper methysticum* (Kava) extract or a matching placebo. This proof-of-concept study assessed the aforementioned outcomes and whether these markers signal the plant's anxiolytic activity.

Results

The results of the first investigation did not reveal group differences in GABA level ($p = .302$). The relationship between GABA and anxiety severity was different for each group; a significant positive correlation in GAD (e.g., HAM-A, $p = .018$) and a negative correlation in healthy controls (e.g., trait anxiety, $p = .019$). The functional task was successful in eliciting regional BOLD signal differences between valent congruency conditions. Two regions exhibited significant group differences (at $p < .05$), showing hyperactivation in GAD and reduced activation in healthy controls. In both groups BOLD signal significantly predicted severity of state anxiety (GAD $p = .027$; HC $p = .041$). Gender, age, and comorbidity in the GAD group also influenced the biomarker-anxiety relationships.

The results of the second study showed that Kava treatment was associated with a reduction to GABA levels at eight weeks ($p = .049$). The treatment was not associated with anxiety symptom, nor fMRI signal change, measured at eight weeks.

Discussion

This research investigated regional brain properties in GAD for biomarker utility, before testing them in a ‘proof of concept’ study using the purported anxiolytic agent, Kava. Metabolic and functional data were successful in producing differences in the dorsal ACC that could be (if replicated in a larger study) be utilised as biomarkers to aid in the management of GAD symptoms. Limitations of the studies were small sample sizes, GABA signal quality and equivocal toolbox results.

The neurobiological effects of Kava have not been directly studied using MRI imaging in humans. The findings of a reduction to GABA levels after treatment may potentially reflect a normalising of the GABA system similar to healthy control data observed in the first study.

GAD is a prevalent psychiatric disorder that is under-diagnosed and under-treated. While a great deal of work is inherent in establishing biomarkers for clinical benefit, this research contributes MRI evidence of biological differences, and insight into the mechanisms of Kava, together with a translational rationale for the study of novel anxiolytics as potential GAD treatments. The outcomes and findings of this research fit well with the current affective disorder literature and exceed contemporary work in the field of GAD biomarker and treatment research.

Declaration

This is to certify that:

- (i) the thesis comprises only my original work within the PhD except where indicated in the preface;
- (ii) due acknowledgement has been made in the thesis to all other material used; and
- (iii) the thesis is less than 100,000 words in length, exclusive of tables, figures, references and appendices;
- (iv) The research conducted herein received ethics approval from the Alfred Hospital Ethics Committee (University of Melbourne) and Swinburne Human Research Ethics Committee (Swinburne University of Technology).

Signed,

Karen Savage

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Preface

Author publications

Peer-reviewed publications arising from PhD – First author

1. **Savage KM**, Firth J, Stough CK, Sarris J. GABA Anxiolytic Phytomedicines: A Systematic Review of Preclinical and Clinical Evidence. *Phytotherapy Research*. 2018. 32 (1): 3 – 18.
2. **Savage KM**, Stough CK, Byrne GJ, et al. Kava for the treatment of Generalised Anxiety Disorder (K-GAD): study protocol for a randomised controlled trial. *Trials*. 2015. 16:493. doi: 10.1186/s13063-015-0986-5
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Conferences, proceedings

1. **International Society for Affective Disorders (ISAD), 9th Biennial Conference, 20th – 22nd September 2018, McGovern Medical School, Houston, USA.**
Poster presentation - Adjunctive Treatments in Major Depressive Disorder: A Double-blind, Randomised, Placebo-controlled Trial using S-Adenosyl Methionine (SAME) and a Combination Nutraceutical

2. **Asia Pacific College of Psychiatry 2016 Conference, 2nd – 5th November 2016, Kaohsiung, Taiwan**
Speaker delegate - Nutraceuticals for Mood and Anxiety Disorders
3. **International Society for Bipolar Disorder/International Society for Anxiety Disorder joint conference Anxiety Disorders Society Annual Conference (IADSAC), 15-17th July 2016, Vrije Universiteit Amsterdam, Netherlands.**
Poster presentation - Adjunctive Treatments in Major Depressive Disorder: A Double-blind, Randomised, Placebo-controlled Trial using S-Adenosyl Methionine (SAME) and a Combination Nutraceutical
4. **Society for Mental Health Research (SMHR), Annual conference, 2-4th December 2015, Brisbane, Australia**
Poster presentation - Kava for the treatment of generalised anxiety disorder (K-GAD): study protocol for a randomised controlled trial.
5. **Students of Brain Research (SOBR) Student Symposium, November 2015, Swinburne University**
Poster presentation - Kava for the Treatment of Generalised Anxiety Disorder (K-GAD): Neurophysiological and Pharmacogenomic Anxiolytic Biomarkers
6. **Asia Australia Mental Health, 26th March 2015 National Centre for Neurology and Psychiatry (NCNP), Kodaira, Tokyo**
Presenter – Kava for the Treatment of Generalised Anxiety Disorder
7. **International Anxiety Disorders Society Annual Conference (IADSAC), 28th & 29th November 2014. St Vincent's Hospital, Melbourne, Australia**
Co-Presenter – Kava for the Treatment of Generalised Anxiety Disorder

Glossary

$^1\text{H-MRS}$	Proton magnetic resonance spectroscopy
5-HT	5-hydroxytryptamine; serotonin receptor
BOLD	Blood oxygen level dependent
CNS	Central nervous system
CSF	Cerebrospinal fluid
DANTE	Delays alternating with nutations for tailored excitation
DSM-III; DSM-IV	Diagnostic and Statistical Manual edition 3; 4
GABA	gamma-Aminobutyric acid/ γ -Aminobutyric acid
GABA+	GABA quantity with macromolecule inclusion
GABA-T	Gamma aminobutyric acid transaminase
GAD	Generalised Anxiety Disorder
GAD1	Glutamate decarboxylase
GADe	Glutamic Acid Decarboxylase enzyme (in order to differentiate from GAD, above)
GAT	GABA transporter
Glx	The combined signal from glutamate and glutamine
GM	Grey matter
H+	Hydrogen ions
Hz	Hertz, or cycles per second
IAPS	International Affective Picture System
MEGA	Mescher-Garwood
MEGA-PRESS	Mescher-Garwood Point-Resolved Spectroscopy
mM	Millimoles
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRS	Magnetic resonance spectroscopy
ms	Milliseconds

NAA	N-acetyl aspartate
NMDA	N-methyl-D-aspartic acid
OCD	Obsessive- compulsive disorder
PLP	Pyridoxal phosphate
ppm	Parts per million
PRESS	Point- resolved spectroscopic sequence
PTSD	Post-traumatic stress disorder
SAD	Social anxiety disorder/social phobia
SLC6A1	Solute carrier family 6
SSRI	Selective serotonin reuptake inhibitor
SV	Single voxel
TOOLBOX	Programs (accessory or stand-alone) for performing editing or processing of neuroimaging signal
TR	Repetition time
TE	Echo time
WM	White matter

1 Chapter One. Introductory Overview

1.1 Overview

The structure of this thesis starts with a short summary to guide the reader on the content. Chapters Two through Five comprise the literature review. This thesis initially reviews generalised anxiety disorder (GAD) aetiology, diagnostics and the current lines of treatment, before examining the neurobiological underpinnings of the disorder. A broad examination of multiple systemic, behavioural and psychological influences on GAD prevalence is discussed in a biomarker context, before specifically examining the literature in the domains of structural, functional and metabolic brain imaging, as modalities bridging GAD cognitive symptomatology with underlying neurobiological function.

An overview of anxiolytic phytochemicals that are gamma-aminobutyric acid (GABA) modulatory in action are presented and discussed, before focussing on *Piper methysticum* (Kava) as the most evidenced phytochemical (plant-based medicine) for use in GAD symptom alleviation.

The thesis reports on two investigations in Chapters Six and Seven, outlining the methodology, procedures, results and subsequent findings. Chapter Eight discusses the findings, limitations and future research plan from the two studies before discussing significance of the results in the broader context of GAD biomarkers. Chapter Nine re-examines the aims and objectives of the thesis before concluding statements. Finally, the reference list, and appendices which contain additional details omitted from the methods, and copies of the candidate's first-authored publications.

The aims of the thesis were to better elucidate and characterise neurobiological function occurring within GAD in order to optimise diagnosis and therapeutic treatment. In addition, the research was conducted to advance our current knowledge on Kava/*Piper Methysticum*'s potential mechanisms of action.

2 Chapter Two. Generalised Anxiety Disorder

2.1 Overview

This chapter examines GAD historical diagnostics, reports on the epidemiological status, and examines GAD clinical features. Secondly it also presents selected theories describing cognitive symptomologies and discusses how these factors define and limit how GAD is diagnosed and treated. A second introductory chapter reviews our current understanding of biomarkers and their challenges and significance specific to GAD research.

2.2 Introduction

The anxiety state is not abjectly abnormal and forms part of the orientation towards environmental threat perception to aid human survival. Similarly, 'worry' as cognitive ruminations over events, problems or scenarios is considered a cognitive strategy to cope with future negative events or attempts at problem solving (Borkovec, Robinson, Pruzinsky & DePree, 1983; Tallis & Eysenck, 1994). However, the movement into persistent expression that is, a) out of proportion with associated events and b) interferes with function, with a severity, frequency and intensity at clinical levels, warrants treatment, due to the impact of quality of life.

Anxiety disorders are ubiquitous and persistent, often with an early onset—excessive worry is often reported from childhood (American Psychiatric Association, 2000)—and are highly comorbid with affective/mood disorders and substance misuse (Brown, Antony & Barlow, 1992; Merikangas et al., 2003; Wittchen, Zhao, Kessler & Eaton, 1994a).

GAD is a chronic affective disorder associated with functional disablement and the prevalence and severity statistics are under-represented in community reports. Several reasons have been raised for this epidemiological gap in GAD: poor diagnostic validity, major changes to hallmark diagnostic criteria, misdiagnosis as common comorbidity such as major depression or social phobia, priority attendance given to comorbid disorders involving mortality risk and lack of attention given to symptoms such as cognitive or psychological distress by the patient and/or practitioner (Baldwin et al., 2010b; Barlow & Wincze, 1998; Bystritsky, Khalsa, Cameron, & Schiffman, 2013; Dugas, Marchand, & Ladouceur, 2005; Stein & Sareen, 2015).

2.3 Definition and Guiding Symptomatology

Numerous contributing factors support the development and trajectory of GAD - biochemical, neurobiological, genetics, environment, stress vulnerability and

psychological profile, among many others. However, GAD is defined through cognitive and somatic symptomatology in clinical and primary care settings. Guidelines via the current Diagnostic and Statistical Manual of Mental Disorders, Edition 5 (DSM-5; American Psychiatric Association, 2013) define leading symptoms of GAD as excessive chronic worry and anticipatory anxiety with the presence of ancillary symptoms such as sleep disturbances, restlessness, edginess/irritability, fatigue, concentration difficulties and muscular tension (DSM-5, 2013; refer Table 1).

Table 1. Generalised Anxiety Disorder Diagnostic Criteria as per Diagnostic and Statistical Manual Edition 5 section 300.02 (F41.1) (DSM-5)

Defining symptoms	Excessive anxiety and worry (apprehensive expectation), occurring more days than not for at least 6 months, about a number of events or activities (such as work or school performance)	The individual finds it difficult to control the worry
The anxiety and worry are associated with three (or more) of the following six symptoms (with at least some symptoms having been present for more days than not for the past 6 months) Note: Only one item is required in children		
Restlessness or feeling keyed up or on edge	Being easily fatigued	Difficulty concentrating or mind going blank
Irritability	Muscle tension	Sleep disturbance (difficulty falling or staying asleep, or restless, unsatisfying sleep)
Other conditions for diagnosis:		
The anxiety, worry or physical symptoms cause clinically significant distress or impairment in social, occupational or other important areas of functioning		
The disturbance is not attributable to a medication condition or substance		
The disturbance is not better explained by another mental disorder:		
- anxiety or worry about having panic attacks in panic disorder		
- negative evaluation in social anxiety disorder [social phobia]		
- contamination or other obsessions in obsessive-compulsive disorder		
- separation from attachment figures in separation anxiety disorder		
- reminders of traumatic events in post-traumatic stress disorder		
- gaining weight in anorexia nervosa		
- physical complaints in somatic symptom disorder, perceived appearance flaws in body dysmorphic disorder		
- having a serious illness in illness anxiety disorder		
- the content of delusional beliefs in schizophrenia or delusional disorder		

Source: APA (2013).

The precise criteria for GAD have changed somewhat in the past 30 years, chiefly within the type, frequency, intensity and severity of ancillary symptoms. GAD became a category in the DSM-III (with several revisions after DSM-III-R and DSM-IV), in line with

the disorder moving from a residual category following elimination of other affective disorder-specific categories to a defined cluster of symptoms with anticipatory anxiety and persistent worry as hallmark symptoms for diagnosis; see Table 2 for changes across DSM editions (APA, 2013; Hunt, Issakidis, & Andrews, 2002; Kessler, Keller, & Wittchen, 2001; Rickels & Rynn, 2001; Schienle, Ebner, & Schafer, 2011; Stein, 2005).

Table 2. Shift in Diagnostic Criteria for Generalised Anxiety Disorder (GAD)

Criterion	DSM-III (APA, 1980)	DSM-III-R (APA, 1987)	DSM-IV (APA, 1994)	DSM-5 (APA, 2013)
Anxiety	Persistent anxiety	Unrealistic or excessive anxiety and worry (apprehensive expectation) about more than 2 life areas	Excessive anxiety and worry (apprehensive expectation) about numerous events; worry difficult to control	Excessive anxiety and worry (apprehensive expectation), worry difficult to control
Duration	1 month	6 months	6 months	More days than not for 6 months
Ancillary symptoms	Unspecified, from 3 of 4 categories	> 6 of 18 categories	> 3 of 6 specific symptoms	> 3 of 6 specific symptoms
Symptom categories	Apprehensive expectation; vigilance; motor tension; autonomic	Vigilance (5); motor tension (4); autonomic (9);	Restlessness; mental tension; fatigue; poor concentration; irritability; muscle tension; sleep disturbances	Restlessness; mental tension; fatigue; poor concentration; irritability; muscle tension; sleep disturbances
Associated features	Mild depressive symptoms	Mild depressive symptoms	Depressive symptoms; somatic symptoms; muscle tension; startle response	Depressive symptoms; somatic symptoms; muscle tension; startle response
Impairment in social and occupational function	Rarely more than mild	Rarely more than mild	Significant distress or impairment	Significant distress or impairment
Exclusions	Not due to another disorder e.g. depression or schizophrenia	Anxiety and worry unrelated to another mental disorder; not occurring during mood or psychosis disorder; not related to medical condition, medication or substance abuse	Anxiety and worry unrelated to another mental disorder; not occurring during mood or psychosis disorder; not related to medical condition, medication or substance abuse	Anxiety and worry unrelated to another mental disorder; not occurring during mood or psychosis disorder; not related to medical condition, medication or substance abuse

2.4 Course and Clinical Features

In primary care and clinical settings, the course of GAD appears to be chronic with a waxing and waning cycle, whereby the frequency, intensity and severity of symptoms are sub-threshold over months or years, with an increasing vulnerability of moving to an acute symptomatic level, that is, threshold GAD, via an increase in symptom frequency and intensity (Angst et al., 2009; Culpepper, 2002; Rickels & Rynn, 2002).

Notably, in typical presentations most patients seek help following the appearance of physical symptoms, typically insomnia, gastrointestinal conditions or headaches (Stein & Sareen, 2015; Stein et al., 2005) and adult patients commonly report the existence of symptoms for several years before seeking treatment (Keller, 2002; Yonkers, Warshaw, Massion & Keller, 1996).

Patient descriptions of cognitive/emotional symptoms in typical care settings include statements of feeling anxious or worried over a number of life situations, which are excessive or out of proportion to the identified situation. With regard to neurological symptoms, patients often report experiences of light-headedness or concern over fainting. When performing normal tasks, patients often report restless fatigue that is mental and/or physical with an inability to relax and difficulties in concentrating or maintaining focus. They may report an inability to relax or 'switch off' and may present with shakiness and/or sweaty palms.

The patient may report physical symptoms such as an elevated heart rate and/or difficulty gaining breath or swallowing ('dry' or 'lumpy' throat). Sleep quality disturbances are commonly reported, with broken cycles and periods of wakefulness and/or early wakening. Finally, but not definitively, gastrointestinal and/or genitourinary issues are reported; patients can present with stomach upset and/or irritable bowel and frequent urination, and issues with libido (Kessler et al., 2001; Rickels & Rynn, 2001; Wittchen et al., 1994a).

2.5 Prevalence and Epidemiology

Anxiety disorders result in marked societal suffering and economic costs (Baxter, Scott, Vos, & Whiteford, 2013; McEvoy, Grove, & Slade, 2011; Waghorn & Chant, 2007; Wittchen et al., 2011). The Australian Bureau of Statistics National Survey of Mental Health and Wellbeing in 2007, using the DSM-IV guidelines, found 1 in 7 (14.4%) of Australians aged 16–85 years had reported anxiety disorder symptoms and of these 14% reporting annually, 2.7% were diagnosed as GAD. The lifetime prevalence in Australia is

reported as 6% of the population reporting GAD, with GAD most likely to occur between the ages of 16 and 54 years, with a 50% higher incidence in females than in males (Australian Bureau of Statistics, 2007; Hettema, Prescott and Kendler, 2001).

There is consensus that life stressors are predictors of vulnerability to GAD, with increased risk associated with divorcee/separated/widowed marital status, racial and sexual minority groups, or of socio-economic status, where within the latter, employment status, access to treatment options and education opportunities are all evidenced as risk factors (Hunt et al., 2002; Kessler et al., 2001).

With regard to GAD among other affective disorders, the most prevalent in Australia are post-traumatic stress disorder (PTSD) (6.4%), social phobia (social anxiety disorder (SAD); 4.7%), agoraphobia (2.8%), panic disorder (2.6%) and obsessive-compulsive disorder (OCD, 1.9%). Within the mood disorders, the prevalence of depression is 6.2%, with unipolar depressive episodes 4.1%, dysthymia 1.3%, and bipolar disorder 1.8% (ABS, 2007).

Qualitatively for GAD, the data reveal high or very high psychological distress, e.g. on measures such as the Kessler Psychological Distress Scale (K-10; Kessler et al., 2002) it is reported as a prevalent symptom in 53.2% of patients with the condition (ABS, 2007; McEvoy et al., 2011).

2.6 GAD and Comorbid Disorders

In line with diagnostic guidelines and trends in primary care settings, a high incidence of comorbidity is reported in the presence of GAD (Noyes, 2001; Wittchen, 1994b). Epidemiological studies converge on data that approximately 50% of patients with 6–12 month prevalence of GAD will exhibit a comorbid mood (namely depression or dysthymia), state anxiety (such as PTSD and phobias) or psychiatric disorder (substance dependence or personality disorder) condition (Allgulander et al., 2003; Castle, 2008; Roy-Byrne, 1996; Stein and Sareen, 2015).

For medical comorbidities presenting with GAD, gastroenterological and gynaecological symptoms are the most prevalent (15% and 10%, respectively) (Kessler et al., 2001; Wittchen et al., 2011). The majority of GAD epidemiological and systematic reviews have identified the high diagnostic comorbidity of GAD with major depressive disorder (MDD), likely due to overlap in the high trait negative affect (Hettema et al., 2006; Hunt et al., 2002; Kessler et al., 2001; Mogg, Millar, & Bradley, 2000; Watson, Clark, & Carey, 1988). A US National Comorbidity Survey reported over 90% of

diagnosed GAD had a comorbid diagnosis of depression (40%–70%), dysthymia (22%) or other mood disorders (Kessler, Chiu, Demler, and Walters, 2005). Similar findings were observed in The Harvard Brown Anxiety Research Initiative, where proportionately, just 17% ‘pure GAD’ prevalence was reported in the GAD cases (Weisberg, Beard, Dyck, & Keller, 2012).

Few studies have directly assessed symptom differences between pure GAD and comorbid sample groups, either delineating both psychiatric and/or neurobiological differences between them and/or addressing the influence on GAD severity, trajectory and/or treatment response. Most previous research has used *a priori* categorised clinical groups. Despite this, research from neuroimaging studies suggests different biological substrates (examined further in Chapter Four).

Epidemiological studies are plentiful (e.g. Australian prevalence: McEvoy et al., 2011). In a prospective epidemiological study (Bruce, Machan, Dyck, & Keller, 2001) a GAD sample group (n = 179) was examined and tracked for existing and developing comorbidities over eight years and reported that between one- and two-thirds of participants had or developed definable comorbidities, and there were very few pure GAD cases (5%) reported. A second related study involved child prevalence (aged 6–11 years) comparing pure and comorbid GAD and healthy controls (n = 41) and found increased functional disablement in the GAD/comorbid group (Yonkers, Bruce, Dyck and Keller, 2003). The data suggested pure GAD participants were more likely to be younger and male, with the environment playing a role in GAD/comorbid-associated factors.

Furthermore, the risk factors are different; with one longitudinal study (Moffitt et al., 2007; n = 1037) comparing GAD, MDD and comorbid GAD/MDD to assess 13 risk measures from domains of family history, childhood behaviour, adolescent self-esteem, and personality traits. Risk factors were unique to each, with commonalities in GAD/MDD and pure GAD groups in adverse family environments (e.g. low socio-economic status, somewhat more maltreatment) and childhood behaviour (e.g. internalising problems, conduct disorders, inhibited temperament). GAD/MDD was most severe, whereby it had earlier onset, more recurrence, greater use of medication and mental health services, and generally elevated risk factor levels across all 13 domains.

The significance here is the effect on appropriate diagnosis and treatment of GAD, where often there is prioritisation in treating the presentations of more acute or intense cognitive or physiological symptoms such as depression, insomnia and suicidal thoughts

that are present with MDD and depression disorders, which in turn is likely to affect diagnosis and treatment (Kessler et al., 2005; Starcevic, 2015).

A prominent debate in terms of GAD diagnosis in the field of psychiatry is its diagnostic reliability (refer to upcoming Section 2.7.2). Kessler, DuPont, Berglund and Wittchen (1999), among others, asserted that GAD should be subsumed under MDD as a subsidiary disorder, suggesting it may be a comorbid status disorder for MDD. Much debate surrounds the definition of MDD, with it being heterogeneous, with long overdue sub-classifications and for which GAD is a proposed subtype. The authors also discussed the feasibility that GAD/MDD is in itself a distinct condition separate from pure cases of each.

2.7 GAD Diagnosis in Primary and Clinical Care Settings

The measurement of GAD in primary care settings involves diagnostic and assessment (such as for symptom trajectory) instruments. The first aim is to promote uniformity and replicability in diagnosis. The second aim is to adequately identify factors affecting symptoms and encourage broad lifestyle assessment for optimal interventions. Despite such intentions, the challenges and limitations of effective GAD diagnosis and treatment using implemented guidelines in clinical settings remains, but a deeper discussion is beyond the scope of this thesis, although the reader is invited to consult the following publications: Baldwin et al (2014), Barlow and Wincze (1998), and Starcevic (2015).

The steps for assessment of GAD are illustrated in Table 3, and are derived from guidelines from National Institute for Health and Care Excellence (2011b), and the World Health Organization: Management of Mental Disorders (1997).

Table 3. Example of the Stepped-Care Approach for Generalised Anxiety Disorder Management

1. Assessment
Symptom constellation, symptom/illness course, treatment history
Lifestyle components - sleep hygiene, life stressors, caffeine use, use of alcohol, substances past and present
Administer test battery/ies of relevance
Comorbid conditions for treatment e.g. depression/mood, suicidality
Physiological conditions e.g. hyperthyroidism
2. Intervention
Education about GAD
Lifestyle strategies
Cognitive/Behavioural strategies

Pharmaceutical strategies
3. Monitoring
Reassessment of strategies from Step 2

2.7.1 Instruments for assessment

The psychometric assessment of GAD in primary/clinical care and research settings are generally administered in a self-reporting or semi-structured interview format.

Screening questionnaires are utilised to assess the condition and make a diagnosis or treatment decision. These questionnaires can delineate comorbid disorders that are common with symptom presentation (such as depression). Treatment in a primary care setting is directed at immediate functioning and acute symptoms, whereas long-term treatment of psychiatric disorders requires more complex approaches. Treatments for GAD are discussed in more detail in Chapter Five.

Knowledge of the sensitivity, specificity and clinical utility of these scales is important for diagnosing GAD. For example, the reliability and validity of the GAD-7 instrument that is commonly used in care settings have been cross-examined in a healthy population (Löwe et al., 2008) to establish normative data, and thus increase construct validity. In contrast, the K-10 instrument (Kessler et al., 2002), which is used extensively in primary care settings particularly in Australia, has yet to be validated or tested in a confirmatory fashion for use in diagnosing GAD, as is the case for several other similar instruments. For a summary of instruments typically utilised for GAD diagnosis refer to Table 4.

Table 4. Psychometric Instruments for the Diagnosis and Management of GAD

Instrument	Utility	Frame	Factors	Method	Analysis	Reference
Generalised Anxiety Disorder 7 (GAD-7)	Screening, severity of GAD	7 question modified PHQ, 4-point Likert of frequency	Sum score < 5: Low 5–10: moderate 10–15: High	Self-report	Sensitivity of 89% and a specificity of 82% for GAD (Swinson, 2006)	Spitzer, Kroenke, Williams, & Lowe (2006)
Penn State Worry Questionnaire (PSWQ)	Worry as trait	16 items, 5-point Likert	Sum score 16–39: Low 40–59: Moderate 60–80: High	Self-report	High internal consistency and good test-retest reliability (Brown, Antony, & Barlow, 1992)	Meyer, Miller, Metzger, & Borkovec (1990)

					but may be two-factorial (Hazlett-Stevens et al., 2004)	
Beck Anxiety Inventory (BAI)	Anxiety symptom frequency past week, cognitive and physical	21 items, 4-point Likert	Sum score Minimal: 0–7 Mild:8–15, Moderate: 16–25, Severe: 30–63; four factors	Self-report	Internal consistency, convergent and discriminant validity (Osman et al., 2002)	Beck, Epstein, Brown, & Steer (1988)
Kessler Psychological Distress Scale (K-10)	Symptom frequency from 4 weeks, confirmatory	10 item, 5-point Likert	Sum score < 20: well; 20–24 mild; 25–29 moderate; > 30 severe	Self-report	Cross-cultural validated but not in GAD (Furukawa et al., 2003)	Kessler et al. (2002); Andrew & Slade (2001)
Clinical Global Impression Severity Scale (CGI-S)	Symptom severity: global improvement or change, therapeutic response	3 item, 2 seven-point severity scales and efficacy index	Qualitative assessment by clinician	Clinician observation	Not validated in GAD	Busner & Targum (2007); Guy (1976); Stein, Fincham et al. (2009)
DASS - Depression anxiety symptom scale; also DASS-21	Depression anxiety, stress factors, past week	3 x 14 item scales (42 items), also DASS-21 item/3 factor	2–5 item subscales, sum scores 7–9 mild 10–14 moderate 15–19 severe 20–42 extremely severe	Self-report	Good discriminant validity, and differentiating anxiety disorders (Brown, Chortipta et al., 1997)	Lovibond & Lovibond (1995)
The Hamilton Anxiety Rating Scale [HAM-A]/Structured Interview Guide for the Hamilton Anxiety Scale (SIGH-A)	Anxiety cognitive and somatic symptoms frequency severity and intensity	14 items, 5-point Likert scale	Sum score <17 mild 18–24 mild to moderate severity 25–30 moderate to severe	Clinician structured interview	Good validity, internal consistency and inter-rater reliability (Shear, 2001)	Hamilton (1959; 1969)

Spielberger State-Trait Anxiety Inventory	State (Y-1) and trait (Y- 2) anxiety instruments	20 items each, 4- point Likert	Sum scores, T- score standard ranges grouped by age	Self-report	Internal consistency .86 to .95; test-retest reliability coefficients .65 to .75 (Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983)	Spielberger, Gorsuch, Lushene, Vagg, Jacobs, (1968; 1977)

2.7.1.1 *Hamilton anxiety rating scale (HAM-A) for the assessment of GAD*

The Hamilton Anxiety Rating Scale (HAM-A) and the Structured Interview Guide for the Hamilton Anxiety Scale (SIGH-A) (Hamilton, 1959 ; Shear et al., 2001) are related instruments for measuring anxiety severity. The aim of the original questionnaire was to measure the quantitative symptoms of cognitive and physiological states related to broad anxiety neurosis and later pertaining to GAD. The SIGH-A was subsequently developed with the aim of greater detail and precision in questioning and scoring. Both instruments are considered to hold good validity, internal consistency and inter-rater reliability (Maier, Buller, Philipp, & Heuser, 1988; Shear et al., 2001).

The SIGH-A, or HAM-A as it is generally referred to, is a structured clinician-rated scale and is based on 14 parameters, including anxious mood, tension, fears, insomnia, somatic complaints and behaviour at the interview. Frequency, intensity and severity of symptoms are quantified. Each item is rated on a 5-point scale, ranging from 0 (not present) to 4 (severe). Total scores can range between 0 and 56, where < 17 indicates mild severity, 18–24 indicates mild to moderate severity and 25 and above indicates moderate to severe.

In clinical trial settings, where the HAM-A/SIGH-A is usually accompanied by broader psychiatric assessment instruments such as the Mini-International Neuropsychiatric Interview (M.I.N.I. 6.0; Sheehan et al., 1998) and the Structured Interview Guide for the Montgomery-Asberg Depression Rating Scale (SIGMA) (MADRS; Montgomery & Asberg, 1979; Williams & Kobak, 2008) for depression to account for the existence and intensity of comorbid conditions such as social phobia or where symptoms may be better subsumed under alternate symptomology.

In this setting, the HAM-A assesses anxiety severity symptomatology initially as a screener; determining level of anxiety at baseline (typically low levels of anxiety [SIGH-A

< 18] being excluded) and assess changes in anxiety over follow-up assessment sessions.

Table 5 provides a version of the HAM-A derived from the SIGH-A line structured interview.

Table 5. Structured Hamilton Anxiety Rating Scale (SIGH-A/HAM-A)

STRUCTURED HAMILTON ANXIETY RATING SCALE (SIGH-A) (HAMILTON, 1959)	
<i>Instructions.</i> I will now ask you a number of questions about different aspects of your anxiety. This will help us learn more about the anxiety you experience.	
What has your mood been like this past week? - Have you been anxious/nervous? - Have you been worrying? - Feeling something bad may happen? - Feeling irritable?	1. ANXIOUS MOOD 0 No anxious mood 1 Mild worry or anxiety indicated only on questioning; no change in functioning 2 Preoccupation with minor events, anxiety on as many days as not 3 Near daily episodes of anxiety/worry with disruption of daily activities; daily preoccupation
Have you been feeling tense? - Do you startle easily? - Cry easily? - Easily fatigued? - Have you been trembling or feeling restless or unable to relax?	2. TENSION 0 No tension 1 Several days of mild tension or occasional (e.g., 1-2) episodes of exaggerated startle or labile mood 2 Muscle tension or fatigue 50% of the time, or repeated (> 2) episodes of trembling, exaggerated startle etc 3 Near daily muscle tension, fatigue, and/or restlessness > 75% of the time or persistent, disruptive symptoms 4 Constant tension, restlessness, agitation, unable to relax in the interview
Have you been feeling fearful (phobic) of situations or events? - For example, have you been afraid of: - The dark? - Strangers? - Being left alone? - Animals? - Being caught in traffic? - Crowds? - Other fears?	3. FEARS 0 No fears 1 Mild phobic concerns that do not cause significant distress or disrupt functioning 2 Fears lead to distress or avoidance on one or more occasions 3 Fears are an object of concern on a near daily basis (75%); patient may need to be accompanied by others to a fearful event 4 Fears or avoidance that markedly affect function. Patient may avoid multiple situations even if accompanied; extensive agoraphobia
How has your sleeping been this week? - Any difficulties falling asleep? - Any problems with waking during the night? - Any problems with waking early and not being able to return to sleep? - Do you feel rested in the morning? - Do you have disturbing dreams or nightmares?	4. INSOMNIA 0 No sleep disturbance 1 Mildly disrupted sleep (e.g., one to two nights of difficulties falling asleep or nightmares) 2 Several episodes of sleep disturbance that is regular but not persistent (e.g., over one half hour falling asleep, or excessive AM fatigue) 3 Persistent sleep disruption (more days than not), characterised by difficulty falling (e.g.m over one hour) or staying asleep, restlessness, unsatisfying sleep or frequent nightmares or fatigue) 4 Nightly difficulties with sleep onset or maintenance, or daily severe fatigue on waking in the AM
Have you had trouble concentrating or remembering things?	5. INTELLECTUAL (COGNITIVE) 0 No difficulties 1 Infrequent episodes of forgetfulness or difficulty concentrating that are not distressing to the patient 2 Recurrent episodes of forgetfulness or difficulty concentrating, or episodes of sufficient intensity to cause the patient recurrent concern 3 Persistent concentration or memory impairment interferes with daily tasks 4 Significant role impairment due to concentration difficulties
Have you been feeling depressed? - Have you lost interest in things? - Do you get pleasure from friends or hobbies?	6. DEPRESSED MOOD 0 No depression 1 Occasional or mild blue or sad mood, or reports of decreased enjoyment of activities 2 Sad or blue mood or disinterest 50% of the time, mood does not generally interfere with functioning 3 Persistent mood or loss of pleasure, mood is significantly distressing to the patient or may be evident to others 4 Daily evidence of severe depression with significant role impairment

Have you been experiencing aches, pains, or stiffness in your muscles? - Have you been experienced muscle twitching or sudden muscle jerks? - Have you been grinding your teeth? - Have you had an unsteady voice?	7. SOMATIC (MUSCULAR) 0 No muscular symptoms 1 Infrequent presence of one or two symptoms, no significant distress 2 Mild distress over several symptoms or moderate distress over a single symptom 3 Symptoms occur on more days than not, symptoms are associated with moderate to severe distress and/or regular attempts at symptom control by limiting activities or taking medications 4 Daily or near daily episodes of symptoms that cause the patient significant distress and lead to restriction of activities or repeated visits for medical attention
Have you been experiencing ringing in your ears, blurred vision, hot or cold flashes, feelings of weakness, or prickling sensations? <i>Has this occurred at times other than during a panic attack?</i>	8. SOMATIC (SENSORY) 0 No symptoms 1 Infrequent presence of one or two symptoms, no significant distress 2 Mild distress over several symptoms or moderate distress over a single symptom 3 Symptoms occur on more days than not, symptoms are associated with moderate to severe distress and/or regular attempts at symptom control by limiting activities or taking medications 4 Daily or near daily episodes of symptoms that cause the patient significant distress and lead to restriction of activities or repeated visits for medical attention
Have you had episodes of a racing, skipping or pounding heart? How about pain in your chest or fainting feelings? <i>(Has this occurred at times other than during a panic attack?)</i>	9. CARDIOVASCULAR SYMPTOMS 0 No symptoms 1 Infrequent presence of one or two symptoms, no significant distress 2 Mild distress over several symptoms or moderate distress over a single symptom 3 Symptoms occur on more days than not, symptoms are associated with moderate to severe distress and/or regular attempts at symptom control by limiting activities or taking medications 4 Daily or near daily episodes of symptoms that cause the patient significant distress and lead to restriction of activities or repeated visits for medical attention
Have you been having trouble with your breathing? For example, pressure or constriction in your chest, choking feelings, sighing or feeling like you can't catch your breath? <i>(Has this occurred at times other than during a panic attack?)</i>	10. RESPIRATORY SYMPTOMS 0 No symptoms 1 Infrequent presence of one or two symptoms, no significant distress 2 Mild distress over several symptoms or moderate distress over a single symptom 3 Symptoms occur on more days than not, symptoms are associated with moderate to severe distress and/or regular attempts at symptom control by limiting activities or taking medications 4 Daily or near daily episodes of symptoms that cause the patient significant distress and lead to restriction of activities or repeated visits for medical attention
Have you had any difficulties with stomach pain or discomfort? - Nausea or vomiting? - Burning or rumbling in your stomach? - Heartburn? - Loose bowels? - Constipation? - Sinking feeling in your stomach? <i>(Has this occurred at times other than during a panic attack?)</i>	11. GASTROINTESTINAL SYMPTOMS 0 No symptoms 1 Infrequent and minor episodes of gastric discomfort, constipation, or loosening of bowels, fleeting nausea 2 An episode of vomiting or recurrent episodes of abdominal pain, loosening of bowels, difficulty swallowing 3 Symptoms more days than not that are very bothersome to the patient or lead to concerns over eating, bathroom availability, or use of medication 4 Daily or near daily episodes of symptoms that cause that cause the patient significant distress and lead to restriction of activities or visits for medical attention

Have you been experiencing urinary difficulties? - For example, have you had: - To urinate more frequently than usual? - More urgency to urinate? - Decreased sexual interest? - FOR WOMEN - Have your periods been regular? - Have you experienced a change in your ability to have an orgasm? - FOR MEN - Have you had trouble maintaining an erection?	12. GENITOURINARY SYMPTOMS 0 No symptoms 1 Infrequent and minor episodes or urinary symptoms or mild changes in sexual interest 2 Urinary symptoms several days during the week, occasional difficulties with sexual function 3 Urinary or sexual symptoms more days than not, amenorrhea 4 Daily urinary or sexual symptoms that lead to distress and medical care seeking
Have you been experiencing flushing in your face? Getting pale? Light-headedness? - Have you been having tension headaches? - Have you felt the hair rise on your arms, the back of your neck or head, as though something had frightened you? <i>(Has this occurred at times other than during a panic attack?)</i>	13. AUTOMATIC SYMPTOMS 0 No symptoms 1 Mild symptoms occurring infrequently 2 Symptoms occurred several times during the week and were bothersome 3 Near daily symptoms with distress or embarrassment about the symptoms 4 Daily symptoms that are a focus of distress and impair function (e.g., daily headaches or light-headedness leading to limitation of activities)
Rate interview behaviour - Fidgeting - Restlessness or pacing - Tremor of hands - Furrowed brow - Strained face - Sighing or rapid respirations - Facial pallor - Frequent swallowing	14. BEHAVIOUR AT INTERVIEW 0 No apparent symptoms 1 Presence of one or two symptoms to a mild degree 2 Presence of several symptoms of mild intensity or one symptom of moderate intensity 3 Persistent symptoms throughout the interview 4 Agitation, hyperventilation, difficulty completing the interview
HAMILTON ANXIETY RATING SCALE TOTAL SCORE	

Source: Hamilton (1959)

2.7.2 Diagnostic reliability

Reliability of a diagnosis is a crucial factor underpinning diagnostic validity in psychiatric disorders (Robins & Guze, 1970) and the reliability of GAD as a diagnosis is poor (Portman, Starcevic, & Beck, 2011; Rutter & Brown, 2015). Diagnostic field trials in psychiatric practice measure the reliability of the psychiatric disorder category with minimal information, effectively one patient interviews (Freedman et al., 2013).

The Research Diagnostic Criteria studies (refer to overarching publication Barlow & Wincze, 1998) were a culmination of several DSM-5 field trials (as well as earlier versions) on at least 20 moods, affective and personality disorders and reported a kappa statistic as a test of reliability (Clarke et al., 2013; Narrow et al., 2013; Regier et al., 2013). The kappa estimate was calculated from the likelihood of the same diagnosis from two clinicians 85% of the time, proportional to prevalence, producing a weighted composite statistic. Field trial results found the most reliable psychiatric disorders were major neurocognitive disorders (0.78), PTSD (0.67) and borderline personality disorder (0.54). GAD was placed on the lower end (0.20), just below antisocial personality disorder – refer to Figure 1.

Results from field trials have in the past served to refine psychiatric disorder categories between DSM versions, prompting debate around the less-reliable categories. Major depressive disorder has been proposed as too broad, and GAD asserted to be more

appropriate as an MDD sub-category; and finally that Mixed Anxiety-Depressive Disorder as a recent ‘compensation’ category may still yet be insufficient to encompass the multiple variations in symptoms and medication response (Fava et al., 2008; Möller et al., 2016; Figure 1). Ultimately, these diagnostic issues do little to support GAD as a distinct disorder to adequately explain the neurobiological, symptomatic and treatment efficacy profiles of the disorder, and to discriminate it amongst affective disorders. A focus on neurobiological markers as evidence may provide support for GAD, and is examined in depth in the next chapter.

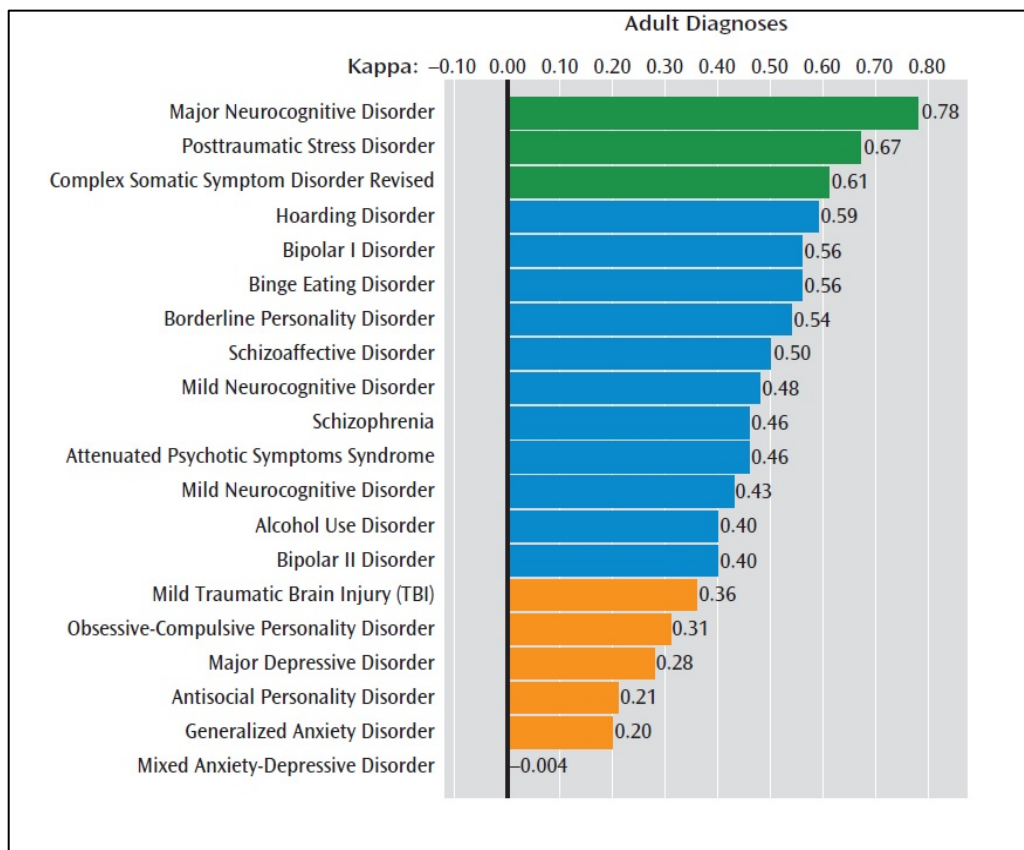


Figure 1. Inter-rater reliability kappa estimates of selected psychiatric disorders from DSM-5 field trials (Adapted from Freedman et al., 2013)

2.8 Cognitive Models Underpinning GAD Symptoms

In investigating the cognitive processes unique to GAD in research settings, it is necessary to construct experimental replication of anxiety cognitions experienced during this state, the re-construction of which depends upon cognitive-emotional

models derived from background theories of both GAD and anxiety trait/state cognitions (Cattell, 1963; Cattell & Scheier, 1961).

While the aim in employing these paradigms is to best replicate regional brain patterns involved in GAD symptoms to meet experimental outcomes, there are limits to the presupposition of a discrete regional function with a singular role demonstrated by a simple paradigm. This type of conclusion of a neurobiology-cognition relationship likely explains equivocal research reporting across studies of similar methodologies and outcome measures. This point is explored further in Chapter Three as a consideration in biomarker utility, particularly in imaging research (Bandelow, Michaelis, & Wedekind, 2017; Etkin & Wager, 2007; Poldrack, 2011).

Despite a simplified inferential approach, in all functional imaging experimental designs, at least one *a priori* model of cognition is employed to quantify neurobiological measurement underpinning regional brain processing (Forstmann, Wagenmakers, Eichele, Brown, & Serences, 2011; Ochsner, Silvers, & Buhle, 2012; Wilkinson & Halligan, 2004). However, without repeated testing of validity and reliability via prospective studies and also across other mood and affective disorders beyond GAD, cognitive theories unique to GAD lack solid evidence (as detailed in a salient discussion by Aikins & Craske, 2001). A critical evaluation of the cognitive, emotional and integrated theoretical models applied in GAD investigations is beyond the capacity of this thesis, and thus a summary is provided here to acknowledge the roles in anxiety neuro-cognition, and thus imaging research, as well as providing context for the functional imaging cognitive model that is applied in the two clinical investigations in Chapters Six and Seven.

There is consensus that GAD is comparatively less well-studied than other affective disorders despite its prevalence. One reason for this difficulty being the complex and evolving nature of cognitive models that serve to best define altered cognitive function in diagnosed GAD (Etkin & Wager, 2007; Mochcovitch, da Rocha Freire, Garcia, & Nardi, 2014; Williams et al., 2016).

In this context, cognitive processes such as the intolerance of uncertainty, negative bias, false beliefs about worry and cognitive avoidance, have all held a determinant role in the diagnosis and maintenance of GAD both in clinical environments and research

settings, whereby anxiety cognitions are primarily characterised by biases in attention and interpretation (Gosselin & Laberge, 2003; Rickels & Rynn, 2001).

The simplest and most encompassing of GAD mental phenomena (and likely the most enduring given the chronic cyclical nature of GAD as outlined in the previous section) are observed cognitive hallmarks of persistent worry and anticipatory anxiety, and a corollary factor, emotional regulation, which is typically coupled to uncertainty tolerance (Behar, DiMarco, Hekler, Mohlman, & Staples, 2009; Schienle, 2011).

To quantify symptoms using instruments such as the HAM-A measure detailed in the previous section, patients must meet at least moderate levels (i.e. score of 2 or above) on item 1 that directly pertains to persistent worry/anticipatory anxiety and item 2 that relates to cognitive (and, to a degree, somatic) levels of edginess, tension, irritability and/or restlessness.

Within the literature, there exists at least three models that emphasise different yet inter-related components of cognitive-emotional processes observed in GAD. Of specific relevance to functional imaging investigations due to the number of task-dependent fMRI paradigms that support them are uncertainty tolerance, emotional dysregulation and/or avoidance models.

The Avoidance Model of Worry (Borkovec et al., 1983; 2004, Behar et al., 2009; Newman & Llera, 2011) implies that the action of worry diminishes negative emotion, fears or anxieties through the rumination of potential scenarios and attempt at problem-solving; however, the frequency and severity is ultimately non-constructive, and limiting in cognitive flexibility or 'free-thinking'. As a result of this cognitive inflexibility, there is reduced emotional processing and avoidance of engagement. The avoidance model consequently also encompasses emotional dysregulation and increased intolerance to uncertainty. Worry is negatively reinforced as a substitute cognition and reinforced by beliefs of a necessary function; 'worrying about worrying' (Borkovec et al., 2004; Stapinski, Abbott, & Rapee, 2010).

The Intolerance of Uncertainty Model (Dugas, Marchand, et al., 2005; Freeston, Rhéaume, Letarte, Dugas, & Ladouceur, 1994), reflects the difficulty in GAD of experiencing uncertain or ambiguous situations. Similar to the Avoidance model, persistent or excessive worry is related to the intolerance of uncertainty, whereby it is a cognitive strategy to gain control over unpredictable situations or unknown outcomes.

The model also incorporates ‘negative bias’ such that appraisal is geared to focus on the negative, erroneous or potentially harmful aspects of a situation, which may be part of survival thinking, but produces bias in assessing situations, as problems are perceived as threats and outcome expectations are negative (Mathews & Macleod, 1985).

In investigative paradigms, gambling tasks are often employed to replicate uncertainty tolerance conditions (e.g. Ladouceur, Gosselin, & Dugas, 2000) and plentiful studies evidence the bias in GAD samples to threat-related imagery in experimental paradigms (e.g. Mogg et al., 2000; for a meta-analysis of studies see Armstrong & Olatunji, 2012); however, negative bias is not unique to GAD, it is also evidenced in other affective and mood disorders (Bar-Haim, Lamy, Pergamin, Bakermans-Kranenburg, & van, 2007; Peckham, McHugh, & Otto, 2010).

Finally, a model of emotional dysregulation is defined through reductions to positive emotions and an increase in negative emotions, such as fear, that, in turn, affect cognitive and behavioural appraisals and outcomes (Hofmann, Sawyer, Fang, & Asnaani, 2012; Mennin, Heimberg, Turk, & Fresco, 2005). Further, the accurate appraisal of situations is reduced, tending to be negative and emotional reactivity is increased.

This model converges on worry as an avoidance strategy because emotional reactivity is arguably an important precursor for constructive thought and development and maintenance of ‘mental fluidity’, which is a hallmark of emotional intelligence (Mayer and Salovey, 1997; Salovey & Mayer, 1990). However, emotional *hyperarousal* is experienced in GAD and in a more persistent and pervasive manner among the anxiety disorders (Decker, Turk, Hess, & Murray, 2008; Mennin, McLaughlin & Flanagan, 2009; Novick-Kline, Turk, Mennin, Hoyt, & Gallagher, 2005; Turk, Heimberg, Luterek, Mennin, & Fresco, 2005).

Further models of note include the Metacognitive Model (Wells, 1999) that delineates further the ‘worry as coping-mechanism’ from the avoidance model into positive beliefs as a prevention strategy and negative beliefs about worry similar to the avoidance model. Although greater insight is arguably concurrent to holding negative beliefs, acknowledging that a state of worrying is ‘out of control and excessive’—very common in GAD—but tends to result in less effective coping strategies characterised as thought-suppression, avoidance and reassurance-seeking behaviours (Behar et al., 2009).

Regarding the application of cognitive and emotional process models in research methodologies, a recent review by Mochcovitch et al. (2014) examined functional imaging studies employing the aforementioned three cognitive models in GAD sample

groups and the degree to which such models were effectively applied in functional magnetic resonance imaging (fMRI) studies. At the time of the review, there were 14 fMRI studies in adult GAD and eight child/adolescent studies employing such paradigms. The review supported the finding that emotional dysregulation is consistent with evidence for prefrontal and anterior cingulate cortices and amygdala connectivity dysregulation (this thesis examines in more detail the anterior cingulate region and its properties associated with anxiety cognitions in Chapter Four).

2.9 Anxiety State Task Paradigms Used in Functional Imaging

This section examines how GAD cognitive models are best operationalised in experimental design paradigms as an attempt to replicate anxiety cognitions or emotional processes in the form of worry induction, response inhibition and/or emotional reactivity to demonstrate attentional bias, as one example. Particular emphasis is given to the International Affective Picture System (IAPS; Lang, Bradley, & Cuthbert, 1997), as it is utilised in the experiments in the thesis investigations.

Common paradigms are emotional variants of the Stroop Task (Stroop, 1935), which was designed to measure response when presented with emotional words of different valence. Studies using the Stroop method predict emotional words to elicit differential responses to neutral words (Williams, Mathews, & McLeod, 1996). In GAD cases, there is an attentional bias towards negative cues (Mathews & Macleod, 1985; Mogg et al., 2000) and it may be feasible to differentiate between anxiety disorders depending upon variances in word type (GAD and Social Phobia: Becker, Rinck, Margraf, & Roth, 2001).

A second common paradigm is the use of 'emotion faces', in varying versions, to elicit emotional response, typically a set of approximately 150 facial expressions of specific emotions are posed and evoked by actors, usually balanced for gender and ethnicity (Gur et al., 2002). In GAD, there is a bias towards negative faces and to level of threat in the stimuli, which is positively correlated to the severity of anxiety (Mogg et al., 2000)

A third regularly utilised paradigm is the use of IAPS in various formats, but principally in studies aiming to utilise emotional reactivity or similar, depending upon research aims. The IAPS is a picture library of approximately 1000 images normatively rated along three scales of emotional affect. These scales are derived from the dimensional conceptualisation of emotional processing proposed by Wundt (1898) and later Osgood, Suci and Tannenbaum (1957). The scale dimensions are valence (pleasant through to unpleasant), arousal (calming through to exciting) and dominance (perception of control through to loss of control). The use of IAPS in studies of attention and emotion involves

the evaluation of pictures selected for their valence, arousal and control levels. The pictures are not readily accessible outside of the methodological research needs, to preserve the novelty of the pictures and maintain research efficacy.

IAPS pictures have been selected for rated levels on these scales via the administration of the Self-Assessment Manikin (Lang, 1980) on sample populations viewing and rating the pictures. The analysis of between-subject and within-subject reliability suggests internal consistency and reliability in picture rating. Picture rating creates a dimensional space, i.e. pictures rated as unpleasant are rated also along the scale of arousal, trending towards high levels, yet pictures rated as pleasant vary across arousal levels, from high to low.

Aversive versus neutral images to generate emotion anticipation have been used in a number of intervention studies in GAD patients. Utilising IAPS in GAD and healthy control groups, Nitschke et al. (2009) reported greater anticipatory activity in the amygdala for all stimuli in the GAD group compared with the controls. Second, in the intervention component of the study (eight weeks of venlafaxine in n=14 GAD patients) there were significantly higher levels of pre-treatment anterior cingulate cortical (ACC) activity in anticipation of both aversive and neutral pictures compared with data following treatment, and higher pre-treatment (venlafaxine) rostral anterior cingulate cortical (rACC) levels predicted greater improvements on the HAM-A scale in GAD patients compared with controls or baseline pre-treatment in GAD.

Leading on from this, a central goal of the current research in this thesis was to evaluate the role of anticipatory anxiety and persistent worry as GAD cognition hallmarks and to assess how these symptoms feasibly represent a broader encompassing of a cognitive behavioural model into neurobiological function within a suitable elicitation paradigm; ostensibly how properties of selected regions associate to GAD cognitions and modulations to such properties following treatment intervention. Further discussion in greater detail is addressed in this thesis in evidence for GAD markers in neuroimaging (Chapter Four) and in the two investigation chapters (Chapters Six and Seven).

2.10 Summary

The history of the diagnosis for GAD and, subsequently, the prevalence of reporting should be viewed in light of the temporal and cultural contexts that represent limitations in understanding GAD nosology. First, GAD has poor diagnostic validity, it has experienced several changes in classification (Barlow & Wincze, 1998), and ambiguous

guidelines risk misdiagnoses of 'outlier' presentations. The poor diagnostic validity of GAD has led to assertions that it is better grouped with MDD and mood disorders owing to symptom overlap and frequent comorbidity and, as such, 'pure GAD' may not be valid (Goldberg, 2010; Hettema et al., 2006).

GAD and MDD exhibit overlap; however, there are distinctions with the imaging literature suggesting biological differences, which are examined in subsequent chapters. However, the lack of reliability of the GAD diagnostic category has ramifications for biomarker research (such as imaging and genetic modalities) using GAD sample populations, which may confer a risk of measurement error in sample groups (Schienle et al., 2011).

There are no current guidelines developed within Australia for the treatment of GAD. Current stepped-care involves antidepressant and benzodiazepine pharmacotherapies, and cognitive behavioural therapy (CBT)—all of modest clinical effect and limited utility for many patients (Gunter & Whittal, 2010; Hoffman & Mathew, 2008; Weisberg, 2009).

Finally, psychiatric drug research is at a global standstill for anxiety disorders such as GAD, which warrants a two-fold need for investigating novel substances and optimising this translational research to individual patients. Before more intensively examining neurobiological markers for GAD, it is vital firstly to establish research criteria and discuss the challenges of biomarkers, which is the subject of the next chapter.

3.1 Overview

This section broadly examines the status of biomarker modelling for psychiatric disorder research, including important considerations and challenges. There is focus on neuroimaging biomarkers thus far evidenced in GAD, that represent *intermediate phenotypes*, or *endophenotypes*. Finally, the use of biomarkers in novel drug research for which there is a paucity in GAD is introduced, which is examined in greater depth in Chapters Four and Five.

3.2 Introduction

Biomarkers, an amalgam of *biological markers*, is a term strictly defined as molecular or gene-level biological measures of systemic state or disease characteristic (Hulka, 1990; Naylor, 2003). The use of biomarkers to distinguish disease or disorder states is not new. Physiological symptoms indicate many systemic states, pathological or otherwise, rather it is in their utility that evolves neurobiological ‘traits and states’ into significant keys for identification and subsequent treatment (Mayeux, 2004; Schulte, 1993). As such, biomarkers are increasingly used in research as functional measures of the biological bases of psychiatric and neurological disorders, as there is an unmet need for gold standard diagnostics in biological psychiatry (Bandelow & Michaelis, 2015; Kapur, Phillips, & Insel, 2012; Venkatasubramanian & Keshavan, 2016).

An important objective is identifying and validating biomarker signatures in GAD and operationalising these findings to offer advantages in the clinical setting for diagnostics, risk assessment and optimisation of stepped-care options: an essential consideration of translational capacity.

3.3 Importance of Biomarkers in Psychiatric Disorders

The World Health Organization’s report on biomarkers and biomonitoring (Aitio et al., 1993) defined biomarkers as ‘a measurable biochemical, physiologic, behavioural, or other alteration in an organism that, depending on the magnitude, can be recognised as associated with an established or possible health impairment or disease.’ As an extension, biomarkers are further defined via modality; from neuroimaging platforms of immune-histochemical staining through to functional imaging; and constrained by advances or limits in these technologies, including methodological approaches, in the

sense of how they are best applied in research (Drucker & Krapfenbauer, 2013; Mayeux, 2004).

Biomarkers have three areas of application in clinical settings: diagnosis and prognosis, patient management and drug/treatment assessment (Hayes, Robinson, Muller, & Wang, 2009; Mayeux, 2004). Biomarker research shifts the focus onto biological mechanisms of disease aetiology, maintenance and trajectory, rather than diagnosis and management through symptoms, and emphasis on symptom treatment. Diagnostically, there is potential to identify disorders before the onset of symptoms, and to identify subtle variations of a disorder or clinically meaningful sub-sets (Kapur et al., 2012; Fernandes et al., 2017b; Singh & Rose, 2009).

3.4 Establishing a Framework for Integrating Biomarker data into Clinical Diagnosis

Multiple agencies worldwide have developed guidelines with the aim of optimal characterisation and utilisation of biomarkers including the United States' Food and Drug Administration and National Institute of Health (NIMH), European Medicines Agency and the European Association for Predictive, Preventive and Personalised Medicine.

In the interest of producing a framework criterion for biomarker development, the NIMH report (2013) produced a statement on the validity of biomarker research in psychiatric disorders with the following defining points:

- Symptoms alone rarely indicate best choice of treatment;
- Cognition, emotion and behaviour are important co-factors;
- Diagnostic categories for psychiatric disorders are insufficient when considering biology;
- Data accumulated thus far is insufficient on conclusions of biomarker utility.

The initiative subsequently generated a paper on Research Domain Criteria (RDoC) framework (Sanislow et al., 2010; see also Cuthbert & Insel, 2013) that emphasised these points in an integrative modelling approach between behavioural neuroscience and psychopathologies. In this model, anxiety is subsumed under a 'Negative Valence System' for which 'units of analyses' are employed to inform a dimensional construct of a given disorder. These units comprise data from genes, molecules, cells, neural circuits, physiology, behaviours and self-reports (NIMH, 2013) and provide evidence of how each unit might contribute to an anxiety disorder.

More recently and specifically aimed at affective and mood disorders, NIMH's '*Research domain criteria Anxiety and Depression*' (RAD project; Williams et al., 2016),

has intended to identify risk factor domains and establish an organisational strategy for underlying neurobiology across these disorders, with a focus on genetics and neural circuitry.

Elucidating neurobiological biomarkers in human anxiety is important for the same reasons that animal models of anxiety and depression have been of use, i.e. enabling an assessment of selected behavioural phenotypes, tracking trajectories of disorders—of particular use in chronic, cyclical conditions of a ‘waxing and waning’ nature such as GAD—and conferring efficient cause-and-effect measurement of drug/treatment intervention.

Given this idea, in examining the components of cognition, mood and emotion in psychiatric disorders, there is a need to examine the underlying structure and function of the brain for associations, and subsequent change, through suitable modalities such as imaging (functional, structural, metabolic) and genetic (inheritance, gene expression) outcomes, as examples. A select summary of research in these modalities relevant to GAD are discussed in further detail in later sections of this chapter.

What is measured as biomarkers via imaging is arguably closer to the exact substrates that underpin and influence disorders, and therefore able to more precisely gauge the relationship between biomarkers and clinical endpoints for the individual (Bandelow et al., 2016; Kapur et al., 2012; Lueken et al., 2016; Williams et al., 2016). There is no question as to the complexity of psychiatric disorders such as GAD, with there being multiple inter-related factors in the aetiology, course and management, which are cognitive-personality, systemic and environmentally-derived, changeable across time and individuals, and therefore difficult to quantify via biological platforms. For a simplified illustration of plausible factors across multiple domains in a model for GAD, refer to Figure 2. The figure illustrates the three domains for which prominent research has been conducted in GAD; systemic, cognitive and environmental. The outer 17 items hold specific evidence for influence in GAD. The inner circle are fields that these findings load onto and represent the numerous and diverse elements that impact aetiology and maintenance of GAD symptoms.

3.5 Challenges and Limitations

The challenges with biomarker research in psychiatric disorders are prolific and only a couple of key points are therefore addressed here. From the above guidelines, several considerations arise within the anxiety disorders, primarily that a strong association between the disorder and marker must exist, and yet such an association is tenuous for

reasons discussed in the next section, as it is more likely that multiple biomarkers exist in an inter-related fashion rather than in a singular marker relationship (Bandelow et al., 2016; Lenzenweger, 2013; Mayeux, 2004).

Moreover, single biomarkers host a degree of heterogeneity—they have a presence across several disorders, for example, limbic system functionality evidenced in panic disorder, GAD and social anxiety. While they are useful for informing about the broader mechanisms underpinning anxiety states that are common to multiple conditions, they provide little discrimination between these disorders, constraining use as uniquely disorder-specific (Maron & Nutt, 2017; Williams et al., 2016).

Given these assertions, it is clear no biomarker can singularly achieve a perfect sensitivity and specificity. Biomarkers cannot arguably be utilised in isolation for psychiatric disorder diagnosis and treatment (Antonijevic et al., 2008; Drucker & Krapfenbauer, 2013). By their nature, affective disorders exhibit cognitive and/or emotional symptoms that must hold a relationship of some kind to underlying neurobiological patterns, but it is equally likely they stem from co-factors to the biomarkers in question.

The inter-relationship of these pathways is multi-directional and dynamic and there are influencing factors difficult to quantify, which is one reason GAD may be clinically challenging to treat. By this logic, biomarkers must form part of an interactive model for both aetiology and symptomology, as well as for diagnosis and treatment, in much the same manner as has been proposed for MDD (Bousman et al., 2009) and psychotic disorders (Freedman et al., 2005). Imaging studies involving functional connectivity, for example, have found greater replicable results where more than one modality has been employed, e.g. functional and structural, together with neuro-endocrinergic measures of stress coupled with functional imaging (Hilbert, Lueken, Muehlhan, & Beesdo-Baum, 2017; Shin & Liberzon, 2010).

Each modality has benefits and shortcomings; in fMRI, which offers non-invasive and high spatial resolution of localised brain activity by proxy, the ratio of oxygenated/deoxygenated haemoglobin (i.e. the haemodynamic blood oxygen level-dependant (BOLD) response) is the consequence of neural activity and it is arguable that this indirect method is imprecise, even that it is not measuring anything valuable (Arthurs & Boniface, 2002; Logothetis, 2002; 2008).

In a second example, the measurement of brain GABA levels derived from spectral data of the brain offers biochemical information, which requires a great deal of data modelling to expose the GABA peaks and there is no standard method for sequencing,

spectral analysis, quality parameters or data modelling of ‘contaminants’ such as macromolecules. This magnifies the risk of measurement error, among other methodological issues (Mullins et al., 2014; Stagg, Bachtiar, & Johansen-Berg, 2011). Both these modalities are examined in greater depth in the next chapter in the GAD context.

Taking the above examples of limitations, there are challenges in establishing the connection among mental states of mood, emotion and affect—essentially that which characterises and differentiates psychiatric disorders—directly to neurobiological mechanisms, and drawing evidence towards establishing unique disorder profiles. Chapter Two defined how the diagnosis and treatment of GAD rely on reported and/or observed symptom type, frequency and severity, and the subsequent limitations. These confounds are not unique to GAD and are associated with the issue of neurobiological inferencing, i.e. issues with reliably deriving data first, to create a model, before concluding observed effects/associations from what is primarily a specific type of data in limited amounts.

As such, while biomarkers aim to be model-free and objective by nature, it is difficult in modalities such as functional fMRI - which rely on tightly-theorised cognitive models in experimental paradigms - to evidence findings. This is related to the point raised in Chapter Two regarding cognitive models for GAD, with paradigms such as Emotion Stroop. There is an inherent risk in applying simplified predictive models to the diagnosis of psychiatric disorders, particularly ‘bottom-up’ diagnostics whereby particular brain patterns are suggested to evidence a particular state/disease/disorder, when they are not reliably replicated nor substantiated as unique to the anxiety state, disorder or disease.

This quandary - inferencing psychological states occurring in cognitive anxiety from brain function data, and vice-versa - has been discussed at length in several studies by Poldrack (2006), Price and Friston (2005), and Sarter 1996, Bernston and Cacioppo (1996). Reverse inference is a known issue—prompting the question relevant to this thesis: how does activation in a region of interest constitute specific anxiety cognitions and do changes imply a significant role of this region in modulating anxiety symptoms?

Many studies similarly operationalise this approach, that is, ‘given the model assumption, the region area shows an effect, therefore this is the cognitive state’. If the ACC region is activated during an anxiety state, should the ACC be activated in other circumstances, concluding that anxiety must be present? This approach is limited in explaining or predicting a behaviour/symptom, and in indicating how big the effect may

be. A common solution would be to induce a state as precisely as practicable and measure the associated brain activity; however, it is then applicable if anxiety cognitions are the only component of ACC activity. This would be assuming a high *positive predictive value*. For regional brain activity to have a high positive predictive value, it must: a) respond *consistently* to the task/state (high sensitivity) and b) respond *only* to the task/state (high specificity) (Bandelow et al., 2016; Kapur et al., 2012; Mayeux, 2004).

A final consideration in biomarker utility in anxiety disorders based on the criteria above is the feature of ‘effect’, i.e. alterations or quantifiable changes in their properties, depending on their magnitude, are associated with the presence or absence of dysfunction or a disorder. Of relevance here is for anxiety disorder diagnostics, with symptom ‘thresholds’ derived from frequency, intensity and severity to enable the differentiation of the disorder from ‘normal’, rather than simply a discrete presence or absence of a marker in a patient. Shortcomings and discussions regarding bias in greater depth are addressed comprehensively in recent reviews by Bandelow et al. (2016), and Maron and Nutt (2017).

This issue also presents a broader problem that biomarker utility experiences with the emphasis on a symptom-driven diagnostic system for psychiatric disorders. A prominent example is data-driven neurobiological findings that evidence commonality or heterogeneity across disorders as well as identify biological subtypes within them; in turn, weakening the validity of classical diagnosis categories. Indeed ‘big data’ in the future may contribute to this trend. This issue is briefly discussed here and is raised in view of the findings of the present study, but for a deeper discussion, the interested reader is invited to examine articles by Abi-Dargham and Horga (2016), Cuthbert and Insel (2013), Kapur et al. (2012), and Venkatasubramanian and Kashavan (2016).

3.6 The Utility of Biomarkers in Intervention Studies

Regarding the measurement of treatment efficacy, biomarkers may best serve as measures of either direct or indirect chemical or metabolic modulation within system substrates. In this sense, biomarker measures hold benefit over symptom severity measures (Shin & Liberzon, 2010), yet in psychiatric disorders, the crucial outcome measure is perceived mental wellbeing quantified through symptom psychometric questionnaires.

With any translational research, there are limitations and challenges, which are highlighted in more detail below, but primarily, the assessment of biomarker capability

requires the amalgamation of a conceptual approach outlined in the points above and large amounts of non-biased data to enable optimal modelling or templating of predictive algorithms that are currently generated via machine learning from this 'big data' (Chen & Butte, 2016; Passos, Mwangi, & Kapczinski, 2016; Stewart & Davis, 2016; Vingron, 2001).

Generally, biomarker findings from intervention studies are derived from group-based data with a need to translate into individual-level relevance. Several constraints come with this approach - sampling can be time consuming and costly and requires deep technical knowledge of the modality - as well as broad knowledge of how to model separate modalities together in a methodologically rigorous, replicable and translational manner (Bandelow et al., 2016; Chen & Butte, 2016; McNaughton, 2014).

The importance of research on biomarker utility in psychiatric disorders has been propositioned over the past three decades (refer to early papers by Hoes (1986), and Sternberg (1984). Within the psychiatric disorder biomarker research literature there is often focus on schizophrenia and mood disorders, mainly depression, with aminergic theories underpinning symptoms and responses to antidepressant treatments (for example genetic measurement of 5-HTTLPR and BDNF as allelic variances of Val66Met for BDNF), as predictors of reduced responsivity to serotonergic drugs being the prominent neurobiological markers thus far assessed (Porcelli et al., 2011; Uher & McGuffin, 2008).

The gene times environment ($G \times E$) studies that were prominent in depression research in the 2000s were equivocal in findings, which may be attributed to methodological processes, such as the way environmental covariates were accounted for (Gottschalk & Domschke, 2017; Tsuang, Bar, Stone, & Faraone, 2004). Nevertheless, explorations of models for disorder aetiology (such as $G \times E$), independent of findings, are necessary for expanding the GAD ontology. Put in the context of the present thesis, the biomarker testing examined the degree to which brain structure and function markers that are unique to GAD are identifiable, and the level to which markers can be effectively modulated via a treatment. The GABA system may confer a more reliable substrate measure in terms of drug development for GAD than the aminergic one has thus far been for depressive disorders (Olivier, Vinkers & Olivier, 2013; Nuss, 2015).

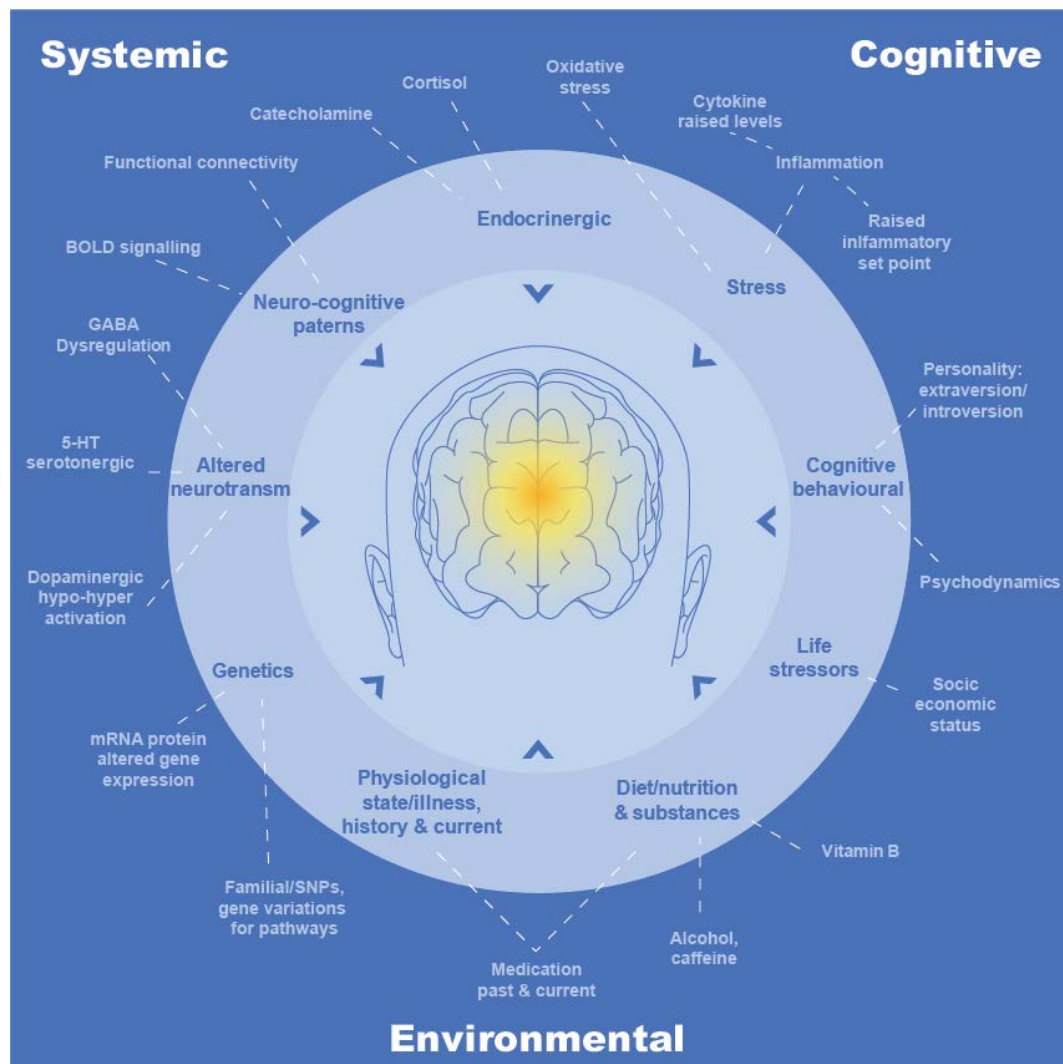


Figure 2. GAD: The multiple influences/factors in the aetiology, trajectory and treatment

A crucial justification for biomarker use within GAD research is that drug development/research has been at a standstill for at least 15 years (Farach et al., 2012; Kapur et al., 2012; Miller, 2010; Murrough, Yaqubi, Sayed, & Charney, 2015; Starcevic 2015). Chapter Five reports on the mainstay treatments for GAD and addresses some of the limitations, in turn supporting the intervention study conducted in Chapter Seven.

There is also justification for pursuing biomarker research from an economic and societal standpoint is where optimising disorder diagnosis, pathogenesis, trajectory and response to treatment is cost-effective and aides risk stratification within primary care settings, with the further benefit of improving future efficacy assessment. Therefore, the use of biomarkers becomes an important objective diagnostic tool and of great potential for improving outcomes because it facilitates targeted treatment and measurable symptom trajectory monitoring (Lueken et al., 2016; Mayeux, 2004; Perlis, 2011).

A final yet crucial benefit, aside from assessing neurobiological processes demonstrably linking biological function and disorder, is the potential to delineate GAD from other anxiety disorders, or isolate GAD/MDD comorbid, or other GAD biological subtype cases. The capacity here is to redefine the putative characteristics of anxiety disorders via evidence derived from neurobiological markers, a prominent example being OCD whereby neurobiological findings enabled the re-categorisation of OCD into a reward-based rather than an affective disorder (Bartz & Hollander, 2006; Pallanti, Barnes, Pittenger, & Eisen, 2017), providing better-targeted intervention and treatment.

3.7 Summary on Biomarker use in GAD

To facilitate biomarker model development, there is a need for efficient, robust and replicable data, and in large amounts. Data are best derived from transparent methodologies in efficacy studies to minimise issues typical observed in psychiatric disorder research and in drug discovery trials (Barabasi, Gulbahce, & Loscalzo, 2011; Klein, 2011; Marx, 2013).

For predictors to be clinically useful, there must be the capacity for application at an individual level. This translational capability presents more challenges; one is that a lot of data is required to generate solid predictive models for which machine-learning algorithms offer this capability, but the ability to exploit such methods is only relatively new. Nonetheless, biomarkers offer great potential in predictive modelling because psychiatric drug development/research is at a standstill, and thus this is one important justification for biomarker discovery. There is at present an unmet need for efficient approaches in identifying treatment efficacy.

GAD is a clinically challenging, prevalent and chronic disorder, feasibly more so because it is ordinarily diagnosed through cognitive-behavioural/psychological and descriptive physiological symptoms and not by biological methods. There is limited understanding of the biology of GAD supported by limited research; however, technological progress across several modalities has improved the body of knowledge, albeit with limited data.

Despite the shortfall GAD remains the most understudied of the anxiety disorders (Bandelow et al., 2017; Starcevic, 2015), reflecting the broader lack of intensive research into affective disorder biomarkers, particularly via longitudinal designs, despite the chronic, prevalent and disabling presentation. Only recently have comprehensive reviews focussed on whether biomarkers can measurably contribute to GAD symptoms. One such review (Bandelow et al., 2016) examined numerous biomarkers for GAD and other anxiety disorders, reporting evidence for associations across monoaminergic,

neuropeptide, hypothalamic–pituitary–adrenal axis, psycho-neuro-endocrinological, cardiovascular and imaging techniques.

Another review (Maron & Nutt, 2017) more specifically examined GAD pathogenesis with a focus on neuroimaging, genetics and neurochemical domains, and the utility of treatment outcome measurements. The latter study highlighted what is a persistent overlap in biomarker evidence between GAD and MDD, reasoning a causative role for depression symptom onset based on neurobiological substrates that underpin the trajectory of GAD symptoms.

Similarly, a systematic review from studies in diagnosed GAD patients (Hilbert et al., 2014) surmised neurobiological evidence from structural and functional connectivity studies, and aminergic substrate evidence from neuro-endocrinergic studies. The review noted limited evidence for the noradrenergic processes, with measures such as cortisol, or from studies using aminergic substrates in GAD (dopamine; serotonin; norepinephrine). Building from these findings, the study drew a model of how GAD psychological and physiological symptoms are maintained by endocrinergic and regional neurobiological substrates (excitatory and inhibitory) with use of the emotion dysregulation and avoidance models. The model is adapted in Figure 3, with some terms and flow components adjusted for clarity. The review asserted the need for integrative modelling in understanding GAD symptom maintenance, and to better discriminate GAD from other anxiety disorders and MDD.

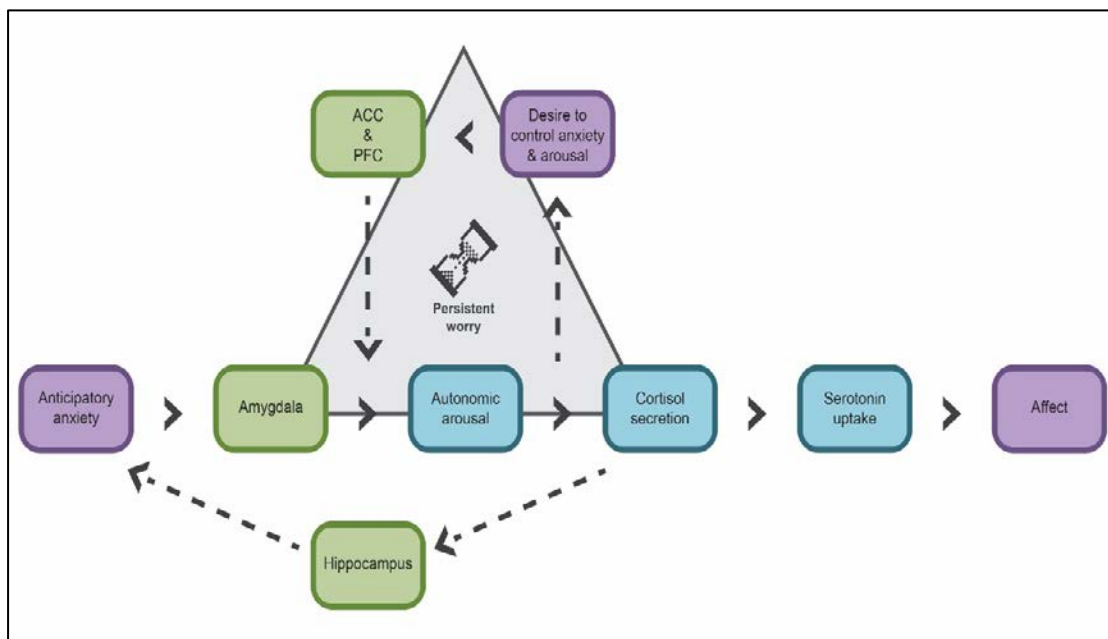


Figure 3. GAD theoretical model of functional regions, aminergic and endocrinergic substrates and cognitive processes (derived from Hilbert et al., 2014)

These reviews have all noted a shortfall in GAD studies from which biomarker evidence can be compiled, that possess sensitivity and specificity so as to have predictive value in GAD diagnosis, and assessment of treatment efficacy. The next section examines in greater detail selected neurobiological components relevant to GAD evidence through neuroimaging, and more specifically the GABA neural substrate and the ACC subregions. Both are identified as having potential as integrative metabolic and structural/functional biomarkers, yet require further examination, justifying the two research investigations undertaken in this thesis.

4 Chapter Four. Neuroimaging Anxiety Biomarkers

4.1 Overview

This comprehensive chapter summarises the current literature relevant to GAD, focussing on neurobiological findings associated with anxiety cognitions derived from neuroimaging. Focus is given to findings from these modalities to understand the general field before discussing the translational utility of neuroimaging research into clinical settings for the diagnosis and treatment of GAD. However, it is recognised that one of the limitations of the thesis is that it is not possible to succinctly detail all biological markers associated with GAD for both discriminant purposes and subsequent modulations from treatments.

The chapter will commence with an overview of research examining the relationship between structural, functional metabolic and other imaging modalities of markers and the pathophysiology of GAD in response to treatment interventions, before examining in detail two biomarker technologies/modalities (functional and metabolic imaging), and two areas of interest for which the two studies (Chapters Six and Seven) investigate: the anterior cingulate cortex via fMRI, and the GABA substrate via spectroscopy.

4.2 GAD – Evidence from Neuroimaging

4.2.1 Introduction

The biological correlations to primary symptoms of persistent worry and anticipatory anxiety in GAD are not clearly understood. Evidence from animal and lesion studies and the increasing body of literature involving neural and metabolic activity implicates brain regions involved in fear and anxiety as principally the limbic and prefrontal cortical areas, and their connectivity to stress response systems such as the hypothalamic–pituitary–adrenal axis (Herman et al., 1997; Martin et al., 2009; Pruessner et al., 2010).

Likewise, investigations of the inhibitory GABA (addressed in Section 4.6.5) and excitatory glutaminergic substrates in these regions have placed them in a central role in neuropathology across anxiety disorders, particularly GAD (Nemeroff, 2003; Nuss, 2015; Nutt, 2001; Puts & Edden, 2012a; 2012b).

4.2.2 Anxiety neurocircuitry

Evidence derived from animal models of fear conditioning, as well as animal and human studies of responses to fear induction and/or emotional response provides insight into anxiety neurocircuitry during fear/anxiety states. There is a focus on the pathological experience of fear and distress in response to threat predictors that is

prevalent within and across anxiety disorders. As such, the regions involved in the perception of threat/context appraisal, fear learning (acquisition, consolidation, retrieval) and extinction, emotional and stress responses are frequently evidenced (Etkin, Egner, & Kalisch, 2011; Shin & Liberzon, 2010; Taylor & Whalen, 2015).

The regions and subsequent circuitry associated with anxiety and fear responses include the amygdala, PFC, hippocampus, anterior cingulate and insular cortices, thalamic and brain stem nuclei, periaqueductal gray and the nucleus accumbens (Davis, 1992; Drevets, 2001; Martin et al., 2009; Vogt, Finch, & Olson, 1992). While these regions are not exclusive hosts of functional fear/stress/anxiety state processes, they are repeatedly evidenced via animal and clinical imaging studies that have examined discrete structural as well as regional functional connectivity in these cognitive/emotional states. Refer to Figure 4 as a summary pictorial regarding the evidence for the role of these areas in GAD.

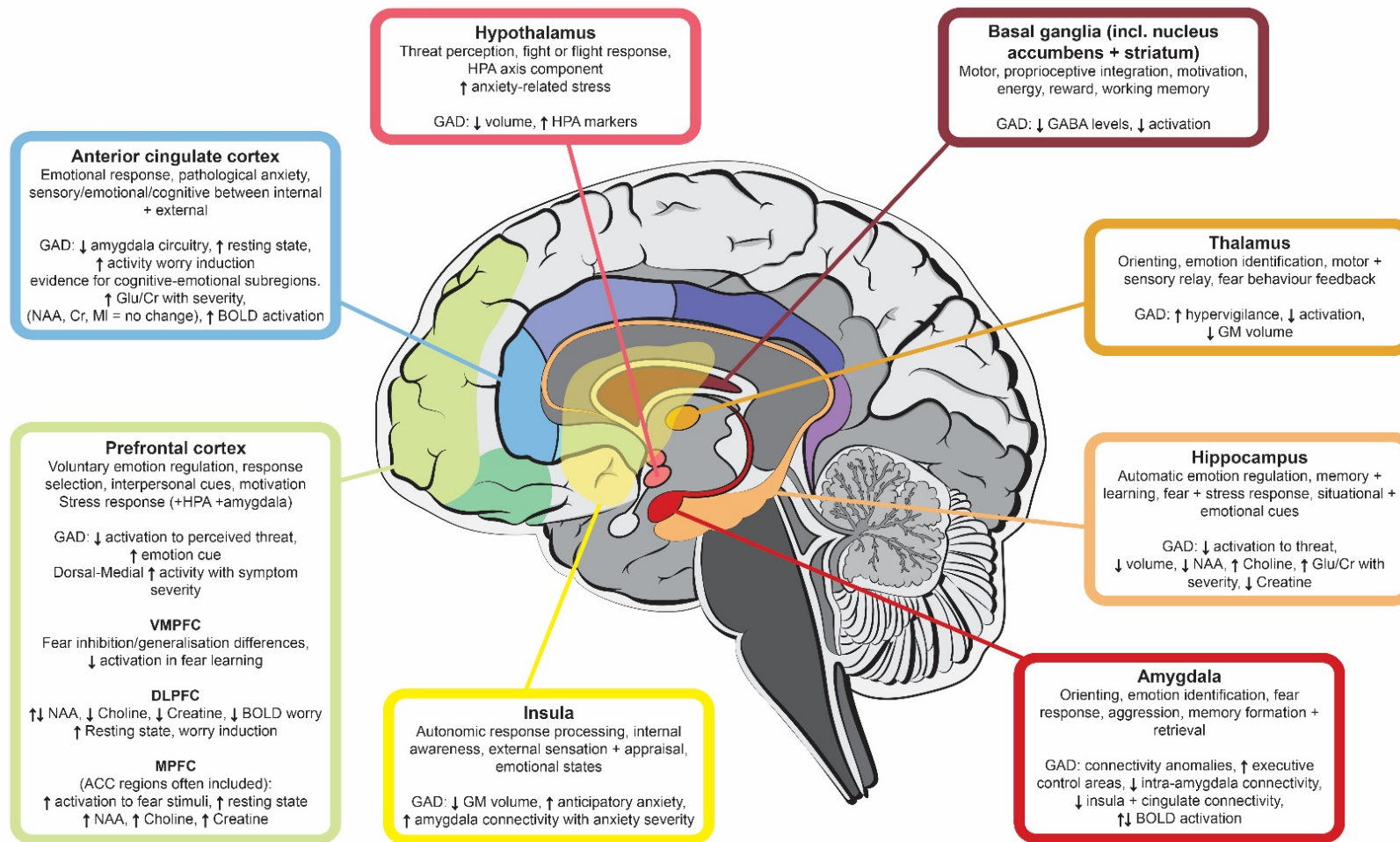


Figure 4. Regions involved in fear/anxiety neurocircuitry based on general findings from neuroimaging studies, emphasising structural, functional and metabolic findings in GAD (Image: author)

The stress response is an important adjunctive consideration given the evidence of an endocrinergic relationship via cortisol, corticotropin-releasing hormone and adrenocorticotrophic hormones to the limbic system (limbic–hypothalamic–pituitary–adrenal axis) and is arguably just as important as fear neurocircuitry (Hilbert et al., 2014; Shin & Liberzon, 2010).

Structurally, there are areas of overlap, including the insula and dorsal-medial prefrontal cortex (dmPFC) associated with stress response mechanisms, and the subgenual anterior cingulate (sgACC) with emotional/mood components (such as sadness affect) (Heim & Nemeroff, 2001; Drevets, Savitz, & Trimble, 2008). This overlap suggest broader mechanisms are contributing to fear and stress neurocircuitry, that could be uniquely functional in GAD, and if considered in conjunction with findings from other markers - such as metabolic, genetic and/or cognitive and behavioural measures – could help establish more dynamic patterns in profiles beyond data observed in single modalities (Martin et al., 2009).

4.2.3 Neuroimaging in the anxiety disorders (notably GAD)

A number of neuroimaging modalities have been used to study anxiety disorder populations including electroencephalography (EEG), positron emission tomography (PET), diffusion weighted imaging (DWI), structural magnetic resonance imaging (sMRI), functional magnetic resonance imaging (fMRI) and magnetic resonance spectroscopy (MRS).

The focus of this review section is on evidence from studies that have employed fMRI and/or MRS, hence reporting alteration of metabolite spectra and blood oxygen level-dependent patterns (BOLD signal; Ogawa and Lee, 1990). Such work seeks to quantify neurobiological processes underlying cognitive and emotional processing and/or characterising relevant symptomatology in GAD.

Neuroimaging is capable of revealing neurobiological markers of mood disorders (Savitz et al., 2013; Shin et al., 2013). Functional imaging of affective brain regions (utilising emotion-cognitive model designs) aims to clarify the contribution of the limbic system, and the role DLPFC and accessory executive regions might play in the cognitive and physiological symptoms typifying conditions such as GAD, and to identify patterns or the course of dysfunction or dysregulation. Furthermore, neuroimaging provides evidence that has contributed to theoretical cognitive models for the diagnosis and the treatment of GAD. Emotional dysregulation, excessive worry, and worry as an avoidance strategy

are typical processes in GAD for which these models aim to assess in imaging investigation paradigms (reviewed in depth by Mochcovitch et al., 2014).

There has been a paucity of neuroimaging investigations of GAD relative to other anxiety and mood disorders, and, as noted by numerous authors, the relationship between anxiety symptoms and underlying neurobiological patterns need further exploration to replicate and expand our understanding of the pathological anxiety state (refer to salient reviews in Duval, 2015; Maddock & Buonocore, 2011; Etkin & Wager, 2007; Mochcovitch et al., 2014; Shin & Liberzon, 2010).

4.2.4 Structural, functional and metabolic imaging evidence

4.2.4.1 *Structural overview*

Studies in anxiety disorder populations have reported differences between healthy controls and across disorders (Ding et al., 2011), from groups with major depressive disorder (Oathes et al., 2015). The evidence suggests delineation of GAD from individuals with social anxiety, phobias, and PTSD (Peterson et al., 2014). Similarly, morphometric studies have reported differences between affective and mood disorders, especially within prefrontal and limbic regions.

A meta-analysis of 24 structural morphometry studies in the anxiety disorders (Shang et al., 2014), found that after controlling for MDD and medication status, GAD, SAD, PD, and phobia groups exhibited reduced grey matter in the ACC and inferior frontal gyri, but increased grey matter in DLPFC. Decreases to grey matter in the middle temporal region with the presence of comorbid MDD, and reduced precentral gyrii grey matter with its absence, suggests that MDD comorbidity is distinguishable through neurobiology. The study discussed structural commonalities consistent with the supporting role of these regions in processes of worry and fear and *ipso facto* anxiety symptoms (see reviews by Drevets, 2008; Goodkind et al., 2015; Shin & Liberzon, 2010). Unfortunately, much of the volumetric evidence reported in this review, and other similar papers, are not specific to GAD, and thus limits conclusions of a consistent and unique profile among the anxiety disorders (Ferreri et al., 2011 and Radua et al., 2010).

Of the research involving adults with GAD compared with healthy controls and/or other psychiatric groups, findings have been equivocal for both whole brain and regional approaches, with regions variously exhibiting increased or reduced tissue volumes. Studies generally concur on grey matter increased amygdalar and basal ganglia volumes and reduced hippocampal volumes, as well as lower white matter volumes in the

prefrontal cortical regions (Etkin et al., 2010; Hilbert et al., 2014; Liao et al., 2014; Moon & Jeong, 2016; Moon et al., 2014; Schienle et al., 2011; Strawn et al., 2013).

One research group has published a series of related structural investigations in GAD compared with controls (initial paper: Moon, Kim, & Jeong, 2014) and reported regional grey matter reductions. The DLPFC volume grey matter was higher in females compared with males, which may be in line with increased prevalence of GAD in females. In a follow-up paper (Moon, Kang, & Jeong, 2015), no differences were found between the GAD group and healthy controls for DLPFC grey or white matter volumes. In a second follow-up paper (Moon, Yang and Jeong, 2015), reduced grey matter volumes were observed in GAD in the hippocampus, midbrain, thalamus, insula and superior temporal gyrus regions compared with the control group. Findings were replicated in a subsequent study where GAD samples had reduced white matter in the midbrain, precentral gyrus, DLPFC and anterior limb internal capsule tract compared to healthy controls (Moon & Jeong, 2016).

Finally, in a recent paper by Moon and Jeong (2017), the authors found reduced midbrain, thalamus, hippocampus, insula and superior temporal gyri grey matter volumes and reduced white matter volumes in the midbrain, anterior limb of the internal capsule, DLPFC and precentral gyrus in the GAD group compared with the healthy controls. These authors have drawn further associations from structural data to metabolic findings, such as reduced DLPFC ratio levels of choline/creatine and choline/NAA ratios in GAD samples compared with controls, and also positive correlations with the ROI white matter volumes in the GAD sample (Moon and Jeong, 2016b).

The reasoning behind the importance of structural findings in GAD is that decreased volumes in areas such as the DLPFC may reflect a shortfall in emotion processing and regulation, and in regions such as the basal ganglia that are associated with persistent worry and rumination (Ball et al., 2013). This evidence is further supplemented by functional or metabolic modality data, which are examined next.

4.2.4.2 Functional imaging overview

Anxiety disorders such as GAD typically exhibit altered cognitive processing, such as sensitized evaluation of the environment, persistent cognitive ruminations/worrying, avoidant behaviour, negative bias in the environment, and error identification and neuroimaging has been used to better understand these altered cognitive states

(Charney, 2003; Engel, Bandelow, Gruber and Wedekind, 2009; Etkin and Wager, 2010 2010; Shin and Liberzon, 2010).

States of fear and panic in the anxiety disorders have been linked to hypoactivation of prefrontal cortical regions, which is subsequently thought to disinhibit amygdalar and other limbic regions; whereas disorders involving more persistent cognitions and ruminations typified in GAD, or negative appraisals of social contexts inherent to SAD - are characterized by a *hyperactivity* of the prefrontal cortex (Etkin et al., 2007; Shin and Liberzon, 2010).

Within this front-limbic connectivity, the anterior cingulate cortex (ACC) function is evidenced largely through experimental anticipation or processing of emotional/valent stimuli; for example, paradigms involving the viewing of phobic material reliably activates the ACC (and the insular cortex) and may therefore indicate state anxiety-specific activations (Hajjak et al., 2003; Klumpp et al., 2012).

With regard to fMRI investigations with exclusively GAD samples, the investigation by Zhao and colleagues (2006) reported significant activations of the ACC region during emotional valence words paradigm, compared with healthy controls. A similar finding was reported in an adolescent GAD sample by McClure et al.(2007), however Blair et al. (2012) reported *hypo*-activation of the dorsal anterior cingulate cortex (dACC) in during both explicit emotion regulation and top-down attentional control tasks in a GAD group compared with healthy controls. These findings were interpreted as a reduced capacity to utilise emotion-regulation networks during these tasks. Etkin et al. (2010) also observed ACC hypoactivity during an emotional conflict task in GAD adults compared with controls, whereby activation levels were negatively correlated with anxiety symptom severity and could be used to *a priori* classify the two participant groups.

These findings fit well with a functional connectivity study by Etkin et al. (2009) where decreased connectivity between amygdala subregions and cingulate, and insular cortices, as well as differential intra-amygdala connectivity in a GAD sample compared with healthy controls was reported, which was reflective of the generalisation of the fear/stress response to miscellaneous situations resulting in the pervasive worry and difficulty with emotional processing that is pertinent in GAD cognitive-control processes.

Further connectivity evidence involving the ACC exists within white matter tracts; Tromp et al. (2012), examining the frontal-limbic ACC-amygdala pathway, reported reduced white matter structural connectivity within the *uncinate fasciculus* that connects the two regions in a GAD group compared with healthy controls, using

diffusion weighted imaging and an aversion anticipatory task during fMRI scanning to cross-reference data. The data may contribute multimodal evidence for frontal-limbic involvement in emotion dysregulation featured in GAD symptoms. The GAD patients without existing comorbid conditions (e.g. MDD) exhibited greater reductions of white matter integrity in these regions, suggesting a capacity to delineate pure GAD as a biological 'subtype'.

These studies are notable for two reasons: firstly, most multimodal investigations that have measured regions associated with anxiety states have reported significant group differences suggesting that delineating markers unique to GAD is demonstrable across more than one measurable outcome; and secondly, biomarker evidence is able to identify differences between GAD and the most common comorbid, major depression, both of which serve to delineate GAD from other affective and mood disorders. As such, the combination of diagnostic categories with multimodal biomarker evidence becomes a crucial step in optimising diagnosis, disease trajectory and treatment of GAD symptoms.

Several reviews have examined the affective disorder neuroimaging literature (Charney, 2003; Duval, 2015; Engel, Bandelow, Gruber & Wedekind, 2009; Etkin & Wager, 2010; Shin & Liberzon, 2010), some exclusively reporting on GAD (Maron & Nutt, 2017; Mochcovitch et al., 2014) and some examining treatment response markers of GAD (Lueken et al., 2016; Shin et al., 2013; Strawn et al., 2012). Specific information regarding structural, functional, and metabolic studies involving GAD samples examining cognitive models relevant to GAD symptomology are summarised in Table 6 with some reviews summarised here to illustrate the overarching findings.

One review conducted by Shin and Liberzon (2010) focussed on anxiety neurocircuitry across several affective disorders. Although the authors noted increased activity in both rostral and dorsal anterior cingulate regions in GAD compared with healthy controls, mixed findings and inconclusive evidence were found in other regions from the reviewed studies. Further, the authors postulated that the evidence for ACC hyperactivation is consistent with non-specific cognitions and emotional processes commonly associated with GAD, rather than for more specific *stimuli-driven* anxiety disorders such as phobias, PTSD and social anxiety, thereby implicating a central role of the ACC region in GAD. Moreover, other reviews based on combining symptoms with biological features identified varied relationships between activation profiles and symptom type and severity, and crucially between trait-based chronic disorders such as GAD and state-based disorders such as specific phobias.

Mochcovitch et al. (2014) conducted a systematic review of functional BOLD signal activation and functional connectivity data from 14 eligible fMRI studies in GAD patients and reported reduced prefrontal and ACC activity and a reduced amygdala-to-cortical associations, implying emotional dysregulation in GAD. The authors delineated the ACC and mPFC regions as exhibiting differential activation levels in GAD patients compared with controls. In addition, activation was modulated during experimental paradigms involving emotion regulation, worry and/or anxiety induction. Finally, activation in these regions was modulated with treatment interventions thereby reducing the difference between GAD patients and controls for fMRI findings. Another review by Duval et al. (2015), reporting on several anxiety disorders, concurred on functional/activation level elevated trend, plus structural volumetric differences associated with GAD, consistent with the aforementioned review (refer to Table 6).

Table 6. Summary of Structural, Functional MRI, PET-SPECT and Metabolic Studies in diagnosed GAD, Including Intervention/Predictive Studies*

Study	Sample	Region/s	Modality	Cognitive model	Task/Measure	Intervention	Result
Andreescu et al. (2011)	7 GAD age 60+	↓ Prefrontal cortex	Task-dependent BOLD	Worry Induction	-	-	GAD patients failed in activating PFC during worry-suppression task.
Andreescu et al., 2015	28 GAD, 31 HC, age 60+	AI, OFC, BNST, sgCING, MPFC, PVN,	Functional connectivity	Worry induction and worry reappraisal	-	Cross-sectional; 12-week intervention in GAD group	GAD greater FC between left anterior insula/BNST/sgACC during induction During reappraisal, GAD greater FC of PVN-amygdala whereas healthy controls had greater DLPFC-MPFC and AI-MPFC FC GAD group following 12-week intervention increased FC DLPFC-PFC regions during worry reappraisal
Ball et al., 2014	28 GAD / 23 HC	↓ DLPFC/DMP FC	Task-dependent BOLD	Emotional dysregulation	Emotion pictures appraisal	CBT	Reduced DLPFC and dmPFC activation compared to HC.
Blair et al., 2008	17 GAD / 17 HC	↑ Lateral PFC	Task-dependent BOLD	Emotional dysregulation	Emotional faces confrontation	-	GAD presented increased responses to angry expressions in the lateral PFC.
Blair et al., 2012	34 GAD, 34 GAD + SAD, 34 SAD, 36 HC	↓dACC ↓Parietal cortex	Task-dependent BOLD	Emotional dysregulation	Emotion pictures appraisal	-	GAD reduced activation in dACC and PC during emotional regulation and top-down-attentional-control, compared with HC.
Buff et al., 2016	21 GAD, 21 PD, 21 SAD and 21 HC	↑DLPFC, INS, CING	Task-dependent BOLD	Threat processing	Emotion pictures appraisal	-	GAD-unique DLPFC, cingulate cortex, dorsal anterior insula increased activity in threat vs. neutral pictures GAD increased FC vLPFC-posterior DLPFC, amygdala-cingulate cortex; anterior insula -cingulate cortex, dorsal anterior insula-mid-DLPFC
Buff et al., 2017	19 GAD, 19 HC	↑ AMYG, BNST	Task-dependent BOLD	Emotional regulation	Direct threat imagery	--	GAD varied phasic and sustained activation in response to threat anticipation in the amygdala, and bed nucleus of the stria terminalis
Cha et al., 2014	32 GAD , 25 HC	↓VMPFC ↑ VTA	Task-dependent BOLD	Fear regulation/ inhibition	Conditioned fear: electric shock		Deficit of vmPFC in recruitment of fear regulation. Increased activation in VTA during fear generalisation
Coplan et al., 2006	15 GAD, 15 HC	↓CHO and CR in CSO	Metabolic	-	Choline Creatine	-	Decreased choline and creatine in centrum semiovale in GAD compared with HC

De Bellis et al., 2000	12 GAD, 24 HC; adolescent /child sample	↑ AMYG	Structural	-	Volumetric	-	Larger volume right and total amygdala in GAD compared with controls
Etkin et al., 2009	16 GAD, 17, 31 HC	↓PFC ↑Amygdala	Task-dependent BOLD	Emotional dysregulation	Resting-state fMRI	-	GAD: disrupted connectivity patterns of the amygdala subregions and a reduced PFC-amygdala connectivity. Greater amygdala grey matter volume
Etkin et al., 2010	17 GAD / 24 HC	↓Amygdala ↓ACC	Task-dependent BOLD	Emotional dysregulation	Emotion faces and words	-	Attenuated signal in the ACC to happy and fearful facial expressions GAD patients failed in activating ACC (negative top-down ACC–amygdala connectivity during the regulation of emotional conflict). No difference in amygdala activation between GAD and HC.
Fonzo et al., 2014	21 GAD, 11 HC	↓AMYG, IN, ACC in GAD ↑AMYG-IN	Task-dependent BOLD	Emotional dysregulation	Emotion faces	CBT, 10 sessions	Pre-treatment GAD greater connectivity in amygdala-insula, and reduced activity in amygdala, insula and ACC in GAD Treatment associated with reduced amygdala and subgenual ACC during fear and angry stimuli, increased insular to happy stimuli
Greenberg et al., 2013	Female 32 GAD / 25 HC	↓ vmPFC Anterior insula	Task-dependent BOLD	Conditioned fear overgeneralisation	Fear response/inhibition – rectangles and electric shock	-	GAD patients showed a deficient ventromedial PFC recruitment during fear inhibition of ‘Generalisation stimuli’ The anterior insula area facilitates fear response to conditioned stimulus
Guyer et al., 2012	18 GAD; 14 SAD; 26 HC; adolescent sample	↑ Caudate, Putamen ↑ nucleus accumbens	Task-dependent BOLD	Behavioural inhibition	Gain/loss monetary incentives	-	Altered putamen activation related to reward processing; Increased caudate activation in GAD compared with healthy controls Increased nucleus accumbens activation in GAD compared with SAD and healthy controls GAD sample had increased loss sensitivity
Hettema et al., 2012	34 GAD twin females, affected and unaffected pairs	↓HIP volume ↑Creatine AMYG, HIP ↓AMYG-PFC FC	Structural Metabolic Functional connectivity	-	Volumetric DTI Creatine	-	Reduced volume of hippocampus in GAD Lower mean anisotropy in uncinate fasciculus FC amygdala/frontal cortex Lifetime GAD associated with increased AMYG creatine levels
Hilbert et al., 2015	19 GAD, 24 HC	↑ BG ↓ DLPFC	Structural	Structural	Volumetric	-	Greater BG grey matter volume Less DLPFC white matter volume

Hoehn-Saric et al., 2004	6 GAD, no controls	↓PFC, ↓striatum, ↓insula ↓paralimbic regions	Task-dependent BOLD	Emotional dysregulation	Emotion sentences	Citalopram	Reduced activation in regions, also reduced differences in responses to worry and neutral stimuli
Holzel et al., 2013	15 GAD / 11 HC	↑Amygdala (increase) vmPFC	Task-dependent BOLD	Emotional dysregulation	Emotion faces Beck Anxiety Inventory (BAI)	Mindfulness based stress reduction (MBSR); Stress management education (SME)	GAD higher amygdala activation in GAD over HC to neutral faces (not angry); subsequent decrease in GAD in both MBSR & SME. VLPFC & amygdala connectivity change correlated with reduction in BAI scores.
Lee et al., 2015	12 GAD, 12 HC	↓ 5HTT in MB ↓DAT in Striatum	2 Ligand SPECT	-	Serotonin transporter (SERT) Dopamine transporter (DAT)	-	In GAD lower serotonin transporter binding in striatum, no differences in midbrain SERT
Makovac et al., 2015	19 GAD, 21 HC	↑Amygdala ↓ gyrus	Structural	-	Volumetric	-	Larger right amygdala volume Reduced supramarginal, precentral, and postcentral gyrus bilateral grey matter volumes in GAD Bilateral insula, bilateral opercular cortex, right supramarginal and precentral gyri, anterior cingulate and paracingulate cortex volumes predicted greater decrease in HRV
Makovac et al., 2016	19 GAD, 21 HC	AMYG-PFC	Functional connectivity	Functional connectivity	Perseverative cognition task; autonomic regulation	-	Lower connectivity between the right amygdala and right superior frontal gyrus, right paracingulate/ anterior cingulate cortex, and right supramarginal gyrus and GAD Decreases in functional connectivity between the L AMYG and subgenual cingulate cortex, and R AMYG and CN predicted magnitude of heart rate variability reduction
Maron et al., 2001	7 GAD, 7HC	- 5HTT in MB	2 Ligand SPECT	-	Serotonin transporter (SERT)	-	No altered serotonin transporter binding in GAD, however binding in midbrain were negatively correlated to reported anxiety levels in GAD

Mathew et al.,2004	15 GAD, 15 HC	↑ NAA DLPFC	Metabolic	-	NAA/Cr	-	Increase in NAA/Cr ratio in right DLPFC in GAD compared with HC
Mathew et al.,2008	15 GAD, 8 HC	↑ NAA/Cr Hip	Metabolic	-	NAA/Cr	8 weeks: Riluzole	Increase in NAA/Cr ratio in hippocampus in responders, conversely decreased in non-responders
Mathew et al.,2010	9 GAD, 10 HC	↓ NAA/Cr Hip with tmt	Metabolic	-	NAA/Cr	12 weeks: paroxetine	Lower NAA/Cr ratio in bilateral hippocampus following 12 week intervention
McClure et al., 2007	GAD, 12 child & adolescent	↑ Amygdala	Task-dependent BOLD	Emotion valence	Emotion faces	8 weeks: Fluoxetine; CBT	Greater pre-treatment activations of amygdala in fearful faces predicted better response outcome.
Mohlman et al., 2017	20 GAD, 16 HC, age 60+	↑ VMPFC + DLPFC, AMYG, IN	Task-dependent BOLD	Worry paradigm	Functional connectivity	-	Worry state associated with increased activation of prefrontal areas in GAD
							In GAD connectivity analysis found circuit ventromedial through dorsolateral prefrontal cortices, converging on the amygdala
							HC had simpler circuit, as well as involvement of orbitofrontal cortex and insula
Monk et al., 2008	17 GAD, 12 HC; child & adolescent	↑ Amygdala	Task-dependent BOLD	Emotion valence	Emotion faces	-	Increased activation of AMYG in GAD Negative connectivity right VLPFC + AMYG suggesting dysfunction/anxiety
Moon, Kim and Jeong, 2014	22 GAD, 22 HC	↓ GM in Hip, MB, THA, INS, STG	Structural	-	Volumetric	-	GAD reductions in the hippocampus, midbrain, thalamus, insula, and superior temporal gyrus compared with the controls
		↑ GM DLPFC female					Higher DLPFC volume in female participants, with positive correlation to HAM-A scoring
		- GM/WM of DLPFC					Gender difference in total intracranial GM volume; n/s difference between GAD + HC
Moon, Kang and Jeong, 2015a	15 GAD, 15 HC	↓ Cho/Cr and Cho/NAA ratios DLPFC	Structural Metabolic	-	Volumetric	Choline/creatine Choline/NAA	No difference between GAD and HC for DLPFC GM/WM volumes GAD lower choline/creatine and choline/N-acetylaspartate ratios GAD choline concentrations negatively correlated with HAM-A and GAD7 scores
Moon, Kang and Jeong. 2015b	17 GAD, 17 HC	↑ VLPFC, PCG, ↓ Hip, MCG, Pu, CN	Task-dependent BOLD Structural	Emotional dysregulation	Emotional valence words, verbal memory	-	In emotional valence condition GAD group significantly increased activity in VLPFC and PCG, but reduced activity in Hip, MCG, Putamen and Caudate Nucleus

		↓ GM in Hip, MB, THAL, IN, STG			Volumetric		GAD group reduced GM volume in hippocampus, midbrain, thalamus, insula, and superior temporal gyrus
Moon and Jeong, 2016	13 GAD, 13 HC	↓ WM GAD ↓ DLPFC Cho/Cr, Cho/NAA	Structural Metabolic	-	Volumetric	Choline/Creatine Choline/NAA	GAD reduced WM in the midbrain, precentral gyrus, DLPFC and anterior limb internal capsule GAD reduced DLPFC Cho/Cr and Cho/NAA ratios WM volume of DLPFC was positively correlated with Cho/Cr and Cho/NAA ratios in GAD
Moon and Jeong, 2017	20 GAD, 20 HC	↓ GM in MB, Thal, Hip, Insula, STG ↓ WM in MB, ALIC, PrG, DLPFC	Structural	-	Volumetric	-	GAD reduced GM volumes in MB, thalamus, Hip, insula, and STG; and reduced WM volumes in the MB, anterior limb of the internal capsule, DLPFC, precentral gyrus
Nitschke et al., 2009	14 GAD / 12 HC	↑ ACC	Task-dependent BOLD	Emotional dysregulation	Affective pictures/ cued anticipation	Venlafaxine 8 weeks	Higher levels of ACC activity predicted greater clinical outcome in GAD patients.
Liao et al., 2013	26 GAD, 25 HC	↑ R Putamen	Structural	-	Volumetric	--	Increased right putamen grey matter volume
Palm et al., 2011	Female 15 GAD / 16 HC	↓ Medial Prefrontal cortex ↓ ACC -Amygdala	Task-dependent BOLD	Emotional dysregulation	Emotion faces		Female GAD showed attenuated PFC and ACC activation, no change to amygdala.
Paulesu et al., 2010	8 GAD / 12 HC	↑ Prefrontal cortex ↑ ACC	Task-dependent BOLD	Worry Induction	Worry induction sentences	-	GAD patients showed hyperactivation in mPFC and ACC during both worry induction and posterior resting state phases while HC showed this response just during the worry phase.
Price , Eldreth and Mohlmann, 2011	16 GAD, 12 HC, 60+ years	↓ Medial prefrontal cortex	Task-dependent BOLD	Attentional bias, attentional control	Emotion Stroop	-	GAD decreased mPFC activation and no changes to amygdala and hippocampus during negative valence condition, in contrast to HC.

Qiao et al., 2017	20 GAD, 20 HC	↑ AMYG, IN, Pu, Thal, PFC	Functional connectivity	-	Resting state	-	GAD stronger functional connectivity in amygdala, insula, putamen, thalamus, and posterior cingulate cortex; weaker for frontal and temporal cortex compared with controls
Schienze, Ebner and Schafer, 2011	Female 16 GAD, 15 HC	↑ AMYG ↑ DMPFC	Structural	-	Volumetric	-	Larger amygdala and dmPFC neural volume in GAD. Symptom severity were positively correlated with dmPFC and ACC volumes Correlation between Penn State Worry Questionnaire scores and ACC/dmPFC volumes
Schlund et al., 2012	10 GAD with MDD comorbid, 10 HC	↓ MPFC, ACC, CING, IN, but ↑ MPFC and IN with ↑ anxiety	Task-dependent BOLD	Emotional valence	Valent self-referential statements	-	Presence of depression associated with hypoactivation in mPFC, ACC, cingulate and insula, but increased anxiety was associated with increased activation of PFC region where patients had GAD as a comorbid condition to MDD.
Strawn et al., 2012	10 GAD, 10 HC, adolescent	↑ L MPFC, R VLPFC R VLPFC – MPFC AMYG – PC, PRE	Task-dependent BOLD	Emotional valence	Functional connectivity	-	Increased activation of the mPFC and right VLPFC Altered connectivity between amygdala/vIPFC and mentalization regions (posterior cingulate cortex, precuneus and medial prefrontal cortex)
Strawn et al., 2013	18 GAD, 28 HC	↑ R PC + PCG ↓ left OG, PC	Structural	-	Volumetric	-	Increased right PC + PCG grey matter volumes Decreased left OG and PC grey matter volumes
Tiihonen et al., 1997	10 GAD, 10 HC, females	↓ GABA-A in temporal pole	Metabolic	-	SPECT by BDZ receptor ligand	-	Decreased GABA-A receptor binding in temporal pole in GAD
Tromp et al., 2012	49 GAD, 39 HC; 21 GAD w/o comorbid, 21 HC	↓ AMYG-ACC connectivity	Functional connectivity DTI	-	Resting state	-	GAD lower mean anisotropy values in bilateral uncinate fasciculus indicating reduced fronto-limbic structural connectivity; greater in pure GAD. GAD decreased functional connectivity between the anterior cingulate cortex and amygdala
Wang et al., 2016	28 GAD 28 HC	↑ DMPFC, DLPFC, PC/PFC	Resting state	-	Functional connectivity	-	GAD higher low-frequency fluctuations in BL-DMPFC, BL-DLPFC and left precuneus/posterior cingulate cortex GAD lower FC in prefrontal gyrus

		↓PFG, PFL, CING					GAD lower PF/limbic and cingulate and higher PF/-hippocampus connectivity were associated with symptom severity
		↑PF/Hip FC					
Whalen et al., 2008	Female 15 GAD	↓ Amygdala ↑ ACC	Task-dependent BOLD	Emotional dysregulation	Emotional faces	Venlafaxine 8 weeks	Pre-treatment greater activity in ACC, and lesser in the amygdala predicted the magnitude of treatment response. No difference in ACC and amygdala activation between GAD and HC No difference before and after treatment in GAD patients
Yassa et al., 2012	15 GAD / 15 HC	↓ Amygdala ↑ BNST	Task-dependent BOLD	Intolerance to uncertainty	Monetary loss task/gambling	-	Decreased activity in the amygdala and increased activity in the BNST in GAD patients comparing to controls.
Yin et al., 2017	20 GAD, 14 HC, adolescent	↓ IFG ACC and VMPFC GAD ↑ Amygdala GAD	Task-dependent BOLD	Emotion valence	Negative vs neutral valence	-	Significant activation of the right inferior frontal gyrus (IFG) in healthy controls, significant activation of bilateral amygdala was activated in GAD patients Decreased activation of right IFG and ACC and vmPFC cortex in GAD patients
Zhao et al., 2006	10 GAD, 10 HC	↑ ACC, DLPFC, STG, PF gyri	Task-dependent BOLD	Emotion valence	Emotion words; visual and auditory	-	Hyperactivation in GAD group in multiple regions such as superior temporal lobe and dIPFC
ACC: anterior cingulate cortex; AMYG: amygdala; BDZ: benzodiazepine; BG: basal ganglia; BL: baso-lateral; BNST: bed nucleus of the stria terminalis; Cho: choline; Cr: creatine; CBT: cognitive-behavioural therapy; CS: conditioned stimulus; DLPFC: dorsolateral prefrontal cortex; GAD: generalised anxiety disorder; GM: grey matter; HC: healthy controls; Hip: hippocampus; IFG: inferior frontal gyrus; IN: insula; MB: midbrain; MPFC: medial PFC; NAA: n-acetylaspartate; OG: orbitofrontal gyrus; PFC: prefrontal cortices; PC: precuneus; PCG: precentral gyrus; SAD social anxiety disorder/social phobia; STG: superior temporal gyrus; VLPFC: ventro-lateral PFC; vMPFC: ventro-medial PFC; WM: white matter							
*Literature current up to February, 2017							

4.2.4.3 Metabolic overview

4.2.4.3.1 PET

PET studies can measure regional GABA-A receptor availability and levels via radiolabelled [18F]-fluoroflumazenil, a competitive antagonist with a high affinity for the GABA-A/BZ receptor and a long half-life, which enables neuroimaging of receptor distribution *in vivo*. PET studies of GABA benzodiazepine bindings have been investigated extensively in schizophrenia (Ball et al., 1998; Kang et al., 2014), but few PET studies have been conducted in GAD samples.

Specific to GABA substrates, one PET review across several anxiety disorders reported temporal-cortical GABA-A receptor reductions in GAD patients compared with healthy controls (Nikolaus et al., 2010). Finally, an earlier study reported reduced GABA receptor densities in the left temporal pole in female GAD patients compared with health controls (Tiihonen et al., 1997).

4.2.4.3.2 MRS

The paucity of metabolite studies via MRS in GAD groups is demonstrated through the brief literature reported below, where a broader summary is provided specific to main metabolites measured via MRS, before focussing on studies involving GAD samples.

Metabolite profile studies have assessed changes in brain indices such as neuronal/axonal integrity (N-Acetylaspartate; NAA), neuronal excitation (glutamate, glutamine/Glx) and inhibition (γ -aminobutyric acid; GABA), oxidative stress (glutathione; GSH), cellular turnover (choline) and inflammation in the brain (MI).

Metabolite profile data across psychiatric disorder groups such as schizophrenia, MDD, bipolar disorder, panic disorder, OCD and substance use including alcohol groups evidence pervasive neuronal dysfunction through NAA reductions, while elevated Glx suggests excitatory dysfunctions in disorders that feature cognitive and perceptual distortions, as seen in schizophrenia and bipolar disorder. Conversely, deficits of Glx in major depression may demonstrate attenuated neurotransmission from reduced glutamate conversion (Johnston, Roberts, Chase & Tower, 1976; Maddock & Buonocore, 2011; Maudesley et al., 2009; Pouwels & Frahm, 1998; Rae, 2014; Rosen & Lenkinski, 2007).

With processes of neuronal excitation/inhibition, decreases to both glutamate and GABA, together with reduced Glx regionally in the ACC were reported in MDD, with increases following SSRI treatment (Auer et al., 2000; for major review, see Luykx et al.,

2012). In bipolar disorder, elevated Glx levels in the frontal brain areas have been reported, regardless of medication status in one meta-analysis (Gigante et al., 2012).

In OCD, there is increasing evidence for a glutaminergic system role in excitotoxicity in OCD and subsequent reported hyperactivity in areas such as the caudate and orbitofrontal cortex (Pittenger et al., 2011; Wu, Hanna, Rosenberg, & Arnold, 2012). In the ACC, reduced levels of Glx have been reported in female OCD subjects (Yucel et al., 2008) across several ACC subregions, and levels correlated to symptom severity. Additionally, MI levels were reported in the OCD sample in this study in both males and females compared with the healthy controls.

Closer to GAD, in panic disorder, reduced GABA levels in regions such as the ACC and basal ganglia have been reported across several studies (Ham et al., 2007; Hasler et al., 2009; Long et al., 2013). In SAD, one study (Pollack et al., 2008) reported elevated whole brain glutamate and glutamine (Glx), and in the thalamus, elevated glutamate with reduced GABA in a SAD sample compared with the healthy control group.

4.2.4.3.3 MRS studies in GAD

Five metabolic studies (1.5, 3, 4 or 7T; up to 2017) were found that compared GAD patients with healthy control sample groups. In Coplan et al. (2006), reduced creatine and choline levels were reported in the centrum semiovale (white matter fibre region) in the GAD group compared with the controls.

One study (Mathew et al., 2004) examined selected regional metabolites in GAD patients and found a 16.5% higher NAA/creatine ratio in the right dorsolateral PFC compared with the healthy controls. The study also reported GAD participants with the occurrence of childhood abuse had higher levels compared to those without abuse occurrence.

In another study, also examining the influence of childhood trauma (Raparia et al., 2016), the GAD and control samples had NAA, creatine and choline levels assessed in the PFC, premotor, somatosensory and parietal cortices. The control group had a significant inverse relationship in all metabolite levels to the degree of childhood trauma, whereas no relationship was observed in the GAD group.

In a related series combining structural and metabolic modalities in GAD in the PFC, several studies reported DLPFC white matter and grey matter volumes, as well as choline/creatine and choline/NAA ratio metabolite levels (Moon and Jeong, 2016; Moon, Kang, & Jeong, 2015; Moon, Kim, & Jeong, 2014; Moon, Sundaram, Choi, & Jeong, 2016). Lower levels of choline/creatine and choline/NAA were reported in the

DLPFC in the GAD sample compared with a healthy control sample (Moon et al., 2015a), with this study also finding a positive correlation between both metabolite ratios and white matter volumes in this region. In a subsequent study, these levels were negatively correlated with anxiety symptom measures (HAM-A and GAD-7; Moon et al., 2015b).

Moon and Jeong (2016), again examining the DLPFC, reported the same data and compared metabolic data with the BOLD signal and found that the choline ratios positively correlated with BOLD signal change in this region. This study reported choline levels were significantly reduced in higher anxiety samples measured via the HAM-A and GAD-7. The study also reported lower levels of the aforementioned ratios in GAD samples compared with healthy controls, suggesting a relationship between these metabolite levels in the PFC with GAD symptom severity.

Finally, in adolescent GAD, Strawn et al. (2012) found no differences in NAA, MI or creatine levels in the ACC region compared with healthy controls; however, they reported a positive correlation between glutamate/creatine and anxiety symptom severity on the Paediatric Anxiety Rating Scale and the SIGH-A HAM-A scale.

By the same authors also in adolescents (Strawn et al., 2013), the ACC region metabolites were compared with healthy controls and these authors found that GLX correlated with symptom severity measured by the HAM-A, yet not exclusively in the clinical group, which suggests metabolite profiles are associated with symptom severity regardless of diagnosis/psychiatric categories.

4.2.4.3.4 MRS intervention studies

There are few studies that report on modulations to metabolite levels as the product of an experimental intervention measured via MRS in GAD. In other groups, a study in first episode psychosis patients (Berger et al., 2008) reported increases to GSH levels in the temporal lobe associated with reductions in negative symptoms as a response to an omega-3 fatty acid supplement, relative to placebo. Also with GSH, Stough et al. (2014) investigated metabolite levels in a healthy but work-stressed sample (ages 18-50) following B vitamin supplementation.

In MDD samples, MRS metabolic intervention studies are plentiful (Etkin & Wager, 2010; Maddock and Buonocore, 2011; Puts & Eddin, 2012b; Rothman et al., 1993; Rae, 2014). Investigations in MDD have reported GABA levels in regions such as occipital and anterior cingulate cortices shown to 'normalise' in samples following successful antidepressant treatment (response and/or remission from symptoms via psychological assessment outcomes, however not in samples where there was 'treatment resistance'

(Sanacora et al., 1999; Bhagwagar et al., 2004). Also in a samples of adults with MDD, Das et al. (2013) similarly found modulation to GSH levels but in the ACC region, associated with N-acetylcysteine supplementation relative to placebo.

In two MRS studies involving SAD, one investigation (Tupler et al., 1997) reported elevated choline and MI in grey matter cortical tissue, and elevated MI ratios with NAA and creatine in subcortical grey matter, mainly the thalamus, compared with the healthy control group. This study followed on from reduced NAA reported in a seminal earlier MRS study (Davidson et al., 1993). No changes were reported as a product of an 8-week benzodiazepine (clonazepam) intervention in the SAD group. The Pollack et al. (2008) study also assessed metabolite changes in a SAD sample following an 8-week intervention, this time an anticonvulsant (levetiracetam), whereby reductions of thalamus glutamate/glutamine (Glx) was reported.

4.2.4.3.5 MRS intervention studies in GAD

In GAD, four intervention studies examining metabolites have been published. One study (Mathew et al., 2008) reported hippocampal levels compared with healthy controls and following eight weeks' riluzole treatment in both responders and non-responders reported increased NAA levels in responders and decreased levels in non-responders, with the increase reflecting normalisation towards levels reported in the healthy control comparator group.

A second study (Mathew et al., 2010) reported lower levels of NAA to creatine ratio in the bilateral hippocampal region in GAD patients following a 12-week dose of the SSRI paroxetine. Finally, Abdallah et al. (2013) reported on NAA/water ratio levels occipital and hippocampal regions in GAD following eight week's riluzole treatment and a healthy control comparator group. Right occipital NAA levels were negatively associated with symptom improvement, whereas reductions with treatment were observed in hippocampal NAA/water ratio levels compared with levels in the control group.

Combined fMRI and metabolic studies in GAD are examined next.

4.2.4.4 Multimodal research in clinical groups (fMRI and MRS)

Specifically involving adults with GAD, few studies have examined the relationship between regional activations and metabolite levels, let alone specifically GABA. Several studies in healthy samples combining the two have reported associations between negative BOLD responses to GABA, suggesting a role for GABA in neuronal inhibition characteristic of the negative BOLD response for which neuronal activity reductions are tightly coupled (Puts & Edden, 2012b; Rae, 2014).

A study by Northoff et al. (2007) reported GABA and BOLD signal correlations in the ACC region, with higher GABA levels measured during resting state associated with a larger negative BOLD signal change in this region during an emotional processing task. The authors suggested that negative BOLD response was underpinned by the neuronal inhibition characteristic of GABA function.

In another study within the dACC, higher GABA levels were associated with increased functional activation of the amygdala depending on stages of the fear network paradigm that was utilised (Levar et al., 2017). A similar study utilised a cognitive-control paradigm to assess dACC BOLD signalling and NAA, creatine and choline levels in a healthy sample (Yucel et al., 2007), as well as intelligence quotient (IQ) data, and reported a relationship between NAA on BOLD signalling conflict monitoring that was mediated by IQ levels.

In a metabolic and functional study Stan et al. (2014) examined the relationship between metabolite levels (glutamate and GABA) and BOLD signal activations in the DMPFC and ACC during emotion regulation in healthy subjects (n=20) during an emotion faces task. The study reported a positive correlation between the pre-task BOLD signal in the pregenual ACC and MPFC glutamate concentration. There was a task-specific negative correlation between BOLD signal in subgenual ACC and MPFC GABA concentration in the sad vs neutral/shape contrast, and in the anger vs shape contrast a negative correlation between age and BOLD signal in pregenual ACC. The results suggest that increased BOLD signalling in the processing of negative emotions may relate to decreased GABA levels and increased glutamate and that age may be a factor in emotion appraisal and self-reference.

Another study utilised additional several stress/anxiety biomarker measures (Levar et al., 2017; n = 70) to examine healthy male dACC region GABA profiles, skin conductance, and BOLD responses and functional connectivity, during fear extinction to fear retrieval. This study reported that dACC GABA levels have a role in extinction learning, and it was involved in inhibiting consolidation of fear extinction.

A combined study (Schmitz et al., 2017) using a thought-suppression paradigm measuring frontal-hippocampal memory retrieval activation reported that increased hippocampal GABA was associated with greater suppression control and a stronger frontal-hippocampal coupling. Conscious control over thoughts resulted in reduced hippocampus activation. The data provide a strong rationale for the inhibitory role of GABA within region-specific cognitive processes, namely memory retrieval.

One recent intervention study examined modulation of task-dependent BOLD signal following the benzodiazepine diazepam (Walter et al., 2016) reporting an inverse relationship between increased ACC activation during a working memory task and plasma drug concentration, which further supports the capacity for GABA to attenuate BOLD signal.

In mood and affective disorder groups, regional correlations in MDD and healthy control samples have been examined to garner a picture of underlying processes in anhedonia (Walter et al., 2009). In the pregenual ACC, no associations were shown between GABA and negative BOLD signals in the MDD groups; however, associations were observed for glutamine and NAA concentrations. In the healthy control group, a negative BOLD response correlated with GABA metabolite levels, in the same manner as the control group in the Northoff et al. (2002) study, which implicates a differential relationship between BOLD signalling and GABA inhibitory processes in these regions in MDD.

A study of MDD that used a matched healthy control (Horn et al., 2010; n = 44) examined GLX and resting-state functional connectivity in the pgACC and left anterior insular cortex regions, reporting that pgACC glutamate and glutamine predicted resting-state functional connectivity between the two regions in the MDD group. Reduced glutamate in the ACC region has also been reported in other depression studies (e.g. Auer et al., 2000; Demenescu et al., 2017) and contributes to the theory raised in Section 4.6 that glutamate substrate processes play a role in the maintenance of depression symptomologies.

Overall, these results suggest that in healthy samples GABA is geared towards the inhibition of activity measured through the BOLD signal, with some functional connectivity to suggest this relationship exists brain-wide (although the evidence is somewhat greater for the relationship between activation and the glutaminergic substrate) and, additionally, evidence suggests that this relationship is measurable during specific cognitive processes, such as hippocampal GABA facilitating unwanted thought inhibition as per Schmitz et al. (2017).

Combined studies in GAD samples are few, and there is therefore limited evidence of metabolic and functional patterns unique to GAD, that might be associated with anxiety symptoms, despite the advantage of conducting multi-modal imaging to better evidence patterns in affective disorders. The aforementioned Moon et al. studies reported positive relationships between the choline ratio and BOLD signal changes in the DLPFC

and contributed some evidence to suggest a correlation of metabolites to symptom measures on several anxiety scales (also seen in adolescents in the Strawn et al. studies).

In healthy samples, GABA profiles and BOLD activation levels indicate a negative relationship, yet in disorder groups, the association is more complex. It is feasible that GABA levels and fMRI BOLD responses that are ordinarily correlated and can be observed during stimulus processing within regions such as the ACC, are indirectly disrupted by mechanisms such as glutaminergic processes.

That mood symptoms and major depressive disorder are common comorbidities in GAD warrants further research as to how the maintenance of these symptoms are modulated by GABA and Glx metabolic profiles, and if they exhibit influence on cognitive function measured through task-dependent BOLD signalling. A review by Puts and Edden (2012b) discusses at length the benefit and inherent need to combine these modalities across mood and affective disorders.

4.3 Biomarkers as Predictors of Treatment Response in GAD

A limited number of intervention studies using GAD samples have been conducted that employ one or more biological measurements such as imaging and that are either randomised and/or placebo controlled, with or without comparator sample groups.

Two reviews of note have examined neuroimaging evidence as predictors of treatment response more broadly across anxiety disorders. Shin and Liberzon (2010) conducted a systematic review across anxiety disorders of structural and functional studies and found that pre-treatment neuroimaging measures predicted the treatment response across OCD, PTSD, GAD and SAD. Specifically, in the three studies that included patients with diagnosed GAD (McClure, Adler, et al., 2007; Nitschke et al., 2009; Whalen et al., 2008) greater pre-treatment regional blood oxygen level-dependent (BOLD) signal activation predicted greater improvement to anxiety on HAM-A and Penn State Worry questionnaires as a function of treatment. In these studies, the activation levels in the medial prefrontal cortex (mPFC) and amygdala regions were significant predictors of treatment response. Specific regions of the PFC included the rostral ACC region where it was related to better venlafaxine/fluoxetine treatment response. Notably, the review did not discuss the significance of change to outcome measures associated with the treatment itself, only regional significance.

A second systematic review somewhat complementary to the first (Ferreira-Garcia, Mochcovitch, Costa do Cabo, Nardi, & Christophe Freire, 2017) examined the broader predictors of pharmacotherapeutic treatment response specifically in GAD, reporting on

22 environmental, personality, genetics and brain function factors. Depression comorbidity was found to be a strong moderator of antidepressant treatment response. Significant predictors of treatment response included neuroticism as a personality dimension, previous treatment history, genetic polymorphisms (such as serotonin receptor gene) and, from functional imaging, activation of the ACC and amygdala regions. The authors discussed a need for more precise examination of predictors to treatment response in GAD, particularly within the domains of genetics and imaging. Both these reviews represent the type of broad assessment that is required for garnering evidence of regional mechanisms associated with GAD and the modelling of influencing factors with which to apply in intervention research.

4.4 Anterior Cingulate Cortex – Structural, Functional and Metabolic Role in GAD

The connectivity of the ACC to the prefrontal-cortical and from lower limbic and hippocampal regions places it in a central role with organisation of affective and cognitive information with processes for sadness recall, selective attention, loss/error/reward, and, importantly, the suppression of negative emotions or cognitions and autonomic processes that underpin anxiety states (Bush et al., 2000; Critchley et al., 2003; Etkin, Egner & Kalisch, 2011; Luu & Pederson, 2004; Vogt et al., 1992).

Further, the region has been proposed to be a 'hub' within the default mode network, of relevance in the processes of self-reference, introspection and adaptation to processes requiring cognitive and attentional control, for example, the detection of 'exclusion' in social contexts, which has ramifications within perceptual as well as behavioural mechanisms (Falkenstein et al., 1991; Greicius et al., 2003; Kawamoto et al., 2015). Lesion studies have had the dual result of identifying the significance of emotion and cognition processes, through the reduction in distress and emotional lability, and autonomic and attentional dysfunction, respectively (Cloninger, 1987; Corkin, 1979).

Yet, despite the importance of the ACC, the regions of greater focus in structural and functional studies of cognition and emotion have been the prefrontal cortical and/or limbic regions such as the amygdala. Thus, the functionality of the ACC region is of notable investigative value for examining the cognitive, affective and autonomic symptoms appearing in GAD, particularly the cognitive components of anticipatory anxiety and negative bias (Buckner et al., 2008; Bush et al., 2000; Cohen et al., 2000; Pizzagalli et al., 2006; Raichle et al., 2001).

4.4.1 ACC anatomical divisions

The ACC is the frontal circle-like portion of the cingulate cortex, which is located anterior to the corpus callosum and forms part of the connectivity between the prefrontal cortical and limbic regions. The ACC is one of four regions of the cingulate cortex and is heterogeneous in terms of cyto-architecture, labelled as Brodmann areas 24, 32, and 33 based on cytology divisions that still need to be clearly elucidated (Brodmann, 1909; Palomero-Gallagher, Mohlberg, Zilles, & Vogt, 2008).

The ACC may be further differentiated into a rostral-pregenua region associated with affective processes and a dorsal-subgenual region associated with cognitive processes evidenced from pre-clinical and human studies (Bush et al., 2000; Devinsky et al., 1995; Etkin et al., 2011; Palomero-Gallagher et al., 2008; Vogt et al., 2005). Evidence here is mainly derived from primate animal models (e.g. Picard and Strick, 1996) and human studies involving emotional appraisal paradigms, such as the Emotion Stroop task, whereby congruent and incongruent stimuli indicate differences within both the rostral and ventral ACC regions (Bush, Luu & Posner, 2000; Mathews, Paulus, Simmons, Neleson, & Dimsdale, 2004; Whalen et al., 1998).

Structurally, the ventral subregion (also termed pregenual, perigenual or rostral in relationship) is positioned anterior to the genu and extends as part of the more dorsal-lateral PFC towards the frontal pole. With reference to the Brodmann areas, BA32 and the inferior portion of BA24 are included (refer Figure 4). The ventral ACC subregion is principally associated with event response, emotional processing and appraisal from the environment. It connects to the amygdala, hypothalamus, anterior insula and nucleus accumbens and, importantly, has parasympathetic connections involving endocrinergic and digestive system functions (Bush et al., 2000; Etkin & Wager, 2011; Koski & Paus, 2000; Pizzagalli et al., 2006).

The dorsal ACC (dACC) subregion is associated with cognitive processes involving the environment such as performance evaluation related to visual stimuli, cognitive control during motor response and reward-based decision making (Asemi et al., 2015; Bush et al., 2002; Hayden & Platt, 2010; Paus, 2001). As such, the region holds connections to the prefrontal and parietal cortices, the visual system, and aspects of the motor system, e.g. the orbitofrontal cortex, nucleus accumbens, amygdala, hippocampus and hypothalamus (Koski & Paus, 2000; Vogt, Finch, & Olsen, 1995).

For emotional and/or aversive stimuli processing specifically, the region has been associated with the appraisal and mediation of fear responses in both animal models

and human studies, which are cognitive components of the fear/stress response. This region forms one of the three core structures of the conscious 'fear network', together with the amygdala and vmPFC (Maier et al., 2012; Milad et al., 2007; Nitschke et al., 2005).

Few (although increasing in number) studies have exploited MRS (or single-photon emission computed tomography- SPECT) to measure metabolic regional variations to aid structural/functional delineations. One study has done so in the ACC region (Dou et al., 2013) and reported inhibition/excitation differences in four subregions, including pregenual ACC from the mid-cingulate cortex in terms of GABA and glutamate levels in healthy samples at 7T.

While neuroimaging investigations involving psychiatric populations have stated the importance of investigating these functionally-specific regions, most findings are equivocal and so may not evidence clear differences between disorders, or ability to distinguish 'normal' fear and anxiety processes, and disorder groups (Koski & Paus, 2000; Mayberg et al., 1997; Nitschke et al., 2009; Palamero-Gallagher et al., 2008).

Specifically, for the prefrontal ACC–PFC regions, the anatomical literature shows at least two aspects of conflicting evidence; first is the tendency of imaging studies to group the dACC region with the adjacent dorsal-medial areas of the PFC (for review, see Kalisch & Gerlicher, 2014), which constrains the evidence for ACC subregion functional specificity. There is a lack of consensus on the precise mapping of the ventral/rostral and dorsal/caudal ACC areas, owing to the relative nature of the regional terminology in the corticolimbic system, particularly as to what constitutes the termination of the rostral area (Stevens, Hurley, & Taber, 2011; Vogt, Hof, & Vogt, 2002). The capacity to precisely map function over these regions in human studies is constrained by the ability to apply invasive techniques to delineate neural structure, function and connectivity in human studies (see a further discussion in Parker et al., 2002).

The second consideration is that the evidence underpinning functional divisions in human studies is largely derived from the Stroop task, with equivocal findings depending on the paradigm variations that are employed. In particular, there is conflicting evidence as to how regions such as the amygdala, dACC/dmPFC and dorsolateral PFC are involved in emotion conflict processing and emotional regulation. Although not extensively examined in the context of anxiety disorders, the use of the Stroop task as a prototypical emotion appraisal task warrants critical consideration. Several review papers have

discussed the validity of valent Stroop task findings at length (Algom, Chajust, & Lev, 2004; Phaf & Kan, 2007; Williams et al., 1996).

For this thesis, the structural and functional rACC/affective and dACC/cognitive subdivisions as presented in Bush, Luu, and Posner (2000), Etkin, Egner, and Kalisch (2011), and Vogt (2005) are utilised (and illustrated in Figures 5 and 6), with consideration for the heterogeneous and integrative role of the dorsal subregion asserted by Etkin, Egner, and Kalisch (2011) in cognitive components of emotional processing that are of relevance to GAD anticipatory–anxiety symptomatology.

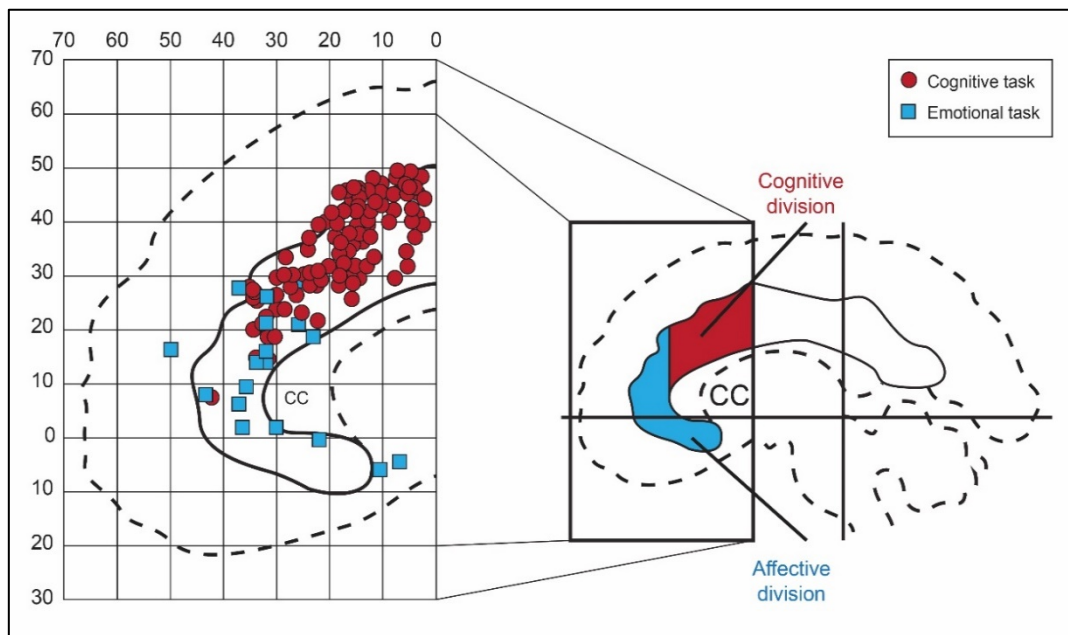


Figure 5. Anterior cingulate cortex functional subregions. Meta-analytic evidence from imaging studies for affective and cognitive functional differences, equivalent to Brodmann areas 24/25 and 32/33 (refer Figure 6). Red dots indicate areas of activation during cognitive paradigm studies; blue squares indicate studies of affective paradigm activations (adapted from Bush, Luu, & Posner, 2000)

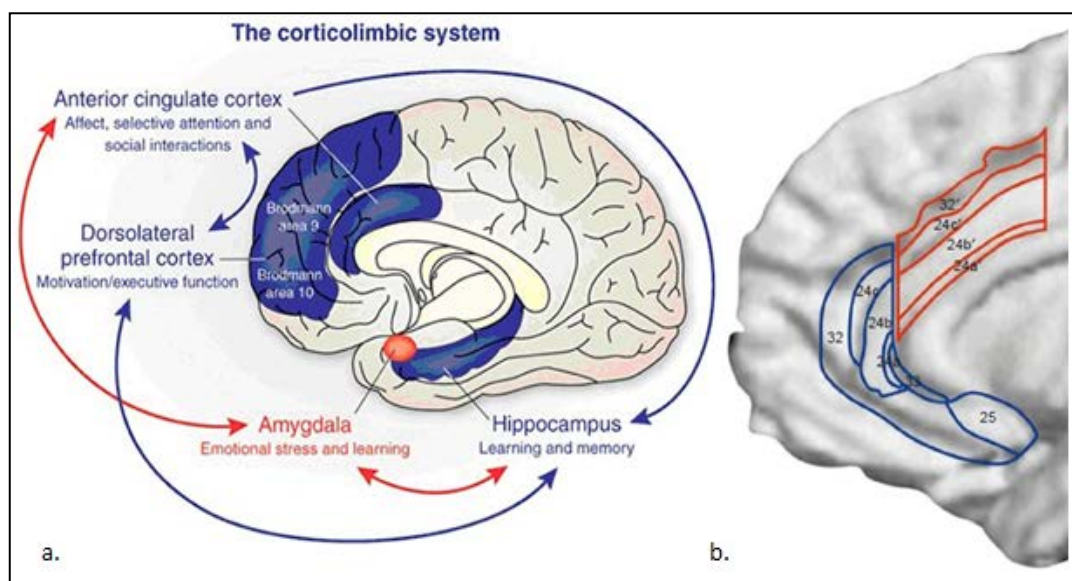


Figure 6. a. Functional anatomy of the corticolimbic system. b. Brodmann labels of the ventral and dorsal subregions of the anterior cingulate cortex (Images: Frontiersin.org; Vogt et al., 1995)

4.4.2 Anterior cingulate function in GAD

Dysregulation within the ACC has been implicated in a number of disorders demonstrating deficits in cognitive, emotional and mood processing, e.g. schizophrenia, autism, bipolar disorder, GAD, social phobia, PTSD, OCD and depression (Hamilton et al., 2015; Luu & Pederson, 2004; Pittenger et al., 2011; Yucel et al., 2003).

The ACC's interface between the internal state and stimulus processing suggests a hallmark role in GAD cognitive/emotional symptomatology. Several studies posit evidence for correlations among ACC regional activation, symptom severity and subsequent response outcome with experimental interventions in GAD groups; however, the research is limited. Nitschke et al. (2009) found that higher levels of pre-treatment ACC activity in anticipatory tasks were associated with greater clinical outcome in patients with GAD treated with venlafaxine and Hoehn-Seric et al. (2004), using a citalopram intervention, found anxiety sentences promoted greater activation in the ACC.

A review of the research by Shin et al. (2013) on the prediction of treatment response in anxiety disorders examined studies by Whalen et al. (2008), Nitschke et al. (2009) and McClure et al. (2007) and found that the mPFC (for which the authors included the rostral ACC) and amygdala activity levels pre-treatment were predictive of the response

to treatment intervention (antidepressants), such that greater levels of activation pre-treatment generally predicted a greater response outcome (refer to Table 6 for details of each study).

Similarly, but using the whole brain (70 regions) random forest plot model of fMRI data collected pre-treatment in GAD and panic disorder patients, Ball et al. (2014) structured a predictive model for response outcome using CBT intervention, i.e., determination of treatment responders. The second aim of the study was to identify CBT responder status via the activations of 70 different brain regions. Although the study's introduction asserted that the 'ACC was identified as a potential predictor of antidepressant medication response in mood disorders' and cites Mayberg et al. (1997) for this evidence (refer to Table 6 for details of this study), in identifying regions as predictors of responder status, the ACC was not a ROI for which activation levels could be used to predict CBT response in the model. The study used negative-valenced images from IAPS in an emotion regulation task where participants were asked to maintain or reappraise responses to the negative images presented. Activations in the hippocampus during maintenance, and in the anterior insula, superior temporal, supramarginal and superior frontal gyri during reappraisal were the regional predictors of response, with greater activations in responders than in non-responders.

The ACC is part of the default mode network that shows predominantly negative BOLD responses during fMRI tasks, which in one cross-modal study (Northoff et al., 2007) was combined with resting-state MRS measurements and the concentration of GABA in the ACC was found to correlate with the levels of BOLD response from activations during an emotional task in the same region.

Directionally, higher GABA levels correlated with stronger negative BOLD responses in the same region and the association was stronger with increased cognitive load during the task (that is, response selection). No association was found with glutamate levels. The authors posited that MRS concentrations of GABA predicted the amount of negative BOLD responses in the ACC during an emotional task.

Because negative changes in the BOLD signal indicate a decrease in activation relative to a given resting state, or 'rest condition', it is feasible that GABA is involved in these metabolic processes, yet it is difficult to conclude that negative changes directly equate to metabolic decreases and that small attenuations can produce measurable changes in GABA concentrations.

During states of emotion processing, GABA levels may increase to effectively reduce the level of neuronal response, as if a threshold level is reached. This would be further supported by the fact that it occurs during higher cognitive load, as an adaptogenic mechanism for energy conservation, also likely reflected in the negative BOLD response. The link between these processes is discussed in greater detail with more cross-modal investigations in Section 4.2. Please also refer to the previous section, which details studies that discuss the alterations of GABA signalling underpinning cognition processes in greater depth.

GABA metabolite levels are significantly higher in regions such as the anterior cingulate (Dou et al., 2013; Ganji et al., 2014). Selected metabolite spectra of the ACC region from a typical MRS is illustrated in Figure 7, sourced from a study by Das et al. (2013). Alterations in cerebral metabolites may reflect changes in various brain micro-structure and neurochemical abnormalities; therefore, MRS is often used to study psychiatric disorders (Berger et al., 2008; Etkin & Wager, 2010; Puts & Eddin, 2012b).

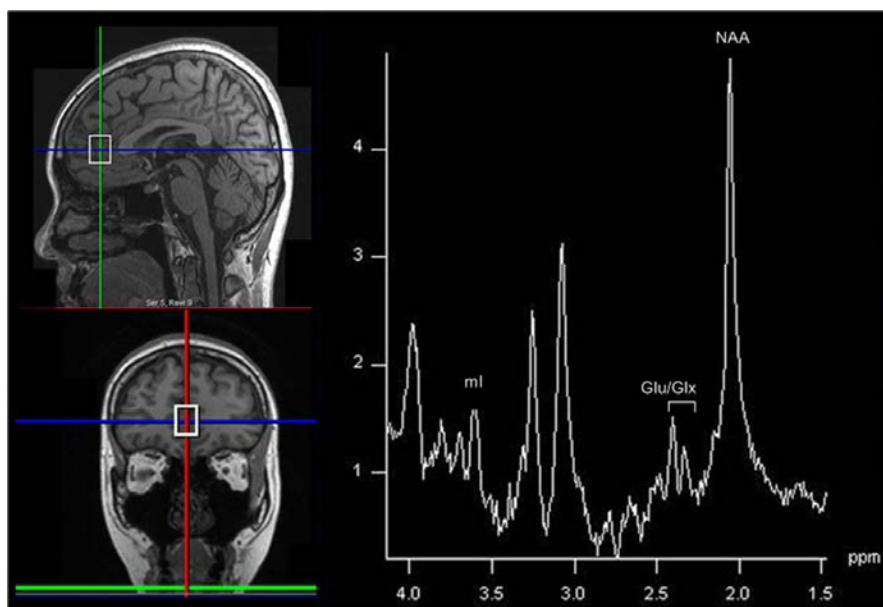


Figure 7. MRS spectra of selected metabolites sourced from the dACC region (white square; source: Das et al., 2013)

For disorders such as GAD, selected cerebral metabolite measurements in regions such as the ACC may provide important biomarkers of the disorder itself and treatment interventions. Of relevance here is glutamate/glutamine (Glx), and GABA for their aforementioned excitatory/inhibitory roles and the ACC region for its central role in anxiety neurocircuitry.

4.5 GABA Substrate in the Brain

The GABA system holds the principle inhibitory role in central nervous system (CNS) functions and it is implicit in a number of neurophysiological processes including motor function, pain, sleep and anxiety (Curtis, Phillis and Watkins, 1959; Jones, 1993; Mohler, 2001; Nemeroff, 2003; Roberts, 1974). GABA (Figure 8) and the functional corollary, glutamate, are the most common CNS neurotransmitters, with over 50% of the brain synaptic connections using the latter and approximately 30%–40% using the former for inhibitory processes via action potential regulation (Paul & Skolnick, 2003; Roberts, 1974).

GABAergic and glutaminergic neurons and glial cells are the main consumers of energy in the cerebral cortex, using 80% of total glucose oxidation (Rothman et al., 1999), which provide a direct relationship between these metabolic pathways and cortical activation (Gruetter, Seaquist & Ugurbil, 2000; Mescher, Merkle, Kirsch, Garwood, & Gruetter, 1998; Shen et al., 1999).

The GABA molecule ($C_4H_9NO_2$) is a non-standard amino acid by virtue of the conformationally flexible amino and carboxylic acid groups it possesses, which benefits its pervasive biological inhibitory function (Hayashi & Nagai, 1956; Killam & Bain, 1957). GABA was a known plant-based substance for 70 years before discovery in the vertebrate CNS (Cooper, Bloom, & Roth, 2003; Roberts & Frankel, 1950).

The role of GABA as a neurotransmitter operates in an inhibitory capacity as an agonist at GABA-A and GABA-B receptors, where the primary function is a reduction to subsequent signalling via neural action potential and/or the release of neurotransmitters. It does not operate in isolation, but in concert with excitatory neurotransmitters such as glutamate and norepinephrine (Hertz, Schousboe, Boechler, Mukerji, & Fedoroff, 1978), although the mechanisms driving production, transmission and reuptake mostly occur separately (Olsen & DeLorey, 1999). GABA is located in every brain region; however, it is highly concentrated in the cortical and limbic areas.

Brain regions comprising the 'anxiety circuitry' are also within these areas; namely, the amygdala, cortex, hippocampus, substantia nigra and striatum regions, and it is the GABA patterns largely within these regions that have been investigated across several disorders involving negative emotions and somatic anxiety/stress responses (Etkin & Wager, 2007; Nuss, 2015).

The utility of pathway enzymes and transport proteins is shown in the pharmacokinetics of several anxiolytic, anticonvulsant and mood-stabiliser drugs (Kalueff & Nutt, 2007; Schousboe, Sarup, Larsson, & White, 2004; Sherif & Ahmed, 1995), such as tiagabine (a GAT-1 inhibitor) which is indicated for anxiety disorders (refer to Chapter Five for information regarding this drug as potential treatment for GAD).

Drugs that exert stimulating or sedating effects often target GABA/glutaminergic substrates. Caffeine, for example, inhibits GABA release and increases glutamate activity, whereas alcohol and sedatives conversely work to increase GABA activity (further details on dietary modulations of GABA is briefly discussed in Section 4.6 in this chapter). Endogenous system GABA acts within brain regions through attachment to postsynaptic GABA-A receptors and opening ionic/chloride ion channels.

An anxiolytic such as a benzodiazepine, action a specific benzodiazepine modulatory site adjacent to GABA-A receptors, enhancing the ion channel opening. In turn, the efficacy of endogenous GABA is enhanced at the postsynaptic GABA-A receptor (Haefely et al., 1975; Tallman, Thomas, & Gallager, 1978).

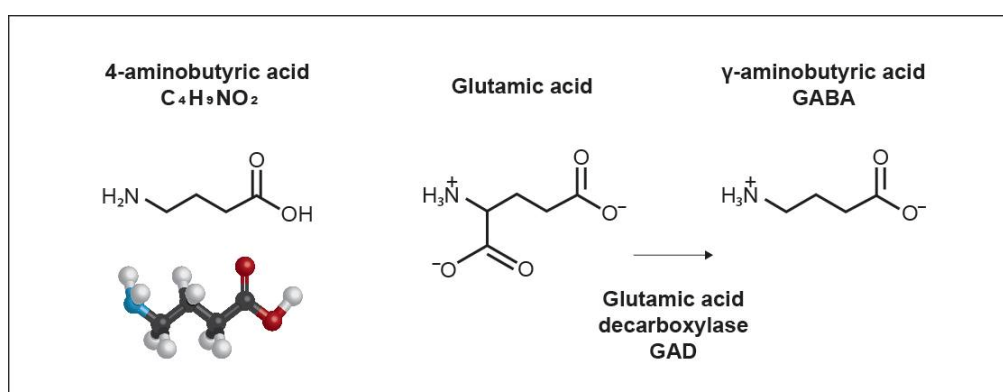


Figure 8. Molecular precursor components of γ -aminobutyric acid/GABA synthesis

4.5.1 GABA pathway components

Several pathway components help reveal the molecular basis for the production, transmission, reuptake and, ultimately, the finely-tuned regulation of GABA homeostasis. Some components are still being elucidated, concurrent to technological improvements in labelled-substrate and spectroscopy instruments, which will likely enable expansion of the current knowledge base regarding GABA system mechanisms and homeostatic mechanisms in the brain.

4.5.1.1 Synthesis

When viewing GABA production from the pre-synaptic level, its origin lies in the GABA shunt. Neural cells do not directly utilise glucose for the GABA/glutamate cycle, rather the metabolism of glucose from the Krebs cycle is achieved by the derivation of α -ketoglutarate via GABA α -oxoglutarate transaminase (GABA-T) to form L-glutamic acid (Martin, 1993; Roberts & Baxter, 1958). Glutamate is generated partially from glutamine at GABAergic synapses and astrocytes release glutamine for neural uptake as the GABA precursor (Rothman et al., 1999).

Thereafter, this component involves conversion from glutamate/L-glutamic acid via L-glutamic acid decarboxylase/glutamate decarboxylase (GADe) to GABA by removal of the carboxylic acid component in the cytoplasm (Kaufman, Houser, & Tobin, 1991; Martin & Barke, 1998; Martin & Rimvall, 1993). GADe is present in two isoforms in adult humans, GAD65 and GAD67, which are thought to synthesise 30% and 70% GABA, respectively (Erlander & Tobin, 1991; Martin & Olsen, 2000).

The smaller isoform GAD65 is most present in axonal terminals, whereas GAD67 is distributed more uniformly. Preclinical research suggests that the concentrations of GADe are maintained via post-translational feedback processes from GABA levels that serve to modulate their expression via mRNA splicing or transcription (Modi, Prentice & Wu, 2015; Rimvall & Martin, 1994; Wei, Jin, Wu, Sha, & Wu, 2003).

GADe requires pyridoxal phosphate (PLP), a form of vitamin B6, as a coenzyme (Balázs, Machiyama, Hammond, Julian, & Richter, 1970; Roberts & Baxter, 1958; Tapia, 1975). In the brain, 50% of the total GADe is present without bound PLP as an inactive apoenzyme, whereby additional GABA is synthesised when required, such as during increased systemic stress (Martin, Martin, Wu, & Espina, 1991; Miller et al., 1977; Patel, de Graaf, Martin, Battaglioli, & Behar, 2006).

GABAergic compounds might interact directly with the PLP-binding site, acting as PLP mimics, or facilitate the binding of PLP to GADe by causing a conformational change; however, the precise mechanisms surrounding the promotion of this activity is unclear. Pyridoxine deficiency is associated with the generation of seizures (Sharma & Dakshinamurti, 1992) and administration of high doses may increase GABA levels, thereby conferring a potential role in disorders that display reduced GABA levels, and a feasible treatment for anxiety symptoms (McCarty, 2000).

4.5.1.2 Transportation

The six known GABA transporter (GAT) proteins (A1/GAT1, A13/GAT2, A11/GAT3, A12/BGT1, A8/CT1, and A6/TauT) are members of a larger, structurally similar neurotransmitter group, the solute carrier family 6 (SLC6; refer to Figure 9) that also comprise transporters for aminergic and creatine, among others (Liu, Lopez-Corcuera, Mandiyan, Nelson, & Nelson, 1993; Scimemi, 2014). GATs have low specificity and, therefore, enact multiple roles in GABA synthesis and transmission during both synaptic and basal activities, particularly within the extracellular GABA uptake 'system'. This is prominently guided by the Na⁺ concentration-dependent GAT-1 (SLC6A1) in CNS neurons and in astrocytes/glial cells, whereby GABA is subsequently recycled (Bröer, 2006; Isaacson, Solís, & Nicoll, 1993; Kristensen et al., 2011; Minelli, Brecha, Karschin, DeBiasi, & Conti, 1995).

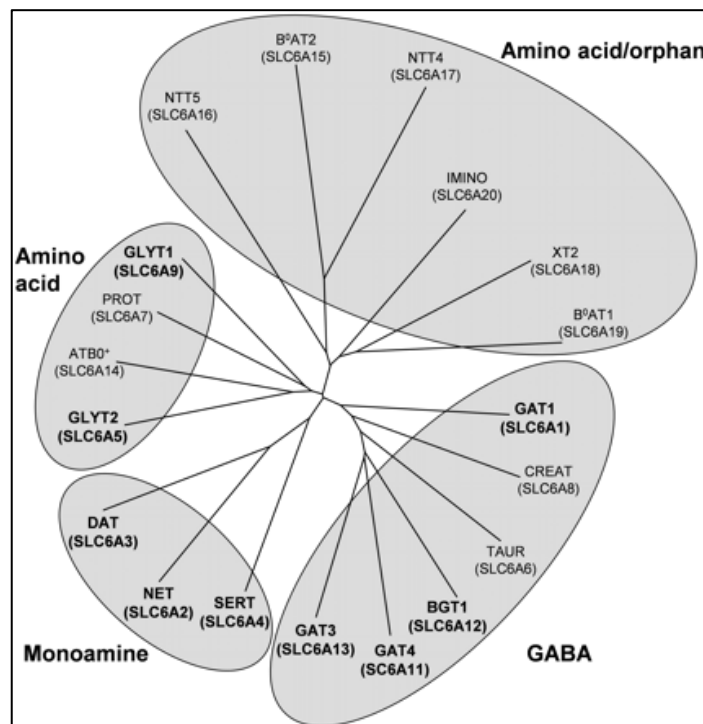


Figure 9. The four solute carrier 6/symporter (SLC6) subfamilies and their isolated genes, indicating functional relationships (Bröer, 2006)

An example of a GATs role in the GABA pathway is evidenced whereby GAT-1 transporter deletion leads to a decreased quantal GABA release in animal models. This generates increased extracellular GABA levels and inhibition of GABA activity, through which behavioural modulations may occur (which are associated via reduced anxiety

and aggression) (Cai et al., 2006; Liu et al., 2007; Zhou & Danbolt, 2013). GATs are a common substrate component for pharmacotherapies and GAT-targeted inhibitors such as tiagabine are supported by both animal and human studies as a mood stabiliser and anxiety treatment. These are discussed more in Chapter Five, which details GAD treatments (refer also the review by Zwanzger & Rupprecht, 2005).

4.5.1.3 GABA Transportation and Reuptake

Following the release into synaptic clefts, GABA will generally bind to GABA receptors as detailed below. The synaptic action of GABA is terminated following catalysis in the cleft or reuptake at the pre-synapse or accessory astrocytes/glia cells by GABA-T, such as 4-aminobutyrate aminotransferase, into succinic semialdehyde and/or L-glutamate (Olsen & DeLorey, 1999; Schechter & Grove, 1980; Tapia, 1975).

GABA-T is present throughout the mitochondrial and plasma membranes and cytoplasmic matrix, and is more concentrated in neurons than in glia, as well as being more concentrated in pre-synaptic terminals (Olsen & Sieghart, 2009; Sherif, 1994). Similar to GADe, GABA-T requires PLP as a co-factor during this step. Modulation of GABA-T enzymes, such as inhibition, produces direct increases to neuronal GABA levels, evidenced from seizure models (Fowler, Beckford, & John, 1975; Meldrum & Horton, 1978; Treiman, 2001).

Finally, succinic semialdehyde is converted to succinic acid via succinic semialdehyde dehydrogenase to re-enter as a usable source of energy in the citric acid cycle (Bak, Schousboe, & Waagepetersen, 2006; Bown & Shelp, 1997).

The 'storage' of GABA relates to both cytoplasmic and vesicular intracellular 'pools' that exist in equilibrium except for during the process of depolarisation (Rae et al., 2014; Waagepetersen et al. 1999; 2001)—refer to Section 4.6 in this chapter for greater detail regarding GABA quantification.

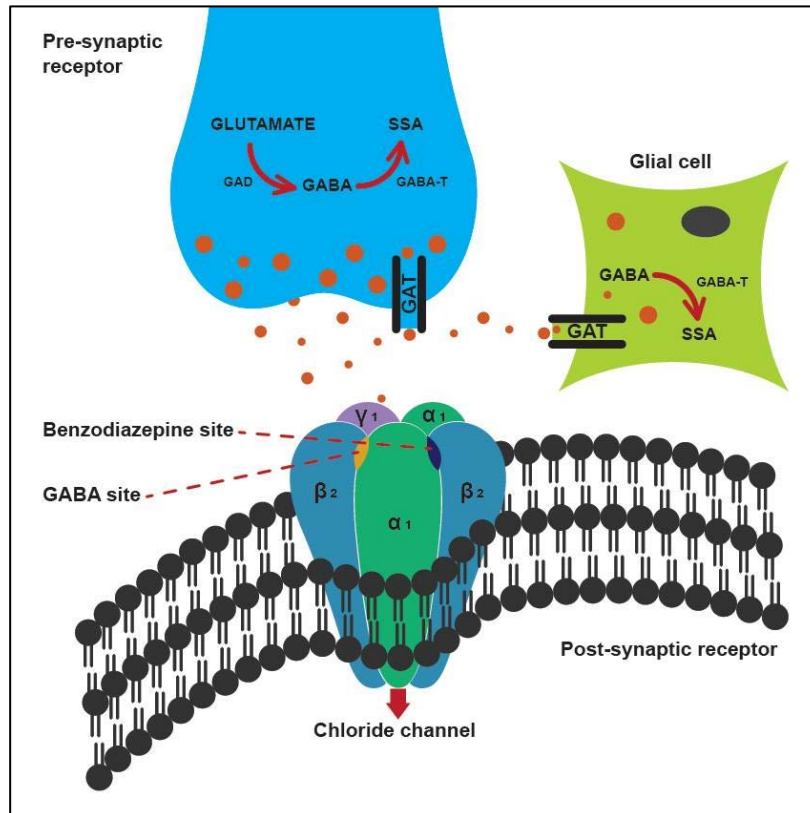


Figure 10. Schematic representation of GABA neurotransmitter components

4.5.2 GABA receptors

GABA-A receptors are not static and experience rapid amendments to their number and composition in response to the cellular homeostatic environment. Further, they are not isolated to synaptic terminals and are extra-synaptic, activated by the GABA present in extracellular spaces (Sigel & Steinmann, 2012). To date, three classes of neuroreceptors belonging to the sys-loop superfamily of ligand-gated ion channels have been isolated that hold the principle inhibitory role in the CNS: GABA-A, B and C (or A-rho) (Miller & Smart, 2010; Whiting, 2003; 2006).

The sys-loop channel family group also includes the nicotinic acetylcholine, glycine and 5-HT₃ receptors. They are composed of pentamer units that are variable in combination with up to 19 subunits (α 1–6, β 1–3, γ 1–3, δ , ϵ , π , θ and ρ 1–3), depending on location and function (Barnard, 2001; Korpi, Gründer, & Lüddens, 2002; Rudolph et al., 1999). However, GABA-A receptors in the brain are generally formed from two α -, two β - and one single γ - or δ -subunits, illustrated in Figure 11 (Hanrahan, Chebib, & Johnston, 2011; Whiting, 2003).

GABA-A receptors are ligand-gated chloride ion channels that are activated by GABA and the agonist muscimol, blocked by bicuculline and coupled to the GABA, barbiturate,

benzodiazepine and picrotoxin binding sites. GABA-B are g-protein-coupled receptors with two subunits and are indirectly involved in transmission via regulation of Ca²⁺ or K⁺ channels, which was discovered via the administration of baclofen (Hill & Bowery, 1981). GABA-C, which is now more frequently labelled as GABA-A-rho after Zhang et al. (2001), receptors are also ligand-gated chloride channels that hold at least three subunits and are the most sensitive to GABA modulation, although less prominent in the CNS. Agonists increase total slow-wave sleep and include picrotoxin (Bettler, Kaupmann, Mosbacher, & Gassmann, 2004; Johnston, Chebib, Hanrahan, & Mewett, 2003).

The role of GABA in anxiety symptoms is associated with GABA-A receptor modulations. Due to GABA-A receptors having a large molecular diversity, there is significant potential for targeted binding by a range of anxiolytic agents which have this affinity. GABA receptors are thus the putative target of anxiolytic drugs via modulating GABA release, reuptake and absorption (Olsen & Sieghart, 2009).

In GABA-A receptors, ionotropic activation triggers the opening of a chloride ion-selective channel, which stabilises or hyperpolarises the resting membrane potential. In turn, excitatory neurotransmitter capacity to depolarise the neuron and generate action potentials are reduced. In addition to the rapid actions of GABA via GABA-A receptors, the compound also modulates neural activity via a second longer pathway via the activation of GABA-A receptors, which belong to the G protein-coupled receptor superfamily (Wasowski & Marder, 2012).

Receptor subtypes and their function in the brain can be identified by the selectivity of certain drugs or compounds that elicit an anxiolytic effect (Olsen & Sieghart, 2009). A variety of compounds are evidenced to act via GABA-A receptors to elicit anxiolysis, including benzodiazepines, barbiturates, sedatives, anti-convulsants, neurosteroids and general anaesthetics. Even so, the majority are non-specific in their subtype activity and are likely to act on any one of numerous allosteric modulatory sites (Eser, Schule, Baghai, Romeo, & Rupprecht, 2006; Jembrek & Vlainic, 2015).

Flumazenil (1,4-imidazo-benzodiazepine) as an example binds to the benzodiazepine site ligand, acting on $\alpha 4\beta 2$ or $\alpha 6\beta 2$ receptors. It has utility in assessing subtypes in ligand binding studies and autoradiography, using [³H]Ro15-4513 in the absence/presence of high concentrations of flumazenil to mask receptors that hold $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunits, as indicated in Figure 11 (Korpi et al., 2002).

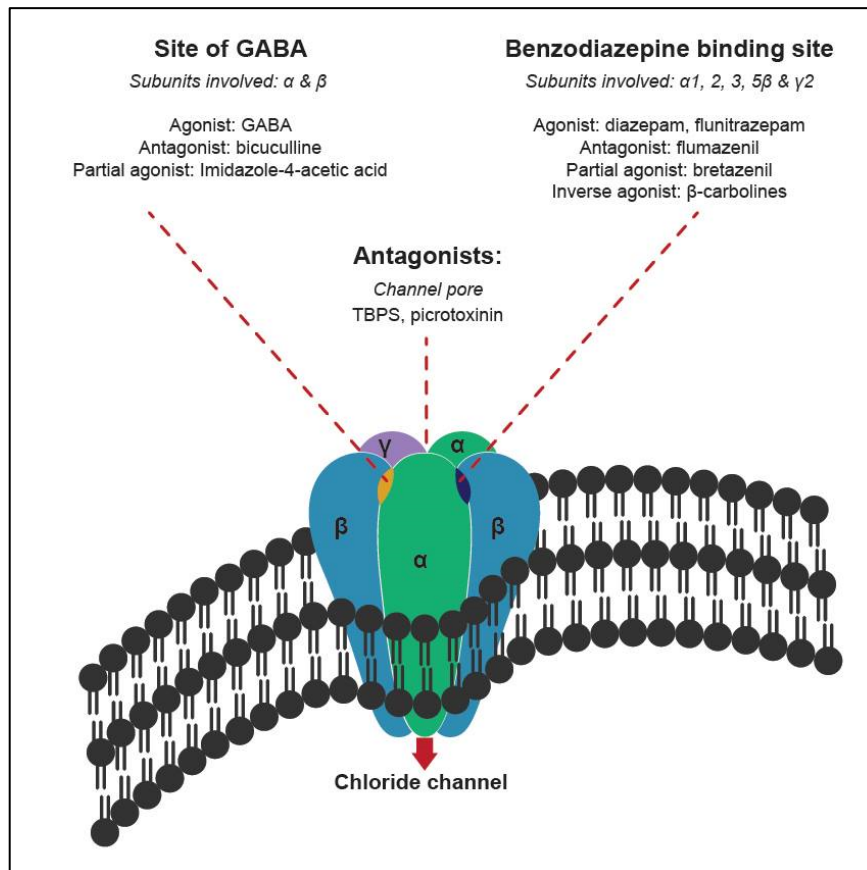


Figure 11. Binding sites and example drug modulators for GABA-A receptor (adapted from Wasowski & Marder, 2012)

4.5.3 GABA substrate connectivity in the central nervous system

GABA physiology interrelates heavily with other neurotransmitter/substrate systems, including dopaminergic (DA), serotonergic/5-HT and cannabinoid systems, and exerts direct and indirect effects (Johnston, 2005; Nemeroff, 2003; Olivier et al., 2013). One example of this relationship in the PFC decreases the activity of glutaminergic projections to the tegmental pedunculo pontine nucleus (an outward projection from the limbic system), and thus GABA neurons are able to indirectly inhibit the midbrain DA cells.

The additional attenuations here are DA neurons in the substantia nigra that receive additional glutaminergic input from the subthalamic nucleus. Direct modulations occur via inhibition through GABA-A and GABA-B receptors on ventral tegmental area dopamine neurons. Finally, both GABA and dopamine neurons share projections in the ventral tegmental area and substantia nigra regions (Sesack, Carr, Omelchenko, & Pinto, 2003; Tam & Roth, 1990).

Studies in humans involving iomazenil (GABA-A benzodiazepine receptor antagonist compound similar to flumazenil) have led to the development of a model for GABA deficits in healthy samples with various symptomatic changes akin to schizophrenia and epilepsy. In an investigation involving healthy controls, D'Souza et al. (2006) paired iomazenil with m-chlorophenylpiperazine (m-CPP), a partial serotonergic agonist of 5-HT(2A/2C) receptors, and produced symptoms of distorted perceptual/dissociative states. It was also reported that GABA deficits in this model increased the sensitivity to serotonergic agents, demonstrating this relationship where it is feasible that GABA deficits increase sensitivity to serotonergic agents. In a recent study, Anh et al. (2015) paired iomazenil with an amphetamine and observed that this induced psychosis-relevant effects in healthy subjects as well as in patients with schizophrenia (refer further the researchers' previous study, Ahn et al. (2011)).

Although GABA level deficit does not generate positive symptoms that are typical in psychosis reported in these studies using iomazenil, it does suggest a vulnerability to these symptoms while demonstrating GABA interrelationship with other substrates. Regional GABA deficits observed in schizophrenia (e.g., Lewis, Hashimoto, & Volk, 2005) via imaging studies (but also from tissue post-mortem models) also suggest it is a contributing factor to the pathophysiology, possibly via benzodiazepine receptor binding.

However regional magnetic resonance spectroscopy (MRS) investigations have reported equivocally on GABA differences in patients with schizophrenia compared to healthy controls, i.e. elevations (Kegeles et al., 2012), reductions (Rowland et al., 2016; Yoon et al., 2010) and also no differences (Goto et al., 2009). It is not clear how regional levels of quantifiable GABA through MRS may signal deficits, nor how it may clearly contribute to the maintenance of psychiatric disorder symptoms.

4.6 GABA Quantification in the CNS

4.6.1 Introduction

Only in the last 20 years has the measurement of GABA metabolite levels been possible in the living human brain (Chang, Cloak & Ernst, 2003). Prior to this, early MRS or high-performance liquid chromatography spectra data were collected on rat brains or post-mortem human brain tissues. The *in vivo* measurement of GABA in humans typically captured via proton magnetic resonance spectroscopy (¹H-MRS) in a 3 tesla (as the typical field strength) scanner, and, while evidenced to be reliable and replicable across time and within regions (Yasen, Smith, & Christie, 2017), there are several challenges in

the acquisition, interpretation and analysis of data (Mikkelsen et al., 2017; Mullins et al., 2014; Rothman, Petroff, Behar, & Mattson, 1993).

MRS quantification of GABA does not wholly measure the GABA activity outlined in the previous sub-section; it will not discriminate intra- or extracellular portions of GABA or account for GABA neuronal numbers, size or, to an extent, neurotransmitter flux (Mullins et al., 2014; Petroff et al., 1989; Puts & Edden, 2012b; Stagg et al., 2011).

The GABA molecule possesses three pairs of protons with distinct J-coupling between analogue pairs, equating to three chemical shifts. The spin system for GABA minus amino protons is shown in Figure 12 and the J-coupling constants for indexed protons and chemical shifts are shown in Table 7.

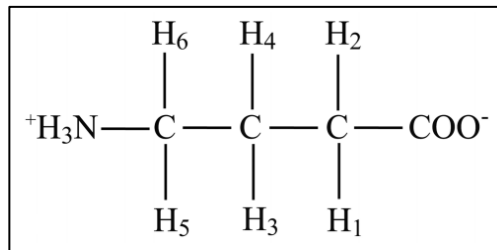


Figure 12. GABA spin system (without amino protons). Derived from Waddell et al. (2007)

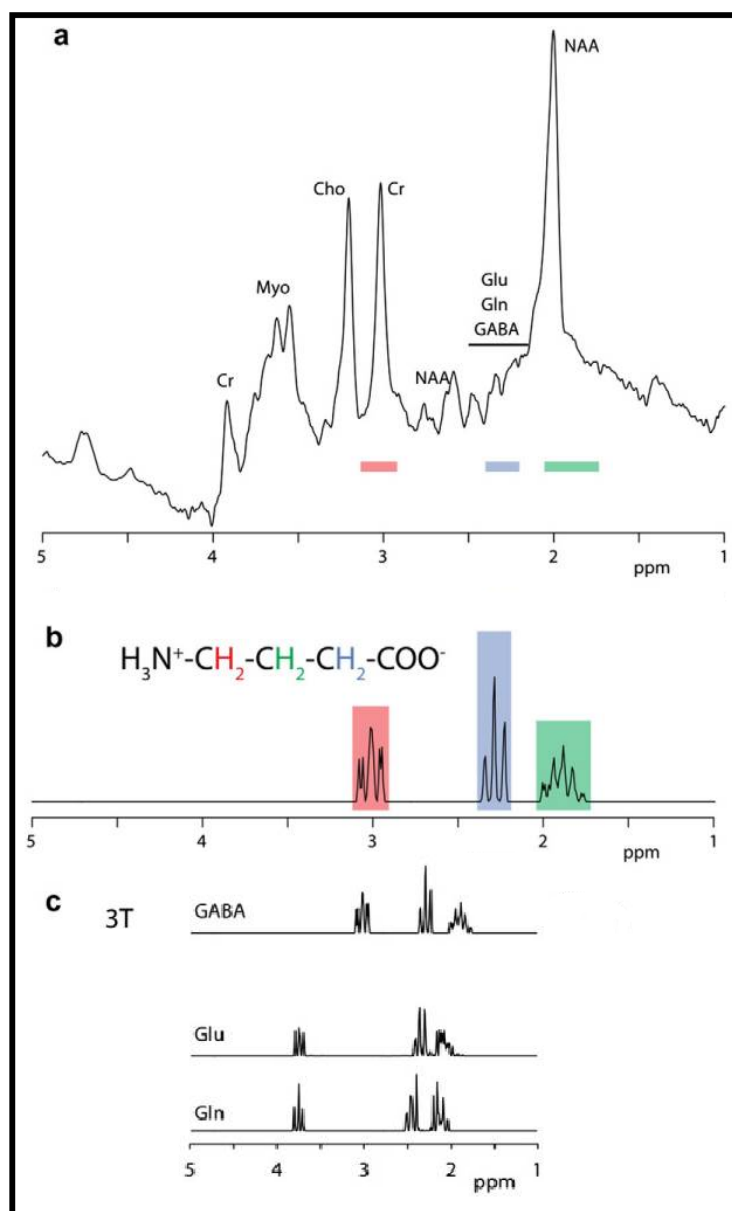


Figure 13. GABA spectra from MRS (a) ^1H -MRS spectra of metabolites acquired at 3T, with peaks corresponding to GABA, N-acetyl aspartate (NAA), creatine (Cr), choline (Cho), myo-inositol (MI), glutamate (Glu) and glutamine (Gln). Colouration corresponds to simulated peak locations in (b); (b) GABA spectra at 3T with CH_2 spins; (c) GABA, glu and gln spectra at a range of field strengths. Derived from Puts and Edden (2012).

Table 5. GABA J-coupling Constants of Indexed Protons

Resonance	Chemical Shift (ppm) ^{1,2}	J-coupling (Hz) ^{2,3}
H1	3.0128	H1-H3 (5.37), H1-H4 (7.13)
H2	3.0128	H2-H3 (10.58), H2-H4 (6.98)
H3	1.8890	H3-H5 (7.76), H3-H6 (7.43)
H4	1.8890	H4-H5 (6.17), H4-H6 (7.93)
H5	2.2840	
H6	2.2840	
¹ relative to ¹ H ₂ O (4.65 ppm).		
² chemical shifts and coupling constants taken from ref 9.		
³ redundant couplings not listed (J _{ij} = J _{ji}).		

Source: Derived from Waddell et al. (2007).

In Chang et al.'s (2003) landmark paper, the GABA concentrations ranged from 0.5 to 1.4 $\mu\text{mol}/\text{cm}^3$ (micromole to cubic centimetre) in neuronal tissue; for example, in cortical grey matter the approximate concentration is ~ 1 mM in humans and GABA lies within the lower sensitivity limits of the current ¹H-MRS methods in standard clinical 3T MRS scanners.

Due to being on the lower end of detection and obscured by frequency locations of metabolites of higher concentration such as creatine, several techniques are applied, such as delays alternating with nutations for tailored excitation (DANTE; Rothman et al., 1993), pulse point-resolved spectroscopy (PRESS; Bottomley, 1987) and Mescher-Garwood methods (MEGA-PRESS; Mescher et al., 1998) during acquisition to enable the proton signal via slice-selective gradient pulses, as well as crucially subtracting higher concentrations and exposing GABA peak frequencies (Figure 13 illustrates this spectra including GABA peaks at 3 tesla).

Editing techniques have the secondary aim of reducing issues inherent to MRS processes such as spectral distortions and more conventional scanner issues, such as motion-related errors (Harris, Saleh & Edden, 2017; Harris et al., 2014; Near, Evans, Puts, Barker, & Edden, 2013). However, there is a 'trade-off' between sensitivity and accuracy and the need for tailored acquisition, editing and filtering refinements, for a given sample size (de Graaf, Mason, Patel, Behar, & Rothman, 2003; Keeler, 2012; Mullins et al., 2014).

GABA levels are typically measured against reference molecules (e.g. via GABA-MRS Analysis Tool/GANNET; Edden, Puts, Harris, Barker & Evans, 2014), utilising total

creatine or total water as the internal reference and, as such, a stable reference molecule level is assumed in the data (example being creatine level assumed at 9 mmol/kg or 7.1 mmol/kg).

Finally, the GABA signal experiences contamination from macromolecules, although it is reportedly quite stable at around 12% (Jensen, Frederick, & Renshaw, 2005; Shungu et al., 2016) and, therefore, the GABA signal is typically indicated as 'GABA+' representing macromolecule signal incorporation.

The need to quantify macromolecule components via symmetric suppression when measuring GABA, and the validity in doing so, is the subject of several discussions; in Edden et al. (2016) and also studies by Edden, Puts, and Barker (2012), Mikkelsen, Singh, Sumner, and Evans (2016) and Shungu et al. (2016). Notably, evidence suggests that macromolecules profiles are variable in clinical groups (such as schizophrenia: Rowland et al., 2016), but the methods with which to apply models of best fit for macromolecule inclusion are highly variable (Harris et al., 2015; Rae et al., 2014).

GABA profiles vary within brain regions and tissues, with higher levels in grey matter than in white matter and little-to-no metabolites present in cerebrospinal fluid (CSF) (Choi, Lee, Merkle, & Shen, 2006; Govindaraju, Young, & Maudsley, 2000; Rothman et al., 1993). As such, tissue correction (segmentation) is a crucial component of the imaging pipeline when assessing voxel GABA levels to prevent the inflation of quantification data that comes from variances in grey and white matter (Ganji et al., 2014; Harris, Puts & Edden, 2015; Kries, Ernst, & Ross, 1993; Mikkelsen et al., 2016).

Despite the importance of processes such as tissue volume segmentation in quantifying GABA, to date, a study or review of regional localised GABA levels at 3 Tesla reporting segmented absolute GABA levels and/or ratios using creatine and/or water, in healthy adults - and across editing toolboxes - is yet to be conducted. Several reviews have focussed on optimising methodological approaches for exposing GABA signal via MRS, for example, Rothman et al. (1993), Puts and Edden (2012b), and Mullins et al. (2014); however, these reviews have focussed on quantification via one acquisition sequence; MEGA-PRESS.

Neuronal GABA is present in cytoplasmic and vesicular regions, and intracellular GABA is generally what is measured via spectroscopy, however the proportions of extracellular GABA are most likely also recorded in total profiles, thus GABA quantifies GABA metabolites as well as GABA neural interactions. That is, intracellular, extracellular and metabolic GABA (Rae et al., 2009).

Extracellular GABA levels can upregulate and downregulate GABA levels as required, and may have a functional role with benzodiazepine receptors whereby homeostatic mechanisms modulate levels in accordance with systemic needs. Evidence from rodent model tissue studies suggests GABA has the capacity to do both in regions such as the cerebellum (Billinton, Upton, & Bowery, 1999; Connor, Tseng, & Hockberger, 1987).

GABA changes measured by MRS is assumed to capture pools associated with *tonic* rather than *phasic* inhibition, thereby such changes are associated with an inhibitory function within a brain ROI rather than just GABAergic synaptic transmission (Dyke et al., 2017; Rae, 2014; Stagg et al., 2011).

GABA levels exhibit stability across diurnal time frames with less than 6% variation (Evans, McGonigle, & Edden, 2010) but are evidenced to reduce with age (Gao, Edden, et al., 2013; Pouwels et al., 1999) and vary between genders, although the latter comparison has limited substantiation in human studies (Rae 2014; Wilson, 1992; Wilson & Biscardi 1997).

In studies involving female samples, hormonal concentrations across the menstrual cycle appear to modulate the GABA profile, particularly during the luteal cycle phase (Backstrom et al., 2003; Epperson et al., 2002; 2006). GABA levels appear most reduced in this phase in non-depressed populations (Amin et al., 2006; Bannbers et al., 2012) and in the prefrontal and occipital cortices of healthy female samples (without hormonal modulation) were most elevated during the follicular to ovulation phases (De Bond et al., 2015; Epperson et al., 2002).

Two reviews on GABA profiling using MRS have raised a methodological need to control for menstrual cycle variances in female participants/patients and/or to utilise the gender covariate in the analysis of MRS data (Chang et al., 2003; O’Gorman et al., 2011). This thesis further discusses the influences on GABA levels that are neuro-steroidal in Section 4.6.7.

4.6.2 Regional GABA levels and modulations in healthy samples

Most evidence for elucidating the role of GABA and its substrate components has been provided through animal models or *in vitro* tissue sampling/post-mortem studies. However an increasing number of imaging studies have also investigated *in vivo* modulation of GABA levels, and the functional corollary, glutamate, in selected brain regions associated with performance, pathology or personality traits, and in behavioural or pharmaceutical interventions (Puts & Edden, 2012b; Martin et al., 1993).

One review paper assessed regional GABA levels but was reported at higher field strength (7 Tesla; Ganji et al., 2014). The quantification techniques or optimisation parameters for ascertaining metabolite concentrations across different studies might constrain comparability, with the issue of high variability raised in several studies (e.g. for LCMoel versus GANNET in Oliveira et al., 2015).

For the current review, and specifically Table 8, an approximate guide to GABA levels across brain regions was derived from studies that used varied field strength MRI data in a healthy control group and/or comparator group to a clinical sample and/or healthy sample baseline prior to an intervention (usually reporting on one or two regions), in which mean concentrations had been collated. Not all studies measuring GABA levels in selected regions could be included due to a) lack of reporting specific mean/standard deviation GABA levels of their healthy group, or b) reported levels were not precise enough to obtain a score, such as using a histogram without actual figures. There was also great variability in the proportions per brain region of GABA.

Table 8. Regional *In Vivo* GABA Concentration Investigations using Healthy Samples at Various Field Strengths

Region	Mean (SD) mM in ~1.5 ppm	Tissue volume segmentation	Group details	Field strength (tesla)	Study
Anterior cingulate	1.90 (0.30)	Yes (but no relaxation correction)	n = 45	3T	Aufhaus et al. (2013)
Anterior cingulate	1.08 (0.22)	Yes	n = 25	3T	Ham et al. (2007)
Anterior cingulate (dorsal)	0.24* (0.10)	NR	n = 8	3T	Long et al. (2013)
Anterior cingulate	0.22 (0.07)	NR	n = 25	3T	Northoff et al. (2007)
Anterior cingulate	1.31 (0.38)	Yes	n = 8	3T	Shibuya-Tayoshi et al. (2008)
Anterior cingulate	0.31* (0.08)	NR	n = 19	3T	Waddell et al. (2011)
Basal ganglia	1.22 (0.22)	Yes	n = 25	3T	Ham et al. (2007)
Basal ganglia	1.64 (0.88)	Yes	n = 8	3T	Shibuya-Tayoshi et al. (2008)
Cerebellar vermis	0.23* (0.06)	NR	n = 19	3T	Waddell et al. (2011)
Frontal cortex (left)	0.4 (0.10)	Yes	n = 9	7T	Ganji et al. (2014)
DMPFC	0.98* (0.25)	Yes	n = 17	3T	Hasler et al. (2009)
Medial-frontal cortex	1.0 (0.10)	Yes	n = 9	7T	Ganji et al. (2014)
Occipital medial	0.8 (0.10)	Yes	n = 9	7T	Ganji et al. (2014)

Occipital (left)	0.3 (0.10)	Yes	n = 9	7T	Ganji et al. (2014)
Occipital cortex	0.24 (0.05)	Yes	n = 31	3T	Godlewska, Near and Cowen (2015)
Thalamus	0.12* (0.07)	NR	n = 9	4T	Pollack et al. (2008)
VMPFC	0.94* (0.18)	Yes	n = 17	3T	Hasler et al. (2009)
Whole brain	1.77 (0.35)	NR	n = 14	2.1T	Goddard et al. (2001)
Whole brain	0.11* (0.07)	NR	n = 9	4T	Pollack et al. (2008)

**Calculated as ratio to creatine; ^Calculated as ratio to water; NR not reported.
Concentration estimates (mM) are regional and where segmented/fractionated, reported GABA values are for grey matter*

Regarding GABA levels associated with emotional-cognitive assessment in non-psychiatric groups, Goto et al. (2009) reported a trend towards the significance of prefrontal region GABA levels that were positively associated with the extraversion trait factor on the NEO-FFI personality questionnaire. Delli Pizzi et al. (2015) reported a positive correlation in a healthy sample between anxiety scored on the Spielberger State-Trait anxiety inventory, state component, and GABA levels in the ventral-medial prefrontal cortex (vmPFC), which suggests that higher state-based levels of anxiety may require increased GABA levels, even in non-clinical samples, possibly as a compensatory process.

4.6.3 Correlations of GABA level to BOLD signalling

The assessment of regional metabolite changes attributable to task-dependent activity as well as endemic or intrinsic activity was summarised in a review using both functional and metabolic modalities (Duncan, Wiebking, & Northoff, 2014). The review examined a total of 35 studies using glutamate/glutamine (Glx) and/or GABA-MRS/PET regionally, together with fMRI to associate activity to metabolite levels. The principle findings were that GABA levels are most evidenced to change with task-dependent activity through a negative correlation to BOLD activity and, for glutamate/glutamine, more so related to functional connectivity between regions, through a positive correlation with both task-related activity and at rest. The review surmised that where GABA is decreased, BOLD activity increases and higher GABA levels were associated with a stronger negative BOLD response in the same region. Negative BOLD responses are tightly coupled to reductions in neural activity throughout the literature; animal models have consistently indicated attenuations to neural activity associated with both negative BOLD signalling and

increases to GABA transmission processes (Devor et al., 2007; Nemoto et al., 2012; Rae, 2014).

In one notable combined study in the review above of metabolic profiles and activation levels during an emotion valence task, Northoff et al. (2007) reported that GABA concentrations measured via MRS at rest predicted the level of negative BOLD responses in the ACC in healthy subjects. Directionally, higher GABA levels correlated with stronger negative BOLD responses in the same region and the association was stronger with increased cognitive load (i.e. during response selection) during the task. No such association was found with task-activation data or glutamate levels. In another study (Muthukumaraswamy et al., 2012), positive BOLD changes were associated with lower GABA in the visual cortex, indicating this relationship is region- and, likely, paradigm-specific.

It is therefore feasible that GABA profile changes reflect the need for adjusted neural signalling used in the processes of brain activity underpinning cognition, which is measured through BOLD signal changes; however, the evidence is region-specific. Given that the above evidence is derived from healthy samples, it is vital that evidence accrues through clinical sample groups, which is examined in the following sections.

4.6.4 GABA levels in psychiatric disorders

The GABA system and its components confer a potential biomarker role in psychiatric disorders based on the evidence thus far from imaging and genomic modalities. Pre-clinical and clinical models contribute evidence within schizophrenia, depression and anxiety disorders, as well as cognitive impairment recently seen in dementia, and epilepsy (Lewis, Volk, & Hashimoto, 2004; Petroff & Rothman, 1998; Schur et al., 2016).

4.6.4.1 *Major depressive disorder*

In MDD studies (for example: Bhagwagar et al., 2004; 2008; Sanacora et al., 1999; Schur et al., 2016), GABA levels in occipital and anterior cingulate areas are reported to normalise following successful treatment as measured by symptom reporting, but not where there was treatment resistance, suggesting a relationship between metabolite levels and reported symptoms. Further, the profile of GABA in these MDD studies suggests that subtypes can be identified via GABA levels, wherein GABA is lower in melancholic diagnostic presentations, and also in treatment-resistant MDD (refer to aforementioned review by Schur et al., 2016).

4.6.5 GABA levels in affective disorders

Imaging metabolic investigations of the GABA substrate function thus far posits a central role across mood and anxiety disorders (Chang et al., 2003; Nemeroff et al., 2003; Nutt & Malizia, 2001; Puts & Edden, 2012b; Rothman et al., 1993).

4.6.5.1 PTSD, panic disorder and social anxiety disorder

For anxiety disorders, MRS studies have reported region-specific variabilities in GABA concentrations (Chang et al., 2003; Rae, 2014). In PTSD, GABA levels in the mPFC were significantly higher than those in the healthy control comparison sample (Michels et al., 2014), and lower in the lateral temporal ACC and parieto-occipital cortices (Pennington et al., 2014). Finally, Rosso et al. (2014) reported reduced insular GABA but no differences from healthy controls in dorsal ACC GABA. When analysing GABA levels against state and trait anxiety, reduced insula GABA was negatively associated in both groups, which suggests a paucity of GABA levels in this region may guide anxiety severity.

For panic disorder, one study reported a 22% reduction in GABA levels (GABA plus homocarnosine) in the occipital cortex compared with healthy controls (Goddard et al., 2001); however, a second study reported no GABA differences in the PFC compared to healthy controls (Hasler et al., 2009). A third study (Ham et al., 2007) reported a reduction in GABA levels in the ACC and basal ganglia compared to healthy controls. These studies suggest GABA region-specific attenuations in those patients with panic disorder.

In one Social Anxiety Disorder MRS study (SAD; Pollack et al., 2008), lower thalamic GABA levels were reported, as were elevated whole brain levels of glutamate and glutamine (yet not whole brain GABA), in the SAD group compared with the healthy controls. Following a benzodiazepine intervention (levetiracetam) in the SAD group, thalamic reductions of glutamine were observed. This study, thus far, is one of a handful of intervention investigations on the anxiolytic modulation of brain inhibitory/excitatory metabolites in anxiety disorders.

4.6.5.2 GABA in GAD

In patients with diagnosed GAD, studies of GABA quantification via spectroscopy are few (discussed in greater detail in Section 4.2 of this chapter), and so GABAergic dysregulation has mostly been implicated using techniques other than MRS quantification. Two studies examined regional benzodiazepine receptor distributions via a receptor-labelled radioligand and found reduced levels in both the medial and

temporal lobes (Tiihonen et al., 1997) and in the peripheral blood mononuclear cells (Rocca et al., 1998) compared with healthy controls. Increased or normalised levels were associated with the response to benzodiazepine treatment in the latter study. GABA-A agonists being anxiolytic, an affinity for GABA-A has been found to predict efficacy of benzodiazepines in GAD sample groups (Schienle et al., 2011).

Regional and peripheral benzodiazepine receptor numbers and associated mRNA levels may offer one type of biomarker for the treatment response to anxiolytic agents acting via benzodiazepine modulation; however, as discussed in the investigations within this thesis, results found in GAD groups are also prevalent in other disorders and, therefore, are not exclusively markers for GAD. Puts and Edden (2012b) posited that while there is evidence that the GABA substrate is most susceptible to anxiolytic interventions and subsequent improvements to anxiety symptoms, there is limited research specifically investigating the role played in neurobiological dysfunction underpinning GAD or attenuations therein as a product of anxiolytic treatment. Modulation of GABA levels from other factors are addressed briefly next.

4.6.6 GABA level changes with treatment interventions

An early intervention study in a healthy sample examined modulations to occipital lobe GABA levels following treatment by the anticonvulsant and GABA transaminase inhibitor vigabatrin in an earlier incarnation of ¹H-MRS (proton nuclear magnetic resonance spectroscopy in conjunction with J editing), reporting dose-dependent elevations from baseline range (1.1 +/- 0.1 $\mu\text{mol}/\text{cm}^3$ to 3.7 $\mu\text{mol}/\text{cm}^3$) following treatment (Rothman et al., 1993).

Similarly, a ¹H-MRS investigation involving acute doses of the anticonvulsant gabapentin (also a GABA-enzymatic, nootropic and non-synaptic GABA modulator) in healthy control subjects in the occipital and medial prefrontal brain regions reported no modulations to GABA or other selected metabolites (Glx, choline or N-acetyl-aspartate), with the data suggesting that GABA level changes are not observed in subjects with 'normal' levels and/or from single doses at 150 mg and 300 mg (Preuss et al., 2013).

In epileptic patients, however, where there is a presumed shortfall or dysfunction in the GABA substrate, intervention studies of GABA modulators reported changes that could be observed as the 'normalising' of GABA levels. Indeed, a number of these studies investigated acute doses of the anticonvulsant vigabatrin (also a GATs enzyme inhibitor), which was found to increase occipital cortex levels (Petroff et al., 1999;

Verhoff et al., 1999), suggesting changes to GABA levels may influence subsequent reported reductions to complex partial seizures. GABA quantification related to interventions are addressed in the next section regarding modulations to levels and also in the next chapter under GAD treatments.

4.6.7 Interventions that Modulate GABA levels for Treatment of Anxiety

4.6.7.1 *Pharmaceuticals*

Pre-clinical and clinical studies have identified multiple components within the GABA substrate that modulate anxiety symptoms, and thus serve as pharmacotherapeutic targets (Nemeroff, 2003; Nuss, 2015; Nutt & Malizia, 2001; Puts & Edden, 2012b; Rothman et al., 1999; Stahl & Moore, 2013).

With the introduction of a GABAergic substance, levels are modulated by targeting pathway components detailed in the previous section: agonist drugs direct to receptor (for example barbiturates); allosteric modulation at GABA-A (e.g. benzodiazepines, steroids); induction of GABA synthesis (e.g. gabapentin, pregabalin, valproate); inhibition of GABA breakdown (vigabatrin, valproate) and synaptic reuptake inhibition of GABA.

GABAergic modulating drugs mostly but not exclusively work as GABA transaminase inhibitors or reuptake inhibitors, such as gabaculine (neurotoxin derived from *Streptomyces toyocaensis* bacteria). The anti-convulsant drug tiagabine also works as a GABA reuptake inhibitor, increasing extracellular GABA levels in regions such as the ventral pallidum in a dose-dependent manner (Finke-Jensen et al., 1992). Topiramate is an anticonvulsant that potentiates GABA-A receptor-mediated activity, with acute doses (1–4 hours) found to increase GABA levels by 70% in healthy subjects and similarly in complex partial seizure patients (Kuzniecky et al., 1998).

However, the precise mechanisms of action of some GABA anticonvulsants are still being elucidated and suggest other kinetic foci such as non-vesicular GABA. A recent pharmacokinetic study examined the viability of MRS to evaluate regional acute drug response to regional GABA profile levels in an animal model using 9.4 tesla MRS and five interventions—vigabatrin, 3-mercaptopropionate, tiagabine, methionine sulfoximine, and riluzole. Results from MRS found a measurable detection of acute dose-dependent changes at levels of 6% in glutamate and 12% in GABA profiles (Waschkies et al., 2014). The acute and chronic pharmacodynamic modulation of GABA is also discussed in the context of anxiety symptom alleviation in Chapter Five.

4.6.7.2 Modulations to GABA levels from Diet or Supplements

For almost a century nutrient research and intervention studies involving nutraceuticals, vitamins and micronutrients have evidenced the role that diet plays in the maintenance and enhancement of cognition and mood, and alleviation of deficits. It is an important finding that in CNS conditions such as mood, affective disorders and cognitive impairment such as dementia typologies are partly attributed to deficits in the nutritional status of neurons (Bourre, 2006; Gomez-Pinilla, 2008; Sarris et al., 2015).

Despite a comprehensive body of literature reinforcing the important role of nutrition in psychiatric disorders, the capacity to derive investigation evidence into workable outcomes is limited by methodological issues such as heterogeneity, sample sizes, replicability and effect of sizing, and a further need to improve the knowledge base regarding biological pathways (Jacka, 2017; Lakhan & Viera, 2008; Petrie & Ban, 1985; Sarris et al., 2012).

It is beyond the scope of this thesis to discuss in detail the literature for nutrient-based nutraceuticals in affective disorders; however, it is relevant to point out the paucity of such research in GAD, and to also highlight some findings that may be relevant for potential use in the alleviation of anxiety symptoms. The interested reader is invited to read the aforementioned 'nutrition in psychiatry' referenced papers. Some notable findings for mood disorders are B group vitamins such as folic acid (vitamin B9), B12 and B6, omega-3 fatty acids and S-adenosyl-L-methionine (S-AMe) as efficacious for the reduction of depression symptoms (Grosso et al., 2014; Miscoulon & Fava, 2002; Sarris et al., 2016).

4.6.7.2.1 Caffeine intake

Caffeine is widely used for psychostimulant properties; however, it also has inflammatory and neuroprotective effects (Stone, Ceruti, & Abbracchio, 2009). The caffeine alkaloid is structurally similar to the endogenous forms of adenosine and acts as an antagonist at adenosine receptors (Dunwiddie & Masino, 2001; Ribiero & Sebastiano, 2010), in turn, influencing a number of substrates to exhibit stimulatory effects.

Adenosine receptor antagonism produces a number of inhibitory systemic effects including respiratory and vasal activities as well as modulations in aminergic substrates including dopamine and acetylcholine that relate to psychostimulant effects (Ferre, 2010; Froestl, Muhs, & Pfeifer, 2014). In the CNS, antagonism at adenosine receptors indirectly influences the GABA system in connected regions governing arousal states (Daly, Shi, Nikodijevic, & Jacobson, 1994; Fredholm et al., 1999).

Specifically, in the GABA system, evidence from animal models suggests caffeine can upregulate GABA-A receptors by 65%, with the presence of caffeine increasing the benzodiazepine binding site GABA-A receptor densities (Shi, Nikodijevic, Jacobson, & Daly, 1993; Wu & Coffin, 1984).

4.6.7.2.2 *Vitamin B in the diet*

B group vitamins are commonly indicated for the alleviation of stress and anxiety, which has been reinforced by intervention efficacy studies (e.g. Long & Benton, 2013; Schlebusch et al., 2000; Stough et al., 2011). The B group, specifically B6 (pyridoxine), B9 (folic acid) and B12 (cobalamin), are thus far evidenced in the literature to modulate cognitive function as they are crucial precursors to neurotransmitters, amino acids and cellular function. Being water soluble, they are not stored and therefore are important in the diet for maintaining normal function. They are inter-related, for example, B12 requires B6 to be utilised. All are vital for amino acid synthesis and protein formation, including plasma homocysteine levels. Pyridoxine hydrochloride is a form of vitamin B6 that occurs in the body as PLP.

Coenzymes in the biosynthesis of GABA, dopamine and serotonin, noradrenalin and melatonin, all play a role in anxiety, however, evidence is less supportive of a direct relationship between B group levels in the diet and anxiety disorders, and there is more likely an indirect relationship, despite deficiencies in sample populations (Kennedy, 2016).

In animal studies, it has been suggested that the increased sympathetic action seen in pyridoxine deficiency may be caused by the reduced production of GABA and serotonin (McCarty, 2000). In the brain, 50% GAD67 is present in inactive apoenzyme form, which is without bound PLP. This form may be subsequently activated during stress, when additional GABA synthesis is required (reviewed by Martin & Rimvall, 1993).

There is also a known association between pyridoxine deficiency and seizures, with the shortfall modulating GABA synthesis, thereby contributing to stress seizures (Sharma & Dakshinamurti, 1992). Administration of this co-factor in high doses is reported to increase GABA levels (McCarty, 2000), and thus pyridoxine may have a role in modulating anxiety although there is limited literature at pre-clinical and clinical levels exploring this hypothesis.

4.6.7.3 *Other compounds modulating GABA levels*

4.6.7.3.1 *Neurosteroids*

Endogenous neurosteroids play a vital role in CNS function (Baxter & Rousseau, 1979). Neurosteroids are endogenous derivatives from pregnenolone with a broad array of both genetic and non-genetic homeostatic mechanisms including stress adaptation, fertility, learning and memory, mood and neural plasticity and excitability regulation (Carver & Reddy, 2013; Majewska, 1992; McEwan, Gray, & Nasca, 2015).

Neurosteroids—allopregnanolone as a common example—are well-evidenced in both GABA- and glutaminergic substrates (but not exclusively) via both fast membranous and slower mRNA-gearred processes (Belelli & Gee, 1989; Eser et al., 2006; Engel & Grant, 2001). In the CNS, neurosteroids such as deoxycorticosterone and testosterone, and metabolites of progesterone such as allopregnanolone are known endogenous ligands that act as positive allosteric modulators at the GABA-A receptor and are therefore implicated in mood and affective function (Backstrom et al., 2003; Dubrovsky, 2005; Turkmen et al., 2011).

In animal rodent models (both male and female), a decrease of progesterone is associated with anxiety behaviour and seizures, as well as benzodiazepine resistance, possibly via increases to alpha-4 subunit proliferation (Gulinello, Gong, & Smith, 2002; Smith, Gong, & Zhou, 2007). Novel neurosteroid drugs appear efficacious in the treatment of epilepsy, anxiety and mood disorders owing to their broad affinity with different GABA-A receptor subtypes (Belelli, Lan, & Gee, 1990; Longone et al., 2011; Rupprecht, 2003).

Examples of human studies within the GABA substrate report attenuations to GABA concentrations and GABA-A receptor sensitivity occurring with steroids such as allopregnanolone and/or oestrogen in patients with severe luteal phase-related symptoms of the premenstrual dysphoric disorder (Backstrom et al., 2011; Halbreich et al., 1996). Such mechanisms, in turn, influence mood, cognitive performance and appetite (Reed, Levin, & Evans, 2008). Allopregnanolone reportedly mediates the anxiolytic effects of progesterone at the GABA-A receptor site, at least in animal models (Bitran, Shiek, & McLeod, 1995) and the levels of both show a positive U-shaped relationship to mood symptoms in females reporting clinical premenstrual dysphoric disorder (PMDD).

In one study, while lower levels of serum progesterone/allopregnanolone were associated with hyperactivation of the amygdala, higher levels were associated with

reduced activity similar to benzodiazepine administration (Backstrom et al., 2014). For neurosteroid action evidenced as a corollary to aminergic anxiety treatments, selective serotonin reuptake inhibitor (SSRI) antidepressants such as fluoxetine and mirtazapine reportedly enhanced the formation of 3 α -reduced steroids such as allopregnanolone (Schule et al., 2011).

One systematic review (Toffoletto et al., 2014) examined oestradiol and progesterone and ovarian hormone levels across 24 imaging studies in healthy females and six studies of PMDD, and found that the hormonal levels across the cycle were associated with attenuations to function in the amygdala, ACC and inferior frontal gyrus regions. A study of GABA levels from MRS in a PMDD sample (Epperson et al., 2001) found lower occipital GABA levels during the follicular phase but higher levels in the mid-luteal phase, conversely in the healthy sample the GABA levels were higher during the follicular phase than the mid-luteal phase.

Reduced GABA levels in the presence of reduced progesterone/allopregnanolone, as known modulators of GABA function, together with mood and affective state symptoms represent one model for the indirect influence that the GABA substrate has within these states, and therefore neurosteroidal mechanisms become a crucial consideration in clinical investigations involving the GABA system, particularly with female participants/patients.

4.6.8 GABA quantification: summary

The GABA substrate concerns not only functional roles of the GABA molecule but a large neurobiological system comprising GABAergic neurons, GABA receptor complexes and associated pathway components. Whole brain GABA quantification *in vivo* or regional is fairly novel, with complex imaging post-processing applied via continually-evolving methods to best expose the GABA resonances for quantification.

GABA quantification is a broad measurement of GABA concentrations at a given point in time, which is thought to generally capture the levels of inhibitory functions, but it does not account for intra- or extracellular GABA differences or GABAergic neuron features, and the finer processes in neurotransmitter flux.

Evidence for GABA modulations in specific psychiatric disorders exists, the most obvious symptomology being epileptic seizures, and is the most evidenced in schizophrenia; however, it is not exclusive to one or two disorders; rather, the dysfunction demonstrates a broad systemic imbalance, implying GABA processes are at

once directly and indirectly involved in disorder symptomologies (Richerson, 2004; Schur et al., 2016; Taylor & Tso, 2015).

Broadly located in the brain, yet difficult to quantify, GABA is a conformationally flexible molecule, with multiple components that have small specificity. Alterations to GABA signalling occur via several direct and indirect processes through GABA synthesis, transportation, receptors and reuptake components of the substrate. Drugs of anxiolytic, anticonvulsant and mood-stabiliser classes are targeted to these components. Finally, in terms of application for anxiety disorder treatments, pre-clinical and clinical studies have identified multiple components within the substrate that modulate anxiety symptoms, and thus serve as pharmacotherapeutic targets (Nemeroff, 2003; Nuss, 2015; Nutt & Malizia, 2001; Puts & Edden, 2012b; Rothman et al., 1999; Stahl & Moore, 2013). Treatments are examined in greater detail in Chapter Five.

4.7 Neuroimaging Markers for GAD – Conclusion

The ACC connectivity from the lower limbic and hippocampus regions to the prefrontal cortical regions places it in a central role in the organisation of affective and cognitive information, where it is evidenced to involve evaluative states, suppression of negative emotion or cognitions and autonomic processes that underpin anxiety (Allman et al., 2011; Bush et al., 2000; Critchley et al., 2003; Etkin et al., 2010; Luu & Pedersen, 2004; Schienle et al., 2011; Vogt et al., 1992).

As such, ACC dysfunction may underpin GAD symptomology, and therefore warrants further research, especially utilising imaging technologies that can probe AAC function. Of particular interest in determining the efficacy of novel anxiolytics in such affective and cognitive processing domains driving GAD symptomatology is the role of GAD1 transporters for which recent neurobiological reviews (see Rae, 2014) suggest have significant influence on GABA levels in limbic regions.

Changes to the properties of GABA constituents as a product of interventions may be measured through MRS and the modulation of activation levels measurable via functional imaging and may be a reflection of the restoration to affective processing functionality, when associated with improvements to affective symptoms quantified by symptom scale measures (Berger et al., 2008; Etkin & Wager, 2007; Mullins et al., 2014; Puts & Edden, 2012b; Rae, 2014; Rothman et al., 1999). This could be a key factor for establishing the efficacy of treatment interventions on a neurobiological level.

To date, however, investigations utilising MRS in anxiety disorders have involved panic disorder, PTSD, social phobia samples, but few within GAD. Further, there is a paucity of intervention research. Given the early MRS evidence linking regional metabolites to symptom profiles in anxiety disorder samples, more research is needed to identify unique GAD profiles associated with both symptom presentation modulations following treatment.

Using MRS to measure GABA metabolite levels is not without challenges; GABA profile levels are difficult to isolate as GABA signal resonances overlap with more abundant metabolites (Govindaraju et al., 2000; Rae, 2014); however, utilising editing techniques (such as Mescher-Garwood point-resolved sequences, or MEGA-PRESS, in the present studies) can optimise the GABA signal against artefacts and macromolecules in generating a clearer GABA profile.

Despite issues in quantifying GABA, it is of high value to exploit MRS to determine the extent to which neural activity is influenced by metabolite profiles, and if there are patterns unique to GAD (Maddock & Buonocore, 2011). The current research also aimed to address the role of GABA in GAD cognitive symptoms, and the association of GABA with symptomatic changes as the product of an intervention.

Similarly, much of the evidence from functional imaging that aims to clarify neurobiological underpinnings of GAD has yet to extensively involve interventions, especially novel substances. Less than 30 studies comprised one systematic review of GAD treatment interventions specific to older adults (Goncalves & Byrne, 2012); one review involved Chinese-specific GAD imaging investigations (Chen & Shi, 2011) with fewer studies utilising functional connectivity, and less in metabolic reviews (such as Etkin & Wager, 2010; Charney, 2003).

Insufficient quantities of original investigations is the likely reason GAD is precluded in many broader anxiety disorder review articles. Where GAD is included, it is often briefly reported or discussed together with other affective disorders. In the Mochcovitch et al. systematic review (2014), the authors noted the paucity of functional imaging research in GAD samples, with 14 studies forming the review (refer also to similar discussion in Shin & Liberzon, 2010).

Meta-analytic approaches for which the current evidence of disorder-specific dysregulation is founded have been based on mapping psychological categories and functional connectivity groups. A valid issue is findings from functional connectivity

studies that identify identical networks across a number of psychiatric disorders insofar as multiple disorders display similar patterns of dysregulation.

The classification of disorders based on neurobiological mechanisms that are observed in pathological anxiety (intermediate phenotypes) would involve extensive re-organisation of current diagnostic categories. Landmark meta-analytic studies such as Kober et al. (2008) asserted that evidence supplied via imaging modalities should be data-driven in establishing neurobiological patterns, and that it is erroneous to utilise *a priori* psychological categories based on symptom reporting for psychiatric disorders.

At this early stage of establishing neurobiological underpinnings of anxiety symptoms, the data are currently posited as a strategy to identify novel targets to treat anxiety symptoms (Olivier et al., 2013; Ressler & Mayberg, 2007).

Review papers in the imaging/intervention/affective disorder investigation domains have converging recommendations for future investigations. In one landmark review, Shin et al. (2013) discussed the optimal methodological approaches in statistical regression analyses, including pre-treatment neuroimaging measures, baseline symptom severity and other clinical, physiological, and cognitive and/or genotype measures, which should be entered as predictors of treatment response and are an essential approach for interpreting imaging data in this context. Further, the authors discussed limitations namely attention to sample sizes, discernment in analysis methods, consideration of pre-treatment symptom severity, requirement for placebo control, diagnostic rigour in the selected disorder/s, presence of comorbid disorders such as depression and, not least, the clinical relevance of group-based findings.

As such, neurobiological evidence with these constraints in mind has the potential to inform and define disorders such as GAD, in turn driving greater objective measurement as an effective adjunct, rather than symptom reporting alone. Regional activation patterns and GABA levels can indirectly bolster GAD prevalence data and contribute to 'precision medicine' through quantifiable treatment responses. This aligns with the discussion in Chapter Three, in which emergent advances in imaging make it an important conduit for supplementing or confirming symptom-based diagnoses in the foreseeable future (Kapur et al., 2012). Finally, there are clear economic and provisional healthcare benefits to the renewal of guidelines for the diagnosis and treatment of pervasive affective disorders such as GAD, the latter of which is examined in the next chapter.

5 Chapter Five. GAD Treatments

5.1 Introduction

GAD remains understudied both as a disorder and in psychopharmacological and psychotherapeutic treatment studies; this having ramifications for the development of treatments (Dugas, 2005; Koerner, 2014; Starcevic et al., 2012). Further, the long-term treatment of GAD patients is challenging (Allgulander et al., 2003; Reinhold et al., 2011), due to the waxing and waning nature of the course of symptoms and the presence of comorbid conditions such as depression that are often given priority treatment.

While there are guidelines for anxiety disorder treatments such as stepped-care methods that stem from national initiatives (e.g. NICE guidelines, ADAA Clinical Practice Review for GAD, APA Practice Guidelines, Royal Australian and New Zealand College of Psychiatrists Anxiety Disorders Guidelines) or geared at clinician level (e.g. Clinical Practice Guidelines: Management of Anxiety Disorders, 2006; Rickels & Schweizer, 1997), only a handful focus on management of GAD (e.g. NICE; illustrated in Table 9). Of these, the first-line treatment is psychotherapy interventions such as CBT and pharmacotherapies such as SSRI antidepressants as monotherapies or combined/adjunctive therapies.

Review papers of GAD treatment interventions have found the efficacy of aforementioned treatments to be moderate at best; with greater efficacy achieved in adolescent GAD whereby a better response to treatment occurs earlier in the disorder trajectory. As GAD symptoms are typically observed from adolescence, adult patients are likely to have experienced the disorder chronically for several years and attempted several treatments, are more difficult to treat and are more likely considered refractory or treatment-resistant (Pollack, 2009; Roy-Byrne, 2015; Samuel, Zimovetz, Gabriel, & Beard, 2011; Van Ameringen et al., 2017).

Clinical practice guidelines exist in Australia for the treatment of panic disorder and agoraphobia (Andrews et al., 2003) and PTSD; but not for GAD. In the United Kingdom, NICE (2011b) have produced GAD-specific guidelines for clinical practice. In the United States, the World Health Organization commissioned written guidelines for the management of mental disorders in 2004. Such guidelines stipulate stepped-care models for anxiety symptom management by 'first-line' and 'second-line' treatments.

Generally, pharmacological interventions are secondary to non-pharmaceutical approaches such as guided self-help and psychotherapeutic courses (Andrews et al., 2003; Baldwin et al., 2014; NICE, 2011b; Starcevic, 2015). Recent initiatives to produce

optimal algorithms for the treatment of GAD in care settings based on better diagnosis have been proposed in Davidson et al. (2010), and recommended in the review by Bystritsky et al. (2013).

Table 9. Stepped-Care Approach for the Management of GAD

Assessment phase
Obtain a detailed history of symptoms and effect on functioning. Ensure that generalised anxiety disorder is the principal or one of the principal diagnoses. Evaluate the patient for common co-occurring mental health conditions (e.g. depression, other anxiety problems and substance-use disorders). Evaluate the patient for suicidal ideation, plans or attempts. Rule out treatable physical conditions such as thyroid and cardiac problems. Use GAD-7 or another suitable measure to gauge severity and track progress.
Step 1. All known or suspected cases of generalised anxiety disorder
Educate the patient and family members about generalised anxiety disorder with the use of self-help sites. Educate the patient about lifestyle changes that can reduce symptoms of generalised anxiety disorder. Discuss strategies for improving the quality and quantity of sleep and encourage regular exercise. Encourage the patient to minimise caffeine and alcohol use and to avoid nicotine and illicit drugs. Monitor the patient's progress with lifestyle changes.
Step 2. Diagnosed generalised anxiety disorder that has not improved after education and active monitoring in primary care
Suggest low-intensity psychological interventions such as individual non-facilitated self-help, individual guided self-help, educational groups and computer-assisted CBT.
Step 3. Generalised anxiety disorder with an inadequate response to Step 2 interventions
Provide choice of a high-intensity psychological intervention or a drug treatment according to patient's preference and then refer patient for individual or group-based CBT (8–16 sessions) or prescription of first-line pharmacologic treatments (SSRIs or SNRIs).
Step 4. Complex or treatment-refractory generalised anxiety disorder
Refer the patient for specialised care by a mental health professional who will prescribe other first-line pharmacologic treatments or adjunctive treatment with a long-acting benzodiazepine (to be avoided among patients who are receiving opioids and among the elderly), buspirone, pregabalin or quetiapine, and who will consider more intensive CBT and other forms of psychotherapy (such as psychodynamic therapy, acceptance and commitment therapy). CBT: cognitive-behavioural therapy; SNRI: serotonin–norepinephrine reuptake inhibitor; SSRI: selective serotonin reuptake inhibitor.

Source: NICE (2001) and Steen and Sareen (2015).

5.2 Psychological Treatments for GAD

Several reviews have assessed psychological interventions for the treatment of GAD in children, adolescents and adults. Findings support the use of CBT as a first-line treatment compared with other practices (Cuipers et al., 2014; Durham & Allan, 1993). Better outcomes are obtained where the therapist/patient relationship is a favourable one (Ardito & Rabellino, 2011; Wright & Davies, 1994). CBT assumes that cognitive reappraisal and emotional regulation strategies can be successfully applied for the

alleviation of anxiety symptoms. These strategies have been associated with modulations to prefrontal-subcortical connectivity (for a review, see Brooks & Stein, 2015).

A meta-analysis from Cuijpers et al. (2014) examined response to psychotherapy treatment, including relaxation therapy, psychodynamic therapy and CBT specific to GAD, finding a large pooled effect size for CBT compared with controls, with low-to-moderate heterogeneity (Hedges $g = 0.84$). A number of recent studies have examined in more detail the outcome of CBT and levels of therapist support in the treatment of GAD (Constantino et al., 2017; Westra et al., 2016).

A recent randomised trial examined the effect of therapist-driven motivational interviewing additional to CBT for the treatment of GAD (Westra et al., 2016). The study found group differences at the longer 6-month and 12-month time points, whereby patients receiving motivational interviewing–held greater worry decline and distress reduction (measured with the Penn State Worry Questionnaire) with a lower drop-out rate than CBT alone. This suggests that use of CBT with higher therapist engagement produced better outcomes in GAD management in clinical settings.

5.3 Pharmacological Treatments for GAD

The current first-line pharmacotherapeutic treatment of GAD involves several classes of antidepressants. Second-line treatments are benzodiazepines and some agents from antihistamine, beta-blocker and anti-convulsant classes (depending on targeted pharmacodynamics). Reviews and meta-analyses assessing efficacy, safety and tolerability of pharmacotherapeutics for GAD are plentiful, such as two Cochrane reviews (Galle & Millichamp, 2011), with some key review findings discussed below. The interested reader is invited to examine any of the following: Allgulander et al. (2003), Baldwin et al. (2010b), Boulenger (1995), Davidson (2001), Goodman (2004), Hidalgo et al. (2007), Mitte et al. (2005) and Rickels and Rynn (2002).

The numerous reviews that assess a relatively small number of drugs for the treatment of GAD symptoms highlight the fact that few drugs are singularly effective. For both adults and adolescents there is high variability in both response and tolerance to these drugs at the patient level. Second, it reflects the paucity in novel drug research as the majority of these drugs have been the mainstay recommendations for over three decades (refer to an in-depth discussion in this issue on anxiolytic drug research by Griebel & Holmes, 2013).

Typical pharmacotherapies used to treat GAD, while efficacious, have a modest clinical effect, as evidenced through small-to-moderate effect sizes for GABA uptake inhibitors (e.g. pregabalin), and for SSRIs and benzodiazepines, as selected pharmaceutical interventions in one meta-analysis (Hidalgo et al., 2007). These findings were reflected in two more recent reviews (Koen & Stein, 2011; Strawn, Geraciotti, Rajdev, Clemenza, & Levine, 2018). In addition, side effects accompany current medications, such as nausea with the SSRI paroxetine, dependency/withdrawal issues and cognitive decrements with the benzodiazepine group (Baldwin et al., 2010a; Hoffman et al., 2008; Papakostas, 2008; Rickels & Rynn, 2002).

Review papers have thus far established effect size analysis of outcomes across double-blinded studies involving: 1) SSRIs: paroxetine, sertraline, fluvoxamine and escitalopram; 2) SNRI1s: venlafaxine; 3) benzodiazepines: alprazolam, diazepam and lorazepam; 4) azapirones: buspirone; 5) antihistamines: hydroxyzine; 6) pregabalin and 7) complementary/alternative medicine: *P. methysticum*, and homoeopathic preparations (Hidalgo et al., 2007; Huh et al., 2011; Koen & Stein, 2011; Mitte et al., 2005; Strawn et al., 2018; Zahreddine and Richa, 2015).

These reviews consistently find moderate-to-small effect size for treatments across adult and adolescent GAD populations. Outside of methodological considerations, the efficacy within GAD patient samples are highly variable when compared to comparator treatment and/or placebo groups and reflects the difficulty in achieving response or remission in GAD with the current array of pharmaceutical treatments.

Recent pertinent reviews conducted by Zahreddine and Richa (2015) examined non-antidepressant treatments for GAD, and concluded that pregabalin and quetiapine are the most promising. This review also discussed the efficacy of other drugs such as zolpidem, beta-blockers and *P. methysticum* (discussed in greater depth under phytomedicine treatments for GAD in the next section), and called for more trials evaluating their efficacy.

In a similar vein, Huh et al. (2011) conducted a systematic review of 36 studies comprising 10 pharmaceutical treatments and found that a tricyclic AD, an antihistamine, and two anticonvulsants (specifically imipramine, hydroxyzine, valproate and pregabalin) showed greater efficacy and were generally better tolerated than current line treatments. Interestingly, none of these treatments are first-line for GAD. Buoli et al. (2013) also reviewed the efficacy of anticonvulsants and atypical antipsychotics, including quetiapine, agomelatine, memantine, ondansetron and

riluzole, and found quetiapine and agomelatine to be most effective based on their review criteria. The interested reader can also view a study on second generation antipsychotics with similar findings for quetiapine by LaLonde and Lieshout (2011). Refer to Table 10 for a summary review of current first-line pharmacotherapeutic treatments for GAD.

5.4 Benzodiazepines in the Treatment of GAD

The effects of benzodiazepines range from anxiolytic to sedative hypnotic and therefore are prescribed for anxiety symptoms, pre-operative anxiety, panic disorder and phobias (Cascade & Kalali, 2008; Hollister, Muller-Oerlinghausen, Rickels, & Shader, 1993; Shader & Greenblatt, 1993).

GABAergic benzodiazepines are arguably the most effective in offering acute relief to anxiety-type symptoms, whereby action within the frontal-limbic system may enhance the control of negative and persistent thoughts and emotions that form a large component of anxiety cognitions, emotional responses and heightened physiological states.

Benzodiazepines are a family of drug consisting of one benzene and seven diapene rings, which are generally classified by structure and elimination half-life following administration. They are defined as allosteric modulators at GABA-A receptor complexes and target the ionotropic GABA-A receptor complex, although not through GABA binding sites, rather to an allosteric histidine residue binding site at the intersections of alpha and gamma subunits (namely H101, H105 and H126). This induces GABA-A receptor conformational change via the chloride channel, hyperpolarising the cell and affinity for GABA (Haefely, 1984; Johnston, 2005; Kelly et al., 2002; Tallman, Thomas, & Gallagher, 1978).

As benzodiazepine receptors have variations defined by alpha subunit isoforms and coupled with differing concentrations across GABA-A receptors, this likely accounts for the differences in the effects of benzodiazepine drugs, additional to their variable pharmacokinetics. They are highly lipophilic and, depending on the route of administration, have a rapid onset and are excreted following cytochrome p450 (CYP450) metabolising and glucuronide conjugation.

Diazepam ('Valium') is an example benzodiazepine drug, where evidence indicates anxiolytic action at the alpha2 GABA-A receptor site and alpha2 and 3 for myotonic and sedating effects (Crestani et al., 2001; Rudolph et al., 1999), reaches peak plasma within one-two hours, has a long half-life (average 60 hours) and produces active metabolites.

By comparison, oxazepam ('Serepax') is a 3-hydroxy benzodiazepine and also a diazepam metabolic by-product but is processed via conjugation to glucuronic acid with no active metabolites. It also has peak plasma within two hours but possesses a shorter half-life (eight hours average; Greenblatt, 1981). Both drugs are indicated for the treatment of anxiety symptoms that exist in GAD (Hollister, 1993; NICE guidelines, 2011b).

The behavioural decrements in psychomotor performance, sedation and memory and cognitive function are associated with benzodiazepine use (Lucki, Rickels, & Geller, 1986; Rickels et al., 1999; Wittenborn, 1979). Addiction and tolerance are also well-known phenomena reflected in rebound symptoms of tension and anxiety, insomnia, sweating, tremors, concentration difficulties and irritability, among others, and tend to be increased with drugs of decreased half-lives, secondary metabolite generation, system accumulation and drug interaction. However, the precise pharmacokinetic mechanisms of tolerance and dependence are yet to be clarified (Nutt, 2001; Moller, 1999; Petursson, 1994; Tan, Rudolph & Luscher, 2011; Woods, Katz & Winger, 1992).

Animal/tissue models suggest modulations to GABA-A receptor subunit compositions via increases to gamma2 subunit phosphorylation and proportion of alpha1-containing GABA-A receptors following chronic administration (diazepam for 14 days; rodent model; Ferreri, Gutierrez, & Gravielle, 2015).

Anxiolytic drug developments (e.g. alpha2- and/or alpha3-receptor subtype partial agonist drugs) are an attempt to negate tolerance and dependency issues (Atack, 2003; 2005), however, effective novel drug development for anxiety disorders is arguably still in shortfall (Altamura et al., 2013; Griebel & Holmes, 2013; Licata & Rowlett; 2008; Stewart et al., 2015).

Table 10. Classes of Drugs Indicated for Treatment of GAD in Australia

Drug	SSRI AD (escit, parox)	SNRI AD (dulo)	Tricyclic AD (Imi)	Atypical AD (Ago)	AC (Preg)	AH (Hydr)	AP (Que)	BDZs	AZ (Bus)
8 – 12 weeks efficacy	++	++	++	++	++	++	++	++	++
6 – 12 months efficacy	++	++	I/D	I/D	++	I/D	++	I/D	I/D
Relapse prevention	++	++	I/D	++	++	I/D	I/D	I/D	I/D
Speed of therapeutic action	0	0	0	+ / ++	++	+ / ++	++	++	0
Cognitive symptom efficacy	++	++	++	++	++	+ / ++	++	0 / +	++
Somatic symptom efficacy	0 / + / ++	0 / + / ++	0 / + / ++	++	++	+ / ++	++	++	0
Depressive symptom efficacy	++	++	++	++	+	0	++	0	0
Overdose safety	+ / ++	0 / +	0	I/D	+ / ++	+ / ++	+ / ++	+ / ++	+ / ++
Withdrawal effect	0 / + / ++	0 / + / ++	0 / + / ++	++	+ / ++	++	0 / + / + +	0	++
Side Effects and tolerability	0 / + / ++	0 / + / ++	0 / + / ++	0 / + / ++	+ / ++	+ / ++	0 / +	+ / ++	0 / + / + +

++ = clear advantage; + = possible/some advantage; 0 = little advantage; Ago: agomelatine; AC: anticonvulsant; AD: antidepressant; AH: antihistamine; AP: antipsychotic; BDZs: benzodiazepines; Bus: Buspirone; dulo: duloxetine; escit: escitalopram; Hydr: hydroxyzine; I/D: insufficient data; Imi: imipramine; Preg: pregabalin; Que: Quetiapine; SSRI: Selective serotonin reuptake inhibitor; SNRI: selective noradrenergic reuptake inhibitor

Source: Adapted from Starcevic (2015).

5.5 Treatment Resistance and Augmentation Strategies in GAD

Treatment resistance, which in GAD is considered to be the failure of two rounds of antidepressants and one psychological intervention, is an important consideration as any intervention in research or clinical settings non-responders occur and influence response and remission rates. The previous chapter discussed the confounds of treating GAD with comorbid mood disorders and trends in diagnoses that occur. While comorbid conditions are a prominent risk factor in anxiety treatment resistance and, in anxiety disorders in Australia this is reported to be depression, dysthymia and alcohol dependence (McEvoy et al., 2011), inadequate treatment and/or compliance of use is also a factor in treatment resistance (Roy-Byrne, 2015). The presence of a mood or substance disorder in GAD is more likely in non-responders and the overall presence of psychiatric or medical disorders is the most common confound to treatment response regardless of which symptomatology is prioritised. In anxiety disorders such as GAD,

treatment resistance is less explored despite a high prevalence and lifetime statistics (Bystritsky, 2013; Nemeroff, 2003; Pollack, 2009).

Remission in GAD is low, and thus relapse prevention is a crucial area of research. A recent systematic review (Mochcovitch et al., 2017) examined the long-term pharmacotherapy treatment of relapse prevalence in GAD. From the eight studies, the review reported that treatment structures maintained after 6 months of remission had better long-term outcomes. Similarly, Baldwin et al. (2011) recommended at least 12 months continued treatment and the need for more research into non-responders of first-line treatments.

Second-line treatment regimens may involve the consideration of comorbidities and subsequently adding treatment for the second condition/s, as well as augmentation strategies, that is, the combination or switching of similar pharmacotherapeutics in the treatment of anxiety disorders, especially where symptoms have been deemed as being 'difficult to treat'.

For GAD, typical augmentation strategies involve treatments from a line of antipsychotics such as beta-blocker class gabapentin or pregabalin to an existing SSRI or tricyclic antidepressant to treat both cognitive and physiological symptom presentations (Bystritsky, 2006; Coplan et al., 1993; Roy-Byrne, 2015). Some studies, such as a recent meta-analysis by Patterson and Van Ameringen (2016), have examined the efficacy of such strategies in treatment-resistant anxiety disorders; however, the findings were noted as limited in sample size and study numbers.

There is a lack of studies assessing combination/adjunctive therapies for GAD among anxiety disorders (Cuijpers et al., 2014). Venlafaxine and relaxation therapy were examined in one crossover study (Zullino et al., 2015), which reported that response occurred in non-remitting patients in each group during the second phase when a second treatment was added.

5.6 Phytomedicines for Anxiety Disorders

The challenge of adequate treatment for disorders such as GAD prompts research into the bioactive constituents of phytomedicines for therapeutic benefit. Plant-derived constituents such as alkaloids, terpenoids/saponins and phenols, including flavonoids and carotenoids, act within the CNS producing a number of modulations to aminergic substrates to produce anxiolytic effect (Graeff, 1988; Nuss, 2015; Durant, Christmas & Nutt, 2009).

The effects of phytoconstituents on GABA pathway components such as GABA-A receptors are complex and warrant extensive research, even for flavonoids such as chrysin and apigenin that are heavily investigated, the precise substrate mechanisms are yet to be clarified. In the literature, general and systematic reviews have examined phytomedicines where traditional use is matched with pre-clinical and clinical models of efficacy; however, the current literature has evidence gaps that impact translational ability.

Some reviews have detailed the GABAergic effect from phytomedicines solely consisting of pre-clinical evidence (Awad et al., 2007; Johnston et al., 2009) or only from the clinical level for the alleviation of anxiety-type symptoms in human studies (Ernst, 2006; Lakhan & Vieira, 2010). Others have more broadly studied phytoconstituents known to modulate multiple neurotransmitter systems involved in anxiety symptomatology (Kennedy & Wightman, 2011; Sarris et al., 2011), which, while exploiting the benefits of synergistic or adaptogenic qualities of some phytomedicines, may not allow for specific and replicable conclusions of GABAergic anxiolytic effects. Similarly, reviews have phytomedicines efficacy of combination herbal formulae, such as *kampo* that is prevalent in Chinese traditional medicine practices (e.g. Liu et al., 2015) or have broader systematic criteria to include supplements and compounds not strictly phytomedicine-derived, such as inositol or 4-hydroxyphenylalanine/tyrosine (Boonstra et al., 2015).

Finally, phytomedicine reviews of GABAergic effect have assessed as treatment for insomnia yet not anxiety (Shi et al., 2014). Reviews specific to GAD have studied it together with anxiety and mood disorders of known comorbidities, such as GAD and MDD, thereby preventing conclusions of purely anxiolytic benefit (Sarris, 2007; 2013; 2017).

Due to these limitations, the author conducted a systematic review of phytoconstituents/phytomedicines (Refer to Appendix E; Savage, Firth, Stough, & Sarris, 2017) compiling a summary list where three levels of evidence could be found: pre-clinical (tissue and animal anxiety models) and clinical human trial evidence acting within the GABA system to achieve an anxiolytic effect via the reduction of anxiety-type symptoms. The review focussed on mapping these phytoconstituents onto the GABA system mechanisms known to modulate anxiolytic activity, and reported on 10 phytomedicines to have sufficient evidence for their use in anxiety symptom alleviation occurring in disorder such as GAD.

Of the phytomedicines collated, *P. methysticum* held the most putative evidence for use as an anxiety disorder treatment, with tripartite-level evidence as stipulated in the review methods criteria and as such is considered a hallmark anxiolytic phytomedicine. This is the most strongly evidenced, particularly for GAD, with clinical investigations now examining GABA metabolite changes via neuroimaging, guided by pharmacogenetic frequencies of GABA pathway constituents to optimise response.

In the review, *Centella asiatica*, *Valeriana officinalis*, *M. recutita*, *Passiflora incarnata* and *Withania somnifera* offered the next most extensive evidence based on the criteria, with at least one clinical affective disorder study reporting efficacy. Additional to these phytomedicines, *H. lupulus*, *S. lateriflora*, *G. biloba*, *Magnolia officinalis* and *Melissa officinalis* held some evidence in human randomised controlled trials involving stress and anxiety outcomes. While promising, again, limited anxiety population sampling is a constraint to the evidence base, despite well-known traditional indications of their use.

With regard to the major GABA modulating phytoconstituents, the terpenoids/sesquiterpenoids and saponins class such as bilobalide, humulene, valerenic acid and withanones from *Withania somnifera* (Ashwagandha or winter cherry) and rosmarinic acids from *Melissa officinalis* (rosemary) were best-evidenced. The flavonoids chrysin, apigenin (4',5,7-trihydroxyflavone from *Matricaria recutita*) and baicalin (7-O-glucuronide from *Scutellera lateriflora*) are also highly evidenced. Flavonoids operate in the CNS through receptor-binding whereby they open chloride channels, hyperpolarise cell membranes and increase the threshold for activity; therefore exerting an anxiolytic effect (Hanrahan et al., 2011; Johnston et al., 2015; Wasowski & Marder, 2012).

Whether the GABAergic constituents from *P. methysticum* (Kava), *Valeriana officinalis* (valerian) and *Centella asiatica* (gotu kola) similarly facilitate binding of PLP to GAD by causing a conformational change, or act as PLP mimics, remains to be elucidated. It may also be worthwhile to determine whether these extracts contain pyridoxine or related compounds for their capacity to influence B group vitamin metabolism (refer to Section 5.7 for *P. methysticum* evidence as an example).

Some phytomedicines also held good evidence however did not meet the three-level criteria. *E. californica*, *Magnolia officinalis* and *P. amurense* had only been trialled in human studies of anxiety as part of a formulation and not monotherapy, thereby limiting their capacity as a treatment option in clinical groups (Garrison & Chambliss, 2006; Hanus, Lafon, & Mathieu, 2004; Kalman et al., 2008; Talbott, Talbott, & Pugh, 2013). Other plant medicines had encouraging pre-clinical data, and a long-standing

record of traditional use, however, lacked human clinical trials, such as *M. citrifolia* (Pawlus & Kinghorn, 2007; Potterat & Hamburger, 2007) and *Z. jujuba* (Choi et al., 2011; Gao et al., 2013).

The translation to phytomedicine utility in medical/clinical settings requires more than a handful of psychiatric samples as evidence to substantiate value in GABAergic effects that may result in symptom alleviation in these disorders. The discussion of these limitations offers up the justification for further efficacy research of phytomedicine extracts and constituents to fill the evidence shortfall.

5.7 *Piper methysticum*— A Review of the Literature

5.7.1 Overview

P. methysticum (Kava) is explored in more detail here with emphasis on the mechanisms relevant for the use in the treatment of anxiety symptoms. *P. methysticum* extract is currently the most highly researched phytomedicine treatment for the alleviation of anxiety symptoms. There is existing tripartite-level (preclinical, animal and human) evidence for Kava efficacy in reducing anxiety, i.e. animal anxiety models and in affective disorder human studies, and mechanisms are evidenced to act within the GABA pathway via numerous tissue studies.

5.7.2 Introduction

The Kava plant (*P. methysticum*) is a perennial shrub of the pepper family *Piperaceae* that is native to the South Pacific (illustrated in Figure 14). The rhizome roots have been used in traditional medicine via cold-water extraction as they possess multiple useful properties including anxiolytic, nootropic and neuroprotective, nociceptive and anti-dysphoria effects (Lebot, Merlin, & Lindstrom, 1992; Pittler and Ernst, 2000; Singh, 1992; Tzeng & Lee, 2015). The main reported psychoactive and physiological effects of Kava are typically anxiolytic/anti-stress (Kinzler et al., 1991; Singh & Singh, 2002) and, at higher doses, sedative/hypnotic (Gleitz et al., 1995; Wheatley, 2001) and analgesic (Jamieson & Duffield, 1990) properties in humans.

The use of Kava as a medicine in extract form (usually delivered via tablets or capsules) has increased globally over the past 30 years. In Australia, one nutraceutical company reported Kava as the second-highest product for sales, with over 1.2 million tablets prescribed or purchased annually (Bensoussan, Myers, Wu & O'Connor, 2004).

The rhizome is traditionally prepared via aqueous extraction; however, ethanolic and acetonic extraction methods are common with increased yield for commercial processes

(Teschke, Genthner, & Wolff, 2009; Teschke et al., 2003). Commercial Kava extracts that employ organic solvents instead of traditional aqueous methods, as well as the inclusion of leaves and stem containing a variable profile (including additional alkaloids), have been associated with hepatotoxic processes, which is discussed in more depth in Section 5.7.6 of this chapter.

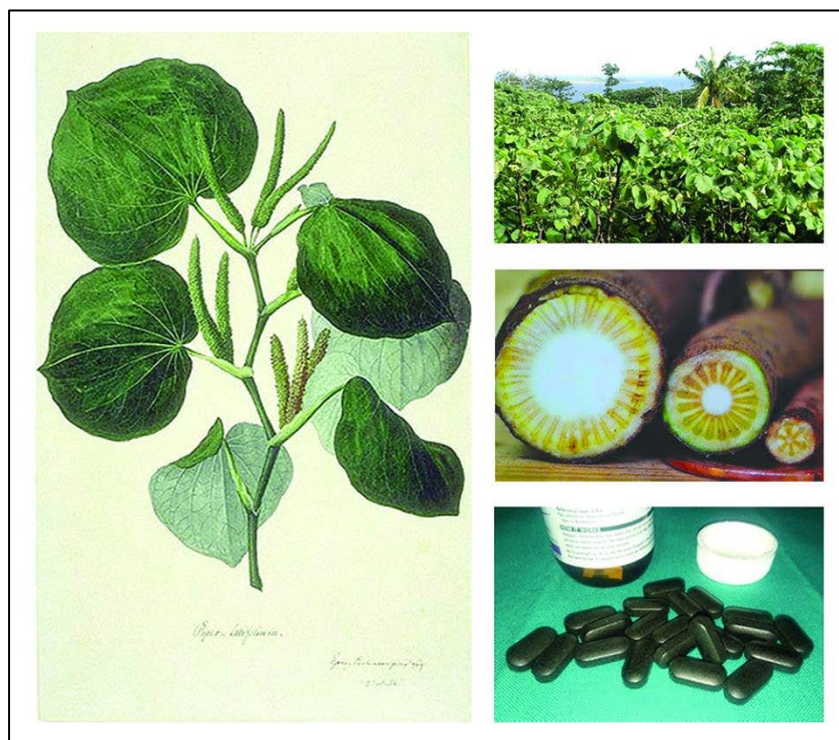


Figure 4. *Piper methysticum*. Clockwise: Illustration of *P. methysticum*; *P. methysticum* crop in Vanuatu; rootstock from which kavalactones are derived; extract in tableted form (images 1-3: open source; 4: author)

5.7.3 Bioactive constituents

The Kava plant comprises a range of bioactive constituents, including pyrones, flavonoids and alkaloids, and lactones. The latter ‘kavalactones’ are the principle components for which *P. methysticum* is mostly utilised, with 18 isolated thus far, and six of which comprise the majority of pharmacodynamic effect: methysticin, dihydromethysticin, yangonin, demethoxyyangonin, Kavain/kawain and dihydrokavain/dihydrokawain (Bilia et al., 2002; Chua et al., 2016; Duffield et al., 1989; Meyer, 1979). New kavalactone dimers have been extrapolated recently, such as yangonindimers A, B and C (Song et al., 2017; Yuan et al., 2017). These kavalactones comprise a basic structure of methoxyl lactone and benzene ring, connected by an ethylene or ethyl bridge (refer to Figure 15; Dharmaratne, Nanayakkara & Khan, 2002).

The main six lipophilic kavalactone constituents have been the subject of most investigations due to their concentrations, comprising up to 96% of the rootstock lipid resin, but also their variable motieity, which may explain the broad range of aforementioned effects. Of these constituents, kawain and dihydrokawain are evidenced to exert the strongest anxiolytic activity (Mathews et al., 2005). Kavalactones are also found in one other species, *Alpinia zerumbet* (shell ginger), in which the constituents dihydro-5,6-dehydrokawain and 5,6-dehydrokawain are also present (Xuan et al., 2015).

Following ingestion, *P. methysticum* extracts reportedly reach peak concentration at 1-2 hours with effects lasting for eight hours. The elimination half-life is approximately nine hours and has a reported distribution half-life of 50 minutes (Saletu, Grunberger, Linzmayer, & Anderer, 1989).

Kavalactones are mainly metabolised *in vivo* by the modulation of phase I enzymes including the CYP450 family of hemethiolate enzymes in the liver, which is involved in the oxidative metabolism of a number of substances (Zhang & Ramzan, 2010; Zou, Harkey, & Henderson, 2002). Kavalactones inhibit several of the P450 enzymes in the liver, for example dihydromethysticin and desmethoxyyangonin both induce CYP3A23, lipophilic compounds (Lim et al., 2007; Mathews, Etheridge, & Black, 2002b; Mathews et al., 2005) and elicit a number of pharmacodynamic interactions with other substances requiring these enzymes.

There is the suggestion of a synergistic effect or enhancement of kavalactones when administered together that is supported through the hepatic mechanisms whereby total kavalactones are required to generate CYP3A23 induction by dihydromethysticin and desmethoxyyangonin (Ma et al., 2004). *P. methysticum* kavalactones exert similar effects on P450s as that of the phytomedicine valerian (*Valeriana officinalis*), with both specifically modulating CYP1A2, CYP2D6, CYP2E1 and/or CYP3A4/5 activity, but not CYP2D6 (Gurley et al., 2005; 2008; Russmann et al., 2005; Wanwimolruk, Phopin, & Prachayasittikul, 2014; Unger et al., 2002).

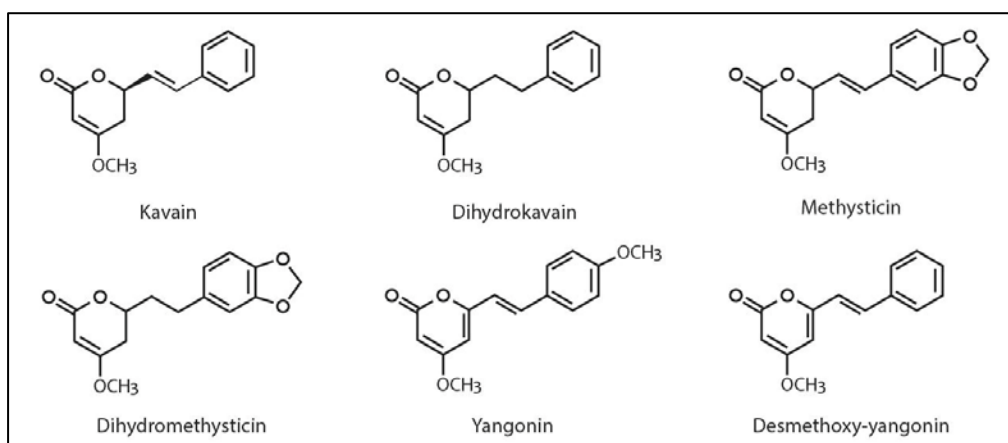


Figure 5. Molecular structures of the six principal kavalactones in *P. methysticum* (image by author)

Both gas chromatography and high performance liquid chromatography are now used for the analysis of kavalactones, although there is some debate as to the accuracy of these processes, as components such as methysticin are temperature sensitive, and limited capacity to isolate constituents via gas chromatography to achieve adequate separation of spectra between yangonin and methysticin (Bilia et al., 2004; Wang et al., 2010).

As with all plant species, constituent profiles vary between crops. Studies of constituents and their effects on selected CNS substrates have found variations between cultivar types (Lebot & Lévesque, 1989). One study found IC₅₀ values ranging from 5 micrograms/ml (Nene cultivar) to 87 micrograms/ml (Mahakea cultivar) root extracts with GABA-A receptors and GABA binding sites from kavain, 7,8-dihydrokavain, methysticin, 7,8-dihydromethysticin, yangonin and 5,6-demethoxyyangonin constituents (Dinh et al., 2001). Tableted versions are typically standardised to 30% kavalactones and constitute quantities of 80 to 250 mg. A typical constituent profile of a tableted kavalactone extract is illustrated in Figure 16 (noting that water is the only allowable extraction method in Australia), and the principle kavalactones are summarised below with some of their mechanisms, with the associated investigations summarised in Table 11.

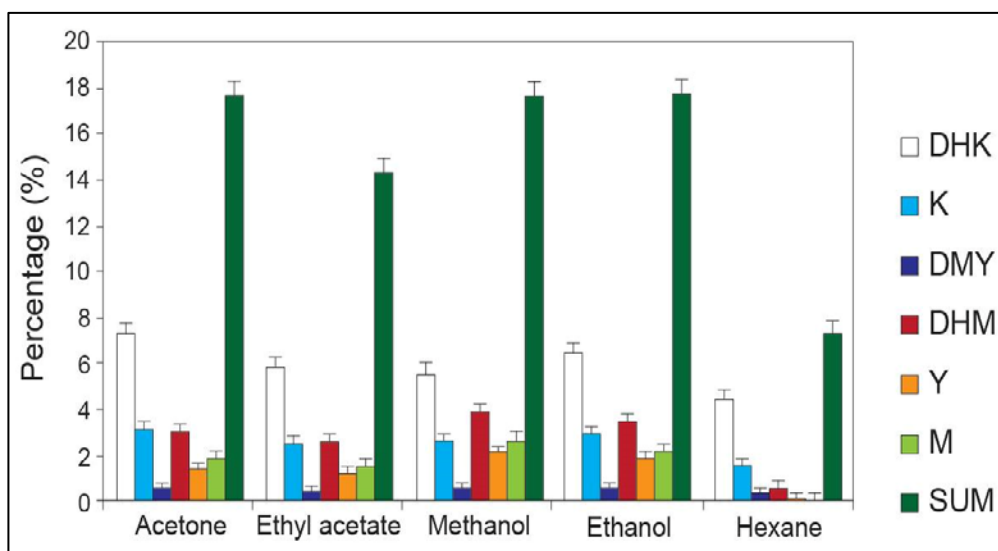


Figure 6. Percentages of six major kavalactones and their total content extracted with different solvents (DHK-dihydrokavain; DHM-dihydromethysticin; DMY-desmethoxyyangonin; K-Kavain; M-methysticin; Y-yangonin).

Adapted from Wang et al. (2015)

Dihydrokavain/dihydrokavain (5,6-dehydrokavain) has been quantified as the highest percentage constituent in many composition analyses. It is differentiated from kavain by its benzene ring substitutes. Animal models of stress report an anxiolytic effect with isolated dihydrokavain in 8-day-old chicks with a measure of distress vocalisation as a 'chick social separation-stress procedure' and a chlordiazepoxide benzodiazepine comparator equitable to chlordiazepoxide in reduction to anxiety in the model, but without sedating effect (Feltenstein et al., 2003).

Kavain is the second highest in concentration from rootstock in most species of Kava. In animal models, kavain reportedly decreases serotonin concentrations and modulates dopamine levels in rat mesolimbic pathways, suggesting these pathways contribute to eliciting Kava's relaxant/euphoric effect (Baum, Hill, & Rommelspacher, 1998).

Methysticin is one of the major kavalactones and is also associated with an anxiolytic effect. It is a derivative of kavain, with an additional five membered ring to the B ring. Neuroprotective properties have been reported together with dihydromethysticin/7,8-dihydromethysticin in a modal of focal cerebral ischaemic damage (Backhauss & Krieglstein, 1992). Both induce hepato-enzyme CYP1A1 and are the strongest kavalactones to do so (Li et al., 2011). Kavalactones may potentiate the effects of other substances by regulating the activities of CYP450 or P-glycoprotein. Further, the kavalactones methysticin and kavain are evidenced as eliciting non-stereo-selective

inhibition of noradrenalin uptake, found to be inhibited mostly by kavain in rat hippocampus and cortical cell synaptosomes in a tissue model (Seitz, Schle, & Gleitz, 1997)

Dihydromethysticin, as with the former two, differs by substitutes on the benzene ring, and all three may enhance NMDA receptor function or voltage-dependent calcium channels (Walden et al., 1997). Both methysticin and dihydromethysticin are believed to exert neuroprotective effects indirectly based on CYP1A1 expression via an AhR-dependent mechanism, typically associated with apoptotic processes (Li et al., 2011, Ma et al., 2004). In animal models of cerebral infarction, effects were similar to the comparator anticonvulsant memantine (Backhauss & Krieglstein, 1992).

Yangonin enhances the binding of bicuculline at GABA-A receptors at 1.0 μ M (Boonen & Haberlein, 1998) and is a potent NF-kappa-beta inhibitor, thus exhibiting anti-inflammatory benefits (Ma et al., 2012). One study reported an affinity of yangonin for the human recombinant CB1 receptor, the only kavalactone that inhibited endocannabinoid system enzymes. The CB1 receptor interaction with yangonin suggests that modulations to the endocannabinoid system might contribute to the cognitive effects (Ligresti et al., 2012). Regarding metabolising processes, yangonin produced a hepato-protective effect against LCA-induced toxicity and the amelioration of cholestasis after yangonin treatment in one recent combined *in vivo* and *in vitro* study (Kong et al., 2018).

Methysticin, kavain and yangonin are also reported to activate NF-E2-related 2 transcription factor and antioxidant enzyme heme oxygenase 1 in neural and glial cell lines in an amyloid model of neurotoxicity (Terazawa et al., 2013; Wruck et al., 2008). Limited research exists on the neuroprotective potential of kavalactone but there is potential for use in oxidative stress and inflammation-related conditions such as Alzheimer's typology from the limited evidence.

Finally, desmethoxyyangonin is reported to increase serotonin and other catecholamine concentrations and increases dopamine levels in the nucleus accumbens of rats (Baum et al., 1998).

Differences in pharmacodynamic properties are further evidenced by altered expression of cytochrome enzymes by each kavalactone. For example, yangonin has a methoxy substitute but only desmethoxyyangonin modulates the expression of CYP3A23 although they are structurally identical. Similarly, methysticin lacks the methyl bridge and dihydromethysticin elicits CYP3A23 activity.

Table 11. Pre-clinical Studies Evidencing Kavalactone Mechanisms of Action

Mechanism of action	Study design	Key findings	Reference
Neuroprotective effects via a model of focal cerebral ischaemia	<i>In vitro</i>	Methysticin and dihydromethysticin associated with a significant reduction of infarct area comparable to memantine	Backhaus and Krieglstein (1992)
Reduced neuronal reuptake of dopamine	<i>In vitro</i>	High doses of Kavain and desmethoxyyangonin increased dopamine levels in rat nucleus accumbens	Baum et al., 1998
Enhanced ligand binding to GABA- α receptors	<i>In vitro</i>	Yangonin, Kavain, dihydrokavain, methysticin, and dihydromethysticin enhanced specific binding of [3H]-bicuculline methochloride (although not [3H]-flunitrazepam)	Boonen et al. (1998)
Kavain elicits enhancement of GABA-A receptor function in a subtype non-selective and flumazenil-insensitive manner	<i>In vitro</i>	Kavain exhibited positive modulation across nine GABA receptor subtypes expressed in <i>Xenopus</i> oocytes: $\alpha 1\beta 2$, $\beta 2\gamma 2L$, $\alpha x\beta 2\gamma 2L$ ($x = 1, 2, 3$ and 5), $\alpha 1\beta x\gamma 2L$ ($x = 1, 2$ and 3), and was greatest at $\alpha 4\beta 2\delta$. Effects not modulated by flumazenil. Non-additive effects in the presence of benzodiazepines	Chua et al. (2016)
GABA-A and GABA-B, benzodiazepine binding sites of individual kavalactones	<i>In vivo</i> / <i>Ex vivo</i>	Mild effects to benzodiazepine site binding from yangonin, dehydromethysticin, desmethoxyyangonin via ligand displacement	Davies et al. (1992)
Modulation of calcium and sodium channels	<i>In vitro</i> / <i>In vivo</i>	Prior to anoxia the sodium channel blockers tetrodotoxin and Kavain preserved vesicular ATP content, prevented both the veratridine-induced increases of Na^+ and Ca^{2+}	Gleitz et al. (1997)
Enhanced binding [3H] muscimol	<i>In vitro</i>	Kavapyrones enhanced [3H]-muscimol binding in a concentration-dependent manner. Maximum potentiation was observed in the hippocampus, amygdala and medulla oblongata	Jussofi et al. (1994)
Voltage-dependent sodium channel inhibition	<i>In vitro</i>	Both kavain and methysticin inhibited voltage-dependent Na channels in acutely dissociated rat CA1 hippocampal neurons leading to a decrease of cellular excitability	Magura et al. (1997)
Inhibition of voltage-dependent calcium channels	<i>In vitro</i>	Kavain interacted with M3 receptor or M3 associated G-protein receptor. Kavain did not affect the prostaglandin pathways and nitric oxide mediated relaxation not observed	Martin et al. (2000)
Reduced neuronal reuptake of noradrenaline	<i>In vitro</i>	Kavain and methysticin inhibited uptake of noradrenaline in rat cerebral cortex and hippocampal synaptosomes	Seitz et al. (1997)
Modulation of brainstem GABA	<i>In vitro</i>	Kavalactones or dihydrokavain significantly reduced rat brain stem nucleus tractus solitarius inhibitory effects induced by muscimol	Yuan et al. (2002)
Reversible inhibition of MAO-B	<i>In vitro</i>	MAO-B reversible inhibition by individual kavalactones: desmethoxyyangonin, methysticin, yangonin, dihydromethysticin, dihydrokavain, kavain	Uebelhack et al. (1998)

Anti-inflammatory actions in COX2 signalling pathway	<i>In vitro</i>	Dehydrokavain, yangonin inhibit H ₂ O ₂ -induced P38 phosphorylation	Rao et al. (2014)
Upregulation of antioxidation enzymes in amyloid-induced neurotoxicity	<i>In vivo/Ex vivo</i>	Methysticin, kavain, and yangonin stimulates ERK1/2 phosphorylation in PC12 cells and subsequently Nrf2 and HO1	Wruck et al. (2008)

Source: Adapted from Sarris et al., 2011

5.7.3.1 Evidence of pharmacodynamic evidence in animal anxiety studies

Animal anxiety model investigations are plentiful (e.g. Garrett et al., 2003; Rex et al., 2002), with the mirrored chamber avoidance and the Elevated Plus Maze the most common paradigms used. The majority of investigations concur in reporting efficacious findings such as reduced entry latencies and increases in time spent in unfamiliar environments compared to the placebo comparators, equating to efficacy.

5.7.4 Neurocognitive research

Only one study has employed imaging via EEG in healthy subjects following kavain doses (Saletu et al., 1989) and included acute doses (200 mg, 400 mg and 600 mg) of kavain and two comparators (placebo and 30 mg clobazam). The study reported improvement on measures such as drive, wakefulness, affectivity, mood and wellbeing compared to the placebo with a 200 mg kavain dose, which was also associated with a decrease of delta and beta activity and increase of alpha activity. All three doses of kavain produced improved intellectual performance (Pauli test), attention, concentration, reaction time and motor speed (rigidity test) compared with the placebo. However, the higher dose (600 mg) of kavain was associated with sedation effects. Encephalotropic effects were reported in the frontal area with kavain, whereas the benzodiazepine comparator was central and parietal. Data from the study contributes to the evidence of differential GABAergic mechanisms of kavalactones from that of benzodiazepines, and reinforce the findings reported in pre-clinical tissue studies on GABA receptor mechanisms.

5.7.5 Evidence for clinical efficacy in treating anxiety and GAD

P. methysticum holds the most plentiful and putative evidence for efficacy in the treatment of anxiety symptoms for a phytomedicine, and there is an abundance of studies over the past 30 years supporting its use. Early clinical investigations following a

double-blinded and placebo-controlled approach include a study by Lehmann et al. (1989) using Kavain ('Cavain') who reported significant effects from a 200 mg daily for 14 days' administration in 'abnormal anxiety'/psychosomatic complaints, using the HAM-A outcome scale and placebo comparator. Another study, by Lindenberg and Pitule-Schodel (1990), found doses of *P. methysticum* Kavain extract (marketed as Neuronika trademark) had equivalent effects to an oxazepam benzodiazepine comparator in anxiety subjects ($n = 38$).

The clinical evidence for Kava efficacy in the treatment of anxiety or mood disorder symptoms has culminated in several recent reviews or meta-analyses for Kava alone (LaPorte et al., 2011; Pittler & Ernst, 2003; Sarris et al., 2011; Singh & Blumenthal, 1997; Stevinson et al., 2002) or with similar phytomedicines (Chiappedi & Bejor, 2010; Lakhan & Viera, 2010; Sarris & Kavanagh, 2009; Sarris et al., 2012). The majority of studies used in reviews report improvements in the HAM-A in Kava mono-preparations relative to placebo where results are significant for Kava.

A Cochrane review and meta-analysis of six randomised controlled trials using Kava mono-preparations (60 mg–280 mg kavalactones) for the treatment of GAD symptoms indicated significant reductions of anxiety on the HAM-A for those in the Kava group compared with the placebo ($p = 0.01$) (Pittler & Ernst, 2003). Similarly, a pooled analysis of six studies using Kava versus placebo in the treatment of GAD symptoms found a significant effect in favour of Kava on the HAM-A (Cohen's d of 1.1; Sarris et al., 2011).

Recent related studies operating as precursors to the current investigation in Chapter Seven include a crossover three-week study using 250 mg daily Kava in patients with generalised anxiety and depressive symptoms (Sarris et al., 2009). Scores on the HAM-A in the treatment group showed a significant reduction with a strong effects size, which reported a reduction in anxiety symptoms using the HAM-A of 10.1 points in the pooled Kava phase compared with a -0.6 to 3.3 point change in the pooled placebo phases (Cohen's $d = 2.24$, $p < 0.0001$) and scores on the MADRS in the treatment group were also significant.

A second related study specifically involved patients with diagnosed GAD, over 8 weeks (Sarris et al., 2013) and involved the taking of 120–240 mg kavalactones daily. This study had similar outcomes including changes in the HAM-A score over the treatment period and a number of biochemical, psycho-social and pharmacogenomic outcome measures. The study found significant reductions in anxiety symptoms using the HAM-A outcome measure over an eight-week period of a mean reduction of 8.5 points for the Kava group

on the HAM-A compared with the placebo ($p = 0.020$; $d = 1.28$). For participants with moderate-to-severe level DSM-5 anxiety (as assessed on MINI Plus), the treatment was more pronounced ($p = 0.020$), with a larger effect size ($d = 0.80$). At the study end, close to one third of the Kava group were classified as remitted with a HAM-A score of 7 or less.

5.7.6 Safety aspects of *P. methysticum* extracts

While *P. methysticum* is listed in Australia by the Therapeutic Goods Association, is available over-the-counter and is not associated with any reports of health issues in this country, concerns over hepatotoxicity have led to withdrawal or restriction in some countries. Many reported cases have involved concomitant ingestion of other compounds with potential hepatotoxicity, that is, other medications and/or alcohol or the consumption of aerial parts of the plant. There is also evidence of hepatotoxicity derived from intervention studies in samples without screening of existing hepatic pathologies, such as those by Escher, Desmeules, Giostra, and Mentha (2001), and Strahl, Ehret, Dahm, and Maier (1998). At the clinical level, a variety of case study data from patients with Kava hepatotoxicity has been gathered (Sarris et al., 2012) and it appears to be an extremely rare occurrence, with probable causation only directly linked in a few cases (refer also to discussion in Sarris, Ng, & Schweitzer, 2011).

As the liver's function in metabolising substances is transformation into water soluble products preventing reabsorption or further reactions, it does so by adding water soluble groups such as the -OH (hydroxy) groups with additional attachments of ionic molecules (conjugation). Phase 1 metabolism involves chemical processes mainly by CYP450 enzymes, such as hydroxylation, which increase the water solubility. During Phase 2, conjugation reactions occur with attachment by large ionic molecules such as glucuronic acid or glutathione, further increasing solubility. During Phase 2, UDP-glucuronosyltransferase (UGT) in the liver transfers glucuronic acid to a metabolite that is more water soluble and can be excreted in the urine. In some cases, CYP450 priming occurs prior to UGT metabolism such as with benzodiazepines and anti-inflammatory drug metabolites, and from other plant bio-constituents including curcuminoids from turmeric (Volak, 2008). Similarly, kavalactones may operate as a substrate for UGTs.

Kavalactone mechanisms on CYP450 induction are related to individual variances in the functionality of the enzyme CYP2D6 (Poolup et al., 2000); thus, the induction of

hepatotoxicity via the concurrent use of other drugs (such as alcohol) are also attributed to increased prevalence (Mathews, Etheridge, & Black, 2002a).

During Phase 1, there is fairly putative evidence that kavalactones inhibit CYP450, in particular 2E1, but also 1A2, 2C19 and 2C9, and slight effects with 2D6, 3A4 and 4A9/11 (Ma et al., 2004; Mathews et al., 2002a; Unger et al., 2002). This inhibition may partly explain the interaction with alcohol via compound effects, possibly resulting in the accumulation of acetaldehyde. It provides evidence that drug/drug interactions (mostly involving the inhibition and induction of P450) are not isolated to Kava use and do not evidence direct hepatotoxicity just from the use of kavalactones (Shord, Shah, & Lukose, 2009).

A contributing factor in hepatotoxicity connected to *P. methysticum*, originally found with German Kava products, was likely due in part to the use of non-water soluble chemical extraction techniques (the traditional solvent method is water-based) using the aerial parts of the plant, roots and stem peelings in commercial crops (these may contain the alkaloid pipermethystine) (Nerurkar et al., 2004). Such raw material may be poorly prepared or potentially contaminated raw material (e.g. the development of mould; Teschke, Qiu, & Lebot, 2011).

In response to these safety concerns, the World Health Organization commissioned a report assessing the risk of Kava products (Coulter, 2007). Recommendation 2.1.3 from this report suggested that products from water-based suspensions should be developed and tested in clinical trials and that these preparations should preferentially be used over acetonetic and ethanolic extracts (also asserted in Teschke, 2010; Teschke, Sarris, & Lebot, 2011; Teschke et al., 2011).

The extract used in the intervention study in this thesis addresses these safety concerns by using a water soluble and standardised formulation of *P. methysticum* from the peeled rootstock of a 'noble cultivar'. Such cultivars are higher in kavain and dihydrokavain and are lower in dihydromethysticin.

5.7.7 Concluding statements on Kava for the treatment of GAD

As detailed in the literature review, there is putative evidence of *P. methysticum* extract efficacy within pathways associated with anxiolytic effect, chiefly the GABA substrate. Each of the six kavalactones exerts slightly different modulations; however, the specific pathway components have only recently been elucidated, such as the

finding that actions involve non-selective subtypes and non-additive benzodiazepine/non-benzodiazepine site mechanisms with kavain (Chua et al., 2016).

Based on the literature thus far, GABA pathways are one principle pathway for kavalactones and there is some evidence for kavalactone modulation within frontal and limbic brain regions (in the one EEG study by Saletu et al., 1989). However, these mechanisms remain largely unknown, as no research has involved direct *in vivo* measurements.

Clinical studies involving relevant neurobiological measures as outcomes will serve to link much of the pre-clinical findings regarding the GABA substrate with anxiolytic modulation by kavalactones to establish putative evidence for a) the pathways of effect that occur with combined kavalactone *P. methysticum* extract, giving insight into pharmacodynamics and b) the cognitive and physiological anxiolytic benefit provided when used in sample populations where GABAergic physiology is dysfunctional, such as GAD.

Although the efficacy data on *P. methysticum* in reducing anxiety is promising, the prescribed use for GAD symptoms cannot be established without larger, confirmatory, investigations that employ measurement of neurobiological patterns in order to strengthen the efficacy evidence via measurable neurobiological biomarkers. As such, the current research detailed in the next two chapters and, more specifically, in the second investigation involving *P. methysticum* aimed to build upon the limitations identified from the current body of literature to clarify how Kava constituents act on the GABA pathway within affective brain regions and how this is connected to reported symptom changes in a GAD sample. Additionally, it has the potential to guide drug development through the improved understanding of pharmacodynamic effects within the GABA pathway system.

GAD is a prevalent disorder that is under-diagnosed and under-treated. While a great deal of work is inherent in discovering anxiety disorder biomarkers for clinical benefit, the two studies in the thesis not only add to the evidence on how anxiolytic substances (such as *P. methysticum*) affect brain functions using available imaging technologies but also expand the literature regarding what these novel substances can tell us about the biology of anxiety disorders such as GAD.

The capacity to selectively determine a response to interventions in psychiatric disorder populations has obvious benefit for patients with GAD. It is therefore timely for further research into the alleviation of symptoms and improvement to quality of life in a

disorder for which there is a high level of psychological distress. From a practitioner point of view, the evidence may solidify *P. methysticum* as a *bona fide* treatment option and could reduce the burden that affective disorders hold within healthcare domains if it is cost-effective and offers better symptom management.

6 Chapter Six. Investigation of the Structural, Functional and Metabolic Markers of Generalised Anxiety Disorder: a Cross-sectional Study

6.1 Overview

As detailed in the introduction chapters, there remains crucial work needed in examining the neurobiology of GAD. The use of phytomedicines with putative GABA-modulating activity also warrants examination via neuroimaging. As such, two studies were conducted and reported in the next two chapters.

To assess the biomarker properties unique to GAD and to establish the patterns of change in such markers, a crucial first step is a comparison of neuroimaging, psychological, cognitive, and lifestyle factors between non-psychiatric ‘healthy control’ and a cohort with GAD. This chapter reports on the first investigation, its main aims, eligibility criteria, measures and procedures for data collection, processing and analysis. The chapter reports subsequent results and discusses findings in terms of the primary aims and in the context of the literature.

Data (demographic, medical and psychiatric) were collected from two groups: GAD patients as per DSM-5 criteria and a cohort-matched healthy control sample group. Data were collected across two imaging modalities for the sample groups: 1) fMRI activation levels during a worry/anticipatory anxiety induction task, and 2) GABA metabolite levels quantified via MRS (functionally for the whole brain, and then both in the dACC as the ROI). Following relevant pre-processing and analyses, both data types were compared between the two groups, before associations between modalities for the ROI were explored. Given the variability in some measures of the sample groups, confounding factors were minimised as much as practicable to construct a methodologically rigorous dataset for comparison purposes.

6.2 Introduction

In structural and functional neuroimaging, the neurobiological features unique to GAD have not been extensively studied (Maddock & Buonocore, 2011; Shin & Liberzon, 2010). Importantly for the present study, fMRI investigations of regions associated with

anxiety cognitive-affective processing such as the ACC have reported equivocal findings, such that review articles in this area concur on the need for further investigations (Engel et al., 2009; Mochcovitch et al., 2014; Taylor & Whalen, 2015). Similarly, regional GABA levels, especially in limbic areas such as the ACC that are associated with anxiety symptoms, have not been specifically tested as potential markers for unique profiling characterisation of GAD.

Regarding the ACC positioning, several studies and review articles examining functional connectivity have identified an inhibitory role of the amygdala that has a central role in cognitive-emotional processes (Cannistraro & Rauch, 2003; Phan et al., 2005). However, in imaging practice there is difficulty functionally imaging the amygdala (Kim & Goettsching, 1975; Tyszka & Pauli, 2016). Structurally, ACC subregions are appropriate to examine owing to the proximity of the sinusoidal ducts providing a solid referencing point, and, functionally, for its connection to subcortical components such as the amygdala. Finally, as further research is warranted into the neurophysiology of the ACC region during anxiety states, exploring functional subregions holds great potential not just in affective disorder imaging research but as findings for general brain neurophysiology. Given the above substantiation and the state of the literature in the area, this multi-modal group-comparison investigation and subsequent methodology represents a strong first-step approach to deriving evidence of biomarkers unique to GAD.

6.3 Main Aim and Hypotheses

6.3.1 Main aim

The main aim of this investigation was to identify metabolic and functional regional brain differences, between GAD patients and healthy controls, with special focus on the properties of the dorsal ACC 'cognitive' subregion. This was measured through a) GABA profile levels representing inhibitory capacity as measured by MRS, and b) task-related BOLD activation. The fMRI BOLD signal was assessed via performance of a paradigm of worry induction/anticipatory anxiety, specifically the appraisal and response to affective pictures coupled with a matched or non-matched cue, detailed in Section 6.3.6 below.

6.3.2 Hypotheses

The following directional hypotheses were investigated using metabolic data:

1. MRS-measured dACC GABA metabolite levels will be significantly different in the GAD group compared with the healthy control comparator, whereby reduced GABA

levels are expected to be seen in the GAD group compared with the healthy control group.

2. GABA levels will have a negative relationship to anxiety levels. That is, in the GAD group, lower GABA levels in the ROI will be associated with increased anxiety levels measured using the SIGH-A/HAM-A anxiety scale. To reinforce this assertion, anxiety level data will also be assessed in the control group.

3. Biological factors such as gender and age, and presence of a comorbid anxiety disorder will influence GABA metabolite levels, whereby comorbidity, gender differences (female > male) and age will increase the negative relationship predicted by the previous hypothesis in the GAD group.

The following directional hypotheses were investigated using functional MRI task-dependent data:

1. Group-based task-dependent BOLD signal amplitudes are expected to be different, with increased levels observed in GAD, compared with healthy controls. Select conditions within the valence-assessment task are expected to generate regional differences observed in the GAD group, from healthy controls.

2. dACC BOLD activity will be significantly different in the GAD group compared with the healthy comparator group during selected contrasts, whereby higher general activation levels will be observed in the GAD group.

3. dACC BOLD activation levels during the task will be associated with the severity of anxiety symptoms (or anxiety levels in the non-clinical group) such that greater activity is associated with increased severity/higher scores.

Finally, the following bi-modal hypothesis was also tested:

1. In the ROI, GABA levels will hold a negative relationship to BOLD signalling, whereby decreased GABA levels will be associated with higher activation levels. As a corollary, increased levels of GABA will be associated with negative BOLD activation, implying reduced neural activity. This relationship is expected to occur in both groups, but be stronger in the GAD group, representing a possible role of GABA in moderating regional task-related processes.

6.4 Methods

6.4.1 Design overview

This study was a cross-sectional and non-intervention investigation of participants aged 18–65 years, with diagnosed GAD ($n = 41$) and a non-medicated, non-psychiatric healthy control group ($n = 35$).

The primary outcome measures were MRS-derived GABA metabolite levels in the dorsal subregion of the ACC as the ROI and the task-dependent BOLD signal as activation level in the ROI (valent IAPS-based task paradigm).

Psychiatric measures were administered across groups to ensure psychiatric status (details under Sub-section 6.3.5—Psychiatric Assessment below). Refer also to the assessment timeline in Figure 17 for a summary of measures.

For details regarding the larger overarching healthy control and GAD group investigations in their entirety (both conceived and managed in part by the author), please refer to the respective protocol document in Appendix F: *The KGAD Study: Kava for the treatment of Generalised Anxiety Disorder*.

6.4.1.1 Participants

Males and females aged between 18 and 65 years, who were free of any unstable and serious medical conditions, and who did not screen for any current psychiatric disorders on the standardised neuropsychiatric interview held at the first session were included in the study as healthy controls. The GAD group was composed of those who met the criteria in Table 12 for current GAD as per the DSM-5 (APA, 2000). Healthy participants were matched in gender and age to the GAD group, owing to the ease of recruiting healthy participants compared to with diagnosed GAD. The inclusion and exclusion criteria for each group are detailed below.

6.4.1.2 Participant recruitment

Participants were recruited through advertisement sources including a dedicated study website, advertisements on social media, posters and brochures in medical clinics and ACL pathology collection centres, websites and venues of local anxiety disorder organisations such as Anxiety Disorders Association of Victoria and research centre websites hosted by the University of Melbourne and Swinburne University of Technology. Potential participants were screened by telephone with reference to the inclusion, exclusion and MRI protocol safety criteria.

Eligible participants received a copy of the Participant Information and Informed Consent Form and written details regarding the first session including instructions for the functional MRI picture task to review prior to the session.

6.4.1.3 Sample size

With an *a priori* population sample size of $N = 80$ ($n = 40$ per group), a 95% confidence interval and 5% margin of error (for a standard alpha of 0.05), and a small to medium effect size (confidence interval reporting was the chosen parameter), a minimum total sample size of $n = 67$ was determined by using G.Power calculation program (Faul, Erdfelder, Lang, & Buchner, 2007) in order to interpret results in this study.

Table 12. Enrolment Criteria for Groups

Healthy Control Group
Inclusion Criteria
• Aged 18 to 65 years old • Fluent in written and spoken English • Has the capacity to consent to the study and follow its procedures • A body mass index (BMI) no greater than 30 as determined by height and weight measurements at baseline or by self-report during telephone screening
Exclusion Criteria
• Known or suspected clinically unstable systemic medical disorder • Current diagnosis of any psychiatric disorder(s) on the Mini International Neuropsychiatric Interview- 6.0 (MINI 6.0). • A lifetime diagnosis of any psychotic disorder and/or personality disorder on the MINI 6.0 or self-reported as diagnosed by a doctor • A history of a major depressive episode or disorder within the last three years as determined by the MINI 6.0 or self-reported as diagnosed by a doctor • A family history (first-degree relative – parent, sibling or child) with a diagnosed psychotic disorder or bipolar affective disorder • Current or recent use (within the past three months) of psychotropic medications for mental health and/or sleep • Recent use of antibiotics within the past six months • Current smoker or recent history of smoking (within past five years) • Current use of vitamins, nutraceuticals, supplements, herbal medicines – for which a two-week washout occurred except for fish oil or other omega-3 supplements such as flax seed oil, requiring a 4-week washout) • Any unstable, chronic or serious medical conditions (e.g. cancer, stroke, immune disorders, cardiovascular disease, acquired brain injuries) • Any medical condition requiring prescription medication (e.g. high blood pressure, cholesterol, arthritis, hypothyroidism) • Pregnant or breastfeeding
Generalised Anxiety Disorder Group
Inclusion Criteria
As above for demographic and medical, plus; • Meets the DSM-5 diagnostic criteria for generalised anxiety disorder based on structured interview MINI 6.0. Note that while the MINI 6.0 uses the DSM-IV criteria, the same criteria are used in the DSM-5

• Presentation with anxiety (SIGH-A ≥ 18) at the time of study entry • Presentation of mild depressive symptoms (SIGMA ≥ 12) at the time of study entry
Exclusion Criteria
• Primary diagnosis other than GAD • Presentation of moderate to severe depressive symptoms (SIGMA ≥ 12) at the time of study entry • Presentation of suicidal ideation (≥ 3 on SIGMA suicidal thoughts domain) at the time of study entry • Current diagnosis of a psychotic disorder (bipolar disorder I, schizophrenia) on structured interview (MINI 6.0) • Current or past diagnosis of obsessive-compulsive disorder or bipolar I or II disorders • Current substance/alcohol use disorder on structured interview (MINI 6.0) • Currently taking an antidepressant, mood stabiliser, antipsychotic, anticonvulsant, warfarin or thyroxine, or regularly using a benzodiazepine or opioid-based analgesic (more than 2 days per week) • Current use of St John's wort • Three or more failed trials of pharmacotherapy for the current GAD episode • Recently commenced psychotherapy (within four weeks of study entry) • Known or suspected clinically unstable systemic medical disorder • Pregnant or breastfeeding or trying to conceive • Not using a medically approved form of contraception (including abstinence) if female and of childbearing age • Unable to participate in all scheduled visits, treatment plan, tests and other trial procedures according to the protocol
Imaging eligibility criteria - exclusions (both groups)*
• Left-handed • Pacemaker • Infusion pumps • Aneurysm clips • Metal prostheses • Metal joints • Metal rods • Metal plates • Metal staples • Non-removable body piercings • Persons who have worked as welders or may have metal splinters in their body • For females: taking hormone-modulating contraception including oral contraceptive pill or implants • Females: regular menstrual cycle to conduct scans within days 1–10
*Further criteria involving scan eligibility via the 'Swinburne Neuroimaging MRI-15 Safety Form' in Appendix D

6.4.1.4 *Female participants*

The study enrolled female participants on day one of their menstrual cycle with the aim of conducting the first scan on approximately day five, and no later than day 10, to ensure minimal modulation of brain metabolites through hormonal fluctuations because some metabolites of progesterone are potent, positive allosteric modulators of GABA-A receptors (please refer to Chapter Four for the reasoning behind phase cycle controlling). The second scanning session occurred exactly eight weeks following the

first scan (thus, day five of the menstrual cycle). Female participants were provided with a menstrual cycle tracker on day one to help with compliance.

6.4.2 Measures

Individual psychiatric, demographic and imaging measures are detailed in separate subsection but are summarised in Table 13.

Table 3. Assessment Timeline Summary

Measure	Week 0	Week 8*
Participant information and informed consent	x	
Demographics	x	
Health and medications questionnaire	x	x
Psychiatric Assessment		
Mini International Neuropsychiatric Interview (MINI 6.0)	x	
Structured interview for the Montgomery-Asberg Depression Rating Scale (MADRS)	x	x
Structured interview for the Hamilton Anxiety Scale (HAM-A)	x	x
Spielberger State-Trait Anxiety Inventory – Trait component	x	
Spielberger State-Trait Anxiety Inventory – State component	x ¹	x ¹
MRI-15 Pre-scan questionnaire	x ¹	x ¹
Edinburgh Handedness Inventory (EHI)	x	
Resting state/structural	x	x
MRS of ACC (GABA/NAA/Glutathione/Creatine/Glutamate-Glutamine)	x	x
Functional MRI during VICT task	x	x
¹ Before and after the MRI scan		
* Applied in the second investigation only; please refer to next chapter		

6.4.3 Demographics

The collection of demographic information including age, gender, education and employment occurred following the signing of the consent form. Health and medical information including height, weight, BMI, substance use, caffeine amounts, alcohol use, familial and personal (comorbid) psychiatric conditions, and duration and indication for current medication/supplement use and use in the previous 12 months were also collected.

6.4.4 GAD comorbidities

Comorbidity has not been typically been accounted for when studying neuroimaging in GAD samples. The prevalence of comorbidity in GAD is higher compared with other affective disorders, with comorbidity amplifying impairment (Noyes, 2001; Nutt et al., 2002). The presence of comorbid conditions was recorded in GAD participants.

Allowable secondary conditions were SAD, panic disorder with and without agoraphobia, and phobic disorder. Major depression was allowable only where no episodes had occurred in the 12 months prior to enrolment. It also considers an overlap in diagnoses in GAD where symptoms might be best explained by a comorbid condition, notably MDD.

Establishing pure GAD, GAD/comorbid and healthy control groups in the present study accounted for the presence of other anxiety disorder symptoms that may generate the observed neurobiological trends. Additional comorbid conditions may imply an increased severity of symptoms, where higher anxiety measures scores may be observed. Due to this, comorbid presence was defined either as a separate GAD group or used as a predictor in linear models.

6.4.5 Psychiatric assessment

6.4.5.1 MINI International Neuropsychiatric Interview

The MINI 6.0 (Sheehan et al., 1998) is a 15-minute brief, structured diagnostic interview for DSM-IV and the International Statistical Classification of Diseases and Related Health Problems, 10th Revision (World Health Organisation, 1992 1192) psychiatric disorders of 25 modules mapping onto DSM-IV diagnoses (APA, 2000). If initial screening questions are answered yes, then systematic exploration of symptoms is undertaken. Scoring involves checking whether a specific pattern of 'yes' responses satisfied the diagnostic criteria (lifetime versus current) for each disorder. The MINI 6.0 was validated against other psychiatric structured interviews such as the SCID (Structured Clinical Interview for DSM-IV; First & Gibbon, 2004) and complemented the diagnoses from the DSM-IV.

6.4.5.2 Montgomery-Asberg Depression Rating Scale

The MADRS (Montgomery & Asberg, 1979) is a clinician-administered rating scale for assessing levels of depression over the past seven days. There are 10 items of cognitive and physiological symptoms; each item is rated on a 7-point Likert scale, where 0 indicates absence of symptoms and 6 indicates extreme presence of symptoms. A score between 0 and 6 is normal/symptom free, 7 to 19 is mild depression, 20 to 34 is moderate depression and over 34 is severe depression. The formalised question format version Structured Interview guide for the Montgomery-Asberg Scale (SIGMA; Williams & Kobak, 2008) was utilised in this investigation to standardise the question format and optimise inter-rater reliability where applicable. Inter-rater reliability for the SIGMA is

reported to be excellent ($r = 0.93$). For the healthy control group, the maximum score was four points throughout enrolment, whereas for GAD the maximal score allowable was 18 following study enrolments.

6.4.5.3 *Hamilton Anxiety Scale*

The HAM-A (Hamilton, 1959) is also a clinician-administered rating scale developed to quantify the severity of anxiety symptomatology in the seven days prior. It contains 14 items of cognitive and physiological symptoms, whereby each item is rated on a 5-point Likert scale, where 0 indicates absence of symptoms and 4 indicates extreme presence of symptoms. A score between 0 and 4 indicates asymptomatic, 5 to 17 mild anxiety, 18 to 24 moderate severity and a score 25 and above severe anxiety. The SIGH-A was used in this study to ensure a standardised questioning format and to facilitate inter-rater reliability. Reliability and validity of the SIGH-A is assessed to be moderate-to-high (Shear et al., 2001). Score requirement in the present study for the healthy control group was four points throughout enrolment, whereas for GAD the group minimum score following study enrolment was 18.

6.4.5.4 *Spielberger State-Trait Anxiety Inventory*

The STAIS and STAIT (Spielberger et al., 1983) are commonly-used, self-reporting questionnaires administered as a measure of transient state-level and more enduring trait-level anxiety symptoms. A total of 20 items assess state anxiety (e.g. 'I am tense'; 'I am worried') and 20 items assess trait anxiety (e.g. 'I am content'; 'I am a steady person'), rated on a 4-point Likert scale. A total of 10 items in the STAIS and nine items in the STAIT were reverse scored, producing a total score assessed against normative ranges specified below. Internal consistency coefficients for the scale ranged from 0.86 to 0.95; test-retest reliability coefficients range from 0.65 to 0.75 over a two-month interval (Spielberger et al., 1983). For the present study, trait anxiety levels were measured via the STAIT at the first visit, but more importantly STAIS state-level anxiety that was administered both pre-scan and post-scan in both sample groups. The normative means and standard deviations are provided in the author manual for gender, age and clinical groups. For the present study, the ranges shown in Table 14 were applied in the sample groups.

Table 14. Normative means and standard deviations (bracketed) for the Spielberger STAIS/T questionnaire

State anxiety/STAIS

Age range (years)	19–39	40–49	50–69
Males – non-anxious	36.54 (10.22)	35.88 (10.52)	34.51 (10.34)
Females – non-anxious	36.17 (10.96)	36.03 (11.07)	32.20 (8.67)
Trait anxiety/STAIT			
Males – non-anxious	35.55 (9.76)	35.06 (8.88)	33.86 (8.86)
Females – non-anxious	36.15 (9.53)	35.03 (9.31)	31.79 (7.78)

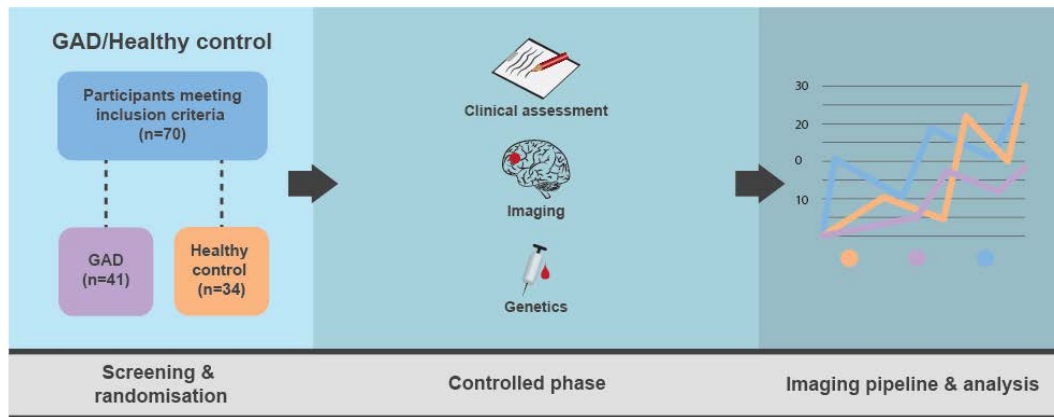


Figure 7. Trial timeline illustration for the cross-sectional study indicating resulting sample sizes. Note this investigation utilised baseline (week 0) data only and no genetics.

6.4.6 MRI Scanning experiments

Assessment of neuroimaging safety and risks, risk management, confidentiality and data access information can be located in Appendix A document. All MRI scanning was performed at the Swinburne University of Technology's neuroimaging facility (Hawthorn, Australia) using a Siemens 3 Tesla Trio MRI scanner (Siemens AG, Munich, Germany) with a 32 channel headcoil.

Table 15. Imaging Order of Measures and Sequencing Details

Sequence	Measure	Detail	Time (mins)
1a	Localiser		2
1b	T1 - MRI	A high-resolution (1 x 1 x 1 mm) T1 weighted scan	10
1c	MRS - PRESS	Single Voxel MRS PRESS sequence TE = 30 ms, TR = 2000, Ave = 128, weak water suppression	5
1d	MRS - MEGA-PRESS	TE = 68 ms, TR = 2000, suppression freq. = 1.95ppm, Ave = 64 TWIX Data	5
1e	MRS – Water	16 averages	2
2a	fMRI - resting-state	EPI sequence, TE/TR=30/2500, 3x3x3mm, 33 slices	6
3a	fMRI VICT 1 35 pictures	EPI sequence, TR=2000, 3x3x3mm	10

4	DTI		6
3b	fMRI VICT 2 35 pictures	EPI sequence, TR=2000, 3x3x3mm	10
Total time			56 minutes
DTI: Diffusion Tensor Imaging; EPI: Echo Planar Imaging; ; MEGA-PRESS: Mescher-Garwood Point Resolved Sequence; PRESS: Point resolved sequence; T1: weighted image proton only; TE: echo time; TR: repetition time; VICT: Valent IAPS Congruence Task			

6.4.6.1 *Magnetic resonance spectroscopy (MRS) of GABA*

MRS assesses non-invasive measurement of metabolite levels in the ROI. The cerebral metabolite of interest was GABA. Alterations in cerebral metabolites may reflect changes in various brain micro-structure and neurochemical abnormalities; therefore, MRS is routinely used to probe brain neurochemistry in vivo (Berger et al., 2008; Etkin & Wager, 2007; Puts & Eddin, 2012b; Rae, 2014; Rosen & Lenkinski, 2007). Only GABA metabolite levels have been investigated here for thesis brevity. Details of parameters, toolboxes and preparation pipelines for use of MRS in this study are detailed below.

6.4.6.2 *Functional task: Valent IAPS congruent task (VICT)*

The model of anticipatory anxiety utilised in the present study followed a similar design to Nitschke et al. (2009), which employed an affective IAPS picture paradigm designed to examine ACC function in a GAD sample. To this end, we used aversive (or negative) stimuli to elicit anticipatory anxiety and persistent worry that are features of GAD to probe the function of dorsal ACC in GAD participants compared to non-anxious control participants.

6.4.6.2.1 *Stimuli*

The stimulus presentation sequence consisted of cue-probe pairs. Cues were 'o' or '-' and probes were stimuli from the IAPS library that were selected with appropriate parameters of valence, arousal/excitement and dominance/control based on IAPS normative ratings (see Table 16; for IAPS picture codes please refer to Appendix B). Neutral pictures were selected to be least arousing/exciting, with scores closest to 5 in terms of pleasantness (i.e. neither pleasant nor unpleasant) and with scores ranging from 2.0 to 4.0 for low dominance/control.

Negative pictures were selected that were the most arousing/exciting, most unpleasant and with high levels of dominance/control to maximise the difference between negative and neutral subject matter. Picture stimuli were edited for brightness,

contrast, mean spatial frequency and 90% quality JPEG files, balanced by category and were quality cross-checked by a fellow staff member unrelated to the investigation.

6.4.6.2.2 *Experimental task design*

The task was programmed in-house using Psychopy (Peirce, 2007), and included two blocks of 35 trials each. Therein, of 50% trials included aversive stimuli and 50% included neutral stimuli. Each trial began with 1000 ms white ‘o’ or ‘-’ cue on a grey background, indicating that a neutral or aversive probe stimulus, respectively, would follow; neutral and probe stimuli were equiprobable.

Cues were immediately followed by pseudo-random and equiprobable 1500, 3000 or 6000 ms blank grey background and then the probe stimulus for 5000 ms followed by a 12 s rest period where a blank screen was presented. The jittered time frame between the cue and probe-stimuli was intended to maximise statistical efficiency (Sakai & Passingham, 2003). After probe-stimulus presentation, the 12 s rest period allowed the hemodynamic response to return to baseline (see Figure 18).

On 50% of trials the cue was informative (congruent trials) whereas the remainder of trials they were not (incongruent trials). This yielded a 2 (Neutral vs Negative) x 2 (Congruent vs Incongruent) design with 4 trial types (Neutral- Negative, neu-neg; Neutral-Neutral, neu-neu; Negative -Neutral, neg-neu; Negative- Negative, neg-neg). Trial order was pseudo-randomised of 25% trial per condition, with the instruction that neither aversive nor neutral trials were presented more than twice in a row.

Participants held a button box in each hand with instructions to press the left hand button for a congruent trial (a ‘match’) and the right handed button for a incongruent trial (a ‘non-match’). Although data related to button responses were collected in the form of response time (milliseconds) and accuracy (percentage correct responses) and will be utilised for future analyses, this data did not form part of the thesis research investigations.

Table 6. IAPS Picture Parameters – Normative Ratings

IAPS group	Valence range (1 = most unpleasant, 9 = most pleasant)	Valence Mean	Arousal range (1 = least arousing, 9 = most arousing)	Arousal mean	Dominance/Control range (1 = least, 9 = most)	Dominance/Control mean
Negative Time 1	Valence < 4.0	2.34	Arousal > 6.0	6.57	Dominance > 6.0	7.00
Neutral Time 1	Valence 4.0 – 6.0	5.01	Arousal < 3.6	2.84	Dominance < 4.0	3.23

Source: Bradley and Lang (1997)

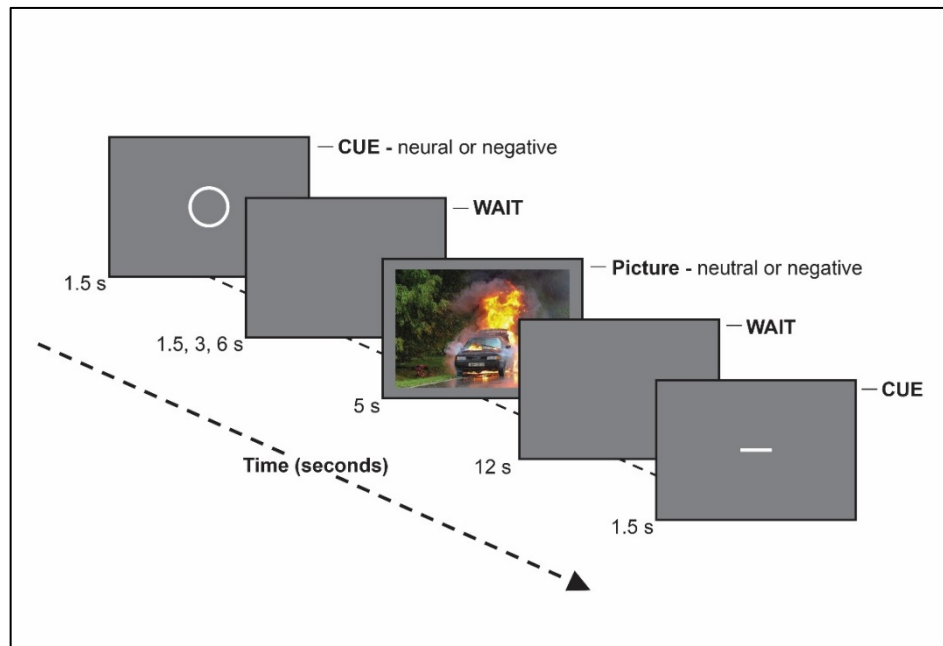


Figure 8. VICT presentation sequence

6.5 Procedure

6.5.1 Screening

Prior to scheduling sessions, participants were pre-assessed via a standardised phone-screen of the above criteria. Any medical anomaly was referred to the trial GP staff for decision regarding inclusion. Potential participants considered eligible over the phone were sent a Participant Information and Consent Form via email or post and asked to attend a baseline session at Swinburne University. Refer to Table 13 for summaries of group inclusion criteria.

6.5.2 Baseline session

Participants arrived fasted and without having consumed caffeine or alcohol in the 12-hour period prior to the morning of the session. Prior to any data collection, informed consent was obtained from each participant. Refer to Table 14 and Figure 17 for timelines of the assessments. Blood was collected onsite by a research nurse for biochemical measures not included in the present investigation. Following blood collection, a standardised breakfast was served to all participants (toast with topping and plentiful water). Demographic, medical and psychiatric data were collected for one

hour before the scheduled scan. Instructions for the functional component of the scanning session were provided to the participant during this time.

6.5.3 Imaging procedure

Prior to scanning, all participants completed and signed an MRI safety questionnaire that screened for MRI contraindication and was administered by a trained radiographer, confirming that all MRI information forms had been read and understood.

Prior to and following each scan, participants completed the self-reporting STAIS-STAIT paper questionnaire state component. Preparation for the scan involved the removal of relevant clothing and metallic items and participants were fitted with earphones for noise reduction and for communication with staff.

6.5.4 MRI methods

6.5.4.1 *Structural MRI*

For each participant a high-resolution 176 (sagittal) slice T1-weighted scan was acquired using a magnetization prepared gradient echo (MPRAGE) sequence (1 mm³ voxels; scan repetition time (TR) = 1900 ms; echo-time (TE) = 2.52 ms; flip-angle = 9°; field-of-view (FoV) = 256 x 256 mm).

6.5.4.2 *Regions of significant activation and the region of interest (ROI)*

The ROI and any regions identified as significantly different between groups in terms of activation levels were identified through the following:

1. Visual inspection and confirmation via Talairach Atlas (Talairach & Tournoux, 1988) from Talairach Client (Version 2.4.3; <http://talairach.org/client.html>); and,
2. Use of MNI coordinates within two anatomical programs: MRICron (Rorden & Brett, 2000; <http://www.nitrc.org/projects/mricron>) and BrainNavigator (http://www.thehumanbrain.info/brain/brain_navigator.php); and,
3. Regions cross-checked by a second researcher to ensure rigour in labelling.

The ROI dACC was located via the application of the MNI co-ordinates: (0, 34, 26 mm). The ROI was defined by the Automated Anatomical Labeling (AAL) atlas included in the Wake Forest University PickAtlas software (WFU Pick Atlas; Maldjian et al., 2003; 2004; Tzourio-Mazoyer et al., 2002) and transformed into Talairach space (Lancaster et al.,

2007) using ICBM2TAL in MATLAB (v8.2, MathWorks, Inc., Natick, Massachusetts, U.S.A.). Refer to Figure 19 for a stereotaxic visual of the ROI.

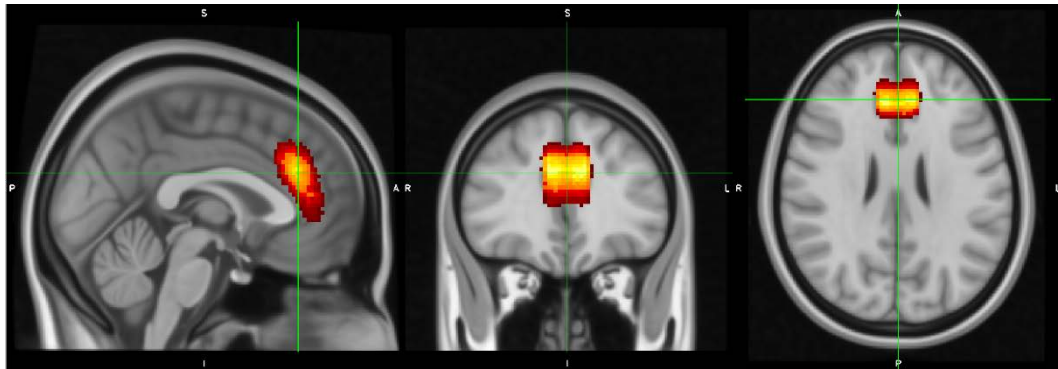


Figure 9. Sagittal T1-weighted HFS-LPH slice showing the placement of the voxel position for the dorsal subregion of the anterior cingulate cortex from combined study participants (dACC; voxel = $1.2 \times 1.2 \times 5 \text{ mm}^3$)

6.5.4.3 MRS acquisition

6.5.4.3.1 Spectra acquisition

Single Voxel MRS was applied to measure the metabolites in the ROI. This is a molecular-based tool that is used to measure the metabolite concentration level within the particular ROI, hence appropriate MEGA-PRESS (see below) sequencing was utilised that involved the use of J-coupling between GABA-3 (peak at 3.01 ppm) and GABA-4 (peak at 1.89 ppm).

Signal separation occurred by implementing frequency-selective inversion pulses via the Mescher-Garwood algorithm (MEGA; Mescher et al., 1998) in a PRESS sequence (Bottomley, 1987). The PRESS sequence (TE = 80, TR = 2000, Ave = 128, weak water suppression) was first conducted to measure the main metabolite, followed by the MEGA-PRESS sequence; TE = 68, TR = 2000, suppression freq. = 1.95ppm, Ave = 64 (see parameters in Uppman, 2010; Wood et al., 2010). Finally, a water-unsuppressed sequence (16 averages) was conducted for quantification. Both the PRESS and MEGA-PRESS sequences were conducted within the dACC to measure metabolites with this protocol (Heberlein & Brown, 2009; Rae, 2014).

The Totally Automatic Robust Quantitation (Tarquin) was employed as the main toolbox to derive the GABA spectra, with LCModel (Provencher, 2001) employed in a similar manner for cross-validation (details below). Tarquin and LCModel have good accord in the literature (Wilson, Davies, Grundy & Peet, 2009). Siemens scanner data

was provided as the time-domain and time-averaged data, with pre-processing performed using the steps below.

6.5.4.3.2 Toolbox-specific processing pipelines

6.5.4.3.2.1 Tarquin toolbox

Using a basis fit, Tarquin (Wilson et al., 2011) utilises a time-domain fitting with a Levenberg-Marquardt algorithm, taken from quantification during short echo time via additional software (AQSES; Poulett et al., 2007). Tarquin eliminates signal-baseline interference by the removal of initial points of free induction decay and a non-negative least-squares projection. The basis set is derived from amplitude estimates of the aforementioned combinations and the baseline fit involves a convolution filter and smoothed residual signal.

Tarquin simulated a PRESS basis set based on data file parameters, removed the residual water signal via an HSVD algorithm (Barkhuijsen, de Beer, & van Ormondt, 1987) and performed a preliminary phase estimation before calibrating the PPM axis based on spectral features before fitting the data. Then, an advance fit on GABA using the Siemens IMA file format on the water suppressed and unsuppressed files and for the edited spectra pre- and post- alignment frequencies and creatine frequency were performed. Goodness of fit checks on signal amplitudes were subsequently performed via output in CSV format and visual inspection of the plots.

6.5.4.3.2.2 LCModel

LCModel (Provencher, 1993; 2001) is a widely used but a costly metabolite quantification program for ^1H -MRS analyses because it includes a metabolite basis set into the fitting model. It models data in the frequency domain using a linear combination of metabolites, lipids and macromolecules as part of the basis signal. The algorithm is a Marquard modification of a constrained Gauss-Newton least-squares method; the baseline fit is set from cubic b-splines.

LCModel toolbox (version 6.1) was used to cross-validate the Tarquin toolbox data and fit and quantify spectra indicating the concentration of metabolites including ratios, i.e. total NAA, creatine, total choline, MI, glutamate, glutamine and GABA, after quality control parameters were applied to the signal-to-noise ratio (SNR), standard deviation (SD) and full-width at half-maximum height (FWHM) (further details in upcoming MRS analysis section 4.4.5.2.1). Data parameters were the MEGA-PRESS pulse sequence. The

internal basis set was ^1H MEGA-PRESS GABA. While the quality of output between editing programs such as Tarquin and LCModel are equitable, differences have been discussed (refer to section 4.5.7 on GABA quantification in Chapter Four).

The output was also visually inspected in the NIfTI file format using the MRICron program (Rorden & Brett, 2000) utilising the high-contrast T2-weighted reference images with corresponding stereoscopic imaging resolution as a quality check.

6.5.4.3.3 Tissue content correction via segmentation

Tissue correction is a crucial component of the imaging pipeline in assessing GABA levels in grey and white matter, as metabolite concentration volume variances have been reported in the literature (Jensen, Frederick, & Renshaw, 2005; Petroff et al., 1989).

Techniques for the correction and analysis of absolute GABA following segmentation is somewhat contentious. MRS technical publications and review papers are equivocal as to how and where corrections are applied to account for variances in metabolites in grey matter, white matter and CSF (e.g. Mullins et al., 2014; Rae, 2014; Wilson et al., 2011) or is to be avoided (Edden & Barker, 2007; Edden et al., 2014). An alpha-correction was applied in one recent study (Porges et al., 2017) in an aging sample that found age-related reductions to GABA levels prior to correction, but not following. Methods of correction can result in inflation of the GABA signal (Mikkelsen et al., 2016) and there is lack of standardised procedures for correction (Harris, Puts, & Edden, 2015).

As most investigations have quantified and reported metabolites with segmentation/volume correction, this was also incorporated across the toolbox data in the present study. The partial volume effect was corrected using high-resolution T1 and an in-house voxel co-registration script (Kirkovski, Suo, Enticott, Yucel and Fitzgerald, 2018). Tissue segmentation scripting used MATLAB via the voxel location mask on the anatomical T1 image and applied to segmented images to derive percentages for grey matter, white matter and CSF for which volume correction data were then used to correct the LCModel and Tarquin output for tissue and water content and relaxation effects.

6.5.5 Statistical Analyses

6.5.5.1.1 Demographic and medical data

Data were analysed using IBM Statistical Package for Social Sciences software (SPSS; v.22, Armonk, U.S.A). All relevant demographic, medical and psychiatric data were

compared across both groups via simple significance testing (t-tests and non-parametric tests were used when parametric criteria were not met) following quality check for outliers, missing data and incorrect data. Significance is reported where $p \leq .05$; and with functional data, $p \leq .005$ or $.001$. Marginal significance will be reported where $p > .05 - p \leq .10$ although defined as not significant. This less orthodox approach was because of reduced sample sizes; that the outcome relationships were somewhat novel to test; and with some components largely exploratory. It is emphasized that these results will not be interpreted as significant. There are many discussions in the literature regarding validity of results reported over the arbitrary .05 threshold, particularly in the context of p -value reliance; for example, examined in an observational study by Pritchett, Powell and Horne (2016; see also Rosenthal and Rosnow, 1989).

6.5.5.1.2 Image analysis

6.5.5.1.2.1 Modelling MRS tissue volumes

Imaging data analysis was conducted using Statistical Parametric Mapping (SPM; v.8, Wellcome Dept. of Neurology, London, U.K.), MATLAB versions 2011a and 2016b software (MathWorks, Inc., Natick, Massachusetts, U.S.A.), LCModel package, and Tarquin (v.4.3.10).

Absolute metabolite concentrations and metabolite ratios and signal-to-noise quality control components were extrapolated from Tarquin and LCModel toolboxes to fit and quantify the concentrations of GABA. Spectra were analysed with each toolbox using a basis set of several metabolites, although only GABA data were analysed and reported here.

The general procedure across GABA toolboxes was as follows: concentrations were determined using tissue water signal as the internal standard and corrected for grey matter fractions in the voxel as required (see procedures in Gasparovic et al., 2006; Harris, Puts, & Edden, 2015; Kries, Ernst, & Ross, 1993). Raw data results were presented in units approximating millimolar concentration. Quality control measures for each toolbox were applied such as SD residue of total GABA from Tarquin and LCModel toolboxes to assess the calculated difference between the original/acquired spectrum and the sum of the estimated components (see below for toolbox-specific parameters).

Relative to the quality parameters, raw scores were cross-checked following conversion to standardised z-scores, where data were excluded if outside ± 3.29 SDs.

6.5.5.1.2.2 Tarquin

All other metabolic signals were quantified from the edit-OFF subspectra in Tarquin using a linear combination of simulated basis sets based on quantum calculations to fit spectra in the time domain (Wilson et al., 2011). Macromolecules were modelled as components of the Tarquin fit. Further, the signal around 3 ppm has a significant contribution from macromolecules as well as GABA, as such GABA and macromolecule concentrations are reported here as GABA+. Concentrations were determined using the tissue water signal as the internal standard and were corrected for grey matter fraction in the voxel. Results are presented in units approximating millimolar concentration. Three components were utilised to assess signal rigor: SBR, SD and 'Fit Q'. SNR averages as a ratio of maximum spectrum-less-baseline over analysis to twice the root-mean-square residuals were assessed as a quality check and scores were discarded if ≤ 2 . SD as a variance estimate value was assessed as a calculated percentage and were discarded if greater than 60%. Percentages were calculated manually using the SD raw score/TGABA raw scores $\times 100$ to achieve percentage values. Fit Q was derived directly from Tarquin output and discarded if < 1 . Finally, Tarquin's TGABA was corrected for tissue proportion in grey matter using the following calculation:

$$1. \text{ TARQUIN GABA REAL: } \text{TGABA} / \text{GM} \%$$

6.5.5.1.2.3 LCModel

Three quality measures were utilised in assessment of the LCModel data: FWHM, SD and SNR. The FWHM ratio means as estimates of linewidth in *in vivo* spectrum were applied for LCModel data where scores were selected if less than 0.15. SD as variance estimate values were selected if greater than 20%. SNR averages as a ratio of maximum spectrum-less-baseline over analysis to twice the root-mean-square residuals were assessed separately, and scores were kept if ≥ 2 .

Once outliers were discarded based on the above parameters and following z-score conversion (where outliers > 3.29 were discarded), the following segmentation calculation was applied, where GABA concentration was calculated proportional to grey matter percentage calculated for LCModel output data:

$$2. \text{ GABA/LCModel/GM: } \text{Real GABA} = \text{G} / \text{GM}\%$$

Volumetric data, GABA toolbox quality variables and GABA metabolite results were reported separately by means and SDs for each group variable of interest (gender, comorbid presence and psychiatric group).

The aforementioned toolbox-specific quality data were subjected to a univariate analysis of variance (ANOVA) to establish differences, if any, between healthy controls and the two GAD groups. Significant results were assessed for inclusion into the general linear model for toolbox-specific group-level analyses. Volumetric data (grey matter, white matter and CSF) were subjected to a factorial ANOVA linear model, with significance at $p < 0.05$, with age, gender and comorbidity as potential predictors of tissue variability.

6.5.5.1.2.4 Predictors of volumetric variance

Brain neuron size, synaptic integrity, neurotransmitter levels and tissue proportions are all amenable to decline with the progression of years (Haug & Eggers, 1991; Kolb et al., 1998). Recent reviews and meta-analyses examining age-related functional data suggests progressive and pervasive brain differences in emotional and cognitive processing from young adults through to elderly years, such as a decrease in grey matter volumes (e.g. Kaup, Mirzakhani, Jeste & Eyler, 2011; Spreng et al., 2010). However, these differences are not necessarily reflective of an overall global decline (Good et al., 2001; Harada et al., 2013). Second, there is also limited literature evidence for a decline in GABA levels as a function of age (Gao et al., 2013); one study found no difference (Aufhaus et al., 2013), yet differences were reported in an animal model (Nunez & McCarthy, 2007). It is feasible that age and gender-related differences would be observable in the samples tested in the present study, potentially affecting results, if not accounted for. For this reason, age and gender were employed in all functional and metabolic group-based analyses to best delineate group-based differences in function and GABA profiles.

6.5.5.1.2.5 GABA group differences

First, correlational analyses were conducted on the GABA level data for both toolboxes, within each group, via Pearson's correlational analysis (significant at $p = < 0.05$; two-tailed) to test for uniformity in trend. Fisher's r -to- z transformations were conducted to assess if differences between group-specific correlations were significant (Fisher, 1921).

6.5.5.1.2.6 GABA factors

Following trend analysis, GABA quantity data were assessed using a linear model for group differences via univariate ANOVA, with significance at $p < 0.05$. F-test and

significance were reported in text where appropriate, with tabulated parameter estimates to provide details of each model.

6.5.5.1.2.7 GABA relationship to psychiatric measures

GABA concentrations were tested for association to anxiety symptom measures SIGH-A HAM-A, Spielberger trait anxiety and state anxiety pre-scan. All measures were conducted in the GAD group, whereas Spielberger STAI was conducted in the healthy controls only. Assessments were performed via bivariate correlation reporting Pearson's correlation matrices within each group (main groups and GAD group differences in comorbid status) with results deemed significant at $p = < 0.05$ (two-tailed), and corrected for multiple comparisons via Fisher's r-to-z analysis (Fisher, 1959).

Finally, in the GAD group, GABA data and anxiety levels via the HAM-A were entered into a GLM analysis of covariance (ANCOVA) and the model tested for predictor factors of anxiety levels including GABA level variance and comorbidity, age and gender, and for associations to psychiatric symptoms. To validate GABA/anxiety association in a non-psychiatric sample, two factorial ANOVAs were run separately for Spielberger trait and state anxiety levels in the healthy control group, controlling also for age and gender.

6.5.6 Functional MRI

6.5.6.1 *fMRI data acquisition*

Task fMRI data were acquired with a T2*-weighted echo-planar sequence (voxel size = 3 x 3 x 3 mm, gap = 0.5 mm; TR = 2000 ms; TE = 30 ms; flip-angle = 90°; FoV = 224 x 224 mm).

6.5.6.2 *fMRI Image pre-processing*

Task fMRI data pre-processing was conducted using Statistical Parametric Mapping (v.12; Wellcome Department of Neurology, London, U.K.) running on MATLAB (version R2016a; MathWorks, Inc., Natick, Massachusetts, U.S.A.). Initially, T1 and fMRI data were manually re-oriented to the AC-PC line within SPM12. The functional time-series data were then slice-time corrected (central slice reference) using sinc interpolation, and then corrected for head motion by aligning all images to a mean image of session-specific time-series data. Session specific T1 images were then co-registered to the corresponding mean re-aligned image, after which Time 2 T1 images were co-registered to Time 1 T1 images; the parameters of this transformation were subsequently applied to Time 2

functional data. T1 images were then segmented into grey matter, white matter, and cerebrospinal fluid (CSF); The forward model of the de-formation from native space to the standardised stereotactic space described by the Montreal Neurologic Institute (MNI152) computed during segmentation was applied to T1 and functional data. Finally, fMRI data was spatially smoothed with a 6x6x6 full-width at half-maximum height (FWHM) Gaussian filter.

6.5.6.3 Modelling physiological noise

Anatomical component correction ('ACompCor'; Behzadi, Restom, Liao and Liu, 2007) was used to generate physiological noise regressors as implemented in the CONN functional connectivity toolbox (Whitfield-Gabrieli and Nieto-Castanon, 2012). This method involves performing principal component analysis (PCA) on the BOLD time-series extracted from 'noise ROIs' (CSF and white matter obtained during T1 segmentation). We also generated regressors (using the CONN toolbox) flagging 'outlier images', defined as images exhibiting either > 2 mm frame-wise displacement from the previous scan or mean intensity > 3 SD from the time-series mean. Cases were removed where scan had >47 outlying images per run (total 318 images per run; 15%). Following removal, 30 healthy control and 39 GAD participant scans were included for analysis.

6.5.6.4 Outlier identification

Following pre-processing pipeline steps, outlying volume quantification was determined using artefact detection tools (ART); a functional was considered an outlier if its frame-wise displacement was >2 millimetres and/or its global signal was more than 3 standard deviations from time-series mean. Cases were removed where scan had >47 outlying images per run (total 318 images per run; 15%). Following removal, 30 healthy control and 39 GAD participant scans were included for analysis.

6.5.6.5 Statistical modelling of fMRI data

The conditions neu-neg, neu-neu, neg-neg, and neg-neu were modelled as separate stick functions convolved with the canonical hemodynamic response function (HRF) supplied with SPM12. Covariates of no interest included motion realignment parameters, outlier scans (1 regressor per outlier, with a '1' coding the outlier and '0's coding each other scan), and the first 3 principal components from the time-series extracted from each of white matter and cerebrospinal fluid ROIs obtained during T1 segmentation.

After parameter estimation, the contrast of most interest, neu-neg > neu-neu was computed for each participant and used in second level modelling. This contrast was selected as it captures the difference between a high conflict valence/response (i.e., non-matched neutral cue followed by negative stimulus picture) and a control condition involving no appraisal of negative stimuli (i.e., matched neutral cue and stimulus).

6.5.6.5.1 *Group level modelling of fMRI data*

Individual contrast images were entered into separate group level random effects t-test models, including one-sample t-test models to assess within-group activation, and independent samples t-test models to probe group differences in the baseline data. Finally, individual contrast images were entered into a Group (HC vs GAD) X Trial (neu-neg vs neu-neu) ANOVA model. We tested the main effects of Group and Trial, in addition to probing the existence of any interactions. Statistical Parametric Maps (SPMs) were thresholded at $p < 0.001$ (uncorrected) at the voxel level and clusters surviving $p < 0.001$ corrected for family-wise error (FWE) using Random Field Theory (RFT) were considered significant (Worsley et al., 1996). More liberal threshold of $p = < 0.005$ (uncorrected) at the voxel level and the same cluster level thresholding were also used to further explore the data.

6.5.6.5.2 *dACC region of interest; group differences*

Within group-specific and between group-level comparisons were then performed for the dACC ROI given the *a priori* interest in the role of the dACC in GAD. The ROI was matched on the AAL atlas via the WFU Pick Atlas and transformed into Talairach space in MATLAB (refer previous section for details). To enable this, a 1 cm spherical/5 mm radius ROI was centered on the dACC; mean contrast estimates were extracted using the Marsbar region of interest toolbox (Brett, Anton, Valabregue & Poline, 2002) and analysed using SPSS: participant BOLD level contrast estimates were extracted and entered into a Group X Time ANOVA.

6.5.6.5.3 *dACC correlation between fMRI task BOLD activation and psychiatric measures*

Pearson's product-moment correlations between dACC ROI contrast estimates and anxiety symptom scores controlling for age (two-tailed tests). In addition, GAD dACC ROI contrast estimates were correlated with HAM-A anxiety scores, Spielberger Trait and State components, whereas in healthy controls, analysis occurred via Spielberger measures only.

6.5.6.5.4 Activation levels in the prediction of anxiety levels within groups

In the GAD group, factorial ANOVAs were performed to examine the prediction of anxiety scores (SIGH-A, and Spielberger trait and state anxiety scores), from BOLD ROI levels, with age, gender and comorbidity in the model. Second, in the healthy control group, factorial ANOVAs were performed with Spielberger trait and state anxiety scores, also with age and gender.

6.5.6.5.5 fMRI task BOLD activation and MRS GABA relationship

Finally, to investigate group differences in BOLD signal from GABA levels, data were entered into a factorial ANOVA, with predictors being GABA levels, group, gender and age. The model was run twice, for each group, with comorbidity an additional predictor in the GAD group model.

6.6 Results

6.6.1 Overview

Descriptive data are reported with a summary in Table 18 for demographics and clinical data, before reporting on metabolic and functional imaging results.

6.6.2 Demographics and descriptive data

The total sample size of all participants was $n = 70$ (GAD $n = 37$; HC $n = 33$), which met conditions for analyses. Due to the matched-sample procedure, there were no significant differences in age between GAD and healthy control groups, $t(68) = -.84$, $p = .405$, 95% Confidence Interval (CI) $[-8.8, 3.6]$, mean ages 36 years (GAD, SD 13.09) and 38 years (healthy control, SD 12.77), or for gender, $\chi^2(1) = .605$, $p = .436$. Similarly, both groups were identical in having a mean of 17 years of education, $t(68) = -.16$, $p = 0.871$, 95% CI $[-1.80, 1.60]$, (GAD SD 3.91; healthy control SD 3.17).

Alcohol use was also not different between groups, $t(68) = -.67$, $p = .505$, 95% CI $[-2.50, 1.20]$, where mean weekly alcohol use was 3–3.5 standard drinks. Caffeine consumption was also equitable across groups, $t(68) = .04$, $p = .965$, 95% CI $[-53.00, 56.00]$, at approximately 135 mg daily.

There was also no difference in medication use (e.g., ibuprofen, paracetamol, salbutamol/Ventolin) between groups, $\chi^2(1) = 2.85$, $p = .119$. However, the healthy control group inclusion criteria stipulated no regular medication or supplement use, but *pro re nata* (*prn*) medication use was recorded.

Medication/supplement use with potential GABA pathway modulation differed between the groups and therefore an association between group and GABAergic medication use, $\chi^2 (1) = 8.06, p = .006$ (Fisher's exact test), was observed. Of the participants in the GAD, 10% ($n = 4$) reported use of benzodiazepines ~ 1 weekly (inclusion criteria stipulated < 1 weekly), and *prn* use of opioid-based analgesics (13%; $n = 5$). For GABAergic supplements, including substances used for anxiety symptom alleviation (e.g. insomnia), such as melatonin and valerian, again no more than ~ 1 per week, 17% of GAD group ($n = 7$) were taken. In the GAD group, GABAergic substance use was not significantly different between genders, e.g. opioid-analgesic use ($p = .245$).

Menstrual cycle deviations with scan dates were not reported in either group, although the reliability of the menstrual cycle was greater in healthy control females. GAD females ($n = 10$) reported greater fluctuations in cycle lengths and anticipated dates of menstruation, based on the supplied chart tracker, resulting in a greater need for rescheduling scan dates to be with days 1–10 in this group (no cases in the healthy control group).

For the presence of psychiatric conditions (assessed using the MINI psychiatric interview), as expected, the healthy control group reported no current or recent psychiatric disorders, hence there was a significant group difference, $\chi^2 (2) = 70, p < .001$. In the GAD group, 57% ($n = 21$) reported a current comorbid condition in the form of a secondary anxiety disorder, e.g. agoraphobia (45%; $n = 17$), SAD (29%; $n = 11$), panic disorder (24%; $n = 9$) and/or PTSD (3%; $n = 1$).

The presence of a mood disorder was also significantly different between the groups, $\chi^2 (1) = 15.98, p < .001$. In the GAD group, 53% ($n = 20$) reported past episodic MDD greater than 12 months prior to enrolment. Of these GAD participants with MDD history, 35% ($n = 7$) were classed as pure GAD as they did not have a current comorbid anxiety condition. In the healthy control group, three participants (9%) reported a history (although not in the past 12 months) of major depression episodes—two female subjects reported post-natal depression, one male subject reported a single episode 20+ years prior. Due to these circumstances, these participants were enrolled as healthy control participants, but their psychiatric history was recorded for demographic purposes.

Finally, due to the high prevalence of comorbid conditions in the GAD group and evidence for the relationship between neurobiological processes distinct to affective disorders, subsequent analyses of imaging and psychiatric data were performed either

with the GAD group split into 'pure GAD' and GAD/comorbid participants, in order to examine differences as a product of comorbidity.

Table 17. Group-specific Demographics Summary

Demographics (Means, standard deviations, n, or % where relevant)	GAD participants			Healthy Control participants			Group	Females	Males
	Group	Females	Males	Group	Females	Males	p-value	p-value	p-value
Subjects (n)	37	19	18	33	20	13	.436	-	-
Age	36.16 (13.09)	37.00 (13.40)	34.00 (13.00)	38.76 (12.77)	43.40 (9.69)	31.61 (13.97)	.405	.127	.537
Education years	17.65 (3.91)	18.36 (3.35)	16.90 (4.39)	17.79 (3.17)	18.45 (3.28)	16.7 (2.90)	.871	.939	.932
Psychiatric measures									
Comorbid total	21	9	12	0	0	0	< .001	< .001	< .001
Comorbid: SAD	11	4	7	0	0	0	< .001	< .001	< .001
Comorbid: PD	9	4	5	0	0	0	< .001	< .001	< .001
Comorbid: AGO	17	6	11	0	0	0	< .001	< .001	< .001
Comorbid: MDD history	20	10	10	3	2	1	< .001	< .001	< .001
SIGH-A ^a	23.05 (3.60)	22.47 (3.27)	23.67 (3.91)	1.45 (1.44)	1.60 (1.39)	1.33 (1.56)	< .001	< .001	< .001
SIGMA ^b	13.59 (2.99)	12.00 (3.32)	14.28 (2.52)	1.18 (2.20)	1.35 (2.43)	1.00 (1.91)	< .001	< .001	< .001
STAIT ^c	51.09 (6.90)	50.83 (8.23)	51.42 (4.97)	28.91 (5.32)	28.85 (5.92)	29.00 (4.44)	< .001	< .001	< .001
STAIS ^d score pre-scan	45.83 (8.60)	48.79 (9.82)	42.53 (5.59)	25.50 (4.55)	24.64 (4.60)	27.00 (4.34)	< .001	< .001	< .001
Medication									
None	24	13	11	30	18	12	< .001	.008	.003
Benzodiazepines	4	2	2	0	0	0	.030	.163	.083
Opioid-based analgesics	5	1	4	0	0	0	< .001	.163	.083
Other (e.g. ibuprofen, paracetamol)	1	1	0	2	1	1	.119	.960	.095
Supplements	7	6	1	0	0	0	.002	.008	.121
Substance use									
Caffeine (mg/daily)	135 (127)	104 (102)	168 (145)	134 (98)	140 (94)	117 (108)	.965	.258	.320
Alcohol (SD/weekly)	3.5 (3.1)	3.6 (3.0)	3.4 (3.2)	3.48 (4.58)	3.0 (4.02)	4.1 (5.63)	.505	.964	.368

p-values of significance derived from independent sample t-tests or chi square tests where appropriate, significant at $p < .05$; ^a Hamilton Anxiety Inventory; ^b Montgomery-Asberg Depression Scale; ^c State-Trait Anxiety Scale, Trait form; ^d State-Trait Anxiety Scale, State form; AGO Agoraphobia; MDD Major depressive disorder; Mg Milligrams; PD panic disorder; SAD social anxiety disorder; SD standard drink/10 grams of alcohol

6.6.3 Clinical psychiatric data

6.6.3.1 *SIGH-A/HAM-A and SIGMA/MADRS*

As expected, significant differences in psychiatric measure scores were reported between groups, for which means and SDs are reported in Table 17. Differences were observed for the SIGH-A as the primary measure for anxiety symptoms; $t(68) = 32.25$, $p < .001$, 95% CI [20.31, 22.89] and the SIGMA for depression symptoms; $t(52) = 12.69$, $p < .001$, 95% CI [20.26, 22.94]. Gender differences comparing male and female in the GAD group or healthy controls were not significantly different on either of these measures. Across groups, males and female mean score differences were reflective of significant group differences (Table 18).

Both the SIGH-A anxiety and SIGMA depression score measures failed the Levene's test for normality, due to clustering of scores around 1 for healthy controls on the measures (SIGH-A: $m = 1.45$, $SD = 1.44$; SIGMA: $m = 1.18$, $SD = 2.20$) and for GAD group mean scores (SIGH-A: $m = 23.05$, $SD = 3.60$; SIGMA: $m = 13.59$, $SD = 2.99$) and, therefore, equal variances were not assumed in the reporting of t-tests.

When examining differences on SIGH-A anxiety and SIGMA depression scales in the GAD group between GAD with comorbid disorders and 'pure GAD', neither measure data were significantly different (SIGH-A: $p = .205$; SIGMA: $p = .478$), although GAD/comorbid participants were slightly (non-significantly) higher on both measures (SIGH-A: $m = 23.71$, $SD = 3.70$; SIGMA: $m = 13.90$, $SD = 3.14$) than pure GAD (SIGH-A: $m = 22.19$, $SD = 3.37$; SIGMA: $m = 13.19$, $SD = 2.83$).

6.6.3.2 *Spielberger trait and state anxiety scales*

For anxiety symptom measures utilising the Spielberger scales, trait anxiety differences were observed between GAD and healthy control groups; $t(68) = 19.56$, $p < .001$, 95% CI [18.68, 25.69], where GAD scores were significantly higher ($m = 51.09$, $SD = 6.90$) than healthy group scores ($m = 28.91$, $SD = 5.32$). No gender differences were observed in the same group. Similarly, no differences were observed between GAD/comorbid and pure GAD participants ($p = .380$), although the GAD/CM group mean score was (non-significantly) higher ($m = 52.05$, $SD = 6.92$) than pure GAD ($m = 49.86$, $SD = 6.93$) participants.

State anxiety was measured via the STAIS component prior to the scanning event and GAD group score differences were significant from healthy controls; pre-scan scores: $t(56) = 10.23$, $p < .001$ (95% CI 16.86, 23.80), whereby mean GAD scores ($m = 45.83$, $SD =$

8.60) were 20 points higher than the healthy control group ($m = 25.50$, $SD = 4.55$). In examining GAD/comorbid and pure GAD groups, no significant differences in scores were observed pre-scan ($p = .499$) between these groups, although again GAD/CM participants had higher scores ($m = 46.67$, $SD = 7.82$) than pure GAD participants ($m = 44.67$, $SD = 9.74$).

Gender differences on the STAIS scores were similarly observed both across groups separately and also between them in GAD. Male between group scores pre-scan were different, $t(23) = 6.91$, $p < .001$, 95% CI [10.88, 20.18] with GAD males significantly higher ($m = 42.53$, $SD = 5.59$) than healthy control males ($m = 27.00$, $SD = 4.34$). Similarly, female pre-scan STAIS scores were also different, $t(31) = 8.51$, $p < .001$, 95% CI [18.36, 29.93,] with GAD female mean score ($m = 48.79$, $SD = 9.82$) significantly higher than healthy control females by more than 20 points ($m = 24.64$, $SD = 4.60$).

In the GAD group, gender scores were significantly different between females and males, $t = -6.26$, $p = .027$, 95% CI [-11.76, -.76], where mean state anxiety pre-scan scores for females ($m = 48.79$, $SD = 9.82$) were 6 points higher than males ($m = 42.53$, $SD = 5.59$). In the healthy control group, mean state anxiety scores were not significant between genders ($p = .252$).

6.6.4 MRS results

6.6.4.1 Tissue volume and quality parameters

Volume correction/segmentation was applied using custom MATLAB code to derive voxel grey matter, white matter and CSF fractions in the dACC to enable tissue correction to best measure GABA level data for Tarquin and LCModel toolbox data. Parameter estimates are reported in Table 18.

6.6.4.1.1 Grey matter proportional percentage (GM %)

Mean GM% for the groups were healthy control 60.67% ($SD = 9.45$), GAD/comorbid 59.81% ($SD = 14.24$) and pure GAD 60.94% ($SD = 11.51$). The ANOVA model did not indicate significant group differences ($p = .732$) or for gender ($p = .423$). Only age in the model was significant, $F(1, 65) = 19.11$, $p = .000$, where grey matter percent decreased by 0.4% with increasing years, 95% CI [-0.65, -0.24].

6.6.4.1.2 White matter proportional percentage (WM %)

Mean WM% for the groups were healthy control 18.67% ($SD = 8.94$), GAD/comorbid 23.00% ($SD = 12.23$) and GAD pure 24.12% ($SD = 10.73$). The ANOVA was not significant for group differences, $F(2, 65) = 2.48$, $p = .091$.

Age in the model was again significant, $F(1, 65) = 5.03, p = .028$, where white matter percent increased by 0.2% with increasing years, 95% CI [0.02, 0.42]. Finally, gender was not significant in the model ($p = .834$).

6.6.4.1.3 Cerebrospinal fluid proportional percentage (CSF %)

Mean CSF% for the groups were healthy control 19.64% (SD = 6.04), GAD/comorbid 15.81% (SD = 5.45) and GAD pure 15.27% (SD = 5.86).

In the CSF% model, group difference was significant, $F(2, 63) = 6.38, p = .003$. However, an interaction was also observed between group and gender, $F(2, 63) = 3.54, p = .035$. Specifically, in males where significant differences in CSF% between pure GAD and healthy control groups ($p = .015$) was 5.38% higher in the healthy control group, 95% CI [1.56, 14.13]. Differences were not significant in either gender between GAD groups (e.g. female GAD groups, $p = .433$). Gender itself was not a main predictor ($p = .422$). Again, age was a predictor in the model, $F(1, 63) = 14.43, p = .035$, whereby CSF% increased by 0.19% for each unit of age, 95% CI [0.09, 0.29].

Table 18. Tissue Volume Analysis of Factors[^]

Tissue volume	B/Coefficient	95% CI	t	p-value*
Grey matter				
Group – Healthy control	0.13	-6.20, 6.47	0.04	.967
Group – GAD/comorbid	-2.10	-9.07, 4.88	-0.60	.550
Gender	-2.12	-7.38, 3.13	-0.81	.423
Age	-0.44	-0.65, -0.24	-4.37	.000*
White matter				
Group – Healthy control	-5.63	-11.81, 0.55	-1.82	.073
Group – GAD/comorbid	-0.32	-7.13, 6.48	-0.10	.924
Gender	-0.54	-5.67, 4.59	-0.21	.834
Age	0.22	0.02, 0.42	2.24	.028*
Cerebrospinal fluid				
Group – Healthy control	1.16	-2.72, 5.04	0.60	.552
Group – GAD/comorbid	0.06	-4.59, 4.72	0.03	.978
Gender	-2.45	-7.69, 2.78	-0.94	.352
Age	0.19	0.09, 0.29	3.80	.000*
Gender*Group - HC	7.84	1.56, 14.13	2.49	.015*
Gender*Group - GAD/cm	2.71	-4.15, 9.59	0.79	.433

*Significant at $p < 0.05$; corrected for multiple comparisons using least significant differences; Significance reported from parameter estimates table
[^]ANOVA model

6.6.4.2 *Toolbox-specific quality signals*

Quality parameters for signal were compared between groups using a univariate ANOVA test for significance to check whether the data was moderated by scanning or signal processing issues. Cases identified were removed across all toolboxes.

6.6.4.2.1 *Tarquin*

Applied quality parameters (exclusions: GABA Fit $Q < 1$ or > 2 ; SNR $< / 2$; SD% $> 60\%$) resulted in three cases being removed from the dataset, one from the healthy control group and two from the GAD participants. A total of 66 cases were utilised in the final dataset.

Tarquin's Fit Q score was not different between the main groups ($p = .395$). The SNR was significantly different between the groups; $F(1,64) = 3.27$, $p = .044$; compared to the pure GAD group ($m = 9.96$, $SD = 3.72$), the GAD/comorbid group SNR was 1.79 units higher ($m = 11.74$, $SD = 4.05$), 95% CI $[-0.84, 4.42]$ and the healthy control group was 1.02 units lower ($m = 8.93$, $SD = 3.79$) than the pure GAD group, 95% CI $[-3.43, 1.39]$.

Post-hoc comparisons revealed this difference to be significant between the controls and GAD/comorbid groups ($p = .013$). The calculated percentage SD was not significantly different between groups ($p = .228$).

6.6.4.2.2 *LCModel*

Applied quality parameters (exclusions: SNR $< / 2$; SD % $> 20\%$, FWHM > 0.15) resulted in two cases being removed from the dataset (one healthy control and one GAD). The two groups were not significantly different with regard to SD% ($p = .295$). LCModel cases totalled 66 cases in the final dataset.

LCModel SNR was significantly different between groups, $F(2, 64) = 3.32$, $p = .043$. It was lowest in the pure GAD group ($m = 8.06$, $SD = 2.57$), 1.69 units higher in the GAD/comorbid group ($m = 9.75$, $SD = 2.40$), 95% CI $[0.06, 3.31]$ and 0.03 units higher in the healthy control group ($m = 8.10$, $SD = 2.36$), 95% CI $[-1.45, 1.52]$. Post-hoc comparisons showed this difference to be significant between the healthy control and GAD/comorbid groups ($p = .020$) and between the GAD/comorbid and pure GAD groups ($p = .042$).

LCModel FWHM was also statistically significant between the groups, $F(2, 64) = 4.54$, $p = .014$. The FWHM score was highest in the pure GAD group ($m = 0.10$, $SD = 0.05$), 0.01 units lower in the healthy control group ($m = 0.09$, $SD = 0.03$), 95% CI $[-0.03, 0.01]$ and lowest by 0.03 units in the GAD/comorbid group ($m = 0.06$, $SD = 0.02$), 95% CI $[-0.06, -0.01]$. Post-hoc tests revealed differences were significant between the healthy control

and GAD/comorbid groups ($p = .022$) and between the GAD/comorbid and pure GAD groups ($p = .006$).

6.6.4.3 *Correlations between toolbox GABA data*

A test for trends in GABA across toolboxes was performed to check relationship consistency between toolboxes for GABA data. Assessments were performed via bivariate correlation reporting Pearson's correlation matrices with results deemed significant at $p < .05$ (two-tailed). Tarquin GABA was moderately positively consistent with LCMModel GABA, $r(66) = .47$, $p < .001$ (Table 19).

Table 19. The p -Values from Bivariate Correlation Tests by Group for GABA Data Across Toolboxes

Toolbox data	1		2	
	(n = 66)		(n = 66)	
	r	p-value*	r	p-value
TARQUIN GABA/GM%	-	-	-	-
LCMODEL GABA/GM%	.47	.000*	-	-
* significant at $p < .05$; r: Pearson's Product Moment Correlation test				

Table 20. Descriptive Statistics for each Group and for Gender for dACC MRS GABA and Toolbox-specific Quality Parameters

Mean (S.D.), n, or % where relevant	Healthy control			pure GAD			GAD + comorbid			F-test	df	p-value*
	Males	Females	Group	Males	Females	Group	Males	Females	Group			
n	12	20	32	12	10	22	6	8	14	-	-	-
dACC GM (% mm ³)	62.84 (7.83)	59.25 (10.31)	66.67 (9.45)	59.83 (17.12)	61.60 (9.01)	60.93 (12.13)	59.25 (12.39)	60.55 (60.55)	59.81 (14.24)	-	-	.732
dACC WM (% mm ³)	15.00 (3.53)	21.05 (10.56)	18.67 (8.94)	27.00 (15.62)	22.40 (6.88)	24.12 (10.73)	22.67 (13.44)	23.44 (11.20)	23.00 (12.23)	2.48	(2,65)	.091 ^a
dACC CSF (% mm ³)	21.54 (6.99)	18.40 (5.14)	19.63 (6.04)	12.73 (3.60)	16.80 (4.61)	15.27 (4.61)	15.81 (5.45)	15.44 (7.12)	15.81 (5.45)	6.38	(2,63)	.003*
Tarquin GABA	8.20 (2.38)	8.94 (3.07)	8.66 (2.81)	9.87 (2.20)	10.10 (6.75)	10.01 (3.45)	9.81 (3.01)	10.25 (1.39)	9.97 (2.49)	-	-	.302
LCModel GABA	3.95 (0.82)	3.96 (0.50)	3.96 (0.63)	3.64 (0.64)	4.12 (1.28)	3.93 (1.08)	3.36 (0.51)	3.63 (0.78)	3.46 (0.62)	2.80	(2,63)	.068 ^a
TARQUIN FIT Q	1.45 (0.21)	1.43 (0.21)	1.43 (0.21)	1.29 (0.09)	1.45 (0.19)	1.39 (0.18)	1.38 (0.13)	1.35 (0.08)	1.37 (0.11)	-	-	.395
TARQUIN SNR	8.70 (3.85)	9.08 (3.85)	8.93 (3.79)	9.17 (3.03)	10.48 (4.21)	9.95 (3.72)	10.60 (3.84)	13.46 (3.96)	11.74 (4.05)	3.27	(2,64)	.044*
TARQUIN SD%	24.58 (8.62)	25.79 (13.62)	25.33 (11.85)	32.47 (15.48)	26.32 (14.05)	28.78 (14.43)	21.23 (13.29)	21.88 (9.32)	21.49 (11.59)	-	-	.228
LCMODEL SD (%)	9.25 (1.86)	8.21 (1.23)	8.61 (1.56)	9.17 (1.17)	9.50 (3.53)	9.37 (2.82)	10.00 (2.04)	8.50 (1.07)	9.40 (1.85)	-	-	.295
LCMODEL SNR	7.83 (2.82)	8.26 (2.08)	8.10 (2.36)	7.67 (2.06)	8.30 (2.91)	8.06 (2.57)	9.08 (1.97)	10.75 (2.76)	9.75 (2.40)	3.32	(2,64)	.043*
LCMODEL FWHM	0.08 (0.04)	0.09 (0.03)	0.09 (0.03)	0.09 (0.04)	0.10 (0.06)	0.10 (0.04)	0.06 (0.02)	0.06 (0.02)	0.06 (0.02)	4.54	(2,64)	.014*

**p*-Values significant at $p < .05$; ^a marginal significance ($p = > .05 - .10$. df = degree of freedom; F-test: analysis of variance for three-group differences ; F-tests and degrees of freedom reported for significant or marginally significant *p*-values only; dACC GM, dorsal anterior cingulate cortex grey matter; WM, white matter; CSF, cerebrospinal fluid; FWHM (full width at half-maximum) as estimate of linewidth in *in vivo* spectrum; GABA, gamma amino-butyric acid; m-m, macromolecules; SNR signal-noise ratio - the S/N derived from offsets in model spectra as a ratio; SD values: assessing noise level and resolution as a guide to the reliability of the estimates.

6.6.4.4 Group differences in GABA metabolite levels

For GABA metabolite group differences in concentration, ANOVA models were run with additional predictors derived from analyses above if applicable. Descriptive statistics are reported in Table 20 and ANOVA model reporting in Table 21. Boxplots illustrating trends are shown in Figure 20.

6.6.4.4.1 Tarquin GABA concentration in grey matter

For GABA in grey matter from Tarquin as the main outcome, the healthy control mean score was 8.66 (SD = 2.81), pure GAD group mean was 9.97 (SD = 2.49) and GAD/comorbid group mean was 10.01 (SD = 3.45). The ANOVA indicated these group differences were not statistically significant ($p = .302$).

6.6.4.4.2 LCModel GABA in grey matter

The ANOVA indicated these group differences were not significant ($p = .068$).

Table 21. ANOVA for Group-level GABA Differences per MRS Toolbox

GABA model	B/Coefficient	95% CI	t	p-value*
Tarquin: GABA in GM				
Group – healthy control	-1.35	-3.50, 0.80	-1.25	.216
Group – GAD/cm	-0.04	-2.42, 2.33	-0.03	.973
LCModel: GABA in GM				
Group – healthy control	0.03	-0.44, 0.49	0.11	.909
Group – GAD/cm	-0.47	-0.99, 0.04	-1.83	.072

* Significant at $p < 0.05$; significance reported from parameter estimates table

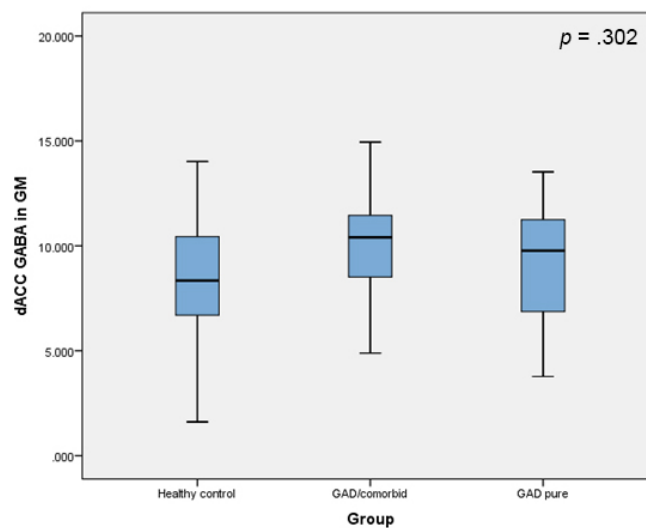


Figure 10. Boxplot for group-based dACC GABA (Tarquin) means and standard deviations

6.6.4.5 GABA and psychiatric measures associations

GABA metabolite concentration data were assessed for the relationship with reported anxiety symptom levels as measured by scales detailed separately below. Output is detailed in Table 22. Healthy control group data for SIGH-A/HAM-A exhibited a ceiling effect to scores as per the study protocol methods, which limits conclusions from these associations. Therefore, healthy control reporting of these measures is displayed in the table only and not discussed in text.

6.6.4.5.1.1 SIGH-A/HAM-A score for anxiety

In the pure GAD group, HAM-A scores were not correlated with GABA. For the GAD/comorbid group, HAM-A scores and Tarquin GABA levels were significant, $r(19) = .54$, $p = .018$, where higher scores were also associated with higher concentrations indicating a strong positive relationship.

6.6.4.5.1.2 Trait anxiety levels via Spielberger STAIT scale

There were no significant associations found for trait anxiety and GABA levels in either GAD group. In the healthy control group, trait anxiety and Tarquin GABA concentration was significant, $r(31) = -.49$, $p = .019$, in a moderate negative trend, where lower scores were associated with increased GABA levels.

6.6.4.5.1.3 State anxiety levels via Spielberger STAIS scale

Anxiety levels as a function of undertaking a brain scan indicated that within the pure GAD group, state anxiety was significantly associated with Tarquin GABA, $r(14) = .54$, $p = .044$, in a positive moderate relationship where higher scores were seen with higher GABA level. No associations were found in the GAD/comorbid group. In the healthy controls, a significant relationship was found for state anxiety scores and Tarquin GABA concentrations, $r(31) = -.52$, $p = .016$, in a moderate and negative fashion, whereby lower anxiety scores were associated with higher GABA levels.

Figure 21 illustrates these trends for each group between Tarquin GABA and state anxiety scores. Inspection of this trend indicated an interaction effect between groups, whereby a positive relationship was observed in clinical anxiety, but in healthy controls it was negative.

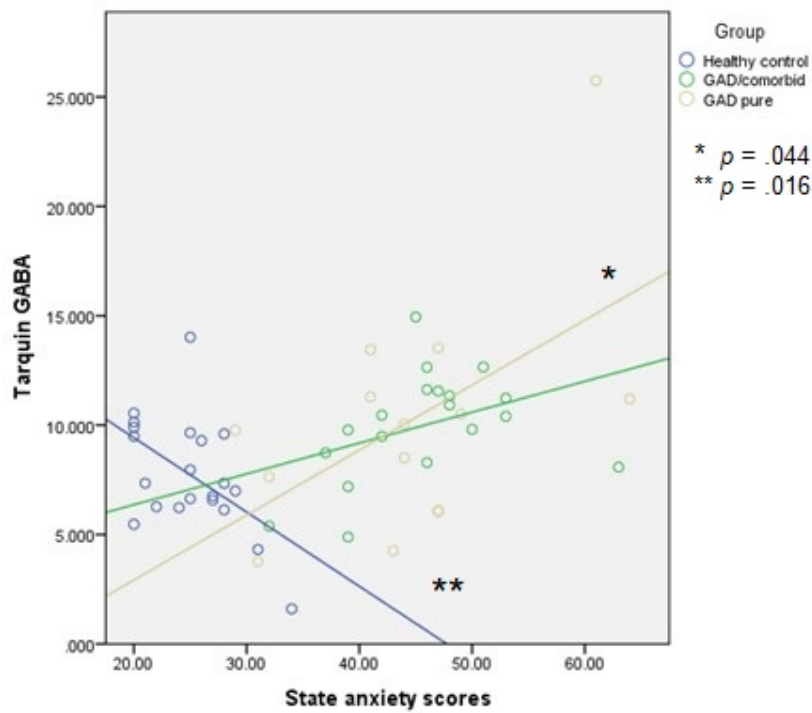


Figure 11. Scatterplot of state anxiety and GABA relationship with trend lines per group

Table 22. Correlation Assessment by Group for Psychiatric Measures and GABA Data

Correlation [^]	Healthy control		Pure GAD		GAD comorbid	
	Pearson's r		Pearson's r		Pearson's r	
	(df = 31)	p-value	(df = 14)	p-value	(df = 19)	p-value
HAM-A						
TARQUIN GABA/GM%	-0.01	.965	0.06	.827	0.54	.018*
LCMODEL GABA/GM%	-0.20	.283	0.25	.352	0.09	.707
STAI Trait Anxiety						
TARQUIN GABA/GM%	-0.49	.019*	0.11	.723	-0.02	.938
LCMODEL GABA/GM%	0.26	.261	0.04	.892	-0.28	.293
STAI score pre-scan						
TARQUIN GABA/GM%	-0.52	.016*	0.54	.044*	0.40	.092
LCMODEL GABA/GM%	-0.11	.656	0.40	.144	-0.28	.245

Significance set as $p \leq 0.05$, df where stated; * significant at $p < .05$

[^]Correlation assessment by group for psychiatric measures and GABA data

6.6.4.5.2 GAD group SIGH-A anxiety predicted by GABA change

To examine GABA metabolite levels as a factor in the maintenance of GAD anxiety symptoms (the primary psychiatric outcome), the HAM-A scores as the main outcome were model tested. Results from main effects and interaction effects of note are

reported in Table 23. Significant main effects for categorical variables were subject to post-hoc comparisons (via Fisher's least significant differences) to better ascertain trends in the data.

6.6.4.5.2.1 Tarquin GABA in grey matter

The model indicated significant main effects, and significant interaction. GABA levels significantly predicted HAM-A anxiety score, $F(1,28) = 6.19$, $p = .019$. Increases to GABA resulted in a 0.85 unit increase in anxiety scores, 95% CI [0.17, 1.53]. However, an interaction effect occurred for comorbidity, $F(1,28) = 4.03$, $p = .05$. While GAD/comorbid participants increased by 0.85 units in HAM-A score with GABA levels, pure GAD participant HAM-A only increased by 0.09 units, 95% CI [-1.54, 0.01]. Figure 22 illustrates the interaction effect between these predictors.

6.6.4.5.2.2 LCModel GABA in grey matter

In the model, GABA levels were not predictive of HAM-A scoring ($p = .145$). Table 23 summarises model statistics for both toolbox analyses.

Table 23. ANOVA Model for Predictors of SIGH-A/HAM-A Anxiety Score in GAD Sample

Source	B/coefficient	95% CI	t	p-value
TARQUIN GABA/GM				
Comorbid_psych	6.48	-1.72, 14.67	1.62	.117
GABA/GM Tarquin	0.85	0.17, 1.53	2.58	.016*
Age	-0.04	-0.14, 0.06	-0.86	.398
Gender	1.06	-1.48, 3.61	0.86	.399
Comorbid*GABA	-0.76	-1.54, 0.01	-2.01	.050*
LCMODEL GABA/GM				
Comorbid_psych	-1.62	-4.29, 1.06	-1.23	.227
GABA/GM LCMODEL	1.19	-0.43, 2.81	1.50	.145
Age	-0.06	-0.16, 0.04	-1.23	.228
Gender	1.22	-1.44, 3.88	0.94	.356

*Significant at $p \leq 0.05$; ^a Marginally significant at $p = .05 - 10$; t: ratio of parameter estimates to standard error; CI: confidence interval

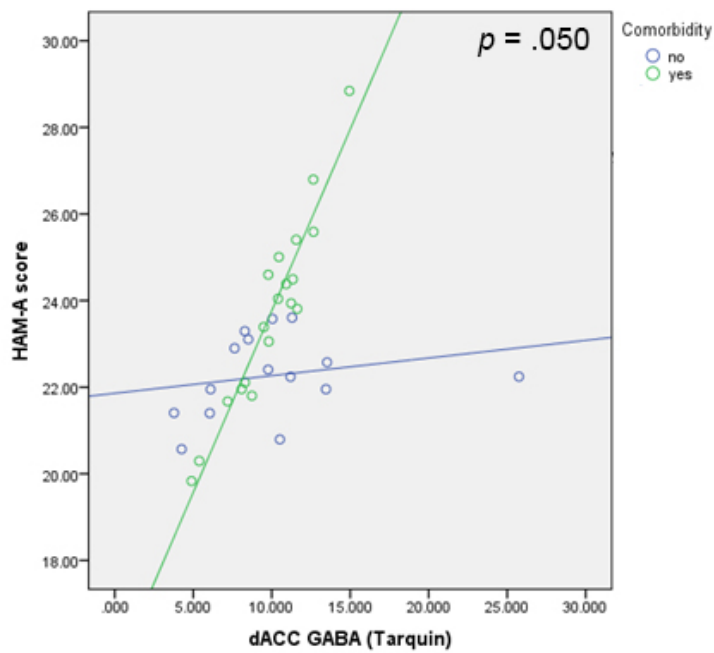


Figure 12. Interaction plot for comorbidity in the prediction of HAM-A anxiety from GABA (Tarquin) in the GAD group

6.6.4.5.3 GAD group trait anxiety predicted by GABA change

6.6.4.5.3.1 Tarquin GABA concentration in grey matter

The model did not indicate GABA level was a significant predictor of trait anxiety in GAD ($p = .919$). Both Tarquin and LCMModel statistics for GAD trait are reported in Table 24.

6.6.4.5.3.2 LCMModel GABA in grey matter

In the model, the GABA level was not a significant predictor ($p = .719$). Age predicted trait anxiety levels, $F(1, 25) = 4.60$, $p = .042$, where increased age showed a 0.23 unit reduction in anxiety scores, 95% CI [-0.45, -0.01].

Table 24. ANOVA Model for Predictors of Trait Anxiety Score in GAD Sample

Source	B/coefficient	95% CI	t	p-value
TARQUIN GABA/GM				
Comorbid_psych	-2.07	-7.97, 3.82	-0.72	.475
GABA/GM Tarquin	-0.06	-1.21, 1.10	-0.10	.919
Age	-0.23	-0.46, 0.01	-1.95	.062 ^a
Gender	-0.70	-6.22, 4.82	-0.26	.795
LCMODEL GABA/GM				
Comorbid_psych	-2.04	-7.32, 3.24	-0.79	.434
GABA/GM LCMODEL	-0.72	-4.77, 3.34	-0.36	.719
Age	-0.23	-0.45, -0.01	-2.14	.042*
Gender	-0.80	-6.21, 4.62	-0.30	.764

*Significant at $p \leq 0.05$; ^a Marginally significant at $p = .05 - .10$; t: ratio of parameter estimate to standard error; CI: confidence interval

6.6.4.5.4 GAD group state anxiety predicted by GABA change

6.6.4.5.4.1 Tarquin GABA concentration in grey matter

The model indicated significant main effects of GABA levels in state anxiety scores, $F(1,28) = 8.89$, $p = .006$. Increases to GABA resulted in a 0.98 unit increase in anxiety scores, 95% CI [0.31, 1.65]. Gender also predicted state anxiety levels, $F(1, 28) = 4.89$, $p = .035$, where the state levels of males were 5.68 units lower than those of females, 95% CI [-10.94, -0.42]. Figure 23 illustrates the trend for GABA on state anxiety via residuals.

6.6.4.5.4.2 LCModel GABA in grey matter

The model indicated significant main effects and a significant interaction. Comorbidity significantly predicted state anxiety, $F(1,28) = 5.54$, $p = .026$. However, comorbidity and GABA levels produced an interaction for state anxiety, $F(1,28) = 4.62$, $p = .040$. GAD/comorbid state anxiety decreased by 4.83 with each unit increase in GABA but increased by 2.5 units in the pure GAD group, 95% CI [0.34, 14.32]. Table 25 summarises the statistics and Figure 24 illustrates this interaction.

Table 25. ANOVA Model for Predictors of State Anxiety Score in GAD Sample

Source	B/coefficient	95% CI	t	p-value
TARQUIN GABA/GM				
Comorbid_psych	-3.11	-8.45, 2.22	-1.20	.242
GABA/GM Tarquin	0.98	0.31, 1.65	2.98	.006*
Age	0.04	-0.17, 0.24	0.37	.715
Gender	-5.68	-10.94, -0.42	-2.21	.035*
LCMODEL GABA/GM				
Comorbid_psych	-29.83	-55.79, -3.86	-2.35	.026*
GABA/GM LCMODEL	-4.83	-10.91, 1.25	-1.63	.115
Age	0.11	-0.11, 0.32	1.02	.316
Gender	-6.20	-11.85, -0.54	-2.24	.033*
Comorbid*GABA	7.33	0.34, 14.32	2.15	.040*

*Significant at $p \leq 0.05$; ^a Marginally significant at $p = .05 - .10$; t: ratio of parameter estimate to standard error; CI: confidence interval

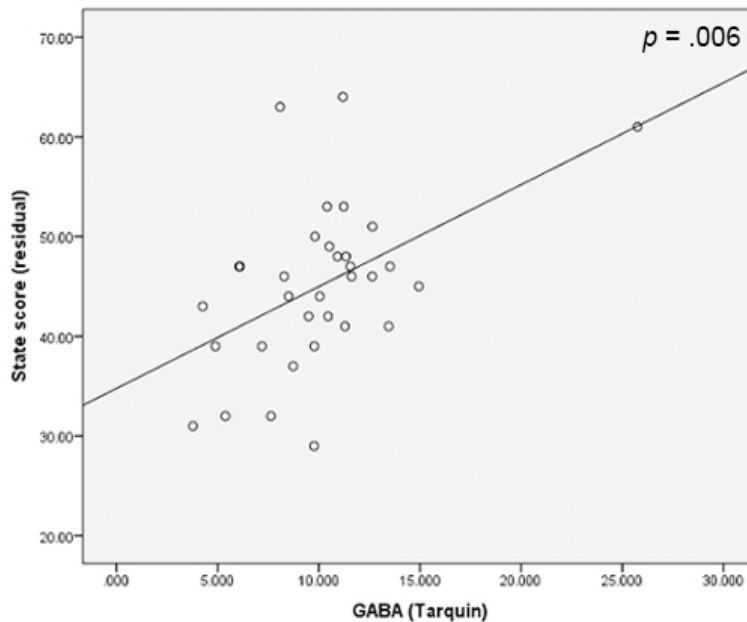


Figure 13. Residual plot for state anxiety predicted by GABA (Tarquin) level in GAD

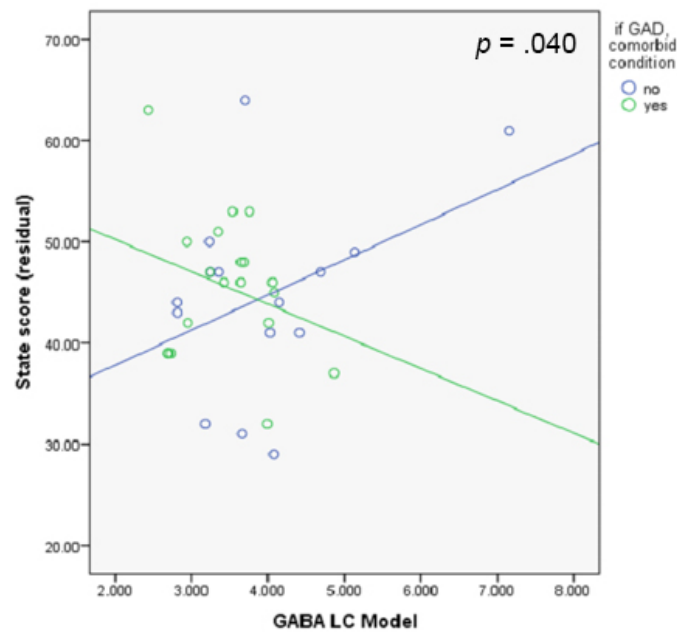


Figure 14. State anxiety predicted by GABA (LCModel) in GAD

6.6.4.5.5 Healthy control group trait anxiety predicted by GABA level

6.6.4.5.5.1 Tarquin GABA concentration in grey matter

In the model, the GABA level significantly predicted trait anxiety level, $F(1,18) = 4.68$, $p = .040$. Trait anxiety scores decreased by 0.90 units for each increase in GABA units, 95% CI [-1.77, -0.03].

6.6.4.5.5.2 LCModel GABA in grey matter

In the model, GABA was not significant ($p = .137$); and no predictors were significant at $p < .05$. Table 26 shows the summary statistics for both toolboxes.

Table 26. ANOVA Model for Predictors of Spielberger Trait Anxiety in Healthy Controls

Source	B/coefficient	95% CI	t	p-value*
TARQUIN GABA/GM				
Age	-0.09	-0.30, 0.12	-0.88	.391
Gender	-1.95	-6.99, 3.09	-0.81	.427
GABA/GM Tarquin	-0.90	-1.77, -0.03	-2.16	.044*
LCMODEL GABA/GM				
Age	-0.17	-0.36, 0.01	-1.95	.068 ^a
Gender	0.62	-4.42, 5.67	0.26	.797
GABA/GM LCMODEL	2.74	-0.96, 6.45	1.56	.137

*Significant at $p \leq 0.05$. ^a Marginal significance at $p > .05$ and < 0.10 ; t: ratio of parameter estimate to standard error; CI: confidence interval

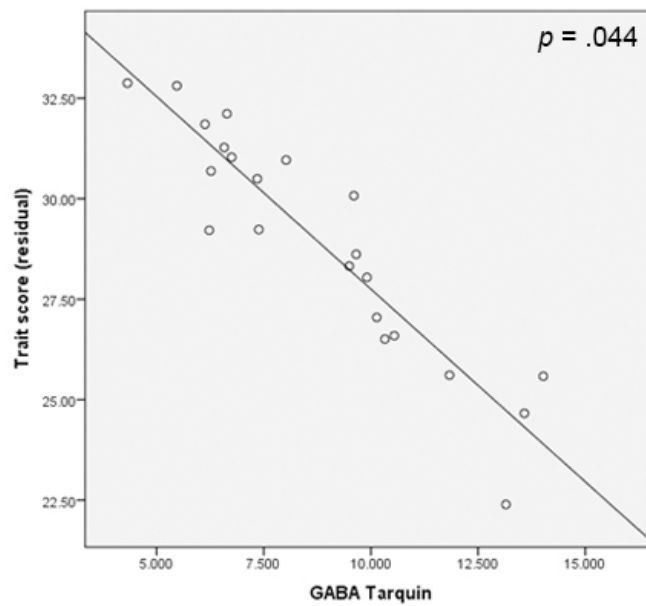


Figure 15. Residual plot for trait anxiety predicted by GABA (Tarquin) level in healthy controls

6.6.4.5.5.3 Healthy control group state anxiety predicted by GABA level

6.6.4.5.5.3.1 Tarquin GABA concentration in grey matter

In the model, GABA level significantly predicted state anxiety level, $F(1,17) = 7.02$, $p = .017$. State anxiety scores decreased by 0.84 units for each increase in GABA units, 95% CI [-1.51, -0.17]. Both Tarquin and LCMoel statistics for healthy control state anxiety are reported in Table 27, and Figure 28 illustrates Tarquin data and state anxiety relationship.

6.6.4.5.5.3.2 LCMoel GABA in grey matter

No predictor was significant in the model; GABA did not predict state anxiety levels ($p = .844$).

Table 27. ANOVA Model for Predictors of Spielberger State Anxiety in Healthy Controls

Source	B/coefficient	95% CI	t	p-value*
TARQUIN GABA/GM				
Age	-0.08	-0.23, 0.07	-1.11	.280
Gender	-0.96	-5.06, 3.14	-0.49	.629
GABA/GM Tarquin	-0.84	-1.51, -0.17	-2.65	.017*
LCMODEL GABA/GM				
Age	-0.07	-0.23, 0.10	-0.84	.411
Gender	0.66	-3.88, 5.20	0.31	.761
GABA/GM LCMODEL	-0.34	-4.01, 3.32	-0.20	.844

*Significant at $p \leq 0.05$. ^a Marginal significance at $p > .05$ and < 0.10 ; t: ratio of parameter estimates to standard error; CI: confidence interval

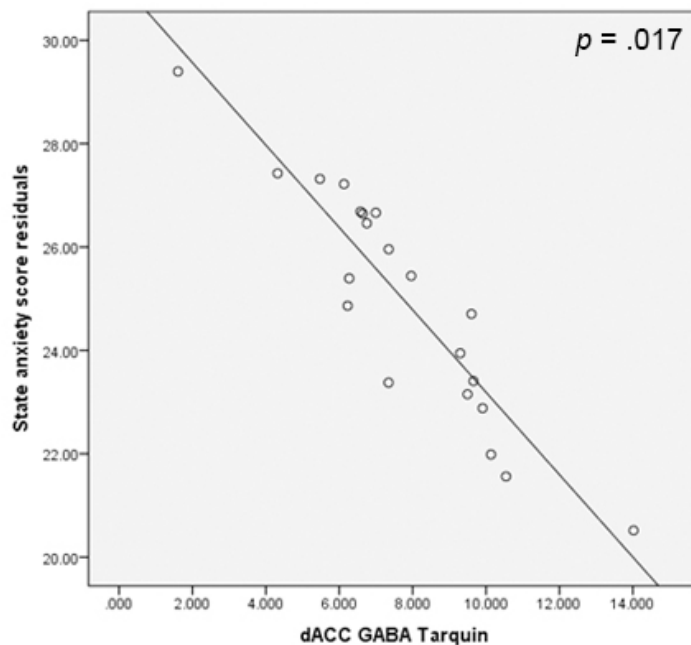


Figure 16. Residual plot for state anxiety predicted by GABA (Tarquin) in healthy controls fMRI results.

6.6.5 fMRI results

6.6.5.1 Whole brain – group-specific activations

For the healthy control group, significant activity was observed in a distributed network including left middle temporal gyrus, right superior parietal lobe, left postcentral gyrus/primary somatosensory cortex, and the left precuneus. At a more liberal threshold ($p < .005$ uncorrected at the voxel level, FWE-corrected at the cluster level), further activation was observed in the right fusiform gyrus and right inferior parietal lobule (see

Table 28 and Figure 27). For the GAD group, significant activity derived from the contrast was found in the right fusiform gyrus and the left inferior occipital gyrus (see Table 29 and Figure 27).

Figure 17. Group-specific cluster-level activation for the selected contrast; a. GAD, and b. healthy control participants

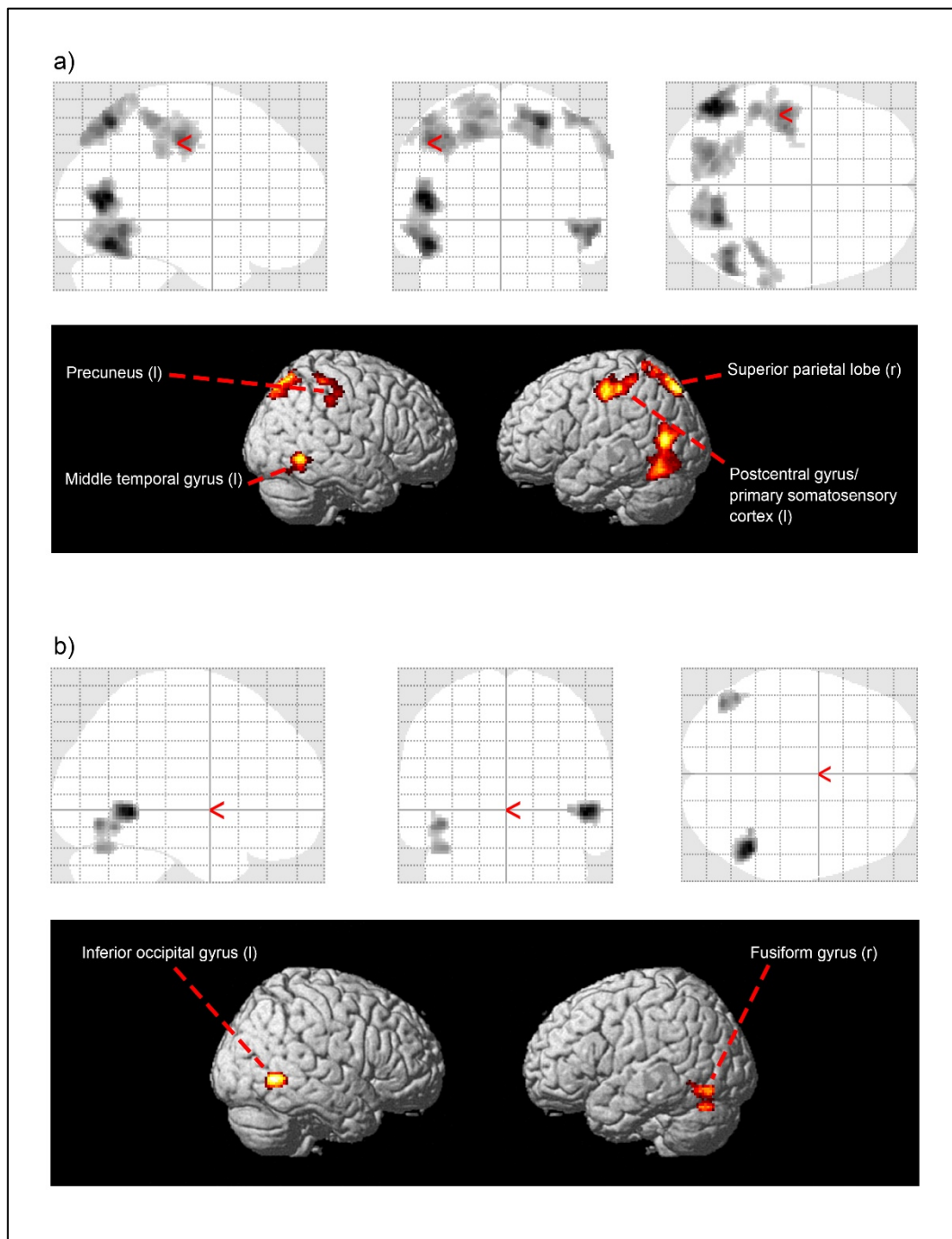


Table 28. Group-specific Cluster Level/Region Statistics for the Selected Contrast

Region	Hemisphere (L/R)	BA	MNI coordinates			Cluster size	t-Value	Cluster sig.
			x	y	z			
Healthy controls								
Middle temporal gyrus	L	19	-48	-72	8	912	7.16	.000*
Superior parietal lobule	R	39	24	-66	56	515	6.53	.000*
Parietal postcentral gyrus/primary sensoricortex	L	1	-46	-22	46	833	5.54	.000*
Parietal lobe/Precuneus	L	7	-18	-72	50	681	5.22	.000*
Middle temporal gyrus	R	37	54	-58	-10	260	5.67	.002 ^a
Inferior parietal lobule	R	40	50	-38	58	221	4.82	.005 ^a
GAD								
Middle temporal /fusiform gyrus	R	37	48	-52	-4	254	6.40	.002 ^a
Inferior occipital gyrus	L	19	-42	-72	-10	211	4.86	.005 ^a
* <i>p</i> -value = < .000; ^a <i>p</i> -value = <.005 threshold; BA Brodmann's area; Reported p-values had family-wise error correction applied; x, y, z: coordinates (millimetres) in MNI space								

6.6.5.2 Whole brain – group comparison

The contrast of between healthy control > GAD groups revealed no significant effects. However, the GAD > healthy controls homologue (see Table 29 and Figure 28) revealed increased activation in the left inferior parietal lobe. No significant effect was observed in left dorsolateral PFC (note that both clusters were significant using *p*FDR < .05 corrected for multiple comparisons).

Table 29. Regional Group Activation Differences (GAD > HC)

Region	Hemisphere (L/R)	BA	MNI coordinates			Cluster size	t-Value	Cluster sig.
			x	y	z			
DLPFC	L	10	-34	40	28	127	4.77	.068
IPL	L	39	-48	-50	46	157	4.30	.031

BA Brodmann's area; DLPFC dorsolateral prefrontal cortex; IPL inferior parietal lobule; x, y, z: coordinates (millimetres) in MNI space

The design matrix was multiplied by the identity matrix to explore the group contrast effects in more detail. This showed that in both regions, GAD exhibited positive BOLD

estimates (activation), while in HC, BOLD estimates were negative (deactivation). This shows that the effects were driven by a combination of increased activation in GAD participants, but particularly by deactivation in controls (refer Figure 28).

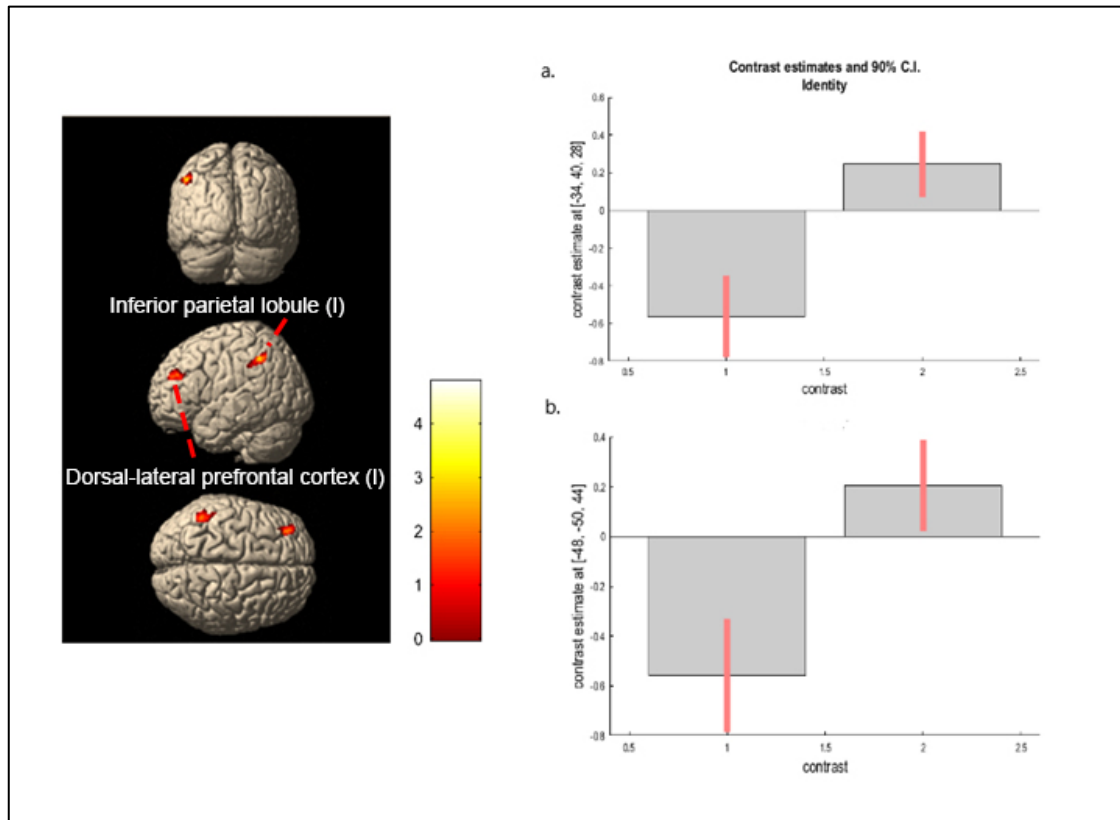


Figure 18. (Left) Regions of significant activation revealed from contrast (subtraction of Neu/Neu from Neu/Neg; $p < .001$, uncorrected). (Right) Mean percent signal change in the a) left DLPFC, and b) left IPL.

By applying more liberal thresholding at the voxel level ($p < .005$) with GAD > healthy control, the model was re-tested. The data revealed significant difference in the mid-cingulate gyrus region (t-value: 4.31; maximal t-value: 2.67; $p = < .005$ uncorrected), again where GAD activation was greater than healthy controls (see Table 30 and Figure 29).

Table 30. Group Differences (GAD > HC) in Task-Dependent Regional Activations Assessed as Whole Brain Significance ($p = < .005$)

Region	Hemisphere (L/R)	BA	MNI coordinates			Cluster size	t-Value	p-value*
			x	y	z			
Mid-cingulate gyrus	L	24	-8	-12	46	400	4.31	.001

* cluster-level significance with uncorrected $p = < .005$; BA Brodmann's area; x, y, z: coordinates (millimetres) in MNI space

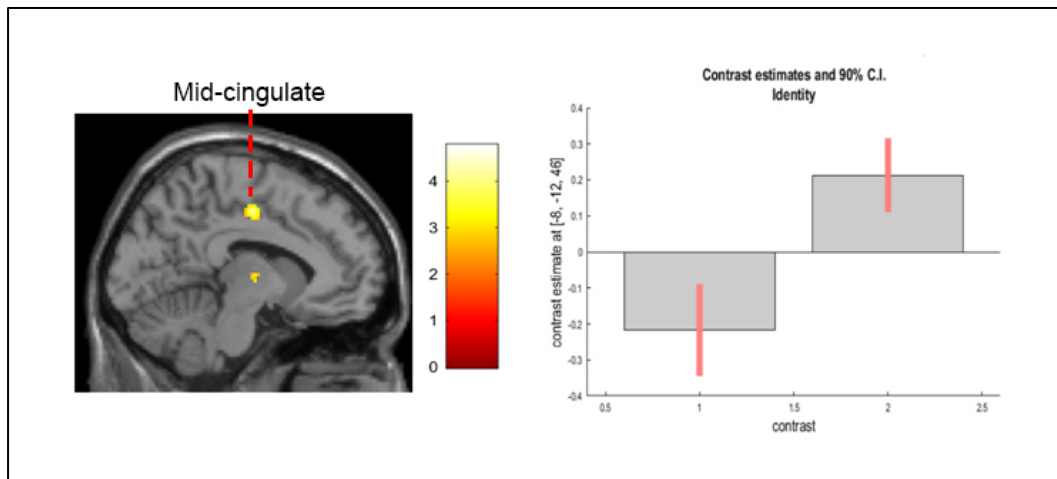


Figure 19. (Left) Mid-cingulate region (I) of significant activation from contrast ($p < .005$), (Right) mean percent signal change in the region

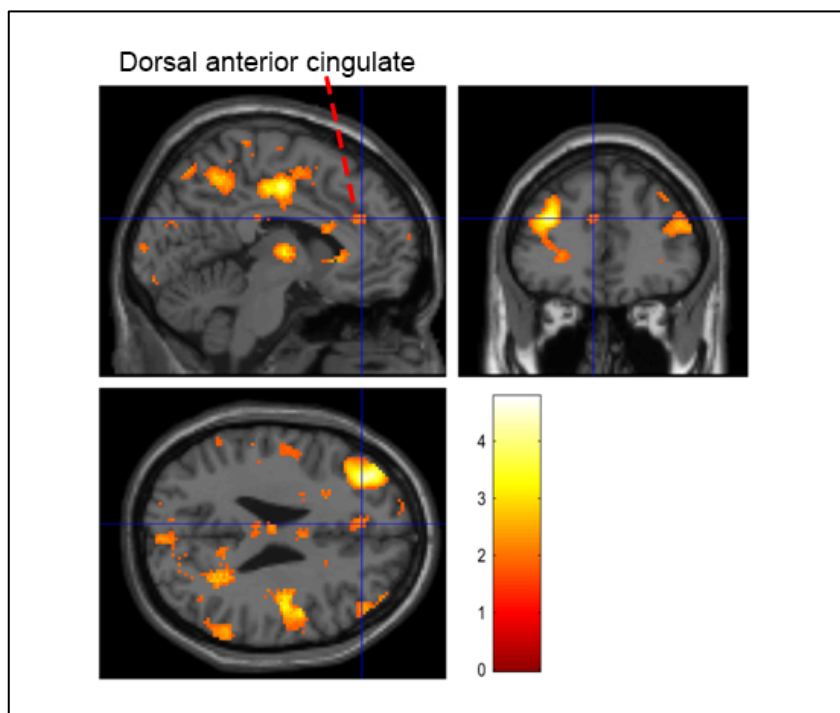


Figure 20. Dorsal anterior cingulate activation ($t = 2.27$, MNI co-ordinates: -6,36,24).

6.6.5.3 dACC – group comparison

For subsequent analyses of the ROI, mean contrast estimates for a 5mm radius, centered on ROI dACC, were extracted and run through SPSS using GLM ANOVA to examine differences between the three groups.

There were no between-group differences, $p = .165$. Although the data were not significant via this model based on p -values, in order to examine the trends via a median test ($n = 54$), 67% of the GAD/comorbid participants were above the overall median, whereas 42% and 36% were above the median in the pure GAD and healthy control groups, respectively (reported in Table 32). The data indicated activation levels were highest in the GAD/comorbid group, followed by the pure GAD group and then the healthy controls (illustrated in groups trends from the aforementioned ANOVA in Figure 31).

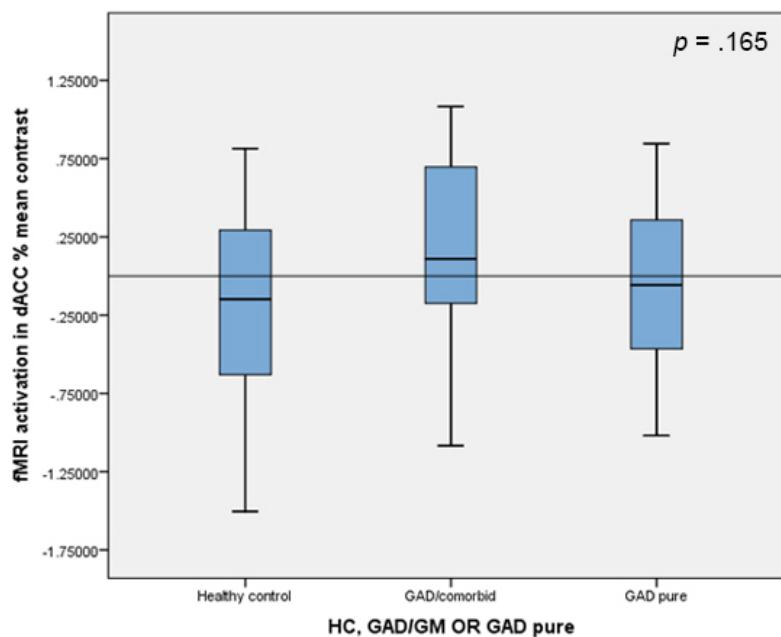


Figure 21. Box plot of data for fMRI activation % mean contrast indicating error bars per group (line is at zero)

Table 31. ROI Function ANOVA Parameter Estimates for Group Differences

Model	B/coefficient	95% CI	t	Sig.
fMRI activation mean contrast				
Group 1 (Healthy controls)	-0.12	-0.50, 0.27	-0.61	.546
Group 2 (GAD/comorbid)	0.21	-0.19, 0.62	1.05	.298
Group 3 (pure GAD)	0	-	-	-

Table 32. Two-way Contingency Table for Groups for ROI fMRI Mean Activation

Overall median =0.00653	Group (n = 54; df = 2)		
fMRI activation in dACC mean contrast	HC	GAD/cm	GAD pure
> Median	10	12	5
< = Median	14	6	7
df: degrees of freedom, HC healthy controls, GAD/cm: GAD + comorbid			

6.6.5.4 dACC task-based BOLD contrast activation and psychiatric measures

6.6.5.4.1 Group activation correlations

Correlations were between individual contrast estimates in the two GAD groups for SIGH-A HAM-A, Spielberger State and Trait scores. The latter two were reported only for healthy controls, due to a ceiling effect in scores for HAM-A for healthy control participants as per the inclusion criteria. Data for this group is shown in Table 33 but are not discussed here. Results indicated that BOLD signal was not correlated with anxiety levels in any group (all $p > .05$).

Table 33. Relationship Between Psychiatric Measures and ROI Task-dependent Activation

Correlation [^]	Healthy control		Pure GAD		GAD comorbid	
	r(df = 25)	p-value	r(df = 14)	p-value	r(df = 20)	p-value
HAM-A	.12	.559	-.17	.601	.17	.501
Spielberger STAIT	.19	.481	-.43	.189 ^a	-.25	.363
Spielberger STAIS (pre-scan)	.07	.797	-.30	.369	-.38	.117

df degrees of freedom; r Pearson's' product-moment correlation, p -value significant at $p < .05$
[^]Correlation assessment by group for psychiatric measures and fMRI activation (dACC mean contrast)

6.6.5.4.2 Activation levels in the prediction of anxiety severity within groups

For the GAD group using HAM-A anxiety scores, no predictors were significant ($p = .947$). The Spielberger trait anxiety ANOVA model was also not significant ($p = .065$). For GAD, the Spielberger state anxiety ANOVA model found that fMRI contrast estimates significantly predicted state anxiety levels, $F(1, 24) = 5.58$, $p = .027$, whereby state scores decreased by 7.35 units for each increase in mean percent activation, 95% CI [-13.78, -0.93] (Table 34). In the model, gender was significant, $F(1,24) = 8.45$, $p = .008$, where males had anxiety levels 9.44 units lower than that of females, 95% CI [-16.14, -2.73]. Please refer to Table 34 for summary statistics.

Table 34. Factorial ANOVAs of BOLD Signal Prediction of Anxiety in GAD

Model 1 GAD group HAM-A	B/coefficient	95% CI	t	Sig.*
Comorbid presence	-1.01	-3.53, 1.50	-0.83	.415
Age	-0.08	-0.19, 0.03	-1.44	.163 ^b
Gender	0.37	-2.22, 2.97	0.29	.770
fMRI activation % mean contrast	0.08	-2.28, 2.44	0.07	.947
Model 2 GAD group - Trait	B/coefficient	95% CI	t	Sig.
Comorbid presence	-3.58	-9.55, 2.39	-1.25	.226
Age	-0.27	-0.55, 0.01	-1.99	.059 ^a
Gender	-2.09	-8.24, 4.06	-0.71	.487
fMRI activation % mean contrast	-5.67	11.72, 0.39	-1.95	.065 ^a
Model 3 GAD group - State	B/coefficient	95% CI	t	Sig.
Comorbid presence	-4.90	-11.51, 1.70	-1.53	.139
Age	0.06	-0.23, 0.35	0.41	.686
Gender	-9.43	-16.14, -2.73	-2.90	.008*
fMRI activation % mean contrast	-7.36	-13.78, -0.93	-2.36	.027*

*Significant at $p < .05$; ^a Marginal significance where $p = .05 - .10$; df degrees of freedom; p -value significant at $p < .05$

In the healthy control group, factorial ANOVA models indicated several significant predictors including fMRI contrast estimates and significant interactions between predictors for trait anxiety levels. For trait anxiety levels, all three predictors were significant in the model; gender $F(1,10) = 7.54$, $p = .021$; age $F(1, 10) = 7.42$, $p = .021$ and fMRI contrast estimates, $F(1,10) = 6.40$, $p = .030$. However, the prediction of anxiety from fMRI contrast estimates was influenced by both gender and age through significant interactions.

Gender and fMRI interaction were significant, $F(1,10) = 5.38$, $p = .043$, whereby trait scores increased in males only by 4.99 units with fMRI signal compared with 16.82 units in anxiety scores in females, 95% CI [0.67, 32.96].

Second, the age and fMRI interaction was also significant, $F(1,10) = 8.36$, $p = .016$, where trait anxiety level increased by 0.20 units with higher fMRI contrast estimates and increasing age, 95% CI [0.14, 1.12]. When observing interaction effects graphically, the trend appeared different in males; with a negative BOLD-anxiety association prior to age 45; which changed after the age of 45 years, where it became a positive trend. Pattern of age as a predictor was not observed in females. Table 35 indicates slopes/coefficients and Figure 32 illustrates the trend in the data for fMRI activation and slopes for age and gender in the prediction of trait anxiety scores.

In the HC model for state anxiety, fMRI contrast estimates predicted anxiety scores, $F(1,25) = 4.66$, $p = .041$, where anxiety scores dropped by 6.86 units for each unit increase (percentage mean contrast change) in fMRI signal activation, 95% CI [-13.41, -0.32]. Age was not significant in the model. Lastly, gender also predicted state anxiety levels, $F(1,25) = 6.54$, $p = .017$, where male scores were 8.31 units lower than those of females, 95% CI [-15.00, -1.62]. Table 35 also indicates the state anxiety model.

Table 35. Factorial ANOVAs of BOLD Signal Prediction of Anxiety in Healthy Controls

Model 1 Spielberger Trait	B/coefficient	95% CI	t	Sig.
Age	-0.43	-0.79, -0.08	-2.72	.021*
Gender	-11.83	33.94, 67.24	-2.74	.021*
fMRI activation % mean contrast	-28.65	-52.97, 4.33	-2.62	.030*
fMRI*Gender	16.82	-0.67, 32.97	3.32	.043*
fMRI*Age	0.63	0.14, 1.12	2.89	.016*
Model 2 Spielberger State	B/coefficient	95% CI	t	Sig.
Age	0.05	-0.24, 0.35	0.37	.715
Gender	-8.31	-15.00, 1.62	-2.56	.017*
fMRI activation % mean contrast	-6.86	-13.41, -0.32	-2.16	.041*

* Significant at $p < .05$; ° Marginal significance where $p = .05 - .10$; df degrees of freedom; p-value significant at $p < .05$

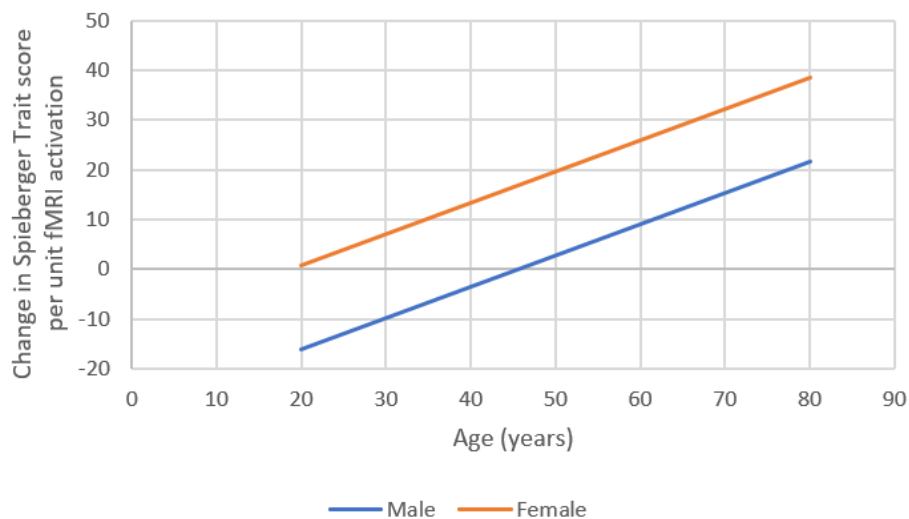


Figure 22. Trait scores in healthy controls, predicted by BOLD signal, gender and age

6.6.6 dACC functional activation and GABA level associations

Finally, to test for associations between fMRI contrast estimates and GABA levels within the dACC as ROI, correlational analyses were performed for GABA toolbox derived values (volume corrected where indicated). Results revealed no association between activation levels and GABA profile (please refer to reported statistics in Table 36).

Table 36. Correlations Between GABA and Functional Data in the ROI per Group

Toolbox data	fMRI activation (% mean contrast)					
	Healthy control		Pure GAD		GAD comorbid	
	r (df = 25)	p-value*	r (df = 19)	p-value*	r (df = 16)	p-value*
TARQUIN GABA/GM%	.07	.727	.07	.835	-.13	.630
LCModel GABA/GM%	.00	.982	.05	.884	.11	.690

*Significant at $p < .05$; df degrees of freedom; r: Pearson's product moment correlation test

Factorial ANOVAs were run to examine whether factors such as group, age, gender, and GABA levels in the ROI influenced task-based activation levels. ANOVAs were run for both GABA toolkit data. No models revealed significant predictors for fMRI activation as a function of GABA levels, psychiatric group, or age (refer to Table 37 for summary statistics).

Table 37. Predictors of BOLD Signal Percent Mean Contrast Change

Model 1 Tarquin GABA/gm%	B/coefficient	95% CI	t	p-value*
fMRI activation % mean contrast				
Group – healthy control	-0.02	-0.42, 0.38	-0.12	.916
Group – GAD/cm	0.24	-0.18, 0.66	1.16	.250
Age	0.00	-0.01, 0.01	-0.28	.782
GABA	0.00	-0.04, 0.05	0.23	.817
Model 2 LCModel GABA/gm%	B/coefficient	95% CI	t	p-value*
fMRI activation % mean contrast				
Group – healthy control	-0.14	-0.53, 0.25	-0.72	.475
Group – GAD/cm	0.17	-0.26, 0.60	0.81	.423
Age	0.00	-0.01, 0.01	-0.06	.950
GABA	0.03	-0.18, 0.24	0.31	.756

*Significant at $p < .05$; df degrees of freedom

6.7 Discussion

This section synthesises the main findings of the first study before addressing these findings against the hypotheses. It then discusses how the findings can be used to define GAD with the existing imaging literature. Limitations stemming from the design and the findings, as well as ramifications, are addressed together with findings in Chapter Seven, and in Chapter Eight (refer also to Figure 39 which illustrates both study findings).

As outlined above, this study examined group differences in GABA levels in the dorsal ACC region, and how GABA levels may be variable with measures of anxiety in both clinical anxiety and healthy controls. Secondly the study examined fMRI activation in: a) regions identified within-group; and b) significantly different between groups from the calculated contrast, c) differences in the ROI (dACC), and d) the relationship between fMRI contrasts in the ROI and anxiety in both GAD and healthy controls.

Finally, the study examined the influence of GABA levels on fMRI signalling (in the ROI) and feasible group differences in this relationship. Although not significant, marginal group differences were observed in GABA levels, with higher levels in GAD, with highest levels observed in GAD/comorbid participants. The relationship between GABA and anxiety displayed opposite patterns in healthy controls (negative) and GAD (positive) in the association analyses. Moreover, all groups showed variations in GABA in the prediction of anxiety levels. In the GAD group, GABA predicted anxiety symptoms on the HAM-A scale in a positive manner, and state anxiety was also positively predicted by GABA, but the pattern differed with comorbidity.

The fMRI data (contrast estimates) indicated increased BOLD signalling in three areas during the contrast (neu-neg) in the GAD group compared to healthy controls, suggesting a pattern of hyperactivation in GAD. In the region of interest this was not significant, however the pattern was similar. fMRI data also predicted state anxiety levels in both groups, and this relationship was modified by gender, age, and comorbidity in the GAD group. Finally, the group differences in the prediction of GABA on fMRI signal were not significant.

6.7.1 Demographic and psychiatric data

6.7.1.1 Group features

General demographic data satisfied the criteria of matched groups; age, education (in years), gender and weekly alcohol and caffeine amounts to minimise confounds unrelated to the main outcomes as best as practicable. A novel observation during the course of controlling for female menstrual cycles and scan scheduling was differences in menstrual cycle regularity in females with GAD compared with healthy controls; females with GAD reported greater fluctuations in cycle length and anticipated menstruation dates, which resulted in multiple rescheduling of scans. All scans were successfully taken within days one through 10, and while this observation was qualitative rather than measured directly, it suggests a relationship of anxiety to biology that warrants further investigation.

6.7.1.2 Medications

In the GAD group, approximately one-third of participants were taking GABAergic substances, either in analgesic, anxiolytic or insomnia indications. Efforts were made to ensure frequency was no more than three times weekly, and while none were taken in the week prior to testing, it is still feasible that GABAergic substances influenced group results by reducing variability.

Although there may have been acute modulations to GABA levels as a product of GABAergic substances, more longer term changes as a consequence of upregulation, such as in receptor densities are also equally likely (greater detail is found in Chapter Four) it should still be feasible to observe group differences, if the relationship between GABA and anxiety symptoms were to hold true, given the GAD group symptom frequency and severities reported through the psychiatric measures.

6.7.1.3 Comorbidity prevalence

In the GAD group, the prevalence of comorbidities was 57%, the most prevalent being agoraphobia, social anxiety, panic disorder and then PTSD. This figure is below the population comorbidity of 65% (NICE guideline, 2011a; Noyes, 2001; Nutt, Ballenger, Sheehan, & Wittchen, 2002; Moffit et al., 2007). Notably, this sample excluded clinical presentations of substance and/or alcohol use disorders, or somatic disorders (e.g. fibromyalgia, Cushing's disease, hyperthyroidism). Additional comorbid conditions may imply an increased severity of symptoms, where higher scores might be observed via the

SIGH-A HAM-A. As such, comorbid presence was defined either as a separate GAD group or used as a predictor in linear models. Separating by this definition produced interesting data for GABA and the relationship to anxiety across several of the psychiatric measures, particularly with the presence of a more state-based secondary anxiety disorder.

Few studies have examined comorbidity with GAD in neuroimaging. The prevalence of comorbidity in GAD is higher compared with other affective disorders, with comorbidity amplifying impairment (Noyes, 2001; Nutt et al., 2002), and so the research is valuable in examining biological patterns that may support differences determined by psychiatric measures.

The prevalence for social anxiety and panic disorder in the literature was replicated in the current study; however, the greater prominence of agoraphobia suggests a stronger prevalence with GAD. While few studies have specifically examined this relationship, many have noted the overlap (e.g. Noyes, 2001; Roth, 1984).

With regard to MDD history, 53% of cases in the present study is similar to the broader prevalence in the literature, and although minimally examined in the literature, the relationship to pure GAD was much lower, with 35% of participants with 'pure GAD' reporting MDD history (Bruce et al., 2001; Wittchen et al., 1994a)

MDD frequency in the present study, although recorded, was not analysed as a separate group, and MADRS scores needed to be less than 12, with no episodes two years prior. Refer to Chapter Eight under limitations and also in the future research section, where this data may be examined in future.

There is suggestion that GAD is a prodromal stage or subtype of MDD and should be subsumed under a revised definition of major depression or mixed anxiety-depression in diagnostic criteria (Borkovec, 2004; Stahl, 1993; Sutherland, Mewton, Slade, & Baillie, 2010). Imaging studies across several modalities supports MDD/GAD differences (refer to review by Martin, Ressler, Binder, & Nemeroff, 2009), such as error-related negativity in functional studies (for example, Weinberg, Klein, & Hajca, 2012); however, evidence also suggests overlap in these patterns (e.g. Oathes, Patenaude, Schatzberg, & Etkin, 2015). This is further discussed with the upcoming imaging results.

6.7.2 Psychiatric measures

The main psychiatric measure (SIGH-A) did not show differences as a function of comorbidity, however these cases scored higher, indicating increased symptom

frequency, intensity and severity in line with the point above regarding GAD comorbidity. Comorbid presence also did not yield differences to state or trait scores, however, suggesting that within-group GAD variances were not captured via these measures. GAD/comorbid group means were, as for the HAM-A, higher than for pure GAD.

In addition, in the state anxiety results, differences found in gender particularly in the GAD group reflect other studies/reviews reporting these trends (e.g. Howell, Brawman-Mintzer, Monnier, & Yonkers, 2001; Lewinsohn et al., 1998; McLean, Asnaani, Litz, & Hofmann, 2011), with increased prevalence and severity in females over males. Taken together with the other results, this suggests that gender symptom profiles are different in terms of frequency, intensity and severity. These differences are interesting components to GAD symptomologies that are less explored within and between gender biology beyond descriptive reporting in the literature. Gender will be an important consideration in future investigations over diagnosis and research regarding treatment interventions, where functional disablement and prevalence are higher in females.

6.7.3 Metabolite data

6.7.3.1 Volumetric findings

In the dACC region, no group differences were observed for grey matter volume. White matter data in the study indicated significant group differences, where the GAD sample had higher levels than the other groups. Age was also found to be a factor for increased white matter volume in the dACC. For CSF levels, significant group differences were found. CSF is typically negatively correlated to the proportion of regional white matter in the literature, where higher CSF reflects a reduced white matter proportion; a trend also occurs with age. In the present study, this negative relationship was also observed; however, the relationship direction was different between psychiatric groups. Healthy controls had significantly lower white matter levels and higher CSF levels, whereas in GAD, higher levels of white matter were observed with lower CSF and the CSF difference was influenced by gender with it only occurring in the male participants.

Although these results are interesting, they cannot be compared to the literature without further processing. The volumetric analysis was for the purposes of partial volume correction in deriving the GABA profile in grey matter and not strictly for structural differences. Moreover, ROI voxel registration and spatial smoothing were not conducted for spectroscopy data. The between-group volume differences in white

matter and CSF warrant investigation following application of further imaging pipeline processes.

6.7.3.2 Quality assessment parameters

Toolbox-specific quality parameters were useful to identify factors that could affect group comparisons of GABA levels. Despite removing scans of spurious quality group differences were still found for these variables.

Both the Tarquin and LCModel SNR data were significantly different between psychiatric groups. The SNR ratio indicates metabolite signal height to noise level and variances would indicate scan-specific events such as increased movement in the scanner, resulting in reduced signal quality. Group differences were not uniform—healthy controls, for example, were lowest in Tarquin, but not in LCModel.

That LCModel and Tarquin measures of quality were different may be due to toolbox assumptions such as an increased emphasis on baseline uncertainty in Tarquin, where values tend to be larger (see Chapter Eight for discussion). However, SD% and Fit Q were not different between groups, as well as manual inspection and removal of problem cases; therefore, it is unlikely that these differences influenced analyses.

FWHM in LCModel was different between groups - the GAD groups had the highest and lowest mean levels, with healthy controls in the middle. Typically, FWHM quantifies the signal decay rate in the time-domain as a result of shimming. In summary, two signal quality parameters across the toolboxes were found to be different among groups, implying a better signal overall in the pure GAD group scans. However, these parameters were not used as covariates in modelling because it was unlikely to influence actual GABA level difference among the groups.

6.7.3.3 GABA group differences

Group-based GABA differences in the dACC, including GAD/comorbidity compared with pure GAD and healthy controls, were not significantly different among groups, with only the secondary GABA toolbox, LCModel, producing a marginal significance. Additionally, the means of the groups were different; Tarquin as the main outcome had elevated levels in GAD groups, whereas LCModel, healthy controls showed higher GABA levels than both the GAD groups. LCModel data was in line with the directional hypothesis of reduced levels in GAD compared with healthy controls. This difference warrants further exploration to ascertain why the toolkits produced contradictory trends, as the results are equivocal.

The literature indicates a pattern of reduced GABA levels in anxiety disorders compared with healthy control samples (refer Chapter Four, Section 4.5.6 for details). Several explanations are put forward for GABA attenuation but the most common relates to a reduction in GABA receptor numbers and/or GABA receptor binding processes (Nikolaus, Antke, Neu, & Muller, 2010; Smith, 2010).

A large body of this evidence is derived from rodent models of acute stress (Braestrup et al., 1979; Tasan et al., 2011) and some evidence exists from PET and SPECT studies in affective disorders, such as panic disorder (Goddard et al., 2001), with just one study supporting reduced GABA binding in GAD (Tiihonen et al., 1997). In panic disorder, where GABA has been studied more extensively, deficits are reported via processes such as GABA-A subunit receptor binding and benzodiazepine reuptake (Bremner et al., 2000; Kaschka, Feistel, & Ebert, 1993; Nutt, 2001).

Evidence for receptor dysfunction is one reason proposed as underpinning clinical phenomena such as increased tolerance to GABAergic benzodiazepines in anxiety disorder patients, and also observed in GAD (Tallman, Thomas, & Gallagher, 1978), measured via eye/visual saccade velocity that is typically reduced following benzodiazepine treatment (Kroboth et al., 1998), thereby suggesting a reduction in GABA-A site sensitivity. One limitation to this conclusion of a possible marker in GAD is that GABA-A dysfunction is not exclusive to GAD, or even affective disorders; and has been reported in schizophrenia, body dysmorphic disorders and major depression samples (Gerner & Hare, 1981; Nuss, 2015).

Conclusions of GABA data group differences, although promising, are limited without toolbox congruence and/or clear significance. Subsequent analyses indicate the inclusion of other factors, such as age and gender and anxiety levels, rather than clinical grouping alone, better clarifies GABA patterns in this region. Limitations of the toolboxes processed are examined further in Chapter 8.

6.7.3.4 Anxiety severity and GABA levels

To examine the second hypothesis of a negative relationship between anxiety severity and GABA level, analyses were performed within-groups using the HAM-A symptomatic scale and STAI scales in GAD, and only STAI scale in the healthy controls.

Group-specific trend tests found significant relationships between anxiety levels and GABA in the ROI, but in a different trend for GAD groups and in healthy controls. Only healthy control group fulfilled the hypothesis of a negative relationship, on both scales.

In the two GAD groups, pure GAD without comorbidity showed a positive relationship between GABA and state anxiety levels. In GAD/comorbid participants, anxiety severity was positively associated with GABA levels but only via the HAM-A, where higher anxiety occurred with higher GABA levels. Neither GAD group showed a significant GABA-trait anxiety relationship.

One influence on anxiety outcomes might be the anxiety scales selected and their method of administration. The HAM-A is researcher-administered and symptom-based, whereas the STAI measures are self-reported and measure more or less enduring levels of anxious mood. The manner in which the clinical groups self-reported anxiety symptoms versus the interview format of the HAM-A (and reporting on subjective mood rather than quantifying anxiety symptoms) may affect the way the data are reported and may reduce the ability to compare data across measures and clinical groups because they may not be quantifying the same anxiety symptom and/or mood phenomena.

That only the GAD/comorbid group showed a significant GABA-HAM-A relationship as the main anxiety outcome, is possibly reflective of symptom severity that this scale captures. The lack of trait anxiety associations suggests that in GAD, where there are already high scores on the scales, there is also little variability in these elevated levels.

The factorial ANOVA models that incorporated other biological predictors, appeared better equipped to establish influences on GABA levels following the simple correlations deriving trends in the data within and across groups. The healthy control group trait and state anxiety was predicted by Tarquin GABA in a negative manner in the ANOVA models that replicated the associations tests, and with no other predictors.

The GAD group showed a significant positive relationship between HAM-A anxiety and Tarquin GABA as the main outcome, and this relationship was amplified by the presence of anxiety disorder comorbidities. Additionally the GAD group showed a significant prediction of state anxiety from GABA change in the same manner, with modifications from gender in Tarquin GABA, and the contradictory trend that was shown with comorbidity, displayed in LCMoel GABA.

There are several explanations for the observed biological results. GAD participants may have less quantities in the substrate, either due to more requirements to inhibit neural signalling and/or we are observing cases where, in the ROI, there is less efficient production of GABA, perhaps a faster step in the metabolic shunt such that in clinical anxiety these metabolic processes are somewhat inefficient within pathway

components, resulting in 'suboptimal' use of GABA, and thus producing a shortage of GABA levels.

If such a relationship in the data is an accurate indicator of GABA in the presence of measurable anxiety symptoms in healthy controls, it suggests a compensatory role of GABA in the ROI where in the presence of increased anxiety cognitions and/or processes related to anxiety, increased GABA is required to maintain adequate inhibition. It is difficult to account for the factor of time; only repeated measures could address whether group levels were an indication of profiles present in participants with higher anxiety or if, in these samples, we are observing anxiety and GABA levels at the time of the scan, for which scan novelty becomes an influencing factor.

In the literature, a positive association for trait (Spielberger) anxiety and GABA/Cr ratio in the vmPFC region in healthy samples has been reported (Delli Pizzi et al., 2015), showing a different trend to the controls in the present study, albeit in a different ROI. Closer to clinical sample evidence, in rodent models, high anxiety-behaviour mice also had higher levels of amygdalar GABA and greater expression of GABA pathway components GAD65 and GAD67 (the influence of which was reported in Chapter Four) than normal anxiety-behaviour mice (Tasan et al., 2011) and mice with knockout $\gamma 2$ subunit of GABA-A receptors showed increased anxiety associated with this reduced function (Earnheart et al., 2007). These studies are congruent with findings in the GAD group in the present study, supporting a compensatory GABAergic mechanism in cases of higher levels of anxiety.

The literature evidence regarding acute modulations to GABA levels is equivocal, mostly suggesting that GABA is fairly invariable over time and not necessarily amenable to state or acute environmental changes. A recent study in healthy males ($n = 29$; Houtepen et al., 2017) that reported state (psychosocial) stress did not attenuate GABA levels (nor glutamate level) in the mPFC (7 tesla) supports this assertion.

However, there is also evidence of variability, where context-dependent anxiety has been shown to modulate mPFC levels (GABA-creatine ratio) in healthy participants, in a shock-threat/non-threat paradigm, where levels were significantly lower prior to, and during, the shock-threat condition than the non-threat condition scans (Hasler et al., 2007). This study evidenced a rapid turnover within the GABA shunt via animal and human metabolic studies, substantiating the claim that it is rapid enough to account for measurable changes observed in the study data. For further analysis here, it would be worth splitting healthy control participants into high and low state and trait anxiety

levels and examining this association with GABA levels repeatedly, which might clarify such a trend in the data.

All significant associations in GAD were positive, which suggests that higher levels of anxiety are associated with systemic increases in GABA in this region, whereas in healthy controls, the relationship was opposite, producing a potential marker of difference. If this region is involved in clinical anxiety cognitions, it reflects a need for higher GABA, possibly due to the need for inhibition of neural signalling that underpins dACC cognitive processes, with this pattern feasibly related to the maintenance of anxiety cognitions.

Finally, the data here, although inconsistent, does suggest a relationship of inhibitory function measured through GABA levels and anxiety levels across both pure GAD, GAD with comorbidity and healthy controls. It also suggests that GABA profiles are variant within a clinical group, but also outside of diagnostic groups, which is much the same as the patterns within the literature. The latter findings prompt investigations with different anxiety scales and the former suggest the methodological framework employed here should be extended to include other affective disorder samples to reinforce the findings observed between GAD and healthy control samples.

6.7.4 Functional task-dependent BOLD signal activation data

This investigation examined neural activity associated with a task that aimed to recruit appraisal and judgement in the processing of emotional stimuli. The intention of the task was to elicit the hallmark traits of GAD, anticipatory anxiety and persistent worry. To this end, a paradigm comprising valence anticipation and appraisal, with maximised expectation effects using a randomised congruence and valence format, and jittered wait time following the cue was used. It was hypothesised that BOLD activation in the dACC region would be greater in the high emotionally valent condition compared to a neutral condition, and further, that this effect would be greater in GAD.

The first analysis exposed several regions within each group that differed in activation between conditions. The contrast analysis found two significantly different regions between the groups, and finally, the region of interest was not significantly different between groups, but analyses of fMRI data and anxiety produced significant group differences.

The Valent IAPS congruency task (VICT) paradigm was successful in eliciting clear task-dependent signalling differences produced by the conditions and contrasts. It was

successful in doing so both within-group and in demonstrating between-group differences between GAD and healthy control groups.

6.7.4.1.1 Within-group contrast level differences

6.7.4.1.1.1 Healthy control findings

The significant activations revealed that contrast condition differences occurred in the left middle temporal gyrus, right superior parietal lobule, left parietal postcentral gyrus/primary somatosensory cortex and the left parietal lobe/precuneus. This network is associated with task processing (Cabeza & Nyberg, 2000; Passingham, Stefan & Kotter, 2002) rather than emotion, fear or stress processing - despite the activation derived from the contrast as thought to indicate heightened anxiety/worry.

In terms of cognitive-emotion processes associated with the identified regions, the middle temporal gyrus has a role in semantic memory processing, visual perception and multimodal sensory integration (Whitney, Kirk, O'Sullivan, Ralph, & Jeffries, 2010). Perceptual memory, occurring with ambiguous stimuli held in working memory and relevant to the current paradigm, is also associated with activation here (Brascamp, Kanai, Walsh, & van Ee, 2010). The superior parietal lobule involvement with spatial orientation and visual input as well as sensory input, is congruent with visual and motor processes involved in performing the task, possibly from hand activity with the button box response (Cabeza & Nyberg, 2000).

The postcentral gyrus as the primary somatosensory region hosts bodily proprioception, location, touch and space feedback, which are also relevant to task-dependent attention and subsequent action (Drevets et al., 1995; Kastrup et al., 2008; Polonara, Fabri, Manzoni, & Salvolini, 1999). Lastly, the parietal lobe/precuneus, which is significant owing to functional association with the aforementioned superior parietal lobule and within the parietal lobe, and because they hold roles in visuo-spatial imagery, episodic memory retrieval and self-processing operations.

The second analysis further revealed the right fusiform (subregion for middle temporal gyrus) and the left inferior parietal lobule. While the functionality of the fusiform gyrus is not fully understood, it has been strongly linked face processing, and is evidenced as a region exhibiting hyperactivation with fearful stimuli in social anxiety disorder (Frick, Howner, Fischer, Kristiansson & Furmark, 2013).

Collectively, most of these regions comprise visual-cortical spatial-sensory network/s that are connected functionally for visual task appraisal and response (Beckmann et al., 2005; Smith et al., 2009).

It was expected that regions that have roles in cognitive processes related to anxiety would exhibit increased activation in the high emotion condition. Such an activation was expected to reflect concern over the correct response, expectation of negative stimuli, judgement of valence severity, as well as individual qualitative judgement of the valent material including memory retrieval and aversion response. It does not imply that regions associated with anxiety were not also activated during the task conditions, however, which is likely to be the case that cingulate, prefrontal regions or limbic regions were recruited more uniformly across all conditions.

6.7.4.1.1.2 GAD findings

The GAD group exhibited increased activation in the right fusiform gyrus and left inferior occipital gyrus. The left inferior occipital gyrus is associated with visual system processing, particularly of faces (Rossion, Schiltz, & Crommelinck, 2003), connected to the striatum and the ipsilateral superior parietal lobule, and also associated with mental imagery and inferential reasoning processes.

Compared to the GAD group, the pattern of whole brain activation observed in the healthy control group could exemplify normal function during the task components, such as stimuli perception, valency and congruency, appraisal and response. This group may have the capacity to adequately recruit these regions in states of anticipation and worry and hence a broader recruitment of regions in at least one condition of valence was observed. The reduced number of activated regions in the GAD group compared with the healthy group may indicate altered processing in GAD, or that these regions are not different in their activation between the contrasted conditions, resulting in minimal contrast activation.

6.7.4.1.2 Across group contrast-level differences

In the first analysis of group differences in the contrast, the DLPFC and the IPL were revealed to be different. Functionally, the DLPFC, is associated with self-reflection and introspection including the monitoring of cognitions and emotions, and DLPFC hyperactivation in GAD has been reported during valent word paradigm (Zhao et al., 2007), in a worry paradigm using an older sample (60 years and above; Mohlmann et al., 2017), and during threat processing (Buff et al., 2016).

These previous findings accord well with the current work where, in comparison to healthy controls, increased BOLD was observed in DLPFC in a high emotional valence condition compared to a neutral condition. That is, compared with the healthy controls, GAD patients may exhibit increased decision-making when presented with valent material. Activity in this area may be consistent with the theory that anxiety affects cognitive performance and may do so through reducing the capacity to consistently maintain and/or fluidly shift attention that these areas are functionally associated. It may do so through the involvement of cognitive traits such as negative bias and it may impede processing of incongruent conditions, hence requiring greater resource recruitment to focus and respond correctly.

The regions may show greater activity as the brain recruits more resources to maintain the same levels of regulation, appraisal and correct response as the healthy controls. It may support the findings summarised in the Mochcovitch et al. (2014) review whereby emotional dysregulation is evidenced through activation differences in prefrontal and anterior cingulate cortices in GAD. In GAD, functional imaging studies indicate that increased PFC activity may have a compensatory role in reducing symptoms (Stein, 2009). Etkin et al. (2010) suggested that PFC regions are unable to 'dampen down' function in accessory regions such as the amygdala and it is this process - or lack thereof - that contributes to anxiety states inherent to GAD cognitive symptoms.

The inferior parietal lobule has roles in visuospatial processing, object use, and cognitive control functions, including within social contexts, and it is often co-activated with the dLPFC (Singh-Curry & Husain, 2009; Zhang & Li, 2014). The literature suggests deactivation during processing of valent tasks in healthy samples, and hyperactivation in GAD during the resting state (Cui et al., 2016) and evidences signalling differences within mood and anxiety disorders such as MDD, and SAD (Goldin et al., 2009; Irle, Barke, Lange, & Ruhleder, 2014; Peng et al., 2015), and subsequently the literature generally supports the observed patterns for the healthy controls and GAD groups in the present study.

A third region, the mid-cingulate was identified as being significantly different in the less conservative second analysis of group differences. This brain area is connected to the amygdala and inferior parietal cortex (Vogt & Palomero-Gallagher, 2012) and has roles in monitoring, decision making and response selection (including motor), motivation and goal orientation. Human and primate studies also suggest it is active in paradigms of social context (Decety et al., 1994; Talairach et al., 1973 1973; Vogt, 1995).

The literature suggests it is not involved in valency appraisal tasks such as counting Stroop, but it is active during non-valent Stroop paradigms, and the appraisal of other's intentions (Frith & Frith, 2003).

In prior GAD studies, posterior mid-cingulate regions exhibited hyperactivation in threat versus neutral valent picture tasks compared with healthy controls, but also compared with social anxiety and panic disorder groups (e.g., Buff et al., 2016). The current findings, together with the literature, suggests the patterns in the mid-cingulate represents a region for symptoms of unique GAD cognitive processes such as negative bias, compared with healthy controls, but also other affective disorders.

Given these observations, the data most likely indicate heightened levels of anticipation of a negative match in cue/stimulus and persistent expectation of an unpredictable condition, and in doing do, could represent the processes underpinning anticipatory anxiety and persistent worry observed in GAD, maintained through elevated BOLD intensity in these regions. However, in healthy participants, the ability for these areas to 'de-activate', or to regulate an internal brain state efficiently by comparison, would be tenable. This might reflect the capacity in healthy samples to 'dampen down' emotional responses to stimuli and focus on the cognitive component of correct response. In affective disorders such as GAD, it may be useful to focus on correct response given the valent material, particularly where the cue and stimuli are incongruent, or they are both negative, suggesting a reduced capacity to regulate internal brain cognitive-emotional states. Furthermore, worry as a cognitive process in GAD may 'compete' for the same prefrontal cortical areas typically involved in adaptive regulation of emotion, through cognitive and behavioural strategies, and a requirement in the current task involving decision-making in a valent context.

Finally, the activation of these three regions (DLPFC, IPL and mid-cingulate) are all connected via a frontal-parietal-limbic white matter tract network. Increased activation in this network in GAD compared with healthy controls implies increased recruitment of resources in that group for performance of this task, feasibly making task processing and/or attention to the task more effortful.

6.7.4.1.3 dACC BOLD signal group differences

The second and subsequent functional hypotheses focussed on the group differences in the contrast of the dACC subregion. As discussed in Chapter Four, this subregion substrates the amygdala and consequently holds a crucial role in emotion regulation, yet

is an important interface between cognition and emotion alone (Mathews et al., 2004) and particularly so during demanding cognitive tasks involving valent elements (see paper by Drevets & Raichle, 1998).

In the present study, significant dACC BOLD signal increases were not observed for each group separately in the between group analyses, although GAD participants did exhibit slightly larger contrast estimates in that region than controls which was consistent with the hypothesis.

One study has examined this region in GAD, and produced different findings. Blair et al. (2012) also employed emotional regulation during the appraisal of valent pictures, reporting reduced dACC activation in GAD compared with healthy controls, as well as differential results for the comorbid GAD/social phobia group. The authors suggested that decreased activation equated to regional deficit during emotional regulation. Their task differed slightly from the current paradigm, as for the picture task using emotion regulation, it was without an additional congruency matching element. Feasibly, the additional cognitive load in the current paradigm contributed to the different results seen in the present study.

6.7.4.1.3.1 ROI BOLD signal and psychiatric measures

In the association analyses, none were significant, however the models exposed fMRI signal as a predictor of anxiety, suggesting that anxiety levels are related to BOLD signal in a different manner for each group, and are amenable to age and gender influences.

In the present study, the fMRI contrast estimates were negatively correlated with state anxiety in both groups, suggesting that acute state anxiety—in this case, in response to undergoing an MRI scan session—feasibly holds a stronger relationship to BOLD signal than does trait anxiety, or anxiety symptoms via the HAM-A in GAD.

The HAM-A model of fMRI signal prediction was not significant in GAD; it is possible the HAM-A, in asking participants to relate symptoms ‘from the past two weeks including today’, quantified through frequency, intensity and severity, recorded a less acute symptom profile, with a greater somatic element, whereas the Spielberger scales collected symptoms of the day and were mostly mood/emotional/cognitive anxiety items. The measure of state anxiety was conducted immediately preceding the scan, but the activation levels were task-dependent. The relationship, therefore, suggests that higher reported state anxiety could be somewhat predictive of affective processing

during the task. In addition, the VICT task was completed approximately 40 minutes after the state anxiety measure.

The fMRI contrast scores in predicting state anxiety were also different with genders in both groups, suggesting that gender distinction in acute anxiety is partly biological and observable across both clinical and healthy groups. The literature supports elevated anxiety levels in females, suggesting that gender differences are a strong biological marker of anxiety severity and probably type, with this trend observed in both clinical and non-clinical samples. There is insufficient research in the literature to indicate there are differences in fMRI signalling in anxiety groups or anxiety paradigms in this region, and as discussed in earlier chapters, the knowledge base of gender-specific diagnoses and treatment within GAD is limited.

Additionally, trait anxiety severity in both groups showed a negative relationship with age; in the literature older samples have lower anxiety than their younger cohorts, although functional disablement tends to be greater (Bandelow & Michaelis, 2015; Wittchen, 2005). The data, as well as the literature suggest that age may have a protective effect on trait-level anxiety. For the healthy controls, the model was successful in delineating trends influenced by factors such as age and gender additional to the BOLD signal; within females the relationship was positive with increased age, yet in males, a negative relationship was observed until middle age (45 years), thereafter a positive relationship was seen. If the BOLD signal within the ROI is an indication of anxiety cognitions, then increased activation in females could contribute to the maintenance of enduring anxiety cognitions.

The pattern in males that shifts with middle age might be an indicator of increased anxiety cognitions, or at least, an increased need in the ROI to recruit resources for optimal task-based performance. BOLD signalling reportedly decreases with age, including within the anterior cingulate (Tsvetanov et al., 2015), although cardiovascular health or volumetric changes represents confounds to the measurement of true neural signalling with older samples (refer to review by D'Esposito, Deouell, & Gazzaley, 2003).

The data suggested state-based mood holds a stronger relationship to our linear contrast estimates that putatively capture affective processes and feasibly is a more reliable indicator of anxiety severity, not only in GAD, but also in healthy controls. Therefore, the dACC region is involved in processes of state-based anxiety, perhaps through the function of self-reference and where the state context was entering the scanner session. Although the results support a prediction of anxiety levels from fMRI

activation in the ROI, impacted also by other biological factors, may be conjecture, due to the region not displaying significant group differences in the previous hypotheses.

6.7.4.1.4 ROI functional activation data combined with GABA metabolite data

In the bi-modal analysis, the hypothesised relationship between the BOLD signal and GABA levels was not found in the ROI. A relationship between regional BOLD activation and GABA levels is indicated in the literature, where it has been proposed that negative BOLD responses are mediated by increased GABA transmission via the inhibitory mechanisms of GABA on neural signalling and subsequent reduction of BOLD activation (Puts & Edden, 2012b; Rae, 2014) and evidenced in multimodal studies within the ACC region. Pairing GABA levels to task-based BOLD signal activation in regions of interest to substantiate findings in each marker is recommended in studies such as Puts and Edden (2012b), with general support for a stronger negative BOLD signal was seen in participants with higher GABA.

Northoff et al. (2007) conducted a combined BOLD signal and GABA study focussed on the ACC region and reported that resting-state GABA predicted a negative BOLD response during the viewing of valent pictures, such that higher GABA levels were associated with a larger negative BOLD response in the same region. This suggests that negative BOLD responses are tightly coupled to reductions in neural activity, which is a mechanism of GABA. In addition, for task-related activation where BOLD signalling elicits positive responses, this signalling is much larger in the presence of reduced GABA levels (Muthukumaraswamy et al., 2012).

The assessment of regional changes in metabolites relative to task-dependent activity and also from endemic or intrinsic activity was examined in one review (Duncan et al., 2014), which demonstrated how bimodal studies might correlate data to gauge cognitive processes. This review examined 35 studies of healthy controls that used Glu/Glx and/or GABA-MRS and PET, together with fMRI, magnetoencephalography (MEG) and electroencephalography (EEG) task data, with the aim of examining the relationship between BOLD activity and metabolite levels. The data were grouped together by region such as the occipital, sensorimotor and visual cortices, the insula and, importantly, ACC/mPFC, with the dACC grouped separately. Seven studies pertained to the ACC/mPFC region, five of GABA and two were of the dACC; however, neither examined GABA (see Table 6). Summary findings were of a region-specific negative relationship between GABA and task-dependent activity and a positive one between

glutamate and inter-regional activation derived from the resting state and functional connectivity data. The review surmised that GABA was reduced where BOLD activity increases and that GABA profile changes reflect GABA substrate signalling, which is used in the process of brain activity underpinning cognition that is also indicated through BOLD signal changes.

Thus, the present study's findings differ with some aspects of the literature, but as with metabolic and functional data separately, there is a paucity of work with clinical groups such as GAD to lend comparisons and the literature is not conclusive on a demonstrable relationship between GABA concentration and task-dependent BOLD signalling. One study (Harris et al., 2015) found no associations in occipital, somatosensory, DLPFC regions between metabolites and activation. Region-specific, in depression, BOLD signalling in the pgACC holds no relationship to glutamate (Walter et al., 2009), which represents one of the few findings consistent with the present study. Most other multimodal GAD imaging research has been conducted in the visual cortex.

6.7.4.1.5 Summary of the functional data

The VICT contrast was successful in exhibiting different patterns for each group. Group-specific activations occurred in regions that are involved in executive function mechanisms involved in task performance. The GAD sample did not exhibit the same pattern in regional activation, which suggests reduced within-group variability in executive function processes relative to context in clinical anxiety. In addition, group data differences were found in the dlPFC, IPL and mid-cingulate, where the healthy control group had reduced activity and GAD group had increased activity, with the former greater than the latter. These regions indicate that the processes that involve the frontal-parietal network between the groups may be different. Where healthy controls were able to variably recruit these regions, the GAD group displayed heightened activation in these regions. Taken together, these results suggest that compared to healthy controls, GAD patients allocated greater cognitive resources, evidenced by greater frontal-parietal network activation, to perform the valent congruency task.

The dACC region, hypothesised to exhibit group differences, was not shown to be different with BOLD signalling. However, the region's activity was more strongly associated with anxiety levels, occurring in different patterns across clinical groups and influenced by age and gender. In both groups, state anxiety held the greatest relationship to fMRI contrast scores, suggesting that a reduced signal is observed with

higher anxiety levels. The pattern of trait anxiety levels from BOLD signal in healthy controls exhibited differences between males and females, with males in particular showing differences in middle age. Thus, the data suggest the dACC region may have a role in the maintenance of anxiety levels both state-based and also more enduring trait levels. Given the function of this region and the parameters of the VICT task, BOLD signalling in this region exhibit a neuro-biomarker of anxiety severity that is not necessarily specific to a clinical group.

7 Chapter Seven. Investigation of GAD Biomarker Modulation from Kava (*Piper methysticum*)

7.1 Overview

This chapter follows on from the previous cross-sectional investigation whereby it details the second investigation, which is an intervention study. This investigation represents a 'proof of concept', as it aims to test the validity of both the functional and metabolic findings via predicted modulations following an anxiolytic intervention (Kava) which is a known GABA pathway modulator.

7.2 Introduction

As detailed in the introduction, phytomedicines and their bioactive constituents with known GABAergic mechanisms such as *P. methysticum* (Kava) possess anxiolytic properties previously shown to be efficacious in alleviating GAD symptoms. However, to date, measuring intervention-based neurobiological modulations through imaging in GAD is absent. Furthermore, biomarker identification as a component of symptom response/remission for the efficacy of anxiolytic phytomedicines such as Kava in human clinical samples has not been reported. As such, this investigation represents a unique and evidenced-based approach to addressing this literature gap for GABAergic phytomedicines, Kava, and GAD biomarkers of treatment response.

7.3 Main Aims and Hypotheses

This investigation assessed MRS GABA metabolite levels and fMRI task-based BOLD activation in the dACC region associated with *P. methysticum* extract treatment in patients with diagnosed GAD compared with placebo for eight weeks. It also assessed whether such changes in fMRI and MRS outcomes are markers of phytomedicine anxiolytic activity.

The following directional hypotheses were investigated:

1. Anxiety symptom severity as measured by the SIGH-A HAM-A will be significantly lower in the active treatment group compared to the placebo group following eight weeks' treatment.
2. The dACC GABA metabolite levels will be significantly reduced at eight weeks in the active treatment group compared to the placebo group, reflecting the effect of *P. methysticum* extract treatment in 'normalising' levels, as observed in the group

differences in the previous, cross-sectional study where healthy control levels were lower than the GAD group.

3. Changes to anxiety symptom levels via the SIGH-A HAM-A as a function of active treatment will be associated with MRS-measured GABA level in the ROI; specifically, anxiety symptoms will decrease with GABA decrease, reflecting normalising' of levels based on the comparison to healthy controls in the previous study.

4. Task-dependent BOLD activation levels will differ in the dACC as the ROI, whereby an increased negative BOLD signal will be associated with *P. methysticum* extract, measured after eight weeks' treatment.

5. Reductions to anxiety symptom levels as a function of active treatment as reported by the SIGH-A will be associated with reduced BOLD activation levels during the anxiety cognition task.

7.4 Methods

7.4.1 Design overview

This was a sub-study that was an eight-week attenuation of a 16-week randomised, double-blinded and placebo-controlled study examining the effects of a 240 mg daily kavalactones dose or matched placebo in participants aged 18–65 years old with diagnosed GAD ($n = 40$; main study $n = 171$) who were non-medicated/untreated for anxiety symptoms. Eight weeks was determined to be a suitable time frame for neurobiological effects of kava and quantification of GABA (Mullins et al., 2014; Sarris et al., 2009). The sample size from this study involved 40 participants who underwent the neuroimaging component. The primary outcome measures were anxiety symptom change as measured via the SIGH-A, BOLD activation levels, and metabolic profile measured using fMRI and MRS, respectively, in the dACC region.

For complete details of the clinical trial investigation, please refer to the published protocol paper (authored by the PhD candidate) for the overarching clinical trial methodology, *Kava for the Treatment of Generalised Anxiety Disorder: An 18-week Double-Blind Randomised Placebo-Controlled Trial*, in Appendix F.

Unless otherwise stated here, the Methods and Procedures used were identical to those used in the first study.

7.4.2 Recruitment and participants

Participants were recruited by the same methods detailed in the previous study.

Inclusion and exclusion criteria for participants are indicated in Table 38.

7.4.2.1 Female participants

The second scanning session occurred exactly eight weeks following the first scan.

Participants were provided with a menstrual cycle tracker accompanying the treatment compliance log sheet to aid in tracking cycles.

7.4.3 Sample size

With a population sample of $n = 40$ ($n = 20$ per group), a minimum sample size of $n = 37$ was anticipated to interpret treatment effect results of a 95% confidence interval and 5% margin of error (for significance of $p < .05$), and the number of analyses in each hypothesis (calculated for repeated measures, using GPower; Faul et al., 2007).

Table 38. Enrolment Criteria for GAD Intervention Study, Including Imaging

Inclusion Criteria
As above for demographic and medical, plus; • Meets the DSM-IV and DSM-5 diagnostic criteria for generalised anxiety disorder (GAD) based on structured interview MINI 6.0. Note that while the MINI 6.0 uses the DSM-IV criteria, the same criteria are used in the DSM-5) • Presentation with anxiety (SIGH-A ≥ 18) at the time of study entry • Presentation of mild depressive symptoms (SIGMA ≥ 12) at time of study entry
Exclusion Criteria
• Primary diagnosis other than GAD • Presentation of moderate to severe depressive symptoms (SIGMA ≥ 12) at time of study entry • Presentation of suicidal ideation (≥ 3 on SIGMA suicidal thoughts domain) at time of study entry • Current diagnosis of a psychotic disorder (bipolar disorder I, schizophrenia) on structured interview (MINI 6.0) • Current or past diagnosis of obsessive compulsive disorder or bipolar I or II disorders • Current substance/alcohol use disorder on structured interview (MINI 6.0) • Currently taking an antidepressant, mood stabiliser, antipsychotic, anticonvulsant, warfarin, or thyroxine, or regularly using a benzodiazepine or opioid-based analgesic (more than 2 days per week) • Current use of St John's wort • Three or more failed trials of pharmacotherapy for the current GAD episode • Recently commenced psychotherapy (within four weeks of study entry) • Known or suspected clinically unstable systemic medical disorder • Pregnancy or breastfeeding, or trying to conceive • Not using a medically approved form of contraception (including abstinence) if female and of childbearing age • Unable to participate in all scheduled visits, treatment plan, tests, and other trial procedures according to the protocol
Imaging eligibility criteria - exclusions

• Left handed • Pacemaker • Infusion pumps • Aneurysm clips • Metal prostheses • Metal joints • Metal rods • Metal plates • Metal staples • Non-removable body piercings • Persons who have worked as welders or may have metal splinters in their body • For females: taking hormone-modulating contraception including oral contraceptive pill or implants • Females: regular menstrual cycle in order to conduct scans within days 1-10
Post-enrolment subject withdrawal criteria
• Change of medication status (e.g., started antidepressants) • Change of psychotherapy status (commenced or ceased) • Increase in depression symptoms (SIGMA > 24) during enrolment • Increase in suicide ideation (> 3 on SIGMA suicidal thoughts domain) during enrolment • Significantly raised liver enzymes (2.5 or above upper normal limit) • Cessation or commencement of effective contraception or pregnancy • Withdrawal of consent • Emergence of adverse events (either by participant or investigators) • Noncompliance with treatment schedule

7.4.4 Treatment

Both the researchers and participants were fully blinded to group allocation. Participants were allocated a treatment code sequentially by a researcher blinded to the randomisation schedule using computerised permuted 3 × 2 block randomisation, for example, ABAAABABBAB. Further information can be viewed in the Appendix F protocol previously detailed in Section 7.4.1.

7.4.5 Measures

An identical timeline of study enrolment and assessments during visits occurred as in the previous study (Table 39); and also illustrated as a timeline (Figure 33).

Table 39. Assessment Timeline Summary

Measure	Week 0	Week 8
Participant Information and Informed Consent	x	
Demographics	x	
Health and medications questionnaire	x	x
Psychiatric Assessment		
Mini International Neuropsychiatric Interview (MINI 6.0)	x	x
Structured interview for the Montgomery-Asberg Depression Rating Scale (MADRS)	x	x

Structured interview for the Hamilton Anxiety Scale (HAM-A)	x	x
Spielberger State-Trait Anxiety Inventory – Trait component	x	
Spielberger State-Trait Anxiety Inventory – State component	x ¹	x ¹
MRI-15 Pre-scan questionnaire	x ¹	x ¹
Edinburgh Handedness Inventory (EHI)	x	
Resting state/structural	x	x
MRS of ACC (GABA/NAA/Glutathione/Creatine/Glutamate-Glutamine)	x	x
Functional MRI during IAPS-based task	x	x
¹ Before and after the MRI scan		

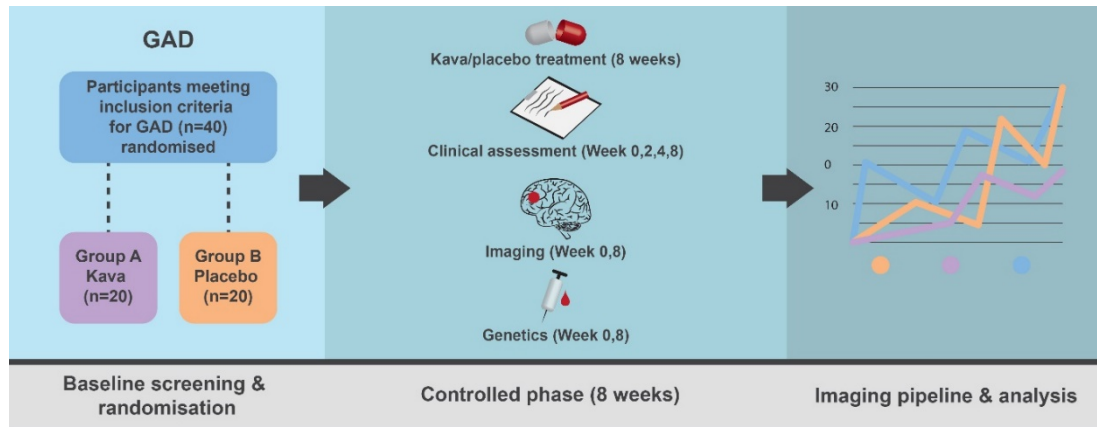


Figure 23. Trial timeline illustration for the intervention study

7.4.6 Neuroimaging

7.4.6.1 Procedure overview

Participants underwent MRI scanning session to investigate the brain's structural, metabolite and task-based BOLD changes prior to, and eight weeks following, the treatment intervention.

7.4.6.2 Scanning experiments

7.4.6.2.1 Magnetic resonance spectroscopy (MRS) of the dorsal anterior cingulate cortex (dACC)

Procedures as per the previous study, section 6.4.6. Importantly, for this study component, as Kava administration has been reported to modulate GABA pathway metabolic processes, changes to the properties of GABA metabolites in the ACC region as a product of the intervention were measured via MRS (Mullins et al., 2014; Rae, 2014).

7.4.6.2.2 Functional task: VICT task

Procedures, stimuli and design are as per detailed in the previous study section 6.4.6.2, with the exception of additional pictures indicated in Table 40. Picture codes are provided in Appendix C.

Table 40. IAPS Picture Parameters – Normative Ratings

IAPS run group	Valence range (1 = most unpleasant, 9 = most pleasant)	Valence mean	Arousal range (1 = least, 9 = most arousing)	Arousal mean	Dominance/Control range (1 = least, 9 = most)	Dominance/Control mean
Negative Time 1	Valence < 4.0	2.34	Arousal > 6.0	6.57	2.0-4.0	3.23
Neutral Time 1	Valence 4.0 – 6.0	5.01	Arousal < 3.6	2.84	1.9-2.8	5.99
Negative Time 2	Valence < 4.0	2.37	Arousal > 6.0	6.53	1.8-2.5	3.17
Neutral Time 2	Valence 4.0 – 6.0	4.96	Arousal < 3.6	2.87	1.7-2.4	5.91

Source: Bradley and Lang (1994)

7.4.7 Assessment of safety and risks

These were identical to the previous study; and are detailed in Appendix A.

7.4.8 Risk management for scans

These were identical to the previous study; and as per above.

7.4.9 Confidentiality and data access

These were identical to the previous study; and as per above.

7.4.10 Ethics and finance

These were identical to the previous study; and as per above.

7.5 Procedure

7.5.1 Baseline session

The first scan was conducted prior to commencing the assigned treatment intervention. In the 12-hour period prior to the scan, participants were instructed against consuming alcohol or caffeinated products (e.g. coffee, green tea, soft drinks and chocolate). Scans were scheduled one hour following commencement of the morning session. Participants fasted for 12 hours prior to the morning visit and were provided with a standardised breakfast one hour prior to the scan time.

7.5.2 Eight-week session

The second and final brain scan took place eight weeks or 60 days (no more than five days allowance) after commencing the treatment intervention. The scanning session occurred on the same day and time as the baseline session to minimise variability in conditions. Additional instructions included no consumption of the study treatment on the day. The assessments were repeated in the same manner as the first visit. Collection of demographic data and the personality questionnaires and order of measures were replicated as per the first visit.

7.5.3 Data analysis

7.5.3.1 *Demographic and medical data*

Data were analysed using SPSS as per the previous study. Prevalence statistics were reported, with a tabulated summary of demographic, medical and psychiatric data. Means and SDs of treatment groups, gender and comorbid status were analysed at baseline and week eight and assessed via independent sample t-tests or chi-square where appropriate. Non-parametric statistics were applied when assumptions for parametric methods were violated.

7.5.3.2 *Hypothesis one: Psychiatric measures following eight weeks of Kava treatment*

Analyses of outcome data in this study were conducted with blinding to group allocations. For analysis methods of the overarching clinical trial, please refer to Appendix F: *Kava for the Treatment of Generalised Anxiety Disorder: An 18-week Double-Blind Randomised Placebo-Controlled Trial*.

The primary analysis assessed treatment group differences for scores on the SIGH-A/HAM-A anxiety measure using a linear mixed factorial model. A linear mixed model (LMM) was chosen as the statistical approach given the degree of missing data that would not be incorporated via repeated-measures ANOVA.

Time points were restructured to create one index variable of time (baseline pre-treatment, week two, week four and week eight), with the dependent variable as restructured SIGH-A anxiety score (also index created from four time points).

The main fixed effects were treatment group, age, gender, comorbid condition and any other variables determined as different between groups that may influence anxiety level outcomes (for example, baseline depression scores, benzodiazepine or alcohol use).

Time and treatment were entered as the main effects, with time x treatment as the *a priori* interaction of interest. The random effect variable was the participant ID.

7.5.3.3 Imaging analysis

Imaging data analysis was conducted using SPM12, MATLAB software and associated MRS toolboxes, detailed in the previous study analyses section.

7.5.3.3.1 MRS

Metabolite concentrations were processed in a toolbox-specific fashion for the Tarquin and LCModel. Results are presented in units approximating millimolar concentration. Partial volume effect was corrected using high-resolution T1 and an in-house voxel co-registration script and volume-specific GABA concentrations were calculated as a function of grey matter and CSF percentage proportion derived from segmentation data.

Outliers derived from z-score conversion that were ± 3.29 SDs were inspected on a case-by-case basis and cross-checked with imaging supervisors to ensure rigour in case selection. Cases that were outliers in either GABA score or quality parameter scores in one toolbox were examined across both toolboxes three toolboxes and removed from both (both baseline and week eight data) if data met $> 33\%$ outlier status.

7.5.3.3.1.1 Tarquin

Processes as per the previous study, section 6.5.4.3.5.2.

7.5.3.3.1.2 LCModel

Processes as per the previous study, section 6.5.4.3.5.3.

7.5.3.4 Hypothesis two: GABA level change following 8 weeks of Kava treatment

To examine the change in GABA levels during the treatment period, a LMM ANOVA was utilised as per the process for the anxiety scores. The main fixed effects were treatment group, age, gender, comorbid condition and any other variables determined as different between groups that may influence anxiety level outcomes (for example, baseline depression scores, benzodiazepine or alcohol use).

Time and treatment were entered as main effects, with time x treatment as the *a priori* interaction of interest. The random effect variable was the participant ID.

Estimated marginal means and parameter estimates were additionally generated to aid details of group differences.

7.5.3.5 Hypothesis three: GABA level influence on anxiety levels following eight weeks of Kava treatment

To examine changes to anxiety levels during the treatment period as a function of potentially changing GABA levels (five measures from three toolboxes), a LMM ANOVA was again utilised as per previous hypothesis two.

The main fixed effects were treatment group, age, gender, comorbid condition, baseline GABA level and any other variables determined as different between groups that may influence anxiety level outcomes (for example, baseline depression scores, benzodiazepine or alcohol use).

Time and treatment were entered as main effects, with a three-way interaction of time x treatment x GABA level as the *a priori* interaction of interest. The random effect variable was the participant ID.

Estimated marginal means and parameter estimates were additionally generated to aid details of group differences.

7.5.3.5.1 Functional MRI

7.5.3.5.1.1 Outlier identification

Following pre-processing pipeline steps, outlying volumes were identified using ART tools as in the previous study. The set threshold was 2 mm and > 3 SDs. Scans with > 47 outlying images per run were removed.

7.5.3.5.1.2 Contrast vectors

The fMRI data were modelled and contrast images (neu-neg > neu-neu) generated session-wise as in the previous chapter, then entered into second level group analyses.

7.5.3.5.1.3 Region of interest

The ROI for the dACC was defined *a priori* using the AAL as per the previous study procedure; mean ROI contrast estimates were extracted from contrast images using the MarsBar Region of Interest Toolbox for SPM12. Randomised groups pre-treatment were assessed for functional differences via t-tests, before conversion of variables into a LMM format for treatment/time-based analyses.

7.5.3.6 Hypothesis four: fMRI difference in activation in the dACC ROI as a function of eight weeks of Kava treatment

To examine any change in BOLD signal activation levels (as percentage signal change) a LMM ANOVA was used. The main fixed effects were treatment group, age, gender and comorbid condition. Time and treatment were entered as main effects, with time x treatment as the *a priori* interaction of interest. The random effect variable was the participant ID. Estimated marginal means and parameter estimates were additionally generated to aid details of group differences.

7.5.3.7 Hypothesis five: fMRI differences influencing anxiety scores as a function of eight weeks of Kava treatment in the ROI

To examine the change in anxiety scores as a function of treatment moderated by BOLD signal activation levels, a LMM ANOVA was used with main fixed effects being treatment group, age, gender and comorbid condition. Time and treatment were entered as the main effects, with time x treatment x fMRI activation interaction as the *a priori* interaction of interest. The random effect variable was the participant ID. Estimated marginal means and parameter estimates were additionally generated to aid details of group differences.

7.6 Results

7.6.1 Descriptive data results

Demographic and descriptive data were compared using t-tests for continuous data and chi-square tests for categorical data. Significance was reported at exact values unless *p*-value significance was .000, where it was displayed as $p < .001$. Baseline demographics were reported as both whole group and randomisation groupings, given the reduced sample size at the second time point. Comparisons involving second time point data (i.e. post-randomisation between treatment and placebo groups) was compared with baseline data grouped by treatment level.

Of the $n = 40$ participants enrolled in the study, only $n = 37$ had viable data at baseline (please refer to next sections for information regarding case removal based on scan quality).

7.6.2 Demographics and frequencies at baseline and week eight

Age: The mean group age was 36 years (SD = 13.09) and differences were not significant between treatment group means at baseline ($p = .965$). Gender age differences were not significant ($p = .507$). Difference in age at week eight was also not significant between the groups (however, assumption for variance was not met on the Levene's test); $t(16.95) = -.348, p = .732$ (Table 41).

Gender: Gender distribution between groups was similar at baseline for treatment groups $\chi^2(1) = 3.25, p = .072$ (Fisher's exact test), for males ($n = 18$) and females ($n = 19$), and at week eight, gender numbers were also not significant between groups; $\chi^2(1) = .737, p = .653$ (Fisher's exact test).

Education: Education levels had a mean 17.65 years at baseline (SD = 3.91), with no significant differences between treatment groups ($p = .181$) and, in examining education by gender, was also not significant.

Table 41. Demographics summary for GAD subjects at baseline (pre-treatment) as whole group, by treatment and by gender.

Demographics <i>Mean (S.D.), n or % where relevant</i>	Group	Group 1 (placebo)	Group 2 (<i>P. methysticum</i>)	Group <i>p-value*</i>	Females	Males	Gender <i>p-value*</i>
Participants (n)	37	17 (9 male)	20 (11 male)	.653	19	18	.072
Age	36.16 (13.09)	36.06 (11.31)	36.25 (14.73)	.965	37.58 (13.40)	34.67 (13.00)	.507
Education years	17.65 (3.91)	18.59 (4.70)	16.85 (2.98)	.181	18.36 (3.35)	16.90 (4.39)	.256
Psychiatric							
Comorbid condition	21	12	9	.110	9	12	.325
Comorbid: SAD	11	6	5	.395	4	7	.247
Comorbid: PD	9	7	2	.034*	4	5	.714
Comorbid: AGO	17	9	8	.324	6	11	.330
Comorbid: PTSD	1	1	0	.460	0	1	.514
Comorbid: MDD	20	9	11	.581	10	10	.746
SIGH-A ^a	23.05 (3.60)	23.29 (4.19)	22.85 (3.10)	.714	22.47 (3.27)	23.67 (3.91)	.320
SIGMA ^b	13.59 (2.99)	14.00 (2.91)	13.25 (3.09)	.456	12.00 (3.32)	14.28 (2.52)	.180
STAIT ^c	51.09 (6.90)	51.57 (7.45)	50.72 (6.64)	.736	50.83 (8.23)	51.42 (4.97)	.813
STAIS ^d score pre-scan	45.83 (8.60)	45.82 (7.33)	45.84 (9.79)	.995	48.79 (9.82)	42.53 (5.59)	.027*
Medical							
Medications	15	7	6	.357	13	11	.330
Benzodiazepines	4	3	1	.367	2	2	.405
Opioid analgesic	5	3	2	.420	1	4	.225
Other (e.g. salbutamol)	1	5	4	.596	1	0	.204
Supplements	9	2	7	.103	6	1	.447
Substance							
Caffeine (mg/daily)	135.54 (127.64)	156.47 (132.96)	117.75 (123.51)	.365	104.42 (102.40)	168.39 (145.44)	.129
Alcohol (SD/weekly)	2.86 (3.08)	1.88 (2.02)	3.70 (3.60)	.073	3.60 (3.00)	3.40 (3.20)	.709

*Significance values derived from independent sample t-tests or Chi Square tests where appropriate and significant where $p < .05$; ^a Hamilton Anxiety Inventory; ^b Montgomery-Asberg Depression Scale; ^c State-Trait Anxiety Scale, Trait form; ^d State-Trait Anxiety Scale, State form

Comorbid conditions: For all GAD participants at baseline, 57% ($n = 21$) reported a current comorbid condition as determined by the MINI questionnaire administered at baseline, the most frequent being agoraphobia (46%; $n = 17$) and then SAD (30%; $n = 11$), panic disorder (24%; $n = 9$) and PTSD (3%; $n = 1$). History of a major depressive episode was reported in 54% of participants ($n = 20$).

Between treatment groups at baseline, the presence of a comorbid condition was not significantly different, $\chi^2(1) = 2.45$, $p = .185$ (Fisher's exact test); however, when examining the prevalence of aforementioned comorbid conditions individually, only panic disorder was significantly different between the two groups, $\chi^2(1) = 4.85$, $p = .034$ (Fisher's exact test). The placebo group at baseline had a higher number ($n = 7$) of participants with panic disorder than the treatment group ($n = 2$).

Caffeine use: Mean caffeine intake at baseline was not significantly different between treatment groups ($p = .365$), with a mean daily consumption of 135.54 mg ($SD = 127.64$). However, large standard deviations in both groups indicated that the amount was highly variable between individuals. Gender differences in caffeine use were not significant for either group. At week eight, difference in caffeine use between groups was also not significant.

Alcohol consumption: Alcohol use at baseline was a mean of 2.86 standard drinks per week ($SD = 3.08$), with no significant difference between baseline treatment groups ($p = .073$), with the placebo group ($m = 1.88$, $SD = 2.02$) lower than the treatment group ($m = 3.70$, $SD = 3.60$). Gender was not significantly different at baseline. At week eight, a significant difference was observed in the number of standard drinks between the treatment groups, $t(18) = -3.33$, $p = .003$, 95% CI $[-7.22, -1.63]$, where the treatment group consumed 6.09 ($SD = 3.73$) mean standard drinks weekly, whereas the placebo group consumed 0.60 ($SD = 3.66$) per week. Using paired-sample t-tests to examine this association, the placebo group change was not significant ($p = .525$), whereas in the treatment group, this change was significant, $t(10) = -2.82$, $p = .018$, 95% CI $[-4.39, -0.52]$.

Medical status: Medical conditions that were reported as ongoing at baseline were categorised as blood pressure aberrations (24%; $n = 9$); irritable bowel symptoms (19%; $n = 7$); eczema, dermatitis or psoriasis (19%; $n = 7$); asthma (13%; $n = 5$) and reflux (8%; $n = 3$). The prevalence of medical conditions was not different between treatment groups ($p = .146$).

Similarly, adverse events/serious adverse events reported during the study enrolment were not significantly different between participants in the treatment and placebo groups ($p = .508$). The active treatment *P. methysticum* extract was well-tolerated, with one participant reporting an adverse event of dizziness and headache that held a probable association to treatment, due to the allocation to the active treatment group.

Concomitant medication in all subjects: Medication (prescription or over-the-counter) was reported by 40% of participants ($n = 15$) and this was not significantly different between groups ($p = .357$). Supplements were reported in 24% of participants ($n = 9$), again without differences between groups ($p = .103$).

Of the medications and supplements that are evidenced to have GABAergic substrate effects, 19% ($n = 7$) reported use in the present study and this prevalence was not significant between treatment groups ($p = .855$). Four participants reported benzodiazepine use of weekly frequency (as *pro re nata*), although one participant reported no use during enrolment and one participant took one dose of alprazolam (Xanax) during enrolment and two participants reported 5 mg diazepam (Valium) use.

No participants reported taking benzodiazepines within the week prior to the brain scans. Eight participants reported use of GABAergic nutraceuticals and/or phytomedicines, the most common being magnesium ($n = 4$; 11%), valerian ($n = 2$; 5%) and melatonin ($n = 2$; 5%). Again, no GABAergic supplements were reported within the week prior to brain scans at either visit.

Other anomalies/reporting: All female participant scans were conducted between days one and 10 of their menstrual cycle, apart from two subjects for whom their cycle was too erratic to monitor precisely, so the most-likelihood time phase was used.

Withdrawals: Attrition of sample size following the baseline visit was 54% ($n = 20$), and were not significantly different between the two groups ($p = .482$) and occurred for the following reasons and frequencies: general non-compliance to study protocol ($n = 7$), non-randomisation (abnormal liver function tests; $n = 3$), lost to follow-up ($n = 3$), side effects to active treatment ($n = 1$), medication status change (antidepressant course, $n = 1$), psychiatric status change (depression symptom worsening, $n = 2$), pregnancy ($n = 1$) and personal reasons/unspecified ($n = 2$).

7.6.2.1 Psychiatric measures – baseline

SIGH-A anxiety score: anxiety symptoms measured by the HAM-A/SIGH-A mean score for the total group pre-treatment was 23.05 (SD = 3.60). For treatment groups at

baseline, the difference was not significant ($p = .714$). Scores between genders at baseline were also not significant ($p = .320$). The presence of a comorbid condition did not indicate significant differences at baseline in SIGH-A scores ($p = .205$), although GAD participants with a comorbid condition reported a higher baseline mean score ($m = 24.00$, $SD = 3.86$) than 'pure GAD' participants ($m = 21.91$, $SD = 3.36$).

MADRS mood score: Depression symptom scoring was reported via the SIGMA for the MADRS, where mean score pre-treatment was 13.59 ($SD = 2.99$). For treatment groups at baseline, the difference was not significant ($p = .456$). Gender differences trended towards significance ($p = .180$) where MADRS score was higher in males ($m = 14.28$, $SD = 2.52$) than in females ($m = 12.00$, $SD = 3.32$) at baseline. GAD participants with and without comorbid conditions were not significantly different at baseline ($p = .478$); although, similar to the anxiety scores, GAD/comorbid groups reported higher levels ($m = 13.68$, $SD = 3.24$) than the pure GAD group ($m = 13.18$, $SD = 2.83$). MADRS scores were required to be 18 or less at baseline for inclusion in the study.

Spielberger Trait scores for anxiety: At baseline, there were no significant differences between treatment groups or genders, although the independent sample t-test for gender differences was significant for Levene's test of homogeneity of variance $F(30) = 4.24$, $p = .048$. However, due to equitable sample size between genders in the groups, no further tests were performed on this data. For GAD/comorbid or pure GAD groups, Trait anxiety scores were also not significantly different at baseline ($p = .380$), although GAD/comorbid participant means were higher ($m = 52.05$, $SD = 6.92$) than pure GAD participants ($m = 49.86$, $SD = 6.93$).

Spielberger STAIS state anxiety measures: State levels of anxiety were measured immediately prior to the baseline scan. There were no significant differences between treatment groups ($p = .995$). There was a significant difference in pre-scan scores between genders; $t(34) = -2.31$, $p = .027$, 95% CI [-11.76, -0.76], whereby mean STAIS scores were five points higher in females ($m = 48.79$, $SD = 9.82$) than in males ($m = 42.53$, $SD = 5.59$). Comorbid condition presence was not significantly different in pre-scan scores ($p = .499$) compared with pure GAD participants. Again, GAD/comorbid participant means were higher ($m = 46.67$, $SD = 7.82$) than pure GAD participants ($m = 44.67$, $SD = 9.74$).

7.6.3 Hypothesis one: Anxiety levels measured via SIGH-A/HAM-A

The LMM ANOVA for change to anxiety symptoms on the HAM-A showed no significant interaction effect for treatment and time, indicating marginal effects, $F(3, 81) = 2.21, p = .093$. Main and interaction effects are reported in Table 42.

Pairwise comparisons indicated that both groups experienced a reduction from baseline to week two, followed by an increase in scores at weeks four and eight in the treatment group. The placebo group dropped similarly at week two and maintained similar scoring at weeks four and eight. Although mean differences appeared significant, differences were small compared to the margin of error.

The difference in mean score between the placebo and treatment groups at week eight was non-significant, with marginal effect ($m = -5.28, p = .092, 95\% \text{ CI } -11.43, 0.87$), which indicated a lower HAM-A anxiety score in the placebo group. Means and parameter estimates are indicated in Tables 42 and 43 and are graphically represented both within and across groups in Figure 34.

Table 42. ANOVA Main Effects Model for SIGH-A Anxiety Level change

Anxiety model	B/Coefficient	95% CI	<i>t</i>	<i>p</i> -value
Treatment	-5.28	-11.43, 0.87	-1.70	.092
Time	6.29	2.09, 10.51	2.96	.004*
Gender	1.45	-2.50, 5.40	0.75	.460
Age	0.03	-0.12, 0.18	0.42	.678
Comorbid	-0.97	-4.94, 3.00	-0.50	.621
Baseline alcohol	0.08	-0.58, 0.74	0.25	.801
Treatment*time	5.20	-0.96, 11.36	1.68	.097 ^a

*Significant at $p < 0.05$; ^a marginally significant at $p = .05 - .10$, CI confidence interval

Table 43. SIGH-A Anxiety Scoring Group and Time Statistics (Estimated Marginal Means)

Time	Treatment group	Mean	Std. Error	df	95% CI
1	A Placebo	22.93	1.81	68.54	19.31, 26.55
	B Kava	23.01	1.65	69.96	19.73, 26.29
2	A Placebo	14.68	1.91	74.71	10.87, 18.49
	B Kava	13.16	1.79	79.56	9.60, 16.72
3	A Placebo	13.04	2.01	80.89	9.04, 17.05
	B Kava	17.13	1.83	82.72	13.49, 20.77
4	A Placebo	11.44	2.21	91.65	7.04, 15.84
	B Kava	16.72	2.06	94.84	12.63, 20.81

Time 1 = baseline/pre-treatment; 2 = week 2; 3 = week 4, 4 = week 8

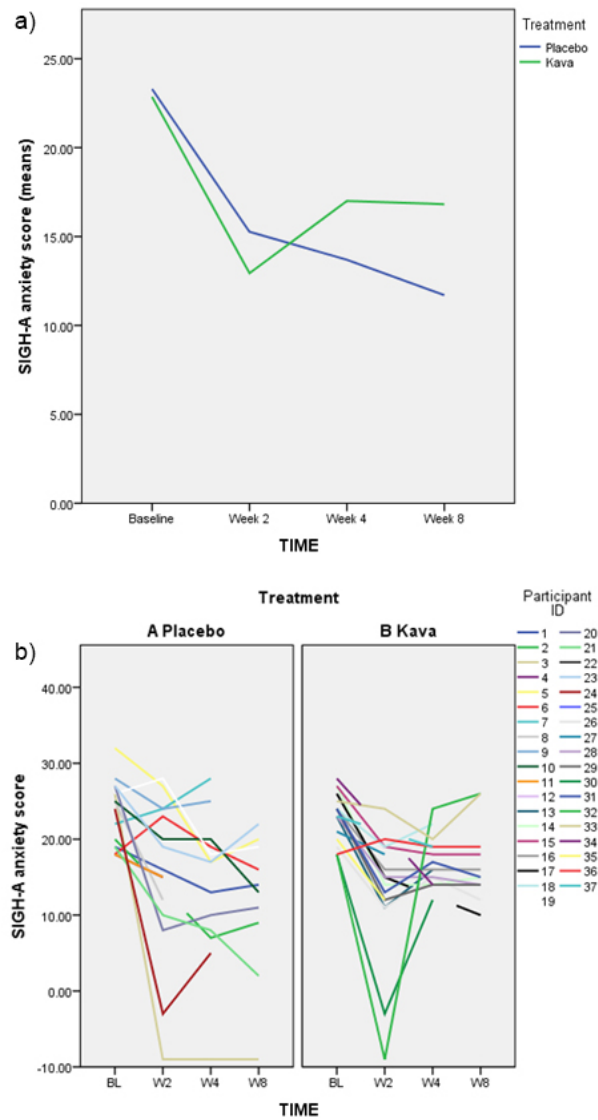


Figure 24. SIGH-A anxiety scores over time for a) Group means; b) Participant scores per group

7.6.4 Metabolite data results

MRS data processes including segmentation data, quality of signal and GABA calculations were performed in the same manner as the investigation methods in the previous study and are summarised in Tables 44 and 45.

7.6.4.1 Segmentation

Assessment of differences in voxel composition for grey matter proportion (GM%) at baseline was not different in terms of treatment groups at baseline ($p = .165$) or for white matter (WM%, $p = .094$) or cerebrospinal fluid (CSF%, $p = .418$). Similarly, there were no significant differences between comorbid presence.

7.6.4.2 *Toolbox-specific quality parameter assessment*

7.6.4.2.1 *Tarquin quality parameters*

Tarquin fit quality score ('Fit Q'; where values >1 $Q < 2$), SNR (where > 5) and calculated Tarquin SD (SD/TGABA; where values $< 30\%$ included) were not significantly different between treatment, comorbid or gender groups at any time point. For GABA/GM, the identification of outlying or missing quality data resulted in nine cases of data loss from 37 cases at baseline and 5 out of 18 week eight cases.

7.6.4.2.2 *LCModel parameters*

For LCModel, SNR (where values $>/ 5$), SD where values $< 30\%$ and where FWHM was > 0.15 . For LCModel grey matter GABA, outlying or missing quality data resulted in six cases of data loss.

LCModel SNR was significantly different between comorbid groups at baseline, $t(34) = -2.03$, $p = .050$, where mean SNR for pure GAD ($m = 8.06$, $SD = 2.57$) was lower than GADS/comorbid group ($m = 9.75$, $SD = 2.40$). FWHM was also significant between these two groups, $t(34) = 2.67$, $p = .050$, where the pure GAD group ($m = 0.10$, $SD = 0.05$) was higher than the GAD/comorbid group ($m = 0.06$, $SD = 0.02$).

FWHM was also significantly different between treatment groups at baseline, $t(34) = -2.29$, $p = .028$, with the placebo group at the pre-treatment/baseline ($m = 0.06$, $SD = 0.02$) lower than the pre-treatment/active group ($m = 0.09$, $SD = 0.05$).

Table 44. ¹H-MRS GABA Toolbox-Specific Data for dACC as ROI, for Comorbid and Treatment Groups

Mean (SD)	Baseline Group	Baseline pure GAD	Baseline GAD/comorbid	Baseline male	Baseline female	Baseline placebo	Baseline active	Baseline psych p-value*	Baseline gender p-value*	Baseline tmt p-value*
Group (n)	37	16	20	18	19	17	20	df: 34	df: 35	df: 35
dACC GM (% ₀ , mm ³)	60.29 (13.20)	60.94 (12.13)	59.81 (14.24)	59.44 (13.62)	61.10 (13.11)	59.41 (12.22)	61.05 (14.25)	.801	.708	.712
dACC WM (% ₀ , mm ³)	23.48 (11.46)	24.12 (10.73)	23.00 (12.23)	24.11 (13.89)	22.89 (8.93)	24.58 (11.86)	22.55 (11.34)	.772	.752	.597
dACC CSF (% ₀ , mm ³)	15.57 (5.04)	15.27 (4.61)	15.81 (5.45)	14.97 (4.18)	16.16 (5.80)	14.53 (3.93)	16.47 (5.77)	.754	.481	.249
TARQUIN GABA/GM	9.99 (3.90)	10.01 (5.27)	9.97 (2.49)	9.83 (2.70)	10.17 (5.01)	10.79 (4.56)	9.19 (3.02)	.976	.804	.236
LCModel GABA/GM	3.67 (0.88)	3.93 (1.08)	3.46 (0.62)	3.45 (0.56)	3.91 (1.10)	3.66 (1.01)	3.69 (0.77)	.116	.130	.932
TARQUIN SNR	10.98 (3.96)	9.96 (3.72)	11.74 (4.05)	10.12 (3.57)	11.89 (4.25)	11.75 (3.70)	10.25 (4.16)	.191	.190	.271
TARQUIN FIT Q	1.38 (0.14)	1.39 (0.18)	1.37 (0.11)	1.35 (0.13)	1.40 (0.15)	1.37 (0.13)	1.38 (0.16)	.729	.320	.786
TARQUIN SD (%)	24.62 (13.20)	28.78 (14.43)	21.49 (11.59)	24.98 (14.64)	24.23 (11.91)	21.28 (6.97)	9.31 (2.83)	.107	.870	.149
LCModel SD (%)	9.39 (2.30)	9.37 (2.82)	9.40 (1.85)	9.72 (1.81)	9.05 (2.71)	9.47 (1.58)	9.31 (2.83)	.975	.392	.843
LCModel SNR	9.00 (2.58)	8.06 (2.57)	9.75 (2.40)	8.61 (2.06)	9.39 (3.03)	9.70 (2.26)	8.37 (2.75)	.050*	.375	.123
LCModel FWHM	0.08 (0.04)	0.10 (0.05)	0.06 (0.20)	0.07 (0.03)	0.08 (0.05)	0.06 (0.02)	0.09 (0.05)	.024*	.695	.025*

* Significant values where $p < 0.05$ are derived from independent sample t-tests, Cr creatine; CSF cerebrospinal fluid; df degrees of freedom; dACC dorsal anterior cingulate cortex; GM grey matter; Fit Err % Fit error; FWHM full width at half-maximum; GABA gamma amino-butyric acid; GABA+ concentrate GABA including macromolecules; ROI Region of interest, SNR signal-noise ratio; SD standard deviation; WM White Matter

Table 45. ¹H-MRS GABA Toolbox-Specific Data for dACC as ROI, Week Eight, for Comorbid and Treatment Groups

Mean (SD)	Week 8 Group	Week 8 pure GAD	Week 8 GAD/comorbid	Week 8 Male	Week 8 Female	Week 8 Placebo	Week 8 Kava	Week 8 gender ^a	W8 psych ^a	W8 tmt
Group (n)	20	9	11	9	11	9	11			
dACC GM (% , mm ³)	58.35 (12.33)	60.00 (7.30)	57.00 (15.54)	54.33 (16.46)	61.64 (6.74)	53.11 (15.89)	62.64 (6.45)	.195	.602	.085
dACC WM (% , mm ³)	23.40 (10.78)	21.00 (7.94)	25.36 (12.68)	25.67 (15.21)	21.55 (5.20)	27.67 (13.90)	19.91 (6.02)	.410	.382	.111
dACC CSF (% , mm ³)	18.01 (6.18)	18.47 (6.91)	17.64 (5.84)	20.24 (7.44)	16.18 (4.49)	19.44 (4.56)	16.84 (7.26)	.148	.774	.362
TARQUIN GABA/GM	10.06 (3.05)	8.58 (2.25)	11.27 (3.17)	11.38 (3.69)	8.97 (1.98)	11.33 (3.55)	9.02 (2.23)	**	**	**
LCModel GABA/GM	3.59 (0.74)	3.23 (0.58)	3.90 (0.76)	3.73 (1.03)	3.48 (0.48)	4.07 (0.75)	3.23 (0.53)	**	**	**
TARQUIN SNR	10.64 (3.73)	10.38 (4.44)	10.85 (3.24)	11.27 (4.00)	10.12 (3.59)	11.72 (3.21)	9.75 (4.03)	.508	.788	.251
TARQUIN FIT Q	1.44 (0.25)	1.45 (0.26)	1.44 (0.25)	1.42 (0.20)	1.46 (0.29)	1.36 (0.15)	1.51 (0.29)	.618	.564	.608
TARQUIN SD (%)	25.05 (13.11)	26.99 (13.26)	23.46 (13.40)	23.37 (9.79)	26.42 (15.66)	26.78 (14.61)	23.64 (12.28)	.756	.948	.184
LCModel SD (%)	9.50 (2.68)	10.44 (3.71)	8.73 (1.10)	9.78 (3.86)	9.27 (1.27)	8.89 (0.78)	10.00 (3.55)	.687	.160	.371
LCModel SNR	9.05 (2.42)	9.00 (2.83)	9.09 (2.16)	9.33 (3.00)	8.82 (1.94)	9.44 (2.13)	8.73 (2.69)	.648	.936	.524
LCModel FWHM	0.07 (0.03)	0.08 (0.04)	0.07 (0.03)	0.07 (0.03)	0.08 (0.04)	0.06 (0.02)	0.08 (0.04)	.519	.455	.240

* Significant values where p < 0.05 are derived from independent sample t-tests, ** Analyses reported as ANOVA elsewhere; ^a t-tests conducted controlling for treatment group; CSF cerebrospinal fluid; dACC dorsal anterior cingulate cortex; GM grey matter; FWHM full width at half-maximum; GABA gamma amino-butyric acid; GABA+ concentrate GABA including macromolecules; ROI Region of interest, SNR signal-noise ratio; SD standard deviation; WM white matter

7.6.4.3 MRS GABA group tests at baseline

GABA data between treatment groups at baseline were not significantly different. The t-tests between treatment groups are reported in Table 46.

Table 46. Baseline Treatment Group Difference T-tests for Significance of GABA Levels

GABA model	df	t	95% CI	p-value*
Tarquin: GABA in GM	32	1.21	-1.10, 4.31	.236
LCModel: GABA in GM	33	-0.09	-0.64, 0.59	.932

*Significant at $p < .05$; CI confidence interval; df degrees of freedom; t t-tests for equality of means

7.6.5 Hypothesis two: GABA level changes as a function of eight weeks of Kava treatment

To assess the effect of treatment on GABA levels over the treatment period, a LMM ANOVA was built with the GABA metabolites outcomes. Results of the parameter estimates for each model are reported in Table 47 and, specific to GABA (LCModel), the significant result is illustrated in Figure 35.

7.6.5.1 GABA concentration via TARQUIN for grey matter

The test for interaction between treatment and time on GABA levels at week eight second time point was not significant in this model. The treatment by itself was marginally significant in the model, $F(1,24) = 3.86$, $p = .061$, where the placebo group had 3.08 unit higher GABA levels than the treatment group, 95% CI [-0.33, 6.49]; however, given no interaction effect with time, this cannot be concluded as a predictor of GABA level. Again, not significant, but an examination of the pairwise comparisons indicated GABA level reduction in the treatment group (3.08 units) and not in the placebo group. This was not significant ($p = .076$), 95% CI [-6.49, 0.33].

7.6.5.2 GABA concentration in grey matter from LCModel

The LCModel's GABA in the grey matter model showed a significant treatment by time interaction, $F(1,21) = 4.36$, $p = .049$. Pairwise comparisons at the second time point indicated that the treatment group was 1.06 units lower than the placebo group and this was statistically significant ($p = .008$), 95% CI [-1.83, -0.28]. Means are plotted graphically in Figure 35, indicating reduced error bars in the Kava (treatment) group.

Table 47. GABA Change Following Eight Weeks of Treatment[^]

GABA model	B/Estimate	95% CI	t	df	p-value
Tarquin: GABA in Grey Matter					
Time	0.51	-2.02, 3.06	0.42	19	.677
Treatment	3.08	-0.33, 6.49	1.82	45	.076 ^a
Gender	0.04	-2.30, 2.38	0.04	21	.971
Age	0.06	-0.03, 0.15	1.43	19	.168
Comorbid	-0.28	-2.69, 2.12	-0.24	23	.809
Baseline alcohol	0.19	-0.21, 0.59	0.98	20	.337
Time*Treatment	-1.23	4.96, 2.49	-0.69	19	.496
LCModel: GABA in GM					
Time	0.48	-0.09, 1.06	1.74	21	.097 ^a
Treatment	1.06	0.28, 1.83	2.75	46	.008 [*]
Gender	-0.28	-0.78, 0.23	-1.14	21	.268
Age	0.01	0.00, 0.03	1.58	19	.129
Comorbid	0.13	-0.39, 0.65	0.53	24	.603
Baseline alcohol	0.05	-0.04, 0.03	1.58	21	.129
Time*Treatment	-0.87	-1.74, 0.00	-2.09	21	.049 [*]

*Significant at $p < 0.05$; ^aMarginally significant at $p = .05 - .10$; B/coefficient unit decimal places are variable due to GABA toolbox level ranges; df degrees of freedom
[^]ANOVA Model from SPSS output following eight weeks of treatment

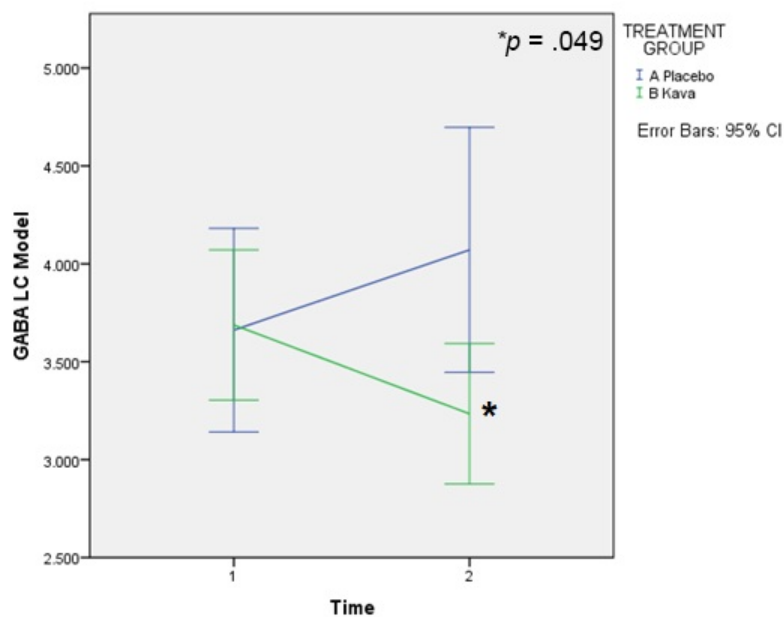


Figure 25. Plotted means per group for GABA (LCModel; baseline and week eight)

7.6.6 Hypothesis three: GABA and anxiety levels following eight weeks of Kava treatment–SIGH-A outcome with GABA levels predictor

The LMM analyses testing the 3-way interaction effect (time x treatment x GABA) was not significant for either GABA model. Tables 48 and 49 report model statistics for the three-way interaction and the contrast (treatment – placebo) as the difference in estimated slope at the second time point. Figure 36 illustrates the estimated slope (change in outcome per change in GABA predictor) and the contrast slope difference for GABA data from Tarquin to illustrate trends.

Table 48. Linear Mixed Model Interaction by GABA Levels and Treatment Across Time

GABA model	MSE	F	df	p-value*
Tarquin: GABA in GM				
GABA*tmt*time	5.90	0.39	3,69	.757
LCModel: GABA in GM				
GABA*tmt*time	5.53	0.34	3,68	.771

*Significant at $p < 0.05$; df degrees of freedom; F ANOVA omnibus F-test; MSE mean standard error

Table 49. Linear Mixed ANOVA Model for SIGH-A HAM-A Contrast (Treatment – Placebo) at Eight Weeks for Each GABA Predictor

GABA model	df	95% CI	t	p-value*
Tarquin: GABA in GM	82	-2.22, 0.90	-0.84	.404
LCModel: GABA in GM	84	-5.15, 11.89	0.79	.434

*Significant at $p < 0.05$; df degrees of freedom; B/coefficient unit t ratio and CI is GABA metabolite at time 4 contrast of treatment - placebo

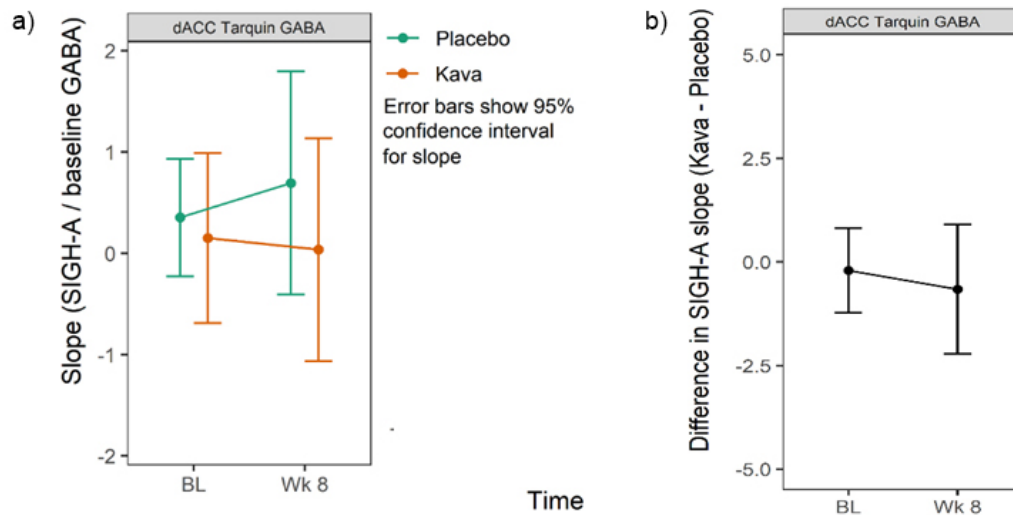


Figure 26. a. Slope (change in outcome/change in baseline GABA), b. Difference in slope, calculated as Kava – placebo

7.6.7 Functional activation in the dACC measured via fMRI

Mean contrast estimates (condition Neu/Neg – Neu/Neu) within the ROI are reported in Table 52. The baseline t-test between treatment groups for BOLD signal activation in the ROI was not significant, $t(29) = 0.16$, $p = .872$, 95% CI [-0.357, 0.419]. Week eight t-tests were not performed as the next section models this data into a linear model with other predictors.

Table 50. Extracted BOLD Signal in the ROI at Two Time Points

dACC BOLD signal, % mean contrast	n	Mean (SD)	df	t-test	p-value
Baseline					
Group	31	0.078 (0.519)	29	0.16	.872
<i>Placebo</i>	15	0.094 (0.494)	-	-	-
<i>Treatment</i>	16	0.063 (0.558)	-	-	-
Gender			28	-1.33	.195
<i>Females</i>	17	0.184 (0.444)	-	-	-
<i>Males</i>	13	-0.071 (0.608)	-	-	-
Comorbidity			28	-1.07	.295
<i>pure GAD</i>	12	-0.052(0.518)	-	-	-
<i>GAD/comorbid</i>	18	0.157 (0.532)	-	-	-
Week 8	n	Mean (SD)	df	t-test	p-value
Group	18	-0.013 (0.716)	-	-	-
<i>Placebo</i>	8	-0.189 (0.709)	-	-	-
<i>Treatment</i>	10	0.127 (0.726)	-	-	-
Gender			-	-	-
<i>Females</i>	9	0.318 (0.605)	-	-	-
<i>Males</i>	9	-0.345 (0.689)	-	-	-
Comorbidity			-	-	-
<i>pure GAD</i>	8	0.099 (0.775)	-	-	-
<i>GAD/comorbid</i>	10	-0.104 (0.692)	-	-	-
*Significant values where $p < 0.05$ are derived from independent sample t-tests, % mean contrast reported to three decimal places due to small unit difference					

7.6.8 Hypothesis four: fMRI difference in activation in the dACC ROI, as a function of eight weeks of Kava treatment

To assess the effect of treatment on BOLD signal over the treatment period, a LMM ANOVA was built with BOLD signal mean contrast estimates as the outcome; fixed effects were treatment group, gender, comorbid presence, age and signal scores measured at baseline, with participant ID as the random effects. Results are collated in Table 51.

The LMM indicated no significant difference between treatment groups over the time period on BOLD signal activation (treatment x time interaction), $p = .423$. In the model, gender had a statistically significant association with signal change, $F(1,25) = 6.20$, $p = .020$, where BOLD signal was 0.47 units lower in males than in females, 95% CI [-0.85, -0.08]. In addition, age predicted BOLD signal, $F(1,29) = 5.09$, $p = .032$. BOLD signal reduced 0.02 units with age, 95% CI [-0.03, 0.00].

Table 51. Mixed Model Statistics for BOLD Signal Change Following Eight Weeks of Treatment

BOLD activation dACC model	B/Estimate	95% CI	t	df	p-value*
Score as contrast % signal activation, week eight					
Time	-0.08	-0.53, 0.38	-0.35	24	.732
Treatment	-0.09	-0.66, 0.49	-0.31	39	.761
Gender	-0.47	-0.85, -0.08	-2.49	25	.020*
Age	-0.02	-0.03, 0.00	-2.26	29	.032*
Comorbid	-0.05	-0.44, 0.33	-0.29	23	.775
Time*Treatment	0.28	-0.42, 0.97	0.81	25	.423
*Significant at $p < 0.05$; CI confidence interval; df degrees of freedom					

7.6.9 Hypothesis five: fMRI differences influencing anxiety scores, as a function of eight weeks of Kava treatment in the ROI

The LMM analysis testing the three-way interaction effect (time x treatment x fMRI signal) was not significant, $F(1,31) = 1.47$, $p = .233$. Tables 52 and 53 report the model statistics for the three-way interaction and the contrast (treatment – placebo) as the difference in estimated slope at both time points. Figures 37 and 38 illustrate the estimated slope (change in outcome per change in fMRI BOLD signal mean contrast predictor) and the contrast slope difference.

Table 52. Linear Mixed Model Interaction Term for HAM-A Anxiety Scores by BOLD Signal and Treatment Across Time

HAM-A scores, week eight	MSE	F	df	p-value*
Time*Treatment*fMRI_dACC	17.50	1.47	1,31	.233
*Significant at $p < 0.05$; df degrees of freedom; F ANOVA omnibus F-test; MSE mean standard error				

Table 53. Linear Mixed ANOVA Model for SIGH-A HAM-A Contrast (Treatment – Placebo) for fMRI BOLD Signal

HAM-A differences scores from fMRI signal predictor model	df	95% CI	t	p-value*
Treatment – placebo time 1	33	-5.62, 6.51	0.15	.882
Treatment – placebo time 2	33	-0.66, 11.42	1.81	.079
*Significant at $p < 0.05$; df degrees of freedom; B/coefficient unit t ratio; CI confidence interval				

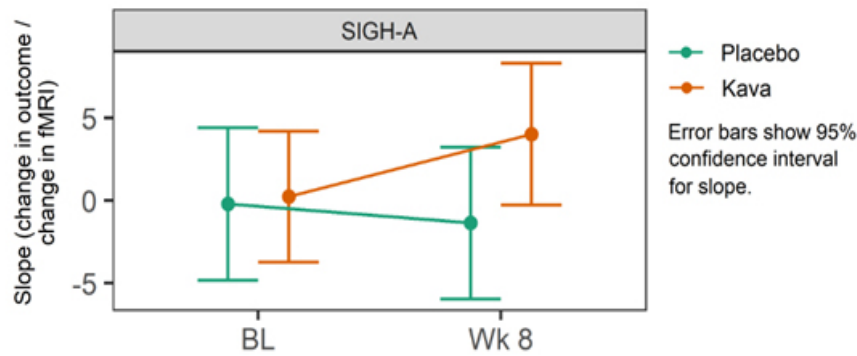


Figure 27. Treatment group slope in anxiety scores for dACC unit change in mean contrast signal

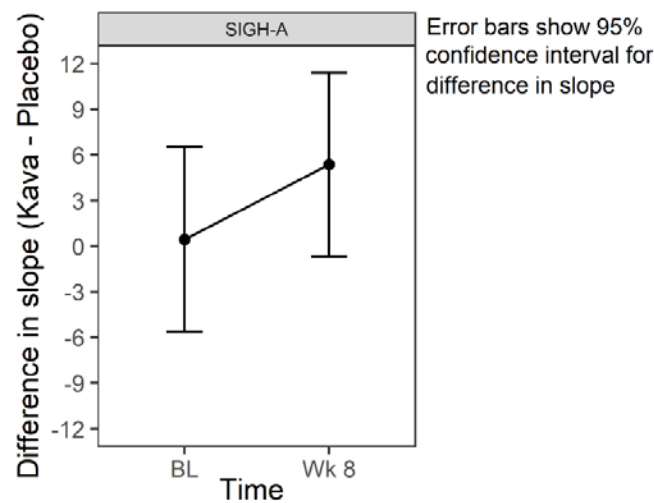


Figure 28. Calculated slope difference in anxiety scores for dACC unit change in mean contrast signal

7.7 Discussion

7.7.1 Overview

This section summarises the main findings of the intervention study and addresses these findings against the psychiatric, metabolic and functional imaging hypotheses. It then discusses how the findings fit with the existing imaging literature specific to treatment interventions in GAD. The limitations of the study methodology and future research considerations are examined in the following Chapter Eight.

The investigation sought to assess how a phytomedicine of known anxiolytic effect namely *P. methysticum* or Kava, would ameliorate anxiety in diagnosed GAD. Specifically, it sought to examine how this may occur using two neurobiological markers

via two imaging modalities: GABA via MRS and task-dependent BOLD signalling as functional activation. Specific attention was given to this activity within the dACC as the ROI deserving further examination within GAD, with evidence substantiating the cognitive-emotional properties of the region feasibly underpinning components of anxiety cognitions prominent in GAD.

First, the study yielded interesting demographic data that, in some respects, informed the neurobiological findings. While both treatment groups were mostly equitable in data at baseline, there were some differences. In terms of comorbidity, just over 50% of the GAD participants also reported a comorbid affective disorder as determined by the MINI questionnaire administered at baseline. This is slightly lower than the epidemiological prevalence of comorbidities in GAD patients, most likely due to selected allowable comorbidities for inclusion into the study.

Panic disorder prevalence was the most common comorbid condition in line with prevalence statistics (e.g. Kessler et al., 2001; Noyes, 2001). Although not significantly different for comorbid presence overall, treatment group panic disorder numbers differed at baseline (placebo $n = 7$; Kava $n = 2$). It is feasible that panic disorder influenced the response to the study treatment by contributing to an overall different biological profile, as is evidenced in the literature through response to GABAergic interventions (Noyes et al., 2001). In terms of the psychiatric profiles, more cases of a state-based anxiety disorder comorbid to GAD might produce higher mean state anxiety scale scores, but comorbidity was not a significant predictor.

At week eight, the sample size was insufficient to partition out comorbid cases and examine pure GAD response over the treatment period, which would better explore the profile of comorbidity. The comorbid presence was not a reflection of severity in the present study within the statistical models, although GAD/comorbid cases did report higher anxiety scores through the HAM-A and Spielberger State and Trait measures.

In terms of gender and psychiatric data, males and females were significantly different in their state anxiety levels immediately pre-scan. Female scores were 6 points higher than those of males and trait anxiety scores were also higher in females by approximately 5 points. The data reflect the findings of previous studies that have shown higher levels in females regardless of psychiatric category. As such, the effect of gender was also a predictor in models.

Finally, for demographic data, alcohol use produced another interesting and possibly moderating finding; higher levels in the treatment group at baseline were reported with

the quantity almost double the standard drink number of the placebo group, although this was not significant. It then became significantly different between groups at week eight. Alcohol use was used as a covariate for this reason, specifically for analyses involving GABA data, due to its direct effect on the GABA system. It is feasible that alcohol use had some effect on treatment response, although increased use may have improved response given alcohol is also GABAergic. However, this was not shown in the ANOVA models and was only shown in the treatment group during the t-tests; therefore, given that the ANOVA models accounted for alcohol differences using covariate, the possible effects found by the t-tests could have been a contribution of alcohol, although this was not shown in the GABA data.

7.7.2 Hypothesis one: Anxiety symptom change following Kava intervention

The first hypothesis, that eight weeks of treatment of 240 mg *P.methysticum* extract would significantly reduce anxiety symptom severity compared with a placebo, where the main outcome was a reduction to HAM-A scores, was not met.

Kava did improve GAD symptoms; however, this also occurred in the placebo group and even though treatment effects were significant, they were not significantly different from the placebo. The most obvious explanation for this was the influence of other factors that were difficult to quantify, such as therapeutic benefit from frequent and more objective monitoring of anxiety symptoms. The placebo effect is a well-known phenomenon in intervention studies, from merely taking a treatment, and it is also likely to come from regular and detailed discussions of symptoms that occurred with study sessions, as inadvertent ‘talking therapy’. While researchers can endeavour to maintain ‘data collection’ mentality during study sessions, the reality is that discussions involving mental health disorder symptoms such as anxiety and depression with researchers will have a therapeutic aspect.

In comparison to the placebo, the treatment did not work to produce anxiety level differences—effectively a treatment with expected efficacy did not occur in the present study given the data. Although effects were noted qualitatively, this did not translate to statistical differences.

In examining the pattern of response in anxiety levels over the treatment period, non-significant effects in HAM-A as a function of treatment between baseline to week eight was found, where the placebo group achieved similar reductions in anxiety scores over the eight weeks. However, the pattern of response was different. HAM-A measurements

at weeks two, four and eight indicated a reduction at week two in both groups before a 'kick back' by a mean of four points for the treatment group. In the placebo group, mean scores of 13 were maintained, which suggests a strong placebo effect as noted above. The non-significant results were not reflective of previous trials involving Kava interventions measured through response by the SIGH-A HAM-A anxiety measure, and additionally where this response pattern was not previously seen in other investigations comprising treatment periods of eight, 12 or 16 weeks (Sarris et al, 2009; 2013; Sarris, LaPorte & Schweitzer, 2011; Savage et al., 2015).

The strain/cultivar of *P. methysticum* that was utilised in the present study will have exhibited a unique profile of kavalactones compared with other *P. methysticum* extract interventions. This unique constituent profile may, in turn, exert differential effects on anxiety symptoms, both somatic and cognitive. While it is likely to produce attenuations in somatic symptoms, cognitive symptoms may have increased, or vice versa, resulting in no change, or even an increase in total scoring. To examine this potential effect further, the individual item responses of the HAM-A questionnaire could be analysed along somatic and psychological lines to determine whether a pattern emerges in responses over the treatment period.

With regard to the measure employed as the primary psychiatric outcome, it is also possible that the treatment elicited symptom changes that were not measurable by HAM-A. It would be of value to perform analyses in a similar manner the STAI data collected, as well as the SIGMA MADRS also administered at each visit; although, the latter measure was primarily used to control for developing depression symptoms, and thus results may be limited here.

Beyond the potential lack of effect in GAD participants on the HAM-A from the phytomedicine tested, explanations for the observed results may be largely methodological. The small sample size in the outcome data (n = 10 treatment; n = 9 placebo) was a recognised insufficiency to reveal a potential effect of treatment, especially given other factors mentioned such as alcohol use and comorbidities. The reduced sample size limits the ability to discriminate the effects of predictors on outcome data and, in turn, preventing rigorous conclusions from the results.

Given this was a sub-study of a larger investigation, the future analyses of the overarching numbers (n = 210) in terms of response in anxiety symptoms to the treatment may produce stronger more reliable results.

Second, there was a large attrition rate in the already small sample size between baseline and the second time point; close to 45% drop-out in numbers between the two time points, significantly reducing week eight data not only on main psychiatric outcomes, but also on the imaging data. This reduction further limits the conclusions because the main outcomes, effects and factors could only be assessed based on a reduced sample, where we cannot assess the status of lost participants. Imputations were applied through statistical modelling, something that a LMM approach can partially account for (refer to discussion in Schafer & Yucel, 2002).

Third, not all imaging data met the quality control for inclusion, with data from three scans removed constituting 20% of the sample at the first time point. Imaging data is discussed in Sections 7.7.3-7.7.6 specific to these outcomes.

A final explanation is that participants who did persist into the second time point were less anxious and more committed and willing or able to continue in the study. Therefore, their symptoms may not have been as severe and they may have seen less improvement in their symptoms. As a corollary, participants with more severe symptoms may have withdrawn from the study due to the procedural pressures of visits and the taking of the study treatment. In psychiatric disorder research, participant session attendance and treatment compliance are difficult to maintain in the presence of symptom severity and high levels of psychological distress. If drop-out is due to tolerance or side-effects, it may impact translational capacity into clinical settings.

7.7.3 Hypothesis two: GABA modulations following the intervention

The second hypothesis that GABA levels would be significantly reduced in the active treatment group following eight weeks of treatment with Kava compared to the placebo group, was partly met with one toolkit.

The metabolic component of the study sought to measure GABA change as a function of eight week's daily dose of 240 mg of *P. methysticum* extract in a GAD sample. GABA was quantified in the grey matter via Tarquin as the main outcome and in the grey matter from LCModel toolbox as cross-validation. All data were segmented for tissue type.

First, while all toolbox data were assessed individually with quality parameters applied, LCModel still displayed baseline group differences with spurious cases removed; SNR and FWHM were different between comorbid groups, with the latter also different between the treatment groups. These results imply that, in general, signal quality was

poorer for the pure GAD group regardless of treatment, although it was still within the acceptable parameters of reasonable quality (derived through best practice by investigators but also compared to the literature). Similarly, the higher FWHM level in the treatment group suggest a marginally reduced signal integrity, yet they were still within normal limits.

GABA metabolite data was not significantly different at baseline for treatment groups. The outcome data for GABA as a function of treatment was only significant in terms of treatment and time in the LCModel data, where GABA levels were significantly lower in the treatment group. Despite this sole toolbox data indicating significance, all GABA data displayed reductions in the treatment group at week eight, whereas levels remained the same in the placebo group.

The results here suggest that the Kava treatment reduced GABA levels in the ROI (dACC). *P. methysticum* as a GABAergic substance should facilitate substrate mechanisms in favour of increased GABA levels, most likely via calcium and sodium channels around the GABA-A receptor sites to perform the indirect enhancement of GABA-A receptor function as well as enhanced ligand-binding at GABA-A. However, the optimal function within the substrate may not be as simple as observing increases or decreases to GABA levels if *P. methysticum* of the observed modulations represents a 'normalising' of function in reducing levels.

A likely explanation is that elevated levels are reflective of a compensatory mechanism, where higher levels indicate an increased need for inhibition via the substrate in the ROI. A reduction to these levels might reflect a normalising of the GABA system and a reduced need for GABA within the ROI.

Data from the previous cross-sectional study indicated elevated GABA levels in GAD participants in the main toolbox examined (Tarquin) and lower levels in healthy controls, and thus this data is congruent with the reasoning that the direction in the GABA trend reflects reductions towards levels observed in the healthy samples. As with the first study, for these conclusions to be rigorous one would expect a uniform trend across the MRS toolbox data and when LCModel was employed to validate the results, the pattern in the GABA levels was not congruent, which prevents solid conclusions of trends from modulatory effects.

The change to the GABA level as a function of the *P. methysticum* extract within the dACC represents a novel finding for several reasons. First, it suggests that the mechanisms within this region are amenable to Kava intervention. *P. methysticum* has

not been tested for measurable changes in brain functions in human samples, either functionally or through spectroscopy, and the present study represents the first investigation of brain-related changes of Kava. Second, GABA measurement from interventions in GAD is not plentiful in the literature and there is insubstantial focus on GABA levels to properly compare with the existing literature. Third, to validate the findings, more than one toolbox was used, which may go part way to clarifying the often-equivocal findings in metabolic studies that do exist, not just in GAD but more broadly in affective disorders.

The functions of this ACC subregion are evidenced as cognitive-emotional feedback between the environment and self, which is most associated with threat-related information, error and incongruency detection and negative anticipation (Bush, Luu, & Posner, 2000; Heilbronner & Hayden, 2016; MacLeod et al., 1988; Mochcovitch et al., 2014). That this region ‘hosts’ prototypical GAD cognitions was the reasoning behind focusing on this region, where it was expected that modulations would be shown in anxiety symptoms.

However, the effect of treatment reflective of reductions to anxiety levels was not shown in the anxiety symptom analysis, which suggests that biological effects are not always translated to symptom improvement using the current measures. The intention was to examine what role the dACC may play in the maintenance of GAD symptoms via measures such as GABA and BOLD activation (examined next). It does not suggest that the Kava treatment was unsuccessful in modulating other brain areas, where modulations may have occurred in several regions—even more globally—as a function of treatment. Further research would ideally examine additional brain regions to determine whether the effects are measurable in other brain areas geared towards relevant GAD cognitive-emotional processes.

Therefore, it is valid to suggest that other substrates may have also displayed measurable changes in profile as a function of treatment such as glutaminergic (glutamate/glutamine) changes. The observed GABA effects could be indirectly reflective of metabolic modulations within other substrates that also warrant future examination.

7.7.4 Hypothesis three: GABA and anxiety modulations following the intervention

Changes to anxiety symptoms levels as a function of the active treatment as reported on the SIGH-A would be associated with MRS-measured GABA level decrease in the ROI via interaction effects in ANOVAs, was not met.

Given the non-significant results with regard to anxiety measures as a function of treatment, the subsequent analysis of modulations to anxiety level as a function of GABA was unsurprising. Similarly, group differences of anxiety levels at week eight assessed through contrasts (slope difference between groups) did not indicate differential effects beyond trends discussed previously at time points between when GABA data were collected. The data here suggest that, in the dACC, a relationship between GAD symptomology and regional GABA levels was not measurable with the outcomes utilised and/or in the models employed to analyse them.

In GAD, measurements of the metabolic relationships to anxiety symptoms as a function of treatment are sparse with which to compare outcomes. Modulations to GABA observed by itself might have elicited symptom changes that were not measurable through HAM-A and analyses with state anxiety, or even SIGMA MADRS for mood might expose an association. In keeping with the main anxiety outcomes, the data were collected but not included in the current methodology. Taken with the observed relationships in the cross-sectional study regarding GABA levels and anxiety in both GAD and healthy control samples, it would be wise to test biological changes against other psychiatric measures.

Finally, also with consideration to improvements to methodology, an introduction of a third arm as an intervention such as a benzodiazepine comparator would have been useful to assess the anxiety/GABA relationship using a substance that is purely GABAergic, given the known activity of *P. methysticum* in other substrates. Some of these elements would broaden the current methodology and findings and are also discussed more specifically in the next chapter.

7.7.5 Hypothesis four: Functional activation modulations following the intervention

Regarding the effect of the treatment predicting task-based BOLD activation reductions (via increased negative BOLD activation in the contrasts) in the ROI, the data did not indicate significant modulations at week eight. In the model, however, both age and gender predicted BOLD signal at this second time point.

Studies in the literature have examined task-dependent activation following interventions in GAD, with the majority examining activation together with symptomatic treatment response to SNRI and SSRI antidepressants such as venlafaxine and citalopram where, generally, reductions to the prefrontal and paralimbic regions, striatum and insula were associated with treatment response (Hoehn-Saric, Schlund, & Wong, 2004; Pollack, Meoni, Otto, & Hackett, 2003).

BOLD signalling changes as a function of GABAergic treatments in anxiety samples are few; one study in healthy samples (pregabalin; Aupperle et al., 2011) suggests reduced activity (in this study, amygdala and insula) associated with treatment.

Further, it is difficult to extrapolate regional activation changes in these investigations as a function of treatment that do not also model symptom response into the outcomes. By virtue of utilising clinical sample groups, the outcomes are associated with clinical symptom changes. It would be constructive in investigations to structure a proportion of the data modelling without psychological outcomes to account for biological effects of treatment (active or placebo).

In the model, both age and gender predicted BOLD activation at week eight, which provides evidence for differences with age and gender in signalling patterns, similarly found in the previous cross-sectional study in modelling group differences. Age and gender represent crucial biological variances that influence anxiety disorder profiles in both diagnosis and treatment and should be factored into all intervention methodologies.

7.7.6 Hypothesis five: Functional activation and anxiety levels following the intervention

Changes to anxiety symptoms levels as a function of the active treatment would be associated with BOLD activation level decrease measured during the anxiety cognition task (correlational SIGH-A and BOLD signal), was not supported.

Given the lack of change to anxiety symptoms and no change to BOLD contrast estimates in the previous models as a function of treatment, the current study indicated there may be little connection between the two for this region. It would be useful to examine whether successful response to treatment and/or pre-treatment signalling levels influenced the relationship between the ROI signal and anxiety severity, which could have more clearly exposed a relationship much as it had done within studies discussed previously.

In the present study, it was not possible to perform such an analysis, which might be applied by separating responders (set level such as 30% reduction to symptoms) from non-responders and gauging BOLD signal change across treatment groups, due to the reduced number of cases in each group measured after the intervention. Given this limitation, the analysis in the present study separated the biological function from the

symptom response, informing both intervention efficacy and appropriate use of neuroimaging measures for symptom change.

7.7.7 Kavalactone mechanisms

The *P. methysticum* extract used in the present study did not meet efficacy expectations in terms of anxiety change but produced measurable biological modulations via GABAergic changes. The profile of this extract was a 'noble' *borugu* strain, obtained exclusively for bottling for this study. The bioactive constituent profile was not an exact replication of previous *P. methysticum* extract studies conducted in the literature as well as in previous research conducted by the candidate and associate investigators using *P. methysticum* extracts, and so consideration to the interpretation of results for Kava 'at large' should be conservative.

Given that constituent profiles will vary within a species as well as displaying within-batch variations, this batch yield produced unexpected effects, that is, a lower rate of response and remission in terms of anxiety measures for the active treatment group than predicted, which was equitable to the placebo group.

The profile of *P. methysticum* utilized in the current study was comprised mainly of kavain and dihydrokavain (refer Chapter Seven results for constituent profile). Furthermore, actions of each kavalactones perform differently upon substrates such as GABA, for example, kavain and desmethoxyyangonin produced increases, whereas yangonin thought to decrease, and dihydrokavain and methysticin producing no changes (Johnston et al., 2009; Spinella, 2001). Individual kavalactones elicit some well-evidenced effects; dihydromethysticin and dihydrokawain are evidenced as neuromuscular blockers and spasmolytic agents in animal models, producing similar effects to benzodiazepine agents such as oxazepam and diazepam (Jussofie, Smiz, & Hiemke, 1994).

The most well-known activity is with the GABA-A receptor complex, where kavalactones do not bind directly to components, such as benzodiazepine receptor, but rather via indirect actions (such as kavain on thromboxane) in turn promoting the effect of GABA as a GABA-A antagonist. They are positive allosteric modulators of chloride channels, increasing chloride influx and are also calcium channel inhibitors, initiating up to 70% reduction (Chua et al., 2016; Gleitz et al., 1995; Magura et al., 1997; Martin et al., 2000). Both these processes serve to produce inhibition of neural firing within substrates and it is at this level that the present study has focused on GABA.

Despite evidence of activation via GABA-A receptors in tissue and pre-clinical studies (for example, Boonen & Haberlein, 1998; Table 12), there is also evidence of little or no

effects within GABA or benzodiazepine receptors sites (Davies et al., 1992; Jussofie, Schmiz, & Hiemke, 1994).

Kavalactones also feasibly block NMDA receptors (Walden et al., 1997; in guinea pig hippocampus), which may explain an alternate (but not unrelated) pathway for anxiolytic activity. They also show the exert effects within aminergic pathways; modulations are also evidenced within DA substrate, with larger doses in the nucleus accumbens in rats via blockage calcium ion channels, as well as blocking the reuptake of 3H-noradrenaline in rats cortical and hippocampal synaptosomes and monoamine oxidase-b inhibition, which antidepressants also exhibit (Baum, Hill & Rommelspacher, 1998; Seitz et al., 1997). There is also preliminary evidence that constituents such as Kavain act like anaesthetics, with some evidence of muscimol binding or transmembrane anaesthetic site activity (Chua et al., 2016). These processes were discussed in Chapter Five and summarised in Table 12.

Additional metabolite data were collected during the scans that were not utilised in this thesis and warrant further exploration for possible modulations, including glutamate and glutamine. Given the purported modulation of the MAO-B receptor substrate of *P. methysticum* via processes such as calcium ion channel blockades, glutamate/glutamine data may indicate modulations and could be cross-referenced for mood improvement measured via the MADRS following the intervention. The glutamate system in the literature has shown promising, albeit somewhat equivocal, linkages to the pathophysiology of depression, where substrate modulators appear to improve symptoms. Use of the NMDA receptor inhibitor ketamine has evidence for efficacy in acute reductions to depressive symptoms (Cornwell et al., 2012; Zarate et al., 2006 2006). Preliminary models also suggest MAO-A inhibition produces antidepressant effects via aminergic pathways, such as the degradation of dopamine and one mechanism via glutamate inhibition (Finberg, 2014; Maher & Davis, 1996).

7.8 Conclusion

Despite some hypotheses not being met, which could be attributed to limitations (refer to Chapter Eight for a greater discussion), and where the study findings might be interpreted as pilot data at best, this research produced both novel results, and a practical methodology to apply multimodal neuroimaging to future GAD investigations. Figure 39 in the next chapter illustrates a summary of the findings from both studies.

The research conducted provided some evidence of a relationship between GABA and metabolic processes within regions associated with emotion stimulus processing. In addition, modulation of the GABAergic system holds an important role in the pathophysiology of GAD cognitions because of how the properties of this region change following anxiolytic interventions that also produce amelioration of GAD symptoms.

The lack of findings for fMRI change reflects in part the results from the first study where differences were not observed between groups in the region of interest within the parameters of the condition contrasts.

The GABA results are also congruent with evidence from *in vivo* studies indicating GABAergic kavalactone modulations. Given the evidence for GABAergic dysregulation in GAD symptomatology, these findings suggest kavalactones play an important role in the amelioration of GAD symptomatology via the modulation of the GABAergic system within the limbic/prefrontal region that serves to guide associated cognitive/affective processes. Therefore, given that GABA neural substrate function in response to a treatment intervention in GAD has not been previously explored, the findings serve to clarify part of the relationship to anxiety symptoms, in terms of patterns of treatment response.

The investigation identified the importance of replicated *P. methysticum* constituent profiles to provide a greater likelihood in replicated findings of previous research in respect to clinical efficacy. Based on the current literature in both pre-clinical and clinical studies, where constituent profiles are indicated (or tested individually), the yields and strains of *P. methysticum* with varied kavalactone profiles clearly elicit different systemic effects, which in human studies may thereby produce differential findings such as the results in the present study (refer Chapter Five for specific research, as well as a systematic review written by the candidate in the Appendix).

The current line of pharmaceutical and psychotherapeutic treatments is not efficient for the treatment of GAD (Starcevic et al., 2012). Demand for evidence-based nutraceuticals exists and *P. methysticum* extract is an example of an affordable, safe and effective option.

8 Chapter Eight. Integrated Thesis Discussion and Conclusions

8.1 Preface

This final chapter will summarise the document as a whole, discuss the findings and the limitations from both the two investigations, and outline the future research directions. It also revisits the aims and objectives, before presenting a final synthesis and conclusion.

8.2 Chapter Two. GAD

The second chapter (Chapter One comprised document introduction) was one of five discussing GAD research that has used neuroimaging. This chapter sought to better understand GAD from several considerations. It did so through discussing the history of the diagnostic category, assessment, and symptoms, as well as factors on the maintenance of symptoms. It also examined the prevalence of GAD, both alone and with typical comorbidities.

8.3 Chapter Three. Biomarkers

This chapter extensively examined how biomarkers might best be utilised in psychiatric disorders and, more specifically, affective disorders, and how selected biological processes could potentially contribute to the identification and maintenance of symptoms unique to GAD.

8.4 Chapter Four. Neuroimaging anxiety biomarkers in GAD

Under the rubric of the structural approach in the previous chapter, this chapter looked at how selected biological processes contribute to the identification and maintenance of symptoms unique to GAD, measured through neuroimaging. It became clear during this review that patients with GAD exhibit symptoms and markers that are common to affective and mood disorders, and that a shortfall exists in research in this area. By extension, this chapter focussed on two biomarkers of interest that might better define GAD among these disorders: GABA substrate function and the ACC subregions, namely the dACC.

8.5 Chapter Five. GAD Treatments

This chapter shifted focus onto treatment interventions for GAD and their involvement in modulating GAD symptoms and markers, mainly focussing on but not limited to pharmacological treatment. It was important to acknowledge the capacity for novel treatments. To this end, *P. methysticum*/Kava was examined in detail, given the evidence for efficacy in GAD, but also an identified need for investigating Kava's biological modulations associated with anxiety symptom improvement.

8.6 Chapter Six. Investigation of the Structural, Functional and Metabolic Markers of Generalised Anxiety Disorder, A Cross-Sectional Study

This chapter highlighted two neuro-biomarkers from Chapter Four and investigated task-based functional signal and GABA metabolic differences using GAD and healthy control sample groups, and associations with anxiety severity. Additionally, it examined factors influencing biological outcomes, such as age, gender and comorbidity in GAD.

The metabolic data showed that there were elevated GAD/comorbid GAD GABA levels compared with healthy controls. Analysis of data across the groups suggested that GABA levels in this region play a role in guiding differences in state-based anxiety symptoms. Data from the toolboxes were not congruent; and while it is probable the methods applied within each toolbox generated the observed GABA profiles, conclusions are limited due to the inconsistent findings.

For the functional data, the IAPS paradigm was successful in producing regional activation changes. These regions were largely overlapping but there were differences for each group, and were areas associated with stimulus perception, appraisal and response. The data suggested a capacity in the healthy sample for greater 'variable' brain function that could be interpreted as cognitive flexibility that comes with efficiently processing emotional and cognitive material, insofar as behaviour can be related back to both functional imaging data and cognitive processes. In contrast, it also implied an invariability or inflexibility in the GAD group to be able to do this.

Second, when groups were compared via the neu-neg > neu-neu contrast, increased activation was observed in GAD sample in the dlPFC, IPL and mid-cingulate regions compared with reduced activation in the healthy group. This suggested again a deficit in regional mechanisms as a product of more effortful cognition in areas associated with memory and cognition, when presented with uncertain conditions in GAD, that we may observe as the pattern that underlies anticipatory anxiety. It is feasible this group may

exert more effortful attentional control due to negative interference effects of the negative stimuli. For further analysis to test this, an examination of group differences in neutral contrast reaction times, versus negative contrast reaction times, would likely reveal an interaction if this were true.

The dACC region, thought to display strong group differences, was not shown to be significantly different, although the trend reflected the other regions of increased activity in GAD. Yet, in both groups, the contrast activation predicted state anxiety in a negative fashion, where an increased signal was seen in lower anxiety. Additionally, in healthy controls, trait levels were predicted by the ROI signal, and gender and age modulated this relationship. The results indicated that state-based mood is a stronger indicator of BOLD signal difference and could feasibly be a more reliable indicator of the anxiety severity.

Finally, in the bi-modal analysis, a predicted relationship between the BOLD signal and GABA levels was not found in the ROI. The results from this study are summarised in Figure 39, together with results from Chapter Seven (left to right in the centre being whole brain, to dACC biomarkers functional to metabolic; and the two groups with reported trends above (GAD), and below (healthy controls)).

8.7 Chapter Seven. Investigation: of GAD Biomarker Modulation from Kava (*Piper methysticum*)

Continuing on from the conclusions in Chapter Five, this investigation employed the same measures emphasised in Chapter Four to test how Kava might modulate both brain activity and GABA profile in the dACC. This study operated as a ‘proof of concept’ investigation, both in establishing measurable changes in GAD neuro-biomarkers and substantiating mechanisms amenable to Kava as a GABAergic intervention. It addressed these questions through GAD symptoms and biological outcomes separately, before examining neuro-biomarkers in the prediction of anxiety symptom changes.

This study found that a daily dose of 240 mg *P. methysticum* extract for eight weeks was not successful in eliciting anxiety symptom improvement at the second time point. However, GABA level reductions were associated with Kava in the dACC region, congruent in GABA from both toolboxes. The functional data did not indicate an effect of treatment on activity in the ROI. Finally, analyses of anxiety over time were not amenable when either GABA or BOLD signalling biological marker change, when they were tested as predictors. Please refer to Figure 39 that illustrates the study’s findings

together with the previous investigation, to form a picture of the biological-anxiety relationship within and between the two groups, and a product of the intervention in this chapter.

8.8 Chapters Nine and Ten. Bibliography and Appendices

These chapters support and supplement the contents of the thesis in several ways. The Bibliography is self-explanatory. The Appendices provide publications written by the candidate. The first, a systematic review on GABAergic phytomedicines, as a method of operationalising GABA function in the context of interventions and to expand the knowledge base on alternative treatments for GAD beyond existing pharmacotherapeutics. The second, a protocol paper for the NHMRC-funded Kava for the Treatment of Generalised Anxiety Disorder (K-GAD; refer Appendix F for protocol paper), of which the investigation in Chapter Seven was a sub-study component. The candidate was instrumental in creating the methodology as a whole and was involved in publications beyond current reported findings.

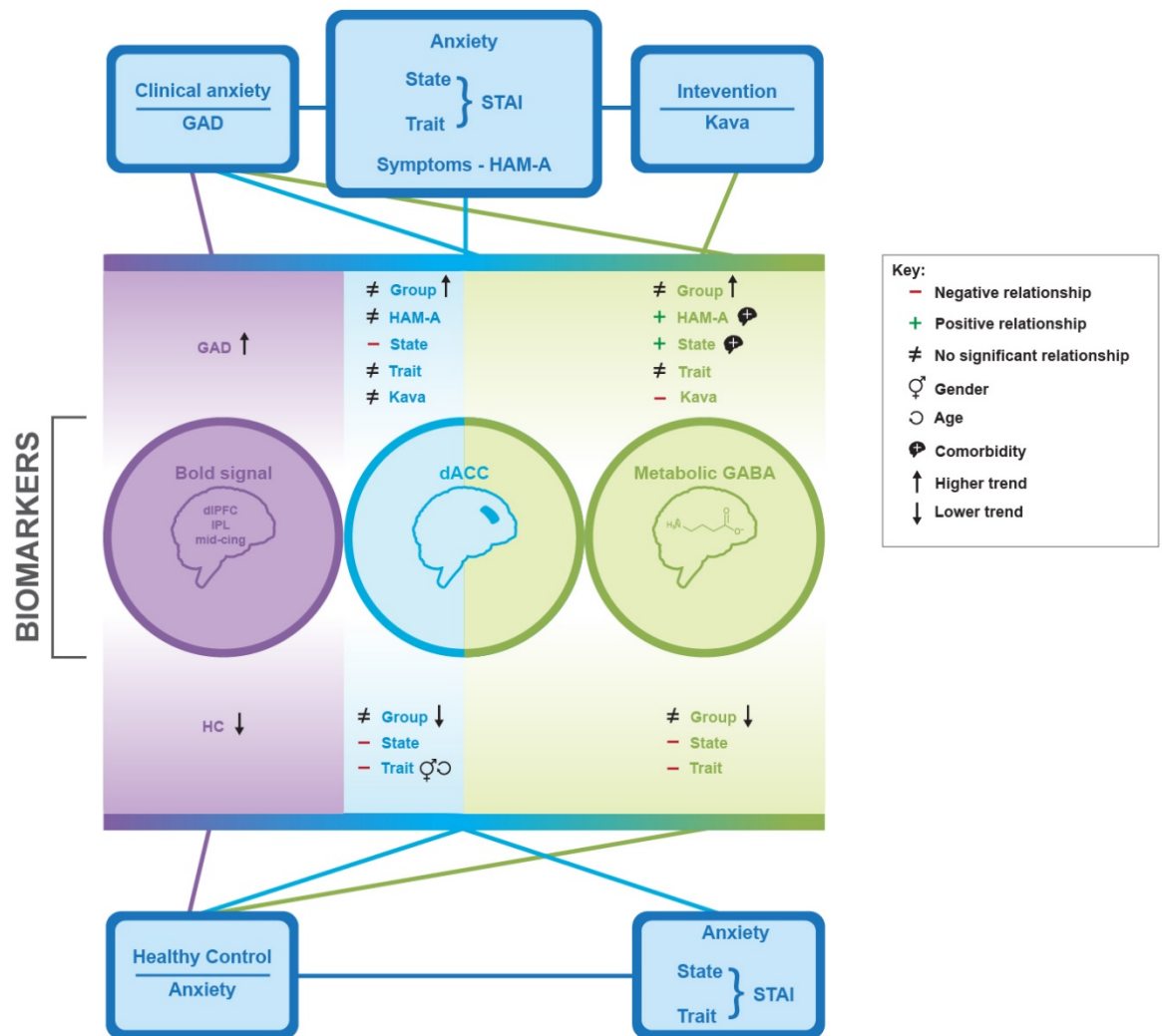


Figure 29. Summary of bimodal imaging and anxiety symptom findings from the investigations. Within and between groups, top: GAD; bottom: healthy controls (relationships are significant, trends were not significant) Lines present samples and measures used for each coloured modality.

8.9 Limitations and ramifications from the Two Investigations

Some methodological limitations were identified from the studies, which contribute to limiting the generalisability of the findings. The latter element is also a product of placement of the research within the literature—the findings from the two investigations do not have enough supporting evidence with which to compare results or even conclude equivocal findings. Therefore, it would be difficult to derive

conclusions about distinctive GAD biomarkers and pre-emptive to make generalisations about their utility.

8.9.1 Sample size

Both studies experienced a limitation in sample size that may constrain findings given the number of analyses. Functional imaging data analyses, in particular, warranted statistical correction for multiple comparisons and, therefore, an appropriate sample size had to be reached to enable adequate power to confirm findings. Fitting GLM models to the limited data quantity risked the appearance of type one errors - one example in the intervention study being treatment x time x GABA assessment in the LMMs, where the observed significant effect at baseline was not statistically feasible.

The first study met adequate sample sizes at the outset ($n = 33$ healthy controls; $n = 37$ GAD); however, once division of the GAD group by comorbidity, followed by the removal of highly variant images in the functional data and GABA cases via application toolbox-specific quality parameters, the sample size became lower than anticipated. In the intervention study, the sample size was more problematic. Participant attrition following baseline was close to 50%. In both studies, there was difficulty with finding suitable and willing imaging candidates within both sample groups. Common issues were handedness and contraceptive medication and, in the intervention study, lost-to-follow-up between the two time points. However, the majority of imaging studies using GAD and healthy control comparator groups have sample sizes within 15 and 25 per group - similar in size to the present investigations. A large sample, while highly sought after, is often not achievable in the time allowable, nor in funding to fit the scale of the project, all of which applied to an extent in the present investigations.

8.9.2 Psychological and lifestyle variables

While the studies attempted to control for participant variances such as psychiatric and medical histories, some factors could not be controlled for, but also for which data were collected but not utilised in the thesis. Personality, psycho-social life events and diet are some examples. The studies did not contain a dietary questionnaire to quantify nutrient indices and it is feasible that nutrient levels could influence the profile of brain metabolites. The intervention study did, however, control for caffeine, alcohol and supplement use, and did implement a schedule for Kava treatment dose and standardised meal occurring on the day of each brain imaging session, all of which to best control for such influences within a randomised controlled trial structure.

Second, life events can greatly affect stress and anxiety levels and contribute to the maintenance of symptoms in affective and mood disorders (Francis, Moitra, Dyck, & Keller, 2012; Kendler et al., 2003). Life events were collected via the Social Readjustment Scale (Holmes & Rahe, 1967) in both samples, but the measure was not utilised in the investigation design. One MRS study illustrated the effect of life stressors on metabolic patterns; NAA metabolite levels in the DLPFC differed within a GAD sample with the presence or absence of self-reported childhood abuse, suggesting a biologically-demonstrable subtype of GAD (Mathew et al., 2004). Although not included in the present study for brevity purposes it is a pertinent example of factors, in this case environmental – playing a role in symptomology that could be examined in the future. Figure 2 in Chapter Three, as well as Figure 39 in Chapter Eight, illustrates how life stressors, among other precipitating/perpetuating factors, can interact in GAD.

Third, personality is a quantifiable construct with evidence of influence in psychiatric disorders, particularly affective disorders, and is demonstrable through emotional-behavioural processing. Additionally, both Figure 2 and Figure 39 illustrate an influence in the literature, as well as existing data available for future research. There is much overlap with scale items from personality inventories discussed in Chapter Two, Section 2.10, including introversion, neuroticism, openness to experience/adventure seeking and anxiety-proneness, with affective disorders (Bienvenu et al., 2004 ; Cloninger, 1986; Clark, Watson, & Mineka, 1994). In the present studies, the Spielberger trait anxiety scale attempted to identify how more enduring trait anxiety might be related to neuro-biomarkers; however, these data were not used as a covariate to control for what may be a substantial influence on the biomarker/psychiatric status relationship. As with life stressor data, personality data were collected in both groups via the Swedish Universities Scale of Personality (Gustavsson et al., 2000), a validated scale within both healthy and psychiatric sample groups, which will be examined in future analyses.

Finally, the investigation in Chapter Six identified that scan novelty would have been an important, but overlooked, variable to collect in both groups (whether it was a first time entering an MRI scanner), as the first experience is very often unfamiliar, uncomfortable and claustrophobic. Therefore, it may have influenced the ability to focus on the functional task and also remain lying still in the scanner with minimal movement. More importantly, given that anxiety levels were main outcomes, this factor was very likely to influence state anxiety level scores collected pre-scan in both groups, but also in the intervention study at the first time point.

8.9.3 Medication and/or substance use

The differences in medication and supplement use between groups in the cross-sectional study feasibly influenced GABA levels in the GAD group. While the healthy control group was medication- and supplement-free, selected concomitant items were allowable in the GAD group. Attempts were made to control for psychoactive substances such as cannabis and substances used for anxiety symptoms (such as benzodiazepines and valerian) were stringently monitored. However, GAD participants might have consumed GABAergic or even opioid substances higher than reported, which may have reduced the differences between the groups in GABA level and, to an extent, BOLD signalling. Similarly, in the intervention study, alcohol use was a prominent substance differing between the treatment groups, and an influence on the GABA profile between baseline and week eight is highly likely. Hence, alcohol use was used as a covariate in the models to best account for a possible effect on outcome measures.

8.9.4 Comorbid conditions

The investigations controlled for comorbid presence by splitting the GAD group into pure GAD and GAD/comorbidity. The method section (6.4.4) detailed which disorders were accounted for by doing so and comorbidity became a significant predictor of some of the biological data. However, there were considerations arising from this approach. Comorbid participants, without accounting for depression prevalence, had higher depression scores, state-based anxiety and anxiety symptom levels on the SIGH-A. Although significant differences were not observed generally, comorbidity was a predictor in some of the modelling anxiety and metabolic data where it was associated with higher scores. This impact of both depression and affective disorders are discussed here.

8.9.4.1 *Depression symptoms and comorbidity*

The two investigations examined and tested the validity of GAD and GAD/comorbid separation. In the limited literature the data concur with the assertion that pure GAD may differ in the neurobiological profiles from GAD plus comorbidities. Typically, comorbidity comprises MDD, as discussed in Chapter One. However, the studies did not account for lifetime MDD as a comorbid condition, which although arguably more 'naturalistic' given the epidemiological prevalence, was a feasible limitation in not clearly defining a MDD/GAD relationship. Indeed, some papers have asserted a need for a separate category for GAD/MDD from pure GAD (e.g. Kessler et al., 1999) or,

tangentially, a need for a separate category within MDD to account for the mixed anxiety-depression MDD-GAD subtype (Watson, 2005).

Depression symptom levels in the GAD group were assessed via use of the MADRS/SIGMA. Second, depression prevalence was validated in inclusion criteria as being no depression episode in the previous 12-month period. Coupled with the emphasis on anxiety outcome measures, in both studies, the depression symptom presence had to be minimised. While the main outcome was anxiety levels obtained from the SIGH-A in GAD, the MADRS results would be very valuable to examine further, which may be achieved via the MADRS linear model in place of the SIGH-A given the significant associations in the t-tests to begin with. A limitation would be the ceiling effect in MADRS scoring; however, the data may be useful to measure depression symptoms as a product of the treatment intervention and against biological measures.

Of the GAD group, 51% ($n = 20$) had a history of major depression and the occurrence of lifetime depression, even without an episode in the previous year, may display a profile different from true 'pure GAD'. It is feasible that participants classed as pure GAD (i.e. no affective comorbid condition) and without an MDD episode in the window period prior to enrolment, by virtue of having a history of MDD, displayed different biological profiles, in turn affecting the data; whether MDD presence in the sample displayed reduced GABA levels (and new ones, such as glutamate).

A recent review/meta-analysis examined MRS GABA in MDD and healthy control sample studies (Godfrey et al., 2018), both whole brain and, more specifically, within the ACC region. Six studies comprised overall effect test of $p = .004$ for the reduction in the ACC compared with healthy controls. Moreover, the review also examined GLX and glutamate to elucidate whether levels behaved as a corollary to reduced GABA in the brain with an emphasis on ACC. Whole brain GLX and glutamate levels were not significantly different; however, in the ACC, GLX was reduced significantly ($n = 12$ studies; $p = .05$), but not glutamate ($n = 9$; $p = .240$). The discussions following both studies emphasised the influence of the glutamate substrate on depression aetiology, maintenance and treatment. Again, it would be of value to examine GLX and glutamate levels as these levels were measured as the basis set in the present study, however, they were not analysed.

8.9.4.2 Affective comorbidity

The second influence under comorbidity is from secondary affective disorders that may have influenced trait, state and symptom anxiety data. For the GAD group, social phobia, agoraphobia and panic disorder were allowable secondary comorbid conditions; however, they were not separated in analyses beyond comorbidity presence. In the first study, increased state anxiety levels were observed in these participants (although not significant). These disorders tend to be more state-specific; therefore, the act of undertaking a scan may have influenced state anxiety levels measured immediately before the scan more so in these cases.

Neuro-biomarker and psychiatric results indicated differences between pure GAD and comorbid groups, which suggests there are measurable differences. The reasoning here is how affective disorders differentially influence biological measures and there is evidence in the literature, for example, social phobia, where increased DLPFC is observed in conditions of anticipatory anxiety (public speaking; Boehme et al., 2014). It is feasible that concerns over performance occurred in participants with social phobia. Similarly, in participants with panic disorder, particularly with agoraphobia, the scanner environment may have also elicited worry and subsequently influenced performance, or at least affected brain-related activation levels during the task.

Comorbidity presence in the present study suggested a different pattern from pure GAD, but one more complex than the present investigations could address. This goes back to the capacity to define GAD uniquely, against definitions of other affective disorders, and supports the argument that while current symptom-based categories are limiting and have a primary basis on psychiatric, behavioural and, to an extent, somatic symptoms, biomarker evidence is still preliminary beyond identifying candidate pathways.

It would be of use to examine metabolic levels and BOLD signalling in pure GAD and other affective disorders separately in a larger study with some form of group prediction. Group prediction through marker assessment has been investigated before in anxiety disorders (Ball, Stein & Paulus, 2014; Goodkind et al, 2015). It has also been investigated in modelling for prediction of treatment response (Lueken et al, 2016; Shin et al., 2013) and provides an argument for biological subtypes, and potential re-categorisation of disorders such as GAD along biological lines. In the context of the present study, an optimal analysis may have been to divide variables by high and low levels and run linear modelling in the form of logistic regression to establish the

influence of secondary affective disorders separately and to identify whether covariate creation was needed in the models.

8.9.5 GABA data between toolboxes

The quantification of GABA as a metabolite across different editing techniques exemplifies differences in toolbox methods to achieve these results. This was to the degree that only congruence was achieved in 50% of the data. While direct comparisons between toolbox data was not a primary outcome, congruence between toolboxes was expected given all data were derived from the same scan sessions.

Despite the continual evolving of acquisition, processing and analyses of MRS, there are a number of elements affecting data quality at scan level that, in turn, affect the data derived from it, e.g. pulse sequence parameters, such as timing; the j-evolution for the GABA spin system, which is TE-dependent (GABA benefits from longer TE); B0 field frequency offset; and macromolecule contamination that can partly be accounted for in post-processing. Therefore, the conclusions were further limited owing to variations in the post-processing pipeline approaches to the data, even prior to statistical modelling.

The aforementioned quality parameters were still different between groups despite attempts in the method and analysis to identify and account for them. There are several possible reasons for this inconsistency; it is feasible that even within the successful scans, aberrations were built into the GABA/grey matter calculations derived from segmentation data, producing inconsistent results on an individual level.

Congruence not being achieved from some of the main GABA outcome analyses was unexpected and suggests the data were not rigorous enough to show trends statistically. A further possibility could be issues with the scan session components, such as movement compensations, localisation and shimming processes, inherent to toolbox-specific pre-processing of the data (and their variances in exposing GABA spectra, such as time vs frequency domain algorithm), and then shown in final quantification data. Manual quality control steps stated in the procedures could only go so far to control for built-in data quality.

A further explanation may be in how Tarquin and LCModel basis set methods will produce variances in data. In terms of technique in quantifying the GABA model, LCModel is user-independent and uses simulations for basis sets, implying larger error potential in applying a model to any data. LCModel can require further steps in pre-processing and can be modified. Tarquin applies a dual Gaussian singlet and an

automatic frequency correction built-in, that whilst it cannot be modified, it is more likely to exclude spurious cases when resolving signal.

In terms of their quality measures, LCModel applies Cramer-Rao Lower Bound (CRLB) as the lowest possible error variance/standard deviation whereas other toolboxes apply residual standard deviations. Use of CRLB in a percentage 'relative' form can contribute to data bias via limit estimations for a group by eliminating valid cases, as opposed to use of an absolute estimate derived from standardised metabolite concentration ranges (Cavassila et al., 2001; Kreis, 2004; 2016).

Tarquin was utilised as the primary method as it has the advantage of short echo time *in vivo* automated analysis and is performed in the time domain using non-negative least-squares to assess the data fit to the model and residual results. Tarquin is understood to overfit to signal (Jenkins, Chandler, Langbein, & Shermer, 2019). Whereas with LCModel, which fits using the frequency domain, there is a limitation in fitting the signal decay that it attempts to accommodate through overestimation. It is possible that precursor factors like delays in receiver initiation from the exact start of the FID, or from the exact centre of the echo, may also affect the accuracy in this manner. However, this impact on GABA quantification seems equivocal in the literature, and both these factors; in terms toolbox-specific resolution of the GABA signal and the parameters applied to the data are possible contributions to different findings of LCModel from Tarquin in some of the results. Although not included in the two studies, it will be of value to cross-validate the Tarquin and LCModel results with data from Gannet, which utilises a peak fitting/integration method, and applies a stricter model in its *Fit Error* quality control, aiding the removal of spurious cases.

Studies that have employed GABA regional comparisons across toolboxes concur on a high level of variability, both within toolbox and brain regions. A common method to assess toolbox variations and bias has been via the creation of a within-subject coefficient of variation, derived from quantification of water and/or creatine ratios, and have estimated variability between 4% and 15% between Gannet, LCModel and AMARES in jMRUI toolboxes (for example Bogner et al., 2010; Mikkelsen et al., 2016; O'Gorman et al., 2011; Puts & Edden, 2012b). This variability itself tends to be region-specific, with data from several studies suggesting the ACC region in particular is subject to 12%–15% variation (Mikkelsen et al., 2016; Near et al., 2013). To ground this significance in the current findings, this variability is most likely reflected in the results, or rather the lack of

coherent results across toolboxes, which the methodology and resulting sample size could not accommodate.

A direct comparison of GABA metabolite data between toolboxes via Bland-Altman analyses may be one approach (Bland & Altman, 1999), although of great value to examine in the future, was beyond the capacity of this thesis other than in addressing how the approach by each toolbox may have influenced the data. Chapter Four of the thesis outlined some of the discriminating features of obtaining GABA data and the merits of the selected toolboxes, and several very good papers have done so that the interested reader may examine (Bogner et al, 2010; Mikkelsen et al., 2016; O’Gorman et al., 2011; Puts & Edden, 2012b), as well as more general reviews on the topic (Mullins, 2014).

8.9.6 fMRI data

Effectively deriving fMRI signal for analysis in any study depends largely on the capacity to understand and apply the fMRI signal to cognitions of interest (refer to discussions in Arthurs & Boniface, 2002; Poldrack, 2006; Logothetis, 2002; 2003). There are several considerations that are broadly applicable. Beyond whole brain group differences in activation and the dorsal anterior cingulate, the investigations in the thesis studies did not examine other brain regions as specific ROIs. In the regions that were different in the contrasts between groups, it may be impossible to conclude whether, in the current studies, this brain region was behaving uniquely as a product of GAD cognitions; the differences in activity profiles between groups might not be GAD-specific.

This relates more broadly to the problem with reverse-inferencing inherent to neuroimaging; in the context of the current designs, it was limited to only looking at brain activity differences between two groups that were then associated with greater or lesser anxiety, that can hope to be unique to one disorder, in this case, GAD compared to healthy controls. Future studies would need to incorporate other anxiety disorder samples to test the assumption that these differences are uniquely observed in GAD.

8.9.7 Intervention treatment

Finally, with regard to the intervention study, the design did not include an active control such as a benzodiazepine, which on some measures may be equitable or better than *P.methysticum*. It would be of use to have a comparison product thought to act similarly on activations and metabolite levels in the ROI. Only a handful of studies have examined GABA levels using MRS in anxiety disorders using benzodiazepines/GABA-a receptor drugs, and fewer have examined them in GAD as a treatment.

With regards to dose-response, the active treatment of 240 milligrams extract daily was uniform for all participants; however, tiered-dosage regime using higher doses might provide a greater therapeutic effect and more accuracy in dose-response, and this could have been implemented in a cross-over design format. The dose selected in the present study was considered optimal based on previous efficacy investigations and general practice in therapeutic dose recommendations. Indeed, the recreational use of kava can amount to 10 to 20 times the dose employed in the intervention study, and higher doses could produce modulations to GABA levels and BOLD signalling in regions of interest.

The investigation has also reinforced the need for repeatedly utilising identical *P. methysticum* profile treatments in investigating efficacy in clinical anxiety. Based on the current literature in both pre-clinical and clinical studies, where constituent profiles are indicated (and/or tested individually), yields and strains of *P. methysticum* that varied their kavalactone profiles clearly elicited different systemic effects, which in human trials are likely to produce variable findings to what is found in similar studies, as was observed in the intervention study (please refer to Chapter Five for specific findings of other investigations).

8.9.8 GAD biomarker identification in the context of the findings

It is appropriate to discuss here after the methodological limitations how the current findings may be limited within the broader aim of identifying GAD biomarkers, which revisits the considerations of reliability, discriminant validity and specificity discussed in Chapter Three. Expanding on the discussion of functional data limitations (8.9.6), how can we evidence an observable difference in brain regional properties is a characteristic of GAD, such that it can reliably (repeatedly) define GAD, not only from healthy controls but from other disorders such as social phobia, panic disorder and MDD?

This question reflects a broader problem with the integration of biomarkers into psychiatry, where the optimal aim is individualised diagnosis and treatment of what are ostensibly biologically and clinically heterogeneous disorders - an integrative and practical 'precision psychiatry' (Fernandez et al., 2017a; Garrod, 2002; Vieta et al., 2015). Adding biological markers of anxiety, together with environmental factors to symptom reporting may be the best avenue for early identification to significantly improve response by early diagnosis and appropriate treatment ('prevention is better than cure'). From this integrative modelling there is ability to test and identify candidate biomarkers, with these models representing one avenue of exploration. It is thus far

clear that there are molecular pathways and neural networks that increase risk, with increasing evidence from structural, functional and metabolic imaging.

Second, crucial to testing these models of markers that may underpin anxiety cognitions in GAD is the assessment of changes in these markers in response to treatment interventions. In terms of improving treatment outcomes, it is feasible that the same treatment applied to 100 patients with diagnosed GAD will work differently in many cases. Biological subtypes might be identified that gear measurable response and, therefore, more detailed data regarding efficacy. Attempts at strategising biomarker research were identified in Chapter Three, via the National Institute of Mental Health initiatives with the launching of the Research Domain Criteria (Insel et al., 2014) and the Precision Medicine Initiative.

One way to operationalise the study of particular biomarkers, such as ROI metabolite levels, is employing markers such as GABA as endophenotypes, which in the future will most likely serve to define subclinical variants more accurately than through symptom reporting alone which clearly have limited utility in discrimination for GAD/non-GAD. The endophenotype, while it is reliable and valid, is proposed similarly to use in genetic research whereby it is intended to reduce heterogeneity. Several authors propose its use as part of a biological diagnostic framework for use in psychiatric disorders; Lenzenweger (2013); Gottesman and Gould (2003), and Chan and Gottesman (2008).

Furthermore, when defining biomarkers, it may be useful to discriminate between the term, endophenotype and intermediate phenotype, when referring to observable symptoms of a pathology in the light of psychopathology and particularly mood and affective disorders. Investigations or reviews of research into putative biomarkers for GAD at the time of writing were in development (Maron & Nutt, 2017; Williams et al., 2016).

Clinically meaningful subsets are where biomarkers are likely to have the most utility. Although the approach is uncommon in intervention research, trends in subsets are often found. In this thesis, GAD with comorbidities was identified as a clinically meaningful subset, although little research in the literature has also done so. One other study has taken this approach using MRS (Hermens et al., 2012) to examine NAA, MI, glutamate and GSH in the ACC region in a young sample ($n = 88$; mean age 22 years) experiencing mood and/or psychotic symptoms (depression, BP or psychiatric disorder as an existing primary diagnosis), where the methodological approach was to categorically identify differences, if any, in metabolite profile to facilitate diagnosis and

the increased likelihood of treatment response within the psychotic disorder prodromal age range.

8.9.9 GAD diagnostic capability

The Kappa rating for GAD diagnostic reliability is 0.20, which is very low. Despite the methodological structuring in the two studies including delineation of pure GAD from cases with comorbidities, the data may contribute better evidence for GAD comorbidity than a capacity to examine and validate activity of neuro-biomarkers in pure GAD.

In terms of testing biomarkers between psychiatric groups, starting with diagnostic categories and not grouped via neurobiology might be somewhat oppositional in the face of evidence that supports biological subtypes. Designing a predictive or dimensional methodology with which to delineate data has been adopted in some studies, and these data-driven approaches have yielded patterns of activation common across multiple disorders. In turn this evidence may serve to restructure a number of disorders based on neurobiology rather than symptom-driven diagnoses.

In one larger structural based meta-analysis (Goodkind et al., 2015) across several mood, affective and psychotic disorders including schizophrenia, bipolar disorder and OCD, in 193 studies found the most pertinent regions to be the dACC (and anterior insula). In the present studies, predicting group based on the measures conducted, it may be of use to examine the data in a different statistical design approach, such as utilising a multivariate pattern classification to determine whether symptoms and brain activation could be used to determine participant diagnostic group (healthy control or GAD, and feasibly also GAD/comorbid). Significant classification of healthy controls and GAD participants could be achieved with both psychiatric, metabolite and activation data, with whole brain data derived from the functional component used to test the prediction.

8.10 Future Research Directions

Improvements or extensions of the methodologies and findings of the present studies and further research that would be of value in future investigations is discussed, before a re-iterated summary statement and conclusion is provided.

8.10.1 Metabolite quantification beyond GABA in the dACC

In both investigations, other metabolites were captured for which post-processing can be applied to expose relevant spectra. It would be of value to assess the relationship between anxiety and mood with Glx (including glutamate and glutamine) profiles, as a notable example. Glutaminergic profiles as a measure of excitatory activity in the region

would complement the current findings because it forms part of the overall substrate, and even though it has not been extensively examined in GAD, it is feasible that glutamate processes make a contribution to anxiety symptoms in the dACC region. The current study also conducted a MR sequence to separate glutamate (Glu) from Glx and will form part of future analyses. Glutamate pathway modulation as a function of Kava is postulated via NMDA mechanisms, but there is no literature evidence beyond pre-clinical models, so the future analyses on these data would provide *in vivo* human evidence.

As previously mentioned, examining the metabolite profile of MDD within GAD given the overlap between the two would be of value. The literature for MDD suggests that GABA levels are correlated to mood symptoms, which would indicate that decreased GABA levels are more strongly evidenced in MDD than in GAD. However, this might be due in part to the greater number of investigations in MDD over GAD. It is also possible that the shared neurobiological features of MDD and GAD may not include GABA levels. Depression may in part involve an aberration within the GABA substrate. For example, in post-mortem studies, there are reductions of both GABAergic neural sizes and densities, as well as decreased GAD enzyme levels in the DLPFC compared with the healthy controls. One recent study, building on a previous investigation reported by the same authors (Gabbay et al., 2012; 2017) found that GABA levels in the ACC were associated with anhedonia presence in an adolescent MDD sample and not with MDD severity, with reduced GABA levels found in this group, suggesting that GABA levels in the ACC are amenable to mood symptom profiles.

Finally, GABA quantification method and toolboxes comparisons are feasible because various formats of the data were collected, including raw, DICOM and RDA (R data) files. Such comparison would help to address the concerns of inconsistency between current popular toolboxes, such as Gannet, Tarquin and LC model.

8.10.2 Functional activation and task-dependent data

The functional task data used for the present study did not examine group differences in response times, correct and incorrect responses or these differences using different conditions within contrasts. Given the somewhat substantial literature findings within affective disorder samples compared with healthy controls of heightened attention to errors or negative aspects of the environment, the data might indicate increased correct responses in the GAD group.

The literature also suggests that increased response times to negative valent stimuli implies greater employment of emotional processing time and/or cognitive interference in working memory. It is therefore likely that the GAD subjects responded slower across all conditions, with less incorrect responses. This data were recorded but not employed for the thesis investigations due to time and data processing constraints, however it will be used in upcoming analyses to substantiate the imaging findings with the purported cognitive processes via behavioural data. Response time and the number of correct responses would be interesting data to examine between GAD and healthy controls to determine whether error-related negativity and/or negative bias translates into performance-based measures. Questions such as whether negative bias increases appraisal time - and are responses more accurate in negative conditions, matched or non-matched - could be explored through remodelling of functional onsets to examine habituation to negative stimuli, and cognitive interference in working memory processes occurring in GAD.

Second, the DLPFC, IPL and mid-cingulate regions that were found to be significantly different in BOLD signalling in the given contrasts between groups would be of value to extract activation and assess against anxiety symptoms, with the same methods to explore the dACC in the current investigations. Furthermore, the thalamic region highlighted on the activation map (Figure 29) is likely part of the same white matter tract network raised in that study's discussion, and it was also differing in activation produced from the contrast, and therefore of interest to explore this functional network further in GAD. The current thesis reported functional connectivity studies within GAD samples (please refer to Table 6 and Section 4.2.4).

8.10.3 Other neuroimaging modalities

Other imaging data were also collected, which can be viewed in the sequence Table 15 in Chapter Six. Resting-state fMRI data was collected, which will be used post-thesis to further examine the default mode network (DMN) or other rs-fMRI network group differences. The MRS voxel of interest (dACC) or task-based findings in the inferior parietal lobule, mid-cingulate and DLPFC will also be selected to test the dACC-seed based functional connectivity network following the same research questions, and group comparisons performed (either clinical or treatment).

8.10.4 Other data

8.10.4.1 Personality

Another potential influence involves the relationship of personality with anxiety (beyond just the use of Spielberger trait component). A self-reported Swedish Personality Questionnaire was administered to both sample groups in the present study and this will be an interesting factor to explore in the future. This can be studied in both cross-sectional and intervention studies to assess factors such as introversion and neuroticism and how they may correlate to activation levels and metabolic level changes.

8.10.4.2 Genetics – SNPs and gene expression

A substantial amount of genetic data in the form of single nucleotide polymorphisms and gene expression formed part of the earlier version of the thesis, measured both cross-sectionally and in the GAD group as a function of treatment responders. Specifically, SLC6A1 (GAD1 enzymatic SNP) will be of particular interest to explore cross-sectionally and in the GAD group as a function of treatment responders.

8.10.5 Kava as an intervention in GAD

The intervention sub-study findings can be viewed as pilot findings at best, particularly due to the reduced sample size. Despite this limitation, the study prompts further research as the results are promising in delineating a unique GAD profile through markers of change following an intervention.

The research conducted provided some evidence of a relationship between GABA and Kava within regions involved in emotion stimulus processing and that modulation of the GABAergic system holds an important role in the pathophysiology of GAD cognitions because of how the properties of this region change following anxiolytic interventions that also produce amelioration of GAD symptoms. This is also congruent with pre-clinical evidence of GABAergic modulations from administration of kavalactones. Given the evidence for GABAergic dysregulation in contributing to GAD symptomatology, it suggests kavalactones may play an important role in the modulation of the GABAergic system within the limbic and prefrontal region that serves to guide associated cognitive and affective processes. Therefore, given that the GABA neural substrate functions in response to a treatment intervention in GAD have yet to be explored, the findings of the present study represent a unique contribution to aetiology and pathophysiology of anxious symptoms for the enhanced provision of efficacious treatment.

Future research should include study designs which test Kava compared to either a benzodiazepine or antidepressant comparator. Also, additional Kava species, that is, more than one Kava treatment, could be used to ascertain whether variable constituents produce different effects both in anxiety symptoms and biologically. Given that the Kava strain used in the present study and, subsequently, the kavalactone constituent profile was unique, it limits comparisons to other studies involving *P. methysticum* standardised products. Therefore, investigations of multiple strains or kavalactone profiles would be of benefit to connect psychiatric and biological responses back to strains.

In summary, future research using data from the two investigations conducted will include:

- Testing the GAD group with and without a prior MDD diagnosis compared with pure GAD and GAD/anxiety comorbidity.
- MRS data from other metabolites, such as glutamate/glutamine, also captured during the scan, both for group differences and against other metabolites.
- Functional data looking at other components of the task and examining in greater detail regions identified from group differences, such as the DLPFC and amygdala and other ACC regions, such as the rostral ACC.

Novel research to implement thereafter in future investigations may include:

- GABA in other regions, e.g. areas such as the DLPFC and amygdala and other ACC subregions, such as the rostral ACC.

9 Chapter Nine: Concluding statements

The research conducted in this thesis makes an important contribution in several ways to the body of knowledge for GAD. Firstly, the results provide evidence of variations in functional and metabolic processes in GAD participants compared with a healthy sample comparison group, and in doing so, meet the overarching objective of identifying biological relationships with anxiety. Furthermore, the research supports the influence of comorbidity in GAD as somewhat biologically distinct. The findings also support the aim to find a relationship between anxiety type and severity (state/trait/symptomatic), and biological features, which may exist outside of an overarching GAD clinical category. In this sense, the aims of the current research have been achieved in a preliminary fashion. GABA levels and some of the fMRI task-dependent modulations were observed to be different in their relationship to anxiety levels, with some considerations, and hence support use of both GABA levels and selected regional task-dependent activation patterns as candidate neuro-biomarkers worth exploring in GAD and GAD/comorbidity.

The research also prompts an expansion of structural, functional and metabolic modalities measurements of these markers as amenable to other treatments beyond antidepressants that had yet to be conducted.

Additionally, the research supports the aims of investigating how other biological factors influence anxiety levels, such as age and gender, and as they also reflect biological variance and will impact the way a disorder such as GAD may be defined and treated. Figure 40 indicates future research foci that build upon the current research and findings.

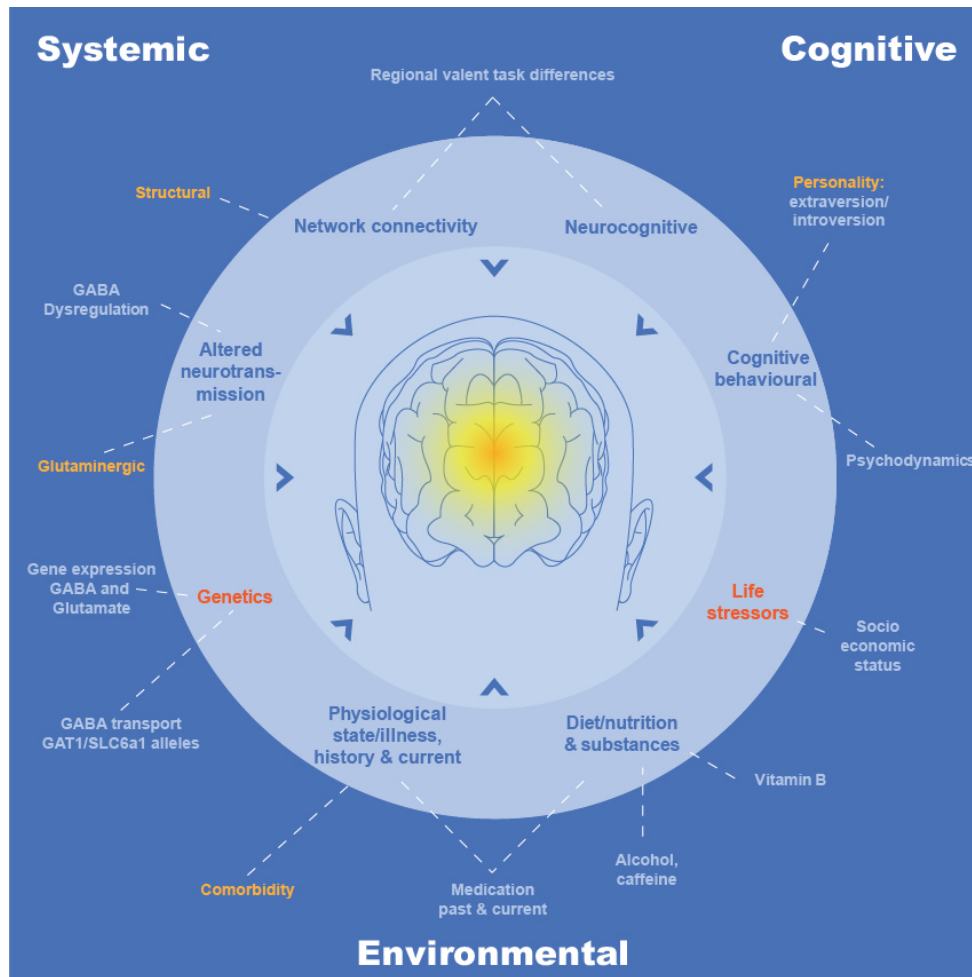


Figure 30. Future research foci that build upon the current research and findings (items in orange are collected data not yet analysed).

The methods and results in the investigations identified a substantial issue in metabolite imaging in that there are no set-standard quality parameters. Papers in the literature are often not clear regarding their parameters used to obtain the data. There is also no set standard for the analysis of GABA levels. This lack of standardisation makes replication of methodologies and results difficult and does not broaden the knowledge base for the use of MRS. Nevertheless, GABA substrate investigations in affective disorders are warranted because GABA substrate properties are a known substantial contributor to anxiety-type cognitive symptoms. As a corollary, a crucial challenge is identifying GABA molecular mechanisms linking aberrations in GABA pathways to the existence of anxiety symptoms. Do whole GABA levels measured regionally contribute to the generated and/or maintenance of anxiety cognitions and produce symptoms? After all, GABA is a smaller part of an allosteric system. Similarly, the dACC region performs a

role within associated regions, both within the ACC and other areas to form a dynamic functional circuitry.

In the literature context, the dACC region is postulated to hold an important role in the maintenance of the cognitive/emotional GAD state and the profile of this region is a potential indicator of response to an anxiolytic intervention, although the evidence is small. Furthermore, given the current knowledge of what the ACC subregions do in anxiety-type symptoms, the biological profile in this region is complex, and the current findings represent insight into how this region contributes to anxiety cognitions.

Relationships were found between anxiety scores, BOLD activity and GABA levels. These relationships were complex and followed a different pattern for GAD compared to healthy controls and, to an extent, varied as a function of age and gender. Increased activation in the region during the contrast, coupled with elevated GABA, might suggest general increased activation of this region in GAD compared with healthy controls, where there were lower GABA and a stronger trend of reduced activation in the region in the healthy controls. Therefore, the function in this area is typically reduced. Given the functions associated with this region, it may support a picture of anxiety cognitions seen in affective disorders such as GAD.

The activation of the other regions (DLPFC, IPL and mid-cingulate) are all connected via the white matter tract network, which was not included in the current analyses. The elevated levels of activation compared with healthy controls implied that there are increased resources required, or a more broader increased activation across multiple regions). A 'GAD brain' may be multitasking constantly, possibly making further task processing and/or attention to the task elements effortful – effectively exhibiting cognitive interference during working memory processes. Further research would ideally examine more brain regions, such as the amygdala, to determine whether the effects are measurable in other brain areas geared towards relevant cognitive-emotional processes.

Furthermore, the trend following Kava treatment in the region to reduce GABA as a function of treatment suggests a 'normalising' in this region. The healthy control sample exhibited lower levels than the GAD sample, which implies this region normally has lower GABA levels.

Biological effects are not always translated to measurable symptom change. This was the case in the intervention study with the effect of treatment not shown in the anxiety symptom analysis but was shown in GABA reductions and not in BOLD signalling. This

suggests that biological effects are not always translated to symptom improvement using the current measures.

Biomarkers can be used to optimise first-line treatment selection as well as guide treatment modifications with disorder progression, severity and presentations. The development of successful biomarkers relies on many factors that have been discussed and addressed to an extent in the investigations, as well as in Chapters Three and Four. The research interface between functional and metabolic properties of brain regions associated with anxiety cognitions will serve to better elucidate the role of such regions and substrates such as GABA. Together with administration of GABAergic anxiolytic treatments to demonstrate how these regions behave during response, would produce an integrative model that is of particular interest to the clinical community in the creation of effective stratified treatment regimes. It is a model template for future novel treatments, particularly plant medicines.

As such, understanding the neurobiological factors affecting responses to *P. methysticum* will add to scientific knowledge and may allow for future judicious clinical application of these interventions. The current line of pharmaceutical and psychotherapeutic treatments is not efficient for the treatment of GAD (Starcevic et al., 2012). Demand for evidence-based nutraceuticals exists and *P. methysticum* extract is an example of an affordable, safe and effective option (Sarris et al, 2013; Sarris, LaPorte & Schweitzer, 2011; Savage et al, 2018).

Epidemiological statistics report that one in four adults experience anxiety disorders (Baldwin et al, 2014; Hunt, Slade & Andrews, 2002). Outcomes are poor and treatment options are limited (Roy-Byrne, 2015; Starcevic, 2015). An influential factor is the paucity of neuro-biomarker data it is unique to GAD and amenable to treatment interventions (Hilbert et al., 2014; Maron & Nutt, 2017; Mochcovitch et al., 2014). As a corollary, our knowledge of the features and maintenance of symptoms in GAD is limited. It is a challenging disorder to diagnose, evidenced by a low Kappa diagnostic reliability of 0.20 (Freedman et al, 2013; see Chapter Two), and subsequently to successfully treat. High comorbidity and a chronic trajectory make substantial contributions to treatment resistance (Brown, Antony & Barlow, 1992).

Increasingly, biomarker investigations in anxiety disorders are producing data that may disrupt the current diagnostic conventions and may support dimensional approaches supported by biological evidence. The way this research is conducted is not flawless; there are contradictions and equivocal findings that can only have utility if

methodologies are rigorous, transparent and replicated. Despite many considerations, multimodal neuroimaging makes a crucial and valuable contribution to the body of knowledge in anxiety disorders such as GAD. The current research findings aimed to satisfy these conditions and in doing so offers an important contribution to the body of knowledge for GAD.

10 Chapter Ten: Bibliography

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11 Appendices

A. Chapter Six Assessment of neuroimaging safety and risks, risk management, confidentiality and data access information

MRI-15 Pre-scan questionnaire

A questionnaire designed by the Swinburne University Neuroimaging Facility (MRI-15; Appendix D) was administered prior to enrolment to ensure eligibility to undergo scans and conducted at both scan visits to ensure continual criteria were met for inclusion. The questionnaire collects basic demographic and medication information and screens for various conditions that could result in moderate or serious injury during MRI scanning. It was administered by the researcher together with the participant in a predominately 'Yes' and 'No' answer format. The MRI-15 was then reviewed by the radiographer conducting the scan.

Assessment of safety and risks

Whilst the study recruited members of the community as relatively healthy, safety issues can - and did arise. A common potential issue is candidates screened who are depressed (and/or suicidal) or presence of psychopathology is determined and may require referral to active treatment or be assessed by one of the medical investigators onsite. In these cases, candidates were offered referral as mentioned, and referred to their GP to discuss presenting symptoms.

Risk management for MRI scans

Numerous precautions, described below, were undertaken to ensure that objects potentially containing ferrous compounds were not taken into the MRI room.

1. Access to the vicinity of the magnet was restricted. The MRI rooms locked, with warning signs on all doors; entry is by authorized personnel or by appointment only.

The MRI room is entered only by participants about to be scanned, and only under the constant supervision of the radiographer.

2. Prior to approaching the magnet, participants were carefully briefed regarding which objects are potentially ferrous and the consequences of bringing such objects into the magnetic field of the scanner. Experienced staff ensured that participants removed all loose metal or magnetic objects (e.g. watches, wallets, etc.) before entering the scanning room.
3. Participants completed a detailed written questionnaire (MRI-15) regarding any surgically or accidentally implanted metal. No participant in this study reported an MRI incompatible implant.

The participants were viewable at all times through MRI control room window with radio contact with the researchers at all times.

Confidentiality and data access

All enrolled participants were allocated a participant code in order to maintain anonymity and for administration. After consent is obtained only the codes were used to identify participants. All raw and analysed data from assessments and questionnaires were linked to the participant code with identifiable information removed. All paper files were kept in locked filing cabinets and all digital files password protected. Confidentiality was only breached in circumstances presenting immediate risk to the participant. Completed data was stored in a de-identified format (by research code only) for access by study researchers as per clinical trial protocols. Only the databank custodian had capacity to link participants to data in the case of withdrawal requests.

Ethics and Finance

The study was conducted in line with the National Statement on Ethical Conduct in Human Research (2007) and both Swinburne Human Research Ethics Committee and University of Melbourne Human Ethics Sub-committee (Faculty of Medicine, Dentistry and Health Sciences) guidelines. The study was also carried out in keeping with applicable local laws and regulations. The project was submitted for ethical review at The University of Melbourne as the primary sponsor and at Swinburne University as the site. Insurance and indemnity was provided by The University of Melbourne as administrator of primary approvals. Swinburne University was also required to source site-specific insurance and

indemnity. The project was also approved by the Neuroimaging Committee at Swinburne University.

The investigation was funded in part from academic and research parties with interest in selected data components therefore providing financial and in-kind support. Professor Jerome Sarris funded the genetics, Swinburne Neuroimaging Director Professor Susan Rossell provided in-kind support for the imaging, and participant payments were covered by researchers at Swinburne University, Monash University, and Deakin University. Research support was provided by Prof. Jerome Sarris, Prof. Susan Rossell, Prof. Con Stough, Prof. Andrew Scholey, and Prof. Greg Murray.

B. Chapter Six IAPS Picture Parameters – Picture Codes

IAPS group	Valence range (1 = most unpleasant, 9 = most pleasant)	Valence Mean	Arousal range (1 = least arousing, 9 = most arousing)	Arousal mean	Dominance/Control range (1 = least, 9 = most)	Dominance/Control mean
Negative Time 1	Valence < 4.0	2.34	Arousal > 6.0	6.57	Dominance > 6.0	7.00
Neutral Time 1	Valence 4.0 – 6.0	5.01	Arousal < 3.6	2.84	Dominance < 4.0	3.23
IAPS picture codes as stimuli						
3053, 6510, 7110, 9413, 6250, 5534, 7038, 3170, 7490, 3063, 7001, 9410, 7050, 3131, 7032, 7012, 3530, 3266, 7003, 2440, 7217, 3100, 5390, 2381, 2980, 3080, 2411, 3030, 7002, 2499, 6312, 1525, 1070, 6260, 5500, 7160, 6563, 1333, 7175, 6263, 3000, 6370, 9412, 7700, 6540, 7053, 1050, 7235, 9414, 7090, 9187, 7010, 6313, 7080, 7705, 3071, 9360, 7161, 9210, 1033, 9635.1, 3005.1, 7009, 6360, 5520, 2811, 2397, 6021, 7140, 2683, 2495, 3212, 2104, 8480, 5120, 2570, 7025, 9520, 3213, 3019, 5972, 8311, 6210, 7187, 9904, 2036, 7040, 2890, 9405, 2200, 9620, 7491, 1932, 6821, 7059, 1304						

Source: Bradley and Lang (1994)

C. Chapter Seven IAPS Picture Parameters – Normative Ratings and Picture Codes for Two Time Points

IAPS run group	Valence range (1 = most unpleasant, 9 = most pleasant)	Valence mean	Arousal range (1 = least, 9 = most arousing)	Arousal mean	Dominance/Control range (1 = least, 9 = most)	Dominance/Control mean
Negative Time 1	Valence < 4.0	2.34	Arousal > 6.0	6.57	2.0-4.0	3.23
Neutral Time 1	Valence 4.0 – 6.0	5.01	Arousal < 3.6	2.84	1.9-2.8	5.99
Negative Time 2	Valence < 4.0	2.37	Arousal > 6.0	6.53	1.8-2.5	3.17
Neutral Time 2	Valence 4.0 – 6.0	4.96	Arousal < 3.6	2.87	1.7-2.4	5.91
IAPS picture codes as stimuli at time 1 (baseline)						
3053, 6510, 7110, 9413, 6250, 5534, 7038, 3170, 7490, 3063, 7001, 9410, 7050, 3131, 7032, 7012, 3530, 3266, 7003, 2440, 7217, 3100, 5390, 2381, 2980, 3080, 2411, 3030, 7002, 2499, 6312, 1525, 1070, 6260, 5500, 7160, 6563, 1333, 7175, 6263, 3000, 6370, 9412, 7700, 6540, 7053, 1050, 7235, 9414, 7090, 9187, 7010, 6313, 7080, 7705, 3071, 9360, 7161, 9210, 1033, 9635.1, 3005.1, 7009, 6360, 5520, 2811, 2397, 6021, 7140, 2683, 2495, 3212, 2104, 8480, 5120, 2570, 7025, 9520, 3213, 3019, 5972, 8311, 6210, 7187, 9904, 2036, 7040, 2890, 9405, 2200, 9620, 7491, 1932, 6821, 7059, 1304						
IAPS picture codes as stimuli at time 2 (week 8)						
2270, 6230, 7100, 2210, b3102, 3500, 8312, 2850, 9360, 9940, 6530, 9252, 1052, 7150, 6250, 6520, 9700, 6231, 6350, 7006, 8485, 6150, 3064, 2480, 9183, 3150, 3103, 9908, 3010, 1300, 3069, 7185, 5530, 7020, 6415, 2102, 7224, 9630, 9921, 6300, 6315, 5510, 7052, 7179, 7547, 9810, 3101, 2038, 2393, 2870, 1201, 9163, 7233, 3550.1, 7004, 7026, 1113, 1931, 2880, 7017, 7710, 5740, 7060, 1930, 9050, 3400, 3059, 3140, 2206, 2221, 9260, 6022, 7950, 7055, 3120, 7034, 6571.1, 5130, 7170, 3250, 5533, 7031, 6560, 7205, 7000, 7030, 4664.2, 3130, 9910, 2190, 6830, 3195, 7056, 7234, 7041, 3068						

Source: Bradley and Lang (1994)

D. Swinburne University MRI-15 Safety Form

PARTICIPANT INFORMATION



MRI Pre Scan Information

WHAT IS AN MRI?

Magnetic Resonance Imaging (MRI) is a medical imaging technique that uses a powerful magnetic field and radio-frequencies to obtain very detailed cross-sectional images inside the body.

IS THERE ANY PREPARATION?

To help us provide an efficient service, you could assist us by: wearing clothing that does not have metal fastenings; not wearing any jewellery and removing all eye make-up (as this can interfere with scans of the head). You will also be required to complete an MRI safety questionnaire before your scan.

CAN ANYONE HAVE AN MRI SCAN?

No. There are some preconditions which can make MRI scanning hazardous. Due to the powerful MRI magnetic field any person with a pacemaker, metal clips on arteries or certain implanted devices cannot have an MRI scan. Women who are pregnant or breast feeding cannot have an MRI scan. It is also advisable for some people with specific medical conditions not to have a scan. These conditions will be rigorously screened for during your pre-assessment for MRI scanning.

IS AN MRI SCAN SAFE?

MRI scanning has been in use as a medical imaging tool for many years and with proper safety controls is commonly regarded by clinicians as a safe procedure. It does not employ ionising radiation (such as x-rays) and hence does not induce an additional cancer risk. It does however entail exposure to electromagnetic fields (EMF) which are much higher than levels recommended by international safety guidelines for general exposure (though still within limits of special guidelines for MRI scanning). Very occasionally, these EMFs may cause some tingling or heating sensations. These effects do not persist after scanning and have no known long term impact on health. Your MRI exposure will be carefully controlled to avoid such effects, and you will be constantly monitored for any signs of these effects and may direct us to stop the scan at any time if you experience uncomfortable sensations. The staff on duty will answer any queries you might have on the day, or if in doubt, call our department before your appointment.

WHAT WILL HAPPEN WHEN I ARRIVE?

The MRI Radiographer or another senior MRI staff member will greet you at the MRI unit waiting room and reception, explain the procedure and ask you questions about previous surgery you may have had regarding implanted metal in your body. You will be asked to leave your valuables (coins, keys, watch, jewellery, credit cards, mobile phones, pagers etc) in a locker. The staff member will guide you on to our MRI scan table. Some equipment may be placed around the body part we will be scanning.

THE SCANNING PROCESS

When we are taking the pictures, you will hear a very loud sound, rather like a vibration, and hearing protection will be provided. When you hear this noise it is important that you keep your body very still as movement will degrade the quality of the image. Usually there are about 4 or 5 different scans, lasting for 2-8 minutes each; and for most studies you will be in the scanner for about 60 minutes. You are welcome to bring along your favourite CD or cassette to listen to, during your scan.

WHAT WILL HAPPEN AFTER THE SCAN?

You can leave immediately after your scan. The images that have been taken will be used to address the research question for the study you have agreed to take part in. In addition they will be examined by a Radiologist. On extremely rare occasions, the radiologist might find an abnormality that is significant and which should be investigated further. If the Radiologist finds such a significant abnormality in your brain, he/she will contact the researcher directly involved in the study. It is then their responsibility to follow up with you; they will speak with your GP who can recommend the most appropriate action.

PARTICIPANT CONSENT



MRI Pre Scan Safety Questionnaire

This questionnaire is designed to screen for various conditions in a potential MRI participant which could lead to moderate or serious injury during MRI scanning. It is VERY important that you complete it as honestly and comprehensively as possible – please ask if you have any questions. This form will be checked by MRI staff on your arrival.

NAME: _____ DATE: _____ ID No. _____

DATE OF BIRTH: _____ HEIGHT: _____ WEIGHT: _____

STUDY/PROJECT NAME: _____

Please circle if any of the following are relevant to you:

Please circle

Do you have or have you ever had a cardiac pacemaker? YES/NO

Do you have an implanted cardiac defibrillator? YES/NO

Do you have an aneurysm clip or been treated for an aneurysm in the head? YES/NO

Do you have a cochlear or stapes implant? YES/NO

Do you have a neurostimulator or spinal cord stimulator? YES/NO

Do you have any implanted electronic or magnetically activated devices? YES/NO

Have you ever had any metal enter your eyes? (Cutting metal, grinding or welding) YES/NO

If yes, was it removed by a doctor? YES/NO

If you answered "Yes" to any of the questions above, please contact MRI on 9214 5514

Please indicate if you have any of the following:

Hip replacement or artificial joint?	YES/NO	Contraceptive IUD?	YES/NO
Pin, plate or screw?	YES/NO	Inflatable breast implant?	YES/NO
Prosthesis -eye, limb, penile implant?	YES/NO	Are you/could you be pregnant?	YES/NO
Implanted coil, filter, shunt or stent?	YES/NO	Medication patches applied?	YES/NO
Eyeliner or other facial make up?	YES/NO	Wire mesh Implanted?	YES/NO
Piercings or any jewellery?	YES/NO	Spine or head shunt?	YES/NO
Hearing aid?	YES/NO	Vascular port or catheter?	YES/NO
Eyelid spring or wire?	YES/NO	Any other implanted metal?	YES/NO
Do you have any tattoos?	YES/NO	Any metal foreign bodies?	YES/NO
Artificial heart valve?	YES/NO	Other concerns?	YES/NO

SIGNATURE _____

PARTICIPANT CONSENT

Are you breast-feeding? YES/NO

Do you suffer from claustrophobia? YES/NO

Do you suffer from epilepsy or ever had a seizure? YES/NO

Do you wear braces, a dental plate or false teeth? YES/NO

Do you suffer from any heart condition that would make you susceptible to an increased risk of cardiac arrest? YES/NO

Have you ever had a surgical operation? YES/NO

If yes, please provide details of body area (head, arm) and medical condition

Have you had a MRI scan before? YES/NO

If yes, where? Clinical purposes or research?

Did you experience any problems while having an MRI scan? YES/NO

Do you have any allergies? YES/NO

If yes, details:

GP details (NAME/ADDRESS/TEL NO)

MRI Pre Scan Consent

I have read the above information and am aware of the risks and benefits of undergoing an MRI examination.

I have been provided with the opportunity to have any questions answered and I therefore give my consent to an MRI scan. I confirm that the questions have been answered to the best of my knowledge.

PARTICIPANTS NAME.....

SIGNATURE..... DATE.....

MRI STAFF MEMBER NAME:

SIGNATURE..... DATE.....

Please empty your pockets of all magnetic items including wallet, bank cards and coins.

You will also need to remove shoes, metal belt buckles and any jewellery you have on.

You will also need to remove your eye glasses the radiographer will provide you with alternatives.

E. Publication 1 (first author): Systematic review

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WILEY

REVIEW

GABA-modulating phytochemicals for anxiety: A systematic review of preclinical and clinical evidence

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Anxiety disorders are chronic and functionally disabling conditions with high psychological stress, characterised by cognitive symptoms of excessive worry and focus difficulties and physiological symptoms such as muscle tension and insomnia. Gamma-aminobutyric acid (GABA) is an inhibitory neurotransmitter within the central nervous system and is a key target of pharmacotherapies in the treatment of anxiety. Although current pharmaceutical treatments are often efficacious, they may cause undesirable side effects including cognitive decrements and withdrawal symptoms. Plant-based "phytochemicals" may provide novel treatment options, to act as an adjunctive or alternative to existing anxiolytic medications. As such, we conducted a systematic review to assess the current body of literature on anxiolytic phytochemicals and/or phytoconstituents. An open-ended search to 5 July 2017 was conducted using MEDLINE (PubMed), Scopus, and Cochrane library online databases and performed in a stepped format from preclinical to clinical investigations. Eligible studies must have had (a) *in vitro* evidence of GABA-modulating activity, (b) animal studies using anxiety models to test an anxiolytic effect, and (c) human clinical trials. Ten phytochemicals were identified as having preclinical investigations showing interaction with the GABA system, in addition to human clinical trials: kava, valerian, pennywort, hops, chamomile, Ginkgo biloba, passionflower, ashwagandha, skullcap, and lemon balm. Collectively, the literature reveals preclinical and clinical evidence for various phytochemicals modulating GABA-pathways, with comparative anxiolytic effect to the current array of pharmaceuticals, along with good safety and tolerability profiles.

KEYWORDS

anxiety, anxiolytic, GABA, herbal, phytochemical, phytotherapy

1 | INTRODUCTION

Anxiety disorders are ubiquitous and persistent conditions following a chronic course with high comorbidity (Baxter, Scott, Vos, & Whiteford, 2013; Bruce et al., 2005). It is estimated over 15% of the population in developed nations will experience anxiety symptoms (which are subsumed under affective disorder diagnostic categories), yet they remain under-diagnosed and under-treated (American Psychiatric Association, 2013; Wittchen et al., 2011). The cognitive and neurophysiological dysfunction that characterises anxiety disorders has been linked to regional dysregulation of excitatory/inhibitory neurobiological pathways, notably in the limbic and prefrontal brain regions (Etkin & Wager, 2007; Martin, Ressler, Binder, & Nemeroff, 2010; Schenke, Hettner, Caceda, & Nemeroff, 2011).

Gamma-aminobutyric acid (GABA) is a nonstandard amino acid that acts as the principal inhibitory neurotransmitter in central nervous

system (CNS) function. Up to 40% of all synapses in the CNS operate for GABA, and GABA receptors are found in every region of the human brain. Thus, GABA systems are implicit in a number of neurophysiological processes, including motor function, pain, sleep, brain development, and importantly for the current review, anxiety. Furthermore, impairments in GABA-mediated inhibition are seen in various neurological and psychological conditions, such as movement disorders, epilepsy, schizophrenia, insomnia, and anxiety disorders (Mohler, 2001).

Preclinical investigations examining the role of GABA substrates in symptoms of anxiety have uncovered several pharmacological targets for treatment of these disorders. These include GABA transporters type 1 or 2, GABA transaminase (GABA-T) or glutamic acid decarboxylase (GAD), and more recently, novel allosteric binding sites on GABA-A receptors (Nuss, 2015; Puthenkalam et al., 2016; Stahl & Moore, 2013). The classes of GABA receptors identified within the human brain include GABA-A, GABA-B, and GABA C (formerly considered

GABA-C; Olsen & Sieghart, 2008). The GABA-A receptor exerts an inhibitory effect when activated, hyperpolarising the neuron and thus reducing the likelihood of an action potential occurring. Therefore, the active site of the GABA-A receptor, along with its various allosteric binding sites (which influence GABA-A receptor activity separately) and receptor subtype units, provides the main target for various anxiolytic, analgesic, and sedative drugs (Ramachandran & Shekhar, 2011).

However, current pharmacotherapeutic treatments that exert anxiolytic effects primarily via the aforementioned GABA-ergic pathways (such as benzodiazepines [BDZs]) provide only a modest benefit for alleviating symptoms in substantial numbers of patients and are noted for negative side effects (such as concentration and memory impairment), along with addiction/abuse issues (Baldwin, Ajel, & Garner, 2010; Hoffman & Mathew, 2008). As such, novel anxiolytic treatments that provide efficacious adjunctive or first-line treatment for anxiety, with fewer adverse consequences from long-term use, would prove highly valuable for patients with these disorders.

Various "phytomedicines" (i.e., whole-plant or plant-extract compounds, thought to play a preventative or therapeutic role in health/disease) have been shown in both preclinical in vitro and animal studies to possess certain pharmacodynamic properties that may confer anxiolytic effects. The pharmacodynamic effects of phytomedicines could be attributable to constituents that may include alkaloids, terpenoids/saponins, and polyphenols, as these molecules have various hypothesised actions within the CNS including binding with BDZ receptor sites (e.g., GABA- α allosteric sites); inducing ionic channel transmission by voltage-gated blockage via alteration of membrane structures; and/or modulating enzymatic processes such as GABA-T or GAD (Awad et al., 2007; Johnston, 2015). Flavonoids in particular are well-known positive modulators of GABA-A receptors (Hanrahan, Chebib, & Johnston, 2011).

Although existing reviews have examined phytomedicines for the treatment of anxiety, the literature thus far has limitations; for instance, covering affective disorders in general, or focusing on several neurotransmitter systems involved in anxiety symptomatology (Sarris, 2007; Sarris, McIntyre, & Camfield, 2013a). Previous reviews have also detailed only preclinical evidence for GABA-ergic effects from phytomedicines, thereby omitting important human studies, thus reducing the clinical applicability of their findings (Awad et al., 2007; Johnston et al., 2009). Others have reviewed both preclinical and clinical evidence for GABA-ergic effects of phytomedicines but focused on isolated conditions (such as insomnia; Shi, Dong, Zhao, Tang, & Zhang, 2014) or lastly, have included combination herbal formulas and/or other nutritional/dietary compounds (Boonstra et al., 2015; Liu et al., 2015), making it difficult to delineate the effects of phytomedicines specifically. Thus, we conducted the first systematic review combining both preclinical and clinical evidence to evaluate how phytomedicines (and/or their constituents) may act within the GABA system to produce anxiolytic effects, while assessing the current level of human evidence.

2 | METHODS

An electronic search was conducted of MEDLINE (PubMed), Scopus, and Cochrane library online databases from inception to 5 July 2017.

The search was not restricted to English, where translation via online journal or digital translation of the publication was adequate. A three-level search strategy was employed to identify phytomedicines with all of the following attributes (a) in vitro studies examining GABA-ergic activity, (b) preclinical animal studies of any GABA-mediated behavioural effects, and (c) human trials reporting anxiety as a primary outcome. To identify in vitro/in vivo studies, the initial primary search terms were "PLANT," "HERBAL," "HERB," "PHYTOMEDICINE" and "GABA," "gamma-aminobutyric acid" and "ANXIOLYTIC," "ANXIOLYSIS," "STRESS," or "ANXIETY." Additionally, individual herbal medicines with both common names and Latin binomial names (where relevant) plus their known bioactive constituents, were specifically searched for. A forward search of papers included in the review was also conducted, using Web of Science and MEDLINE cited reference. A further search of grey literature was conducted to identify unpublished data.

Eligible in vitro studies were those that applied preclinical methodologies towards assessing GABA-related actions of phytomedicines, for example, *Xenopus laevis* oocyte cell line models or ex vivo brain tissue investigations. Phytomedicines with any indicated GABA-ergic activity were eligible at this stage, including those that demonstrated direct GABA-A or GABA-B receptor binding (at either active or allosteric sites), ionic channel or cell membrane modulation, GABA transaminase or GAD inhibition, and/or phytomedicines examined in interactions with known GABA receptor antagonists, such as flumazenil.

We then sought for preclinical studies that used established animal models of anxiety, along with related behavioural paradigms (e.g., grooming behaviour, maze models, or light/dark exposure models) to examine in vivo anxiolytic effects of phytomedicines found to have evidence of GABA-ergic activity revealed in Stage 1.

In the final stage of the search, to identify human trials of relevant phytomedicines, we conducted a further search using the terms: "Anxiety," "Generalised Anxiety Disorder," or "Anxiety Disorder," in addition to anxiety disorders as defined by Diagnostic and Statistical Manual of Mental Disorders, Edition 5 (American Psychiatric Association, 2013), for example, panic/agoraphobia, social phobia, and post-traumatic stress disorder, along with "AND" terms for a list of the phytomedicines (using both common names and Latin binomial names) that had been identified by the earlier search stages to have GABA-mediated anxiolytic effects in in vitro/in vivo studies. Human trials with any design were eligible (including randomised, open-label, or single arm studies), provided that the total sample size was ten or more participants (case studies were also excluded). In cases where a recent (i.e. since 2010) review (and meta-analysis of human trials) had already been conducted on the three levels of evidence for a specific phytomedicine included here (i.e., *Piper methysticum* and *Withania somnifera*), the review was used preferentially over detailing all the individual studies (although updated with a subsequent relevant research).

"Traditional" use of selected phytomedicines was cross-referenced with British Herbal Medicine Association Pharmacopoeia (British Herbal Medicine Association, 1996) and Martindale Complete Drug Reference (Brayfield, 2014). The dosing regimen of phytomedicine products consisted mainly of a single dose unless otherwise specified as multiple doses.

3 | RESULTS

3.1 | Overview

The literature search found 1,328 articles relevant to initial search parameters, for which 1,212 were excluded following a check against criteria for preclinical or clinical phytomedicine studies. Ten identified phytomedicines (key constituents or whole plant preparations) met inclusion criteria and were reviewed. Three phytomedicines found to be lacking one of the three areas of evidence (preclinical tissue and animal and clinical human studies) are later discussed in brief in Section 4. Refer to Figure 1 for the selection processes flowchart.

Of the animal models employed, most being rodent type, the elevated-plus maze (EPM) was most typical, consisting of open and closed trajectories along with a timed measure of arm entry exploration, which reflect animal anxiety levels (Lister, 1987; Walf & Frye, 2007). Other paradigms included timing durations spent in light/dark and open field environments, and measuring grooming, conflict avoidance, and social behaviours. Anxiety measures used in the clinical studies were Hamilton Anxiety Scale (HAM-A; Hamilton, 1959); Spielberger State-Trait Anxiety Inventory (Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1983); Erlangen anxiety, Tension and Aggression Scale (Galster & Spörl, 1979); the Befindlichkeitsskala Well-being Self-rating Scale (von Zerssen, 1976), Anxiety Sensitivity Index (Reiss, Peterson, Gursky, & McNally, 1986), and the Clinical Global Impressions Scale (Guy, 1976). Various biomarkers were also used as proxy measures of physiological anxiety, including hormones (adrenaline, cortisol, adrenocorticotrophic hormone, and noradrenaline levels) as well as neurophysiological data—skin conductance, heart rate, and blood pressure.

3.2 | Anxiolytic phytomedicines with preclinical and human evidence

3.2.1 | *Centella asiatica* (Gotu cola/kola, pennywort)

Centella asiatica is a native Asian herbaceous species used commonly as a whole plant extract in Indian Ayurvedic and Chinese Traditional Medicine (Diwan, Karwande, & Singh, 1991; Gohil, Patel, & Gajjar, 2010). Modern indications are for dermatotic, antimicrobial, nociceptive, cognition-enhancing, anxiolytic, and anti-depressive purposes (Anukunwithaya, Tantisira, Tantisira, & Khemawoot, 2016; Lokanathan, Omar, Ahmad Puzi, Saim, & Hj Idrus, 2016). The chief constituents are triterpenoid glycosides (saponins), sterols, flavonoids, tannins, and stearic acids. Investigations of constituents have chiefly involved the saponins brahmoside and brahminoside and the asiatic and ursolic acids (Brinkhaus, Lindner, Schuppan, & Hahn, 2000; James & Dubery, 2009).

GABA-ergic effects of *C. asiatica* (and constituents) were revealed in studies analysing GABA-T and GAD activity. One study (Awad et al., 2007) reported *C. asiatica* significantly increased GAD activity in vitro by more than 50% ($p < .001$) in male Sprague–Dawley rat brain assays. In a further in vitro study of GABA-A subtype receptor modulation (Hamid et al., 2016), the asiatic acid constituent was found to be negative modulator of GABA-induced currents for $\alpha 1 \beta 2 \gamma 2L$, $\alpha 2 \beta 2 \gamma 2L$, and $\alpha 5 \beta 3 \gamma 2L$ receptors in *Xenopus* oocyte tissue. Further, in an ex vivo study using Charles Foster rats (Chatterjee, Chakraborty, Pathak, & Sengupta, 1992), *C. asiatica* extract was found to increase whole brain levels of GABA.

In rodent models, the constituent triterpenoid extracts of *C. asiatica* (madecassoside and asiaticoside) have been shown to be as effective as diazepam for alleviating behavioural symptoms of

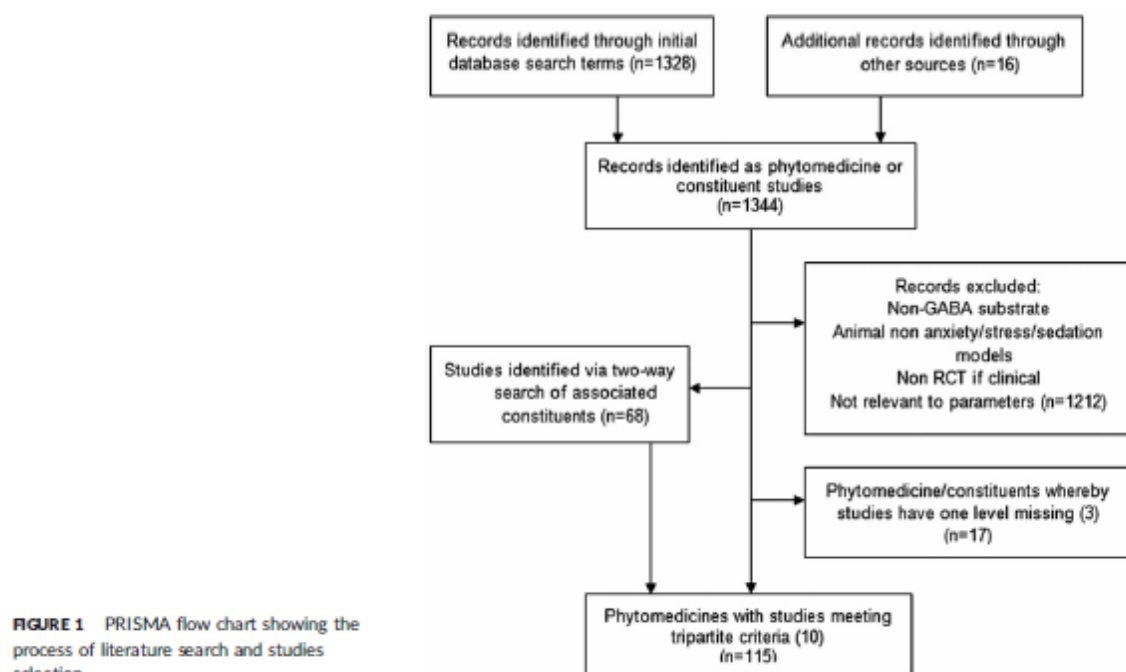


FIGURE 1 PRISMA flow chart showing the process of literature search and studies selection

anxiety in chronic stress settings of EPM, open-field, and dark-light box tests, as well as acute stress models (i.e., immobilisation stress test) in adult male imprinting control region mice (Wanasuntronwong, Tantisira, Tantisira, & Watanabe, 2012). Another study (Wijeweera, Amason, Koszycki, & Merali, 2006) reported significant anxiolytic effects in EPM tests from both methanol and ethyl acetate extracts of *C. asiatica*, along with the isolated asiaticoside constituent in male Sprague-Dawley rats. A final animal study (Ceremuga et al., 2015) investigated the anxiolytic effects of an injected peritoneally asiatic acid extract in four male Sprague-Dawley rat comparison groups. Asiatic acid significantly increased EPM measures of open-arm latency and mobility speed and latency (all indicating reduced anxiety). Further, the investigation reported that administration of GABA receptor antagonist flumazenil attenuated the anxiolytic effects of asiatic acid, thus indicating that the anxiolytic effects of asiatic acid are due to GABA-ergic benzodiazepine receptor activity.

A human study examined the effects of an acute dose of *C. asiatica* extract (12 g) on measures of stress and anxiety, including acoustic startle response, against a placebo comparator in 40 healthy adults (Bradwejn, Zhou, Koszycki, & Shlik, 2000). The results showed significant attenuation of acoustic startle response in the treatment group. Another clinical investigation conducted an open-label study of 500 mg of the phytomedicine twice daily for 2 months in a sample of 33 patients with GAD (Jana, Sur, Maity, Debnath, & Bhattacharyya, 2010). The study found significant improvements in anxiety following 60 days of treatment ($p < .01$) on the HAM-A scale, along with significant reductions in secondary measures of stress and depression.

3.2.2 | *Humulus lupulus* (Hops)

The flowering cones of *Humulus lupulus* (Cannabaceae) have sedative/hypnotic, antibacterial, anti-inflammatory traditional indications; however, the plant is chiefly cultivated worldwide for alcoholic beverages derived from malted grain, namely "beer" (Katsiotis, Langezaal, & Scheffer, 1989). Modern medicinal indications exist for anxiety and insomnia (Blumenthal, 2009; Martin, McCallum, Stofer, & Eichinger, 2002), mostly associated with principal terpenoid and flavonoid alpha and beta acid constituents, such as humulene and myrcene.

In vitro studies of myrcenol (found in the *H. lupulus* plant) have demonstrated GABA-A receptor response potentiation in *Xenopus* oocytes (Aoshima et al., 2006). Additionally, a combination study (Muller, Schumacher, Brattstrom, Abourashed, & Koetter, 2002) investigated how hops extract and *Valeriana officinalis* influenced binding at adenosine receptors within rat cortical and striatum membranes and found that although *V. officinalis* in combination with hops affected this process, hops alone did not—indicating that the mechanism through which hops produces sedative effects may be through GABA receptor activity instead. Preclinical research further supported GABA-ergic effects of *H. lupulus* (extract 0.11–0.65 mg/mL) in male Sprague-Dawley rat brains (Awad et al., 2007), through inhibiting GAD activity.

Animal models examining effects on sedation and anxiety have also been conducted: one investigation (Franco, Sanchez, Bravo, Rodriguez, Barriga, Romero, et al., 2012a) found that *H. lupulus* extract reduced nocturnal activity in an avian model (common quail) and preserved an activity/rest cycle that matches regular circadian

rhythms. Similarly, an investigation using female U.S. Naval Medical Research Institute (NMRI) mice found that *H. lupulus* extract reduced locomotor activity and reduced body temperature consistent with sedating properties (Schiller et al., 2006). However, a preclinical study of *H. lupulus* specific constituent xanthohumol reported no evidence of anxiolysis via GABA-ergic mechanisms of this particular component using male Sprague-Dawley rats, suggesting effects occur by a different constituent or pathway (Ceremuga, Johnson, Adams-Henderson, McCall, & Johnson, 2013).

In human studies, one investigation (Franco, Sanchez, Bravo, Rodriguez, Barriga, Romero, et al., 2012b) measured sleep quality, circadian rhythms, and anxiety/stress levels of 17 "work stressed" shift workers for a regular seven nights (control period) and then following 14 days of nightly ingestion of a 330 ml alcohol-free beer. The study found significant reductions in state anxiety versus the control condition. Aside from this study, the anxiolytic effects of *H. lupulus* have not been widely examined, with the majority of studies focusing on sedating and hypnotic properties instead. As such, the anxiolytic potential of *H. lupulus* in anxiety symptom alleviation is yet to be substantiated through clinical research, despite clear indications of GABA-ergic mechanisms from preclinical research.

3.2.3 | *Ginkgo biloba* (Maiden hair)

The ginkgo tree (*Ginkgo biloba*) is one of the oldest angiosperm species, with the nuts and leaves being used for a range of therapeutic effects (Shizhen, 1990). Active constituents are mainly flavonoids quercetin and catechin, unique terpenoids ginkgolides and bilobalides, and proanthocyanidins (van Beek & Montoro, 2009). Tissue studies of human endothelial cell lines indicate that constituents from the leaves as standardised extract have potent neuroprotective and anti-apoptotic properties, as well as vasculatory benefits via nitric oxide (NO) enhancement (Koltermann et al., 2007). Modulations to cholinergic and monoaminergic pathways have also been evidenced in female Wistar rats and NMRI mice models ex vivo, thereby offering benefit for cognition and mood (Fehske, Leuner, & Muller, 2009).

Preclinical studies were located investigating GABA-ergic mechanisms of action; however, to date the findings are not entirely clear. The ginkgolides A, B, C and bilobalide were reported to operate as antagonists at recombinant $\alpha 1 \beta 2 \gamma 2 L$ GABA-A receptors expressed in *Xenopus* oocytes in tissue investigations, and to exact mixed antagonist action involving similar models with recombinant human $p1$ GABA-C receptors (Huang et al., 2003, 2004, 2012). Additionally, an increase of GABA in hippocampal, cortical, and striatum tissue in male ddY strain mice assayed ex vivo was reported in the presence of bicuculline and picrotoxin, thought to occur via potentiating GAD and thus operating as an anticonvulsant (Sasaki, Hatta, Haga, & Ohshika, 1999; Sasaki, Hatta, Wada, Ohshika, & Haga, 2000). A tissue study also reported that both the ginkgolides and bilobalide selectively inhibit GABA-A receptors to the same extent as the picrotoxin comparator in Sprague-Dawley rat brain tissue ex vivo when exposed to ginkgolides A, B, C, and J and bilobalide (Ivic et al., 2003). The study discussed structural similarities of *G. biloba* constituents to the GABA-A receptor antagonist, picrotoxinin. One investigation examined further the GABA-A mechanisms of action for bilobalide in rat

hippocampal tissue and reported a blocking of GABA-induced chloride uptake in these cells at low potency. In turn, GABA was found to partially antagonise bilobalide's inhibitory actions (Kiewert et al., 2007). One recent study reported that both bilobalide and ginkgolide B selectively inhibited structurally-varied GABA-A positive modulator agent actions when tested in *Xenopus* oocytes (Ng, Duke, Hinton, & Johnston, 2017).

In anxiety rodent models, ginkgolides A, B, C, and bilobalide (individually and combined) were tested using the EPM paradigm with mice (Kuribara, Weintraub, Yoshihama, & Maruyama, 2003). The study found significant dose-dependent relationships between ginkgolide A with reductions in anxiety (i.e., increased open arm time). However, these beneficial effects were not blocked by flumazenil, suggesting that these constituents exert anxiolytic effects through alternate routes to GABA receptors. A further preclinical study of bilobalide treatment found increased locomotor activity in open field and EPM tests and reduced latency in Morris water maze, using male Kunming mice, with glucocorticoid receptor modulation as a hypothesised mechanism of action (Ma et al., 2012). One human study testing *G. biloba* extract at doses of either 480 or 240 mg or placebo in 170 patients with GAD reported significant improvements on HAM-A scale in the treatment groups after 4 weeks, with greatest effects in the high-dose condition (Woelk, Arnoldt, Kieser, & Hoerr, 2007).

3.2.4 | *Matricaria recutita*/*Matricaria chamomilla* (Chamomile, German chamomile)

Matricaria recutita, an Asteraceae (daisy) family flowering plant, is plentiful throughout Eurasia and has been utilised for millennia, chiefly as tea, for calming/sedative effects (McKay & Blumberg, 2006). The bioactive constituents include sesquiterpenes, coumarins (herniarin, umbrelliferone, among others), and flavonoids including apigenin, luteolin and rutin, and phenylpropanoids (chlorogenic and caffeic acids; Srivastava, Shankar, & Gupta, 2010).

Tissue studies report that certain *M. recutita* constituents modulate the function of GABA receptors via ligand effects on the central BDZ site, with a reduction of GABA-activated Cl⁻ currents, with evidence of action in other (possibly monoamine) pathways in Sprague-Dawley rat brain assays (Avallone, Zanolli, Corsi, Cannazza, & Baraldi, 1996; Avallone et al., 2000). Principally, the flavonoid apigenin has been investigated—present also in other plant species—with anxiolytic effects as an inverse BDZ agonist (Johnston, 2015; Viola et al., 1995), potentially biphasic action via receptor subunits similar to BDZs (Campbell et al., 2004; Hanrahan et al., 2011) as the activity on BDZ receptors is somewhat equivocal (aforementioned Avallone et al., 2000). Animal models have supported this, reporting sedative and anxiolytic effects. For instance one study (Shinomiya et al., 2005) found administration of the extract improved sleep latency in male Wistar rats and that these effects were attenuated from co-administration of flumazenil, thus implicating GABA-ergic BDZ receptor modulation as the likely mechanism when measured *ex vivo*. Another study further reported significant reductions in anxiety behaviours in male Balb/c mice via in various testing paradigms, including open field, EPM, and social interaction paradigms (Can, Demir, Kıyan, & Demirci, 2012).

In humans, a double blind randomized controlled trial (RCT) with 57 GAD patients showed significantly greater reductions in anxiety

(HAM-A) among those undergoing *M. recutita* treatment (220 mg, one to four times daily) than those in the control condition, after 8 weeks (Amsterdam et al., 2009). A post hoc analysis of the same sample found that *M. recutita* may also reduce co-morbid depression (HAM-D) in anxious patients (Amsterdam et al., 2012).

Three further human studies reported short- and long-term (i.e., 8 and 38 weeks) effects of treatment for anxiety with 1500 mg (500 mg capsule 3 times daily) in a sample of 179 patients with GAD. At 8 weeks, 58% of patients met the criteria for "clinical response" to *M. recutita* treatment, with significant reductions in mean anxiety (GAD-7) across the entire sample (Keefe, Mao, Soeller, Li, & Amsterdam, 2016). For the long-term study, those who were "responders" were randomly assigned to continued active or placebo treatment for a further 26 weeks. GAD relapse rates were 25.5% in the placebo group and only 15.2% in the continued treatment group (Mao et al., 2016). Although the difference in relapse rates did not reach statistical significance, GAD-7 ratings were significantly improved in the continuation versus placebo conditions. Another clinical investigation in 34 patients with DSM-IV primary insomnia found significant improvements over placebo from 28 days of 270 mg *M. recutita* twice daily to ratings of fatigue, sleep quality and latency, but with no significantly relevant changes in symptoms of anxiety (Zick, Wright, Sen, & Arndt, 2011).

3.2.5 | *Melissa officinalis* (Lemon Balm)

Lemon balm is reported in Theophrastus' *Historia Plantarum* (350–c. 287 BC), thus holding longstanding use for millennia. It is native to Eurasia as a member of the Lamiaceae (mint) family, with a number of phytochemicals including phenolic acids, terpenes, rosmarinic and caffeic acids, eugenol acetate, and tannins. Indications are for antibacterial, anti-stress, hypnotic, and gastrointestinal symptom benefit (Shakeri, Sahebkar, & Javadi, 2016; Ulbricht et al., 2005).

In vitro studies have indicated various GABA substrate activity for *M. officinalis* constituents such as elevation of GABA levels, owing to inhibition of GABA-T, as observed in male Sprague-Dawley rat brain homogenised tissue samples (Awad et al., 2007; Awad, Muhammad, Durst, Trudeau, & Amason, 2009). An aqueous extract of *M. officinalis* (rosmarinic acid) inhibited GABA-T activity (IC₅₀ = 0.35 mg/ml) in the former study (Awad et al., 2007) and replicated in the latter study (Awad et al., 2009). An additional investigation of the triterpenoids, ursolic and oleanolic acids found that these constituents also increase GABA-A BDZ receptor affinity in rat brain tissue (Salah & Jager, 2005). A neurotropic tissue study (Yoo et al., 2011) reported decreased GABA-T levels in the dentate gyrus of C57BL/6 J mice, along with an increase in total GABA levels, following extract 50/200 mg/kg daily for 3 weeks. Finally, in radio-ligand/electrophysiological studies using male Wistar rat cortical cultures *in vitro*, *M. officinalis* extract and selected constituents have shown trans-ocimene inhibited [35S] (TBPS) binding (set radio ligand) at GABA-A receptor level, in a dose-dependent fashion (Mahita et al., 2014), building upon previous similar findings in preclinical investigations (Abuhamdah et al., 2008; Huang et al., 2008).

Rodent model studies have reported a dose-dependent response on the EPM with C57 BL/6 J mice using the extract "Cyracos" (Naturex, *M. officinalis* L. Extract), with principal constituents

rosmarinic acid and triterpenoids oleanolic and ursolic acids (Ibarra, Feuilleire, Roller, Lesburgere, & Beracochea, 2010). Similarly, a second study (Taiwo et al., 2012) used EPM and open field measures to show significant anxiolytic effects of *M. officinalis* extract in male and female Wistar rats, which matched those of the diazepam comparator.

In contrast, one preclinical study (Raines et al., 2009) reported the constituent luteolin confers additive or synergist effects in the presence of a GABA-A agonist but not alone; reporting no differences in anxiety behaviours using an EPM model with male Sprague-Dawley rats between the constituent and a flumazenil group, both administered with a GABA-A BDZ agonist, suggesting that anxiolysis also occurs via different mechanisms.

In human investigations, an open-label study (Cases, Ibarra, Feuilleire, Roller, & Sukkar, 2011) in a self-reported "stressed" sample, showed a 15–42% reduction in insomnia and mild-to-moderate anxiety symptoms over 15 days from 600 mg per day of Cyacos (standardised to 7% rosmarinic acid and greater than 15% hydroxycinnamic acid derivatives). In a healthy sample study (Kennedy, Scholey, Tildesley, Perry, & Wesnes, 2002) a laboratory stress-induction paradigm showed improvements in mood and attention from *M. officinalis* extract. Several similar studies with other laboratory-based paradigms have also reported improvements from *M. officinalis*, which are consistent with anxiolytic-like effects (Kennedy et al., 2003; Kennedy, Little, & Scholey, 2004; Scholey et al., 2014). Finally, a double-blind RCT in 100 school age females (Akbarzadeh et al., 2015) reported efficacy of 1200 mg of *M. officinalis* extract daily over 3 months for reducing stress and tension associated with premenstrual dysphoric disorder.

3.2.6 | *Passiflora incarnata* (Passion flower)

Passiflora incarnata is native to the Americas having been utilised for millennia for conditions of nervousness, anxiety, and sleep issues (Krenn, 2002; Meier, 1995; Miroddi, Calapai, Navarra, Minciullo, & Gangemi, 2013). The chief identified constituents include alkaloids such as chrysin, flavonoids such as schaftoside, isoschaftoside and swertisin, and phenolic compounds (Wohlmuth, Penman, Pearson, & Lehmann, 2010). GABA-ergic modulation could feasibly occur through number of processes, including affinity with GABA-A and GABA-B receptor subtypes and GABA reuptake inhibition, and also via positive allosteric modulation of the GABA-A receptor complex through BDZ sites (Appel et al., 2011; Nassiri-Asl, Zamansoltani, & Shariati-Rad, 2008).

A combined in vitro/in vivo study (Elsas et al., 2010) used a pentetazetazol-induced seizure procedure in a CF-1 mice model to examine effects of *P. incarnata* extract, which was reported to induce direct GABA-A currents in CA1 hippocampal pyramidal neurons. Another combined in vivo/in vitro study (Grundmann, Wang, McGregor, & Butterweck, 2008) utilised a *P. incarnata* extract (as homoorientin, orientin, vitexin, and isovitexin) in male BL6/C57 J mice using an EPM model to show behavioural anxiolytic effects matching those of diazepam from moderate-strength doses. Furthermore, the findings that flumazenil acted as an antagonist against *P. incarnata* demonstrates GABA-mediated pathways for the anxiolytic effects. A related study (Grundmann, Wahling, Staiger, & Butterweck, 2009)

examined effects in a similar rodent EPM model using three strengths of *P. incarnata* with diazepam comparator, finding a U-shape trend dose-response efficacy, in favour of the middle dose (375 mg/kg).

Several in vivo studies investigated both dose and constituent optimisation; the first (Dhawan, Kumar, & Sharma, 2001) used a Swiss mice (male and female) EPM model to delineate potency of different plant components, reinforcing use of the flower for maximal anxiolytic effects. A second study (Sampath, Holbik, Krenn, & Butterweck, 2011) used differential EPM performances in male C57BL/6 J mice to ascertain optimal fractionalisation methods, and a third (Wolfman, Viola, Paladini, Dajas, & Medina, 1994) used anxiolytic and sedative paradigms (EPM, hole board and horizontal wire) in male CF1 mice to examine the constituent chrysin (5,7-dihydroxyflavone) with a diazepam comparator, reporting no significant differences in anxiolytic effect, but an increase to head-dipping and myo-relaxant properties via horizontal wire test in the BDZ group. Finally, a recent study (Aman et al., 2016) observed anxiolytic and sedative effects of *P. incarnata* in staircase and open field tests, with BALB/c mice and female Sprague Dawley rats, which were antagonised by pentylenetetrazole, indicating the behavioural effects of *P. incarnata* occurred through GABA-ergic mechanisms; however, precise mechanisms were again unclear.

Seven studies involving human populations were found; four examined effects on preoperative anxiety in RCTs (Adanargun, Cuvras, Dikmen, Aslan, & Yuksel, 2012; Dantas, de Oliveira-Ribeiro, de Almeida-Souza, & Groppo, 2017; Kaviani, Tavakoli, Tabanmehr, & Havaei, 2013; Movafegh, Alizadeh, Hajimohamadi, Esfehiani, & Nejafar, 2008) all reporting significant benefits from *P. incarnata* treatment in comparison to placebo conditions and/or non-inferiority to BDZ comparators. Most recently, this effect was observed in a double-blind RCT of 40 dental surgery patients (Dantas et al., 2017), showing 260 mg of *P. incarnata* orally administered 30 min before dental surgery reduced subjective anxiety along with physiological indicators (i.e., blood pressure and heart rate) to the same extent as midazolam, with fewer cognitively-impairing side effects.

The BDZ-like efficacy has also been observed for psychiatric disorders. First, a double-blind RCT in 36 patients with GAD (Akchondzadeh et al., 2001) found no significant difference between *P. incarnata* extract (administered as liquid drops) to oxazepam comparator after 4 weeks. Second, another investigation (Mori, Hasegawa, Murasaki, Yamaguchi, & Ito, 1993) found no difference between extract ("Passiflamin" 90 mg) and mexazolam comparator groups for anxiety measures in patients with "neurosis."

3.2.7 | *Piper methysticum* (Kava)

Kava (*Piper methysticum*) is a plant native to the South Pacific, where the roots have been used in traditional medicine for a range of conditions via its anxiolytic, nootropic and neuroprotective, nociceptive, and dysphoric actions (Singh, 1992; Singh & Singh, 2002; Tzeng & Lee, 2015). The chief bioactive constituents are the six lipophilic kavalactones, of which kava and dihydrokava are evidenced to exert the strongest anxiolytic activity. Mechanisms evidenced in preclinical models primarily involve modulation of GABA receptors, and although the exact mechanisms are not clear, these models suggest several avenues beyond an allosteric mode of action in the GABA substrate, such as rapid upregulation of

GABA-A receptor function, blockade of voltage-gated sodium ion channels, enhanced ligand binding across GABA-A receptor subtypes, and reduced excitatory neurotransmitter release via blockade of calcium ion channels (Gleitz, Beile, & Peters, 1995; He, Lin, & Lian, 1997; Jussofi, Schmitz, & Hienke, 1994; Magura, Kopanitsa, Gleitz, Peters, & Krishtal, 1997; Mathews et al., 2005). Table 1 details these studies further. Abundant animal anxiety model investigations exist (e.g., Davies, Drew, Duffield, Johnston, & Jamieson, 1992; Garrett, Basmadjian, Khan, Schaneberg, & Seale, 2003; Rex, Morgenstern, & Fink, 2002), reporting anxiolytic effects (such as reduced entry latencies and increased time in unfamiliar environment) in rodent EPM and other paradigms.

A number of clinical reviews published have systematically examined the mechanisms and efficacy of *P. methysticum* in the clinical treatment of anxiety, including an early Cochrane review (Pittler & Ernst, 2003), along with more recent reviews of preclinical and clinical investigations (LaPorte, Sarris, Stough, & Scholey, 2011; Sarris et al., 2013a; Sarris, McIntyre, & Camfield, 2013b). From human clinical studies these reviews indicate *P. methysticum* is superior to placebo in the treatment of anxiety symptoms. For instance, a pooled effect size calculated for HAM-A scores across 6 RCTs found large, significant reductions in anxiety in favour of the extract (Cohen's $d = 1.1$) (Sarris, LaPorte, & Schweitzer, 2011). A subsequent RCT has also confirmed these findings (Sarris et al., 2013c) observing moderate effects on reducing anxiety reported with the HAM-A in a GAD sample ($n = 75$) treated with either *P. methysticum* (120/240 mg kavalactones), compared to placebo, for 6 weeks.

Through combining human evidence alongside preclinical (in vitro and in vivo) studies, reviews have also found strong indication that the anxiolytic effects of *P. methysticum* are due (at least in part) to the extract's multiple GABA-ergic actions (Sarris et al., 2013a, 2013c). Again, the conclusions drawn from these earlier reviews have been supported by recent studies, whereby GABA-ergic mechanisms are investigated in more detail; in a recent tissue study, examining actions of a specific *P. methysticum* phytoconstituent "kavain" on expressed *Xenopus* oocytes (Chua et al., 2016) found that positive allosteric modulation occurred across all GABA-A receptor subtypes examined ($\alpha 1\beta 2$, $\beta 2\gamma 2L$, $\alpha \beta 2\gamma 2L$ ($x = 1, 2, 3$, and 5), $\alpha 1\beta \gamma 2L$ ($x = 1, 2$, and 3), and $\alpha 4\beta 2\delta$), and where this action was not affected by administration of flumazenil, implicating sites other than BDZ receptors.

3.2.8 | *Scutellaria lateriflora* (Scullcap, Blue Skullcap)

Scutellaria lateriflora is native to America and Europe with use in Native American practice and Western medicine for anti-anxiety, relaxant, and antispasmodic effects (Mills & Bone, 2000). Identified chief bioactive constituents include a number of flavonoids: scutellarin A, baicalin, baicalein, apigenin, oroxylin A, and wogonin (Li, Wang, Smillie, & Khan, 2012; Zhang, Lian, Li, & Stringer, 2009). Preclinical studies have found several constituents of *S. lateriflora* have GABA-ergic properties, including baicalin and baicalein, wogonin, and apigenin, as these constituents act at the BDZ site of GABA-A receptor via positive allosteric modulation (Awad et al., 2003; Wang, Xu, Ren, Tsang, & Xue, 2008; Xu et al., 2006). Animal studies have also shown behavioural changes that reflect these GABA-mediated anxiolytic properties. For instance, one investigation (Awad et al., 2003) observed *S. lateriflora* extract significantly

increased open field entries, head dips, and time spent in open arms in an EPM paradigm with male Sprague-Dawley rats.

Two human studies using *S. lateriflora* extract were found, both in non-clinical samples. One double-blind crossover study ($n = 43$; Brock, Whitehouse, Tewfik, & Towell, 2014) reported significantly greater mood improvement after 2 weeks from 350 mg extract (three times per day) than placebo comparator. Although there were no differences in anxiety outcomes between groups; this was ascribed to a "floor effect" as these healthy volunteers were mostly non-anxious at baseline. Similarly, an earlier briefly-detailed pilot investigation reported clinically significant reductions in anxiety and stress from various strengths of *S. lateriflora* in 19 healthy volunteers (Wolfson & Hoffmann, 2003).

3.2.9 | *Valeriana officinalis* (Valerian)

Valerian is a perennial plant native to mainly Europe, with the root extract utilised medicinally for millennia as a sedative and anxiolytic. The bioactive constituents include alkaloids, flavonones, and terpenes (specifically the sesquiterpene valerenic acid; Houghton, 1988). The results of preclinical studies have indicated that *Valerian* spp. modulates the GABA-ergic system through potentiation of GABA release and inhibition of reuptake or degradation, via inhibited GABA-T activity in brain tissue, from male Sprague-Dawley rats, female Swiss-Webster mice, and male Wistar rats, respectively (Awad et al., 2007; Ortiz, Nieves-Natal, & Chavez, 1999; Santos et al., 1994).

Valerenic acid as the principle constituent was identified as a subunit-specific allosteric modulator of GABA-A receptors, specifically acting as an agonist at $\beta 2/3$ subunits in male RjHan:Wl rat cortical tissues and in *Xenopus* oocytes tissue investigations, likely to interact with the loreclezole binding pocket (Becker, Felgentreff, Schroder, Meier, & Brattstrom, 2014; Khom et al., 2007, 2016; Trauner et al., 2008).

The in vitro findings are reflected through in vivo studies; with animal models demonstrating anxiolytic properties of valerian extracts with female NMRI mice and male Sprague-Dawley rats on the EPM (and also anti-depressant effects via forced swim test model; Hattesoil et al., 2008), that it is equitable to BDZ comparators in female Hooded Wistar rats (Murphy, Kubin, Shepherd, & Ettinger, 2010). There is a strong indication that these behavioural effects are due to valerenic acid and valerenol constituents modulating GABA-A receptors, specifically $\beta 3$ subtypes from one combined in vitro and in vivo study involving human embryonic kidney cell lines, and in $\beta 3$ point-mutated mice in EPM and light-dark choice behavioural tests, using valerenic acid and valerenol extracts (Benke et al., 2009).

In human studies, one investigation (Kohnen & Oswald, 1988) showed that valerian extract reduced subjective feelings of anxiety among healthy adults in a stress-inducing scenario and that these effects occurred independently of the beta-blocker propranolol. Additionally, another study (Ahmadi, Khalili, Abbasian, & Ghaeli, 2017), in a double-blind RCT with 51 HIV-positive patients, found that 530 mg of *V. officinalis* extract daily for 4 weeks significantly reduced the anxiety that commonly occurs as a neuropsychiatric side effect of antiretroviral therapy.

In affective disorder samples, a placebo-controlled, 8 week clinical study of *V. officinalis* root extract (765 mg per day) among 31 adults with obsessive-compulsive disorder (DSM-IV diagnosed) found the extract significantly reduced symptoms of the disorder (Paksereht, Boostani,

TABLE 1 List of selected phyto-medicines with preclinical (in vitro and animal) and clinical evidence

Botanical name	Common name	Active constituents	GABA substrate mechanism	Preclinical animal anxiety models	Clinical group
<i>Centella asiatica</i>	Gotu kola, pennywort	Asiatic acid triterpenoids	Stimulation of glutamic acid decarboxylase; selective GABA-B agonist Awad et al. (2007); Chatterjee et al. (1992); Hamid et al. (2014)	EPM, open-field test, dark-light box Cerenmuga et al. (2015); Wanuntornwong et al. (2012); Wijeweera et al. (2006)	Acoustic startle response Bradwejn et al. (2000); Jana et al. (2010)
<i>Humulus lupulus</i>	Hops	Terpenoids and flavonoids: humulene, myrcene, and xanthohumol	Glutamic acid decarboxylase inhibition Awad et al. 2007	EPM, avian model of nocturnal behaviours Cerenmuga et al., 2013; Franco, Sanchez Bravo, Rodriguez, Barriga, and Juarez (2012a); Schiller et al. (2006)	Menopausal females: "work stressed" healthy sample Franco, Sanchez, Bravo, Rodriguez, Barriga, Romero, et al. (2012b)
<i>Ginkgo biloba</i>	Maiden hair, Ginkgo	Terpenoids: bilobalides, ginkgolides A, B, and C	GABA-A and GABA-C receptors antagonist; increased GABA levels and glutamic acid decarboxylase enzyme activity Huang et al. (2003, 2004, 2006, 2012); Mc et al., 2003; Kiewert et al., 2007; Ng et al., 2017; Sasaki et al., 1999, 2000	EPM, open field, water maze Kuribara et al. (2003); Ma et al. (2012)	Generalised anxiety disorder Woelk et al. (2007)
<i>Matricaria recutita</i> / <i>Matricaria chamomilla</i>	Chamomile, German chamomile	Flavonoids, Sesquiterpenoids, coumarins, phenylpropanoids: apigenin, luteolin, rutin, herbicarin, umbelliferone, chlorogenic and caffeic acids	GABA-B2D receptor ligand binding Avalione et al. (1996, 2000); Johnston (2015); Viola et al. (1995)	EPM, open field, social interaction Can et al. (2012); Shinozaki et al. (2005)	Generalised anxiety disorder, insomnia Amsterdam et al. (2009, 2012); Keefe et al. (2016); Mao et al. (2014); Zick et al. (2011)
<i>Melissa officinalis</i>	Lemon balm	Flavonoids, volatile oils, triterpenoids: rosmarinic acid, oleanolic acid, ursolic acid	GABA-T inhibition; GABA-A BDZ receptor affinity; elevation of brain GABA levels Awad et al. (2007); Awad et al. (2009); Abuhamdan et al. (2008); Huang et al. (2008); Mahita et al. (2014); Raines et al. (2009); Salah and Jager (2005); Yoo et al. (2011)	EPM, open field rodent models Ibarra et al. (2010); Taiwo et al. (2012)	Stress induction paradigm in healthy controls; general anxiety; sleep disorders; premenstrual dysphoria Akbarzadeh et al. (2015); Cases et al. (2011); Kennedy et al. (2002, 2003, 2004); Scholey et al. (2014)
<i>Passiflora incarnata</i>	Passionflower	Flavonoids: chrysin, b-carboline alkaloids	GABA-A BDZ positive allosteric modulation; GABA-A and GABA-B receptor subtype affinities Anan et al. (2016); Dhawan et al. (2001); Elias et al. (2010);	EPM, hole board, horizontal wire rodent models Anan et al. (2016); Dhawan et al. (2001); Elias et al. (2010);	Generalised anxiety disorder; pre-surgery Anxiety Akbarzadeh et al. (2001); Aslanoglu et al. (2012); Dantas

(Continues)

TABLE 1 (Continued)

Botanical name	Common name	Active constituents	GABA substrate mechanism	Predclinical animal anxiety models	Clinical group
<i>Piper methysticum</i>	Kava	Kavalactones: kavain and dihydrokavain	GABA-A receptor positive modulators, ligand binding at all subtypes, also NaCl, Ca ²⁺ gated ion channels Bastier et al. (2013); Boonen and Haberen (1998); Chua et al. (2016); Davies et al. (1992); Gielitz et al. (1995); He et al. (1997); Jusioff et al. (1994); Magura et al. (1997); Martin et al. (2002); Mathews et al. (2005)	EPM	Generalised anxiety disorder; phobia; social phobia; nonpsychotic anxiety; stress in healthy samples; menopausal anxiety LaPorte et al. (2011); Pittler and Ernst (2003); Sarris (2007); Sarris et al. (2011, 2013a, b, c); Singh & Singh (2002)
<i>Scutellaria lateriflora</i>	Scullcap	Flavonoids: baicalin, baicalin, scutellarin A, apigenin, oroxylin A, wogonin	GABA-A receptor positive allosteric modulation Awad et al. (2003); Wang et al. (2008); Xu et al. (2006)	EPM, open field	Healthy sample; general anxiety
<i>Valeriana</i> spp.	European or Mexican valerian	Sesquiterpenoids: valerenic acid, valepotriates, alkaloids, flavonones	Glutamic acid decarboxylase stimulatory effect; GABA-A receptor binding, specifically GABA-A chloride channel receptor complex B2 or B3 subunit (N265 M); GABA-A receptor allosteric modulation Awad et al. (2007); Becker et al. (2014); Khom et al. (2007, 2016); Ortiz et al. (1999); Ramhariter and Miltzer (2009); Santos et al. (1994); Trauer et al. (2008)	EPM, light/dark choice test, seizure model Benke et al. (2009); Khom et al. (2016); Hattisohl et al. (2008); Murphy et al. (2010)	Children with anxiety; HIV adults; healthy sample driving; OCD; generalised anxiety disorder Ahmadi et al. (2017); Andreolini et al. (2002); Gutierrez et al. (2004); Pakseresht et al. (2011); Thomas et al. (2016)
<i>Withania somnifera</i>	Ashwagandha, Winter Cherry, Indian Ginseng	Glycosides alkaloids and steroidal triterpenoid lactones: Glycowithanolides, withanone, withaferin A, withanolides A.D. and G, withanine, withasaminin, withanolides	Ionotropic GABA-A receptors; GABA _A 1 receptor agonists Candelario et al. (2015); Yin et al. (2013)	EPM, social interaction; feeding latency, unfamiliar environment	Generalised anxiety disorder; general anxiety; chronic stress; bipolar disorder Andrade, Aswath, Chaturvedi, Srivasa, and Raguram (2012); Audy et al. (2008); Cooley et al. (2009); Chandrasekhar, Kapoor, and Anishetty (2012); Chengappa et al. (2013); Khyati and Ayup (2013)

Note. BZD = benzodiazepine; EPM = elevated-plus maze; GABA = gamma-aminobutyric acid; GABA-T = gamma-aminobutyric acid transaminase; GAT = gamma amino transferase; HIV = human immunodeficiency virus; OCD = obsessive-compulsive disorder.

& Sayyah, 2011). Another RCT compared a mixture of isolated valepotriates (from *V. officinalis*), diazepam, and placebo over 4 weeks in 36 patients with GAD (Andreatini, Sartori, Seabra, & Leite, 2002). Although there were no significant differences between groups on total symptom scores, valerian appeared to be relatively more effective for psychic symptoms of anxiety (according to HAM-A subscales), whereas diazepam performed better for State-Trait Anxiety Inventory trait anxiety.

Two further studies examined the anxiolytic and sedating effects in a safety context: The first investigated driving performance and sedating effects with placebo comparator, finding no detrimental effects from valerian (Thomas et al., 2016). The second in a similar design ($n = 10$), assessed subjective sedation and psychomotor/cognitive performance effects of *V. officinalis* extract, diazepam and placebo control, finding decrements only occurred from the BDZ comparator (Gutierrez, Ang-Lee, Walker, & Zacny, 2004).

3.2.10 | *Withania somnifera* (Ashwagandha, Indian ginseng, winter cherry)

Withania somnifera is an alkaloid-rich plant from the nightshade family, traditionally utilised in Indian Ayurvedic medicine for the adaptogenic or "rasayana" properties of the root extract (Mishra, Singh, & Dagenais, 2000). The constituents include glycosides (withanolides), alkaloids (withanine, ashwagandhine, ashwaganidhine, and somniferine), and steroidal triterpenoid lactones, which are related to the ginsenosides, hence the common name "Indian ginseng". Tissue studies investigating GABA-ergic actions of withaferin, withanolides-A, and methanol extract of *W. somnifera* have found these constituents are GABA-A and GABA-p1 receptor agonists via *Xenopus* cell lines that also included transplanted Sprague-Dawley rat brain GABA channels (Candelario et al., 2015; Yin, Cho, Park, & Han, 2013). In rodent models using EPM, social interaction and feeding latency/unfamiliar environment paradigms, withanolides have been shown to have equivalent anxiolytic effects to BDZ comparators (lorazepam) in male Charles Foster rats (Bhattacharya, Bhattacharya, Sairam, & Ghosal, 2000; Kaur et al., 2016).

In human studies, a recent systematic review (Pratte, Nanavati, Young, & Morley, 2014) captured all previous studies of *W. somnifera* with anxiety/stress outcomes (e.g., Auddy et al., 2008; Chandrasekhar et al., 2012; Chengappa et al., 2013). This identified five human RCTs with 400 participants (range = 39–130). All five trials reported at least one significant benefit of *W. somnifera* in comparison to control conditions for anxiety and/or stress related outcomes. Doses ranged from 125–1200 mg per day, over 6 to 16 weeks. Since this review, one further eligible study has been conducted: a double-blind RCT in 52 participants with chronic stress, comparing 600 mg of *W. somnifera* extract per day to placebo capsules over 8 weeks (Choudhary, Bhattacharya, & Joshi, 2017). The study showed significantly greater reductions in stress outcomes from the extract, corresponding with significant decreases in salivary cortisol (a stress/anxiety biomarker).

4 | DISCUSSION

An increasing number of phytomedicines and their bioactive constituents with evidenced GABA mechanisms have been studied in the treatment of generalised anxiety symptoms in addition to clinically

diagnosed anxiety disorders. Despite the increased use of phytomedicines, with up to 80% of the world's population using some form of herbal medicine (World Health Organization, 2003), most still lack rigorous investigations of clinical efficacy. This is a common observation from previous reviews of CNS mechanisms of phytomedicines for affective disorders (including GAD) (Ernst, 2006; Lkhan & Vieira, 2010; Liu et al., 2015; Sarris, 2007). In particular, there are a paucity of clinical investigations for phytomedicine use in the treatment of GAD, especially given the epidemiological prevalence of this particular disorder (Australian Bureau of Statistics, 2007; Wittchen et al., 2011).

In the current review, *in vitro* and *in vivo* animal studies both revealed GABA-mediated anxiolytic effects from various phytomedicines (as whole extracts and/or isolated constituents). This was demonstrated via a range of mechanisms, which include direct and allosteric interaction with GABA receptor, up-regulation of relevant agonists, and/or interactions with antagonist drugs such as flumazenil or pentylenetetrazole. However, there is also evidence that some of the phytomedicine constituents included in this review modulate ionotropic GABA receptors independently or only partially from flumazenil-sensitive BDZ sites such as that occurring with diazepam (cf. Johnston, 2005). For example, chrysin is known to be a partial agonist at the central BDZ binding site, but apigenin competitively binds to GABA-A BDZ site, also appears to act on both flumazenil-sensitive and -insensitive components, and the effects of Kavalin are also not blocked by flumazenil, which suggest a different site of action (cf. Johnston, Hanrahan, Chebib, Duke, & Mewett, 2006; Hanrahan et al., 2011). In most cases, the precise sites of GABA-A receptor action are still to be ascertained, because subtype specificity for phytoconstituents has not been fully investigated in tissue and animal models.

Despite the need for preclinical research of GABA pathway mechanisms and subtype-selectivity behaviours, the next level of preclinical studies involving rodent models have demonstrated these GABA system effects result in reduced anxiety/stress related behaviours in established paradigms such as the EPM, light/dark box, and open fields. However, for some of these plant medicines there is currently a deficit of studies examining their anxiolytic potential for clinically diagnosed anxiety disorders.

Of the phytomedicines reviewed, *P. methysticum* has the largest evidence base for use in the anxiety disorders, with tripartite level of support from *in vitro*, *in vivo*, and human clinical studies. The number of positive findings from human studies of *P. methysticum* within randomised, well-controlled trials also supports its use as a treatment for various anxiety disorders and associated symptoms, demonstrating broad clinical utility (Sarris et al., 2011; Sarris et al., 2013b). Thus, *P. methysticum* remains the hallmark anxiolytic phytomedicine (minor concerns over liver toxicity notwithstanding). It is the most strongly evidenced for generalised anxiety, with clinical investigations now examining GABA metabolite changes via neuroimaging, and whether individual gene differences in GABA pathway polymorphisms influence treatment response (Savage et al., 2015).

V. officinalis, *C. asiatica*, *M. recutita*, *G. biloba*, *P. incarnata*, and *W. somnifera* offer the next most extensive evidence for reducing anxiety through GABA-related pathways; all with preclinical studies of GABA-ergic action in rodent animal models, with human studies

showing clinical efficacy of these extracts in people with diagnosed anxiety disorders. *V. officinalis*, particularly the constituent valerenic acid, has been identified as a potent allosteric modulator at GABA-A receptors. Interestingly, *V. officinalis* research involving dose-response effects suggest that mechanisms promote anxiolysis over sedation, with no decrements to cognitive function, even at high doses.

H. lupulus, *S. lateriflora*, and *M. officinalis* also have some promising preclinical evidence, along with human studies of anxiolytic effects in people experiencing stress and anxiety symptoms. However, the lack of evidence showing significant effects in patients with diagnosed anxiety disorders means that the clinical utility of these particular phytomedicines has yet to be shown.

With regard to phytochemical classes of the aforementioned plant medicines, the major GABA-modulating compounds were found to be terpenoids/sesquiterpenoids and saponins classes, such as kavalactones, bilobalide, humulene, valerenic acid, along with the withanones from *W. somnifera*, and the rosmarinic acids from *M. officinalis*. The flavonoids chrysin, apigenin (*M. recutita*), and bacalin (*scutellaria*) are the next most evidenced for influencing GABA systems. As they have been extensively studied, flavonoids are well-known positive modulators of GABA-A receptors, operating in the CNS by competitive BDZ site binding and indirectly opening chloride channels; hyperpolarising cell membrane and increasing the threshold for activity, thus exerting an anxiolytic effect (Johnston, 2015; Wasowski & Marder, 2012). It should be noted that despite the broad range of preclinical studies exploring the actions of these phytoconstituents on GABA type A receptors and their subtypes, the mechanisms of anxiolytic effect are complex and warrant a great deal of further research in order to inform the development and evaluation of targeted phytochemical compounds for anxiety disorders.

One limitation recognised in this systematic review is the narrow eligibility criteria, which included only those phytomedicines with three levels evidence (in vitro, in vivo, and human studies) for GABA-mediated anxiolytic effects. Although this adds strength to the review by providing focused conclusions on anxiolytic efficacy linked to GABA-based mechanisms of action, the strict criteria forced omission of other phytomedicines, such as *Morinda citrifolia* and *Zizyphus jujuba*, which may also potentially act to reduce anxiety via these pathways (Gao, Wu, & Wang, 2013; Pawlus & Kinghorn, 2007) but lack sufficient investigation for inclusion in this review.

Most notably, *Magnolia officinalis* has preclinical studies showing positive allosteric modulation of GABA-A receptors from its bioactive constituents, resulting in animal behaviours consistent with reduced anxiety (Han et al., 2011). However, to our knowledge no current human studies of the plant for anxiety to date, other than investigations within a herbal combination formula. Although combination formulas of various phytomedicines have demonstrated efficacy for reducing anxiety in humans (e.g., "Relora"; Talbott, Talbott, & Pugh, 2013), the extent to which the observed anxiolytic effects are due to individual components cannot be established. Further, it is recognised that formulas that combine multiple active ingredients may be synergistically more effective—acting through independent but complementary pathways to confer maximal effects (Bourin, Bougerol, Guillon, & Broutin, 1997).

Future research should aim to extend beyond animal models to investigate neurobiological mechanisms of anxiolytic action in human samples with technologies such as functional magnetic resonance imaging and ¹H magnetic resonance spectroscopy to examine metabolite and neurobiological changes resulting from phytomedicine intervention. Specifically, investigating GABA profile level changes in regions of the brain associated with anxiety disorders would further elucidate the neurobiological activity of anxiolytic phytomedicines, especially regarding the interaction with GABA-ergic pathways and the resultant clinical activity. Given the existing evidence for GABA-modulating anxiolytic phytomedicines, continuing to distill the underlying operative pathways and further evaluating these compounds in rigorously designed trials (particularly in anxiety disorder samples) could ultimately lead to novel and evidence-based treatments for people with anxiety disorders; thus serving a major unmet need.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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F. Publication 2 (first author): KGAD protocol

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STUDY PROTOCOL

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Kava for the treatment of generalised anxiety disorder (K-GAD): study protocol for a randomised controlled trial

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Abstract

Background: Generalised anxiety disorder (GAD) is a chronic and pervasive condition that generates high levels of psychological stress, and it is difficult to treat in the long term. Current pharmacotherapeutic options for GAD are in some cases only modestly effective, and may elicit undesirable side effects. Through targeted actions on the gamma-aminobutyric acid (GABA) pathway, the South Pacific medicinal plant kava (*Piper methysticum*) is a non-addictive, non-hypnotic anxiolytic with the potential to treat GAD. The evidence for the efficacy of kava for treating anxiety has been affirmed through clinical trials and meta-analyses. Recent research has also served to lessen safety concerns regarding the use of kava due to hepatotoxic risk, which is reflected in a recent German court overturning the previous kava ban in that country (which may in turn influence a reinstatement by the European Union). The aim of current research is to assess the efficacy of an 'aqueous noble cultivar rootstock extract' of kava in GAD in a larger longer term study. In addition, we plan to investigate the pharmacogenomic influence of GABA transporters on response, effects of kava on gene expression, and for the first time, the neurobiological correlates of treatment response via functional and metabolic imaging.

Methods/Design: This clinical trial is funded by the Australian National Health and Medical Research Council (APP1063383) and co-funded by MediHerb (Integria Healthcare (Australia) Pty. Ltd). The study is a phase III, multi-site, two-arm, 18-week, randomised, double-blind, placebo-controlled study using an aqueous extract of noble kava cultivar (standardised to 240 mg of kavalactones per day) versus matching placebo in 210 currently anxious participants with diagnosed GAD who are non-medicated. The study takes place at two sites: the Centre for Human Psychopharmacology (Swinburne University of Technology), Hawthorn, Melbourne, Australia; and the Academic Discipline of Psychiatry (The University of Queensland) based at the Royal Brisbane and Women's Hospital, Herston, Brisbane, Australia. Written informed consent will be obtained from each participant prior to commencement in the study. The primary outcome is the Structured Interview Guide for the Hamilton Anxiety Rating Scale (SIG-HA). The secondary outcomes involve a range of scales that assess affective disorder symptoms and quality of life outcomes, in addition to the study of mediating biomarkers of response (assessed via genomics and neuroimaging).

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Discussion: If this study demonstrates positive findings in support of the superiority of kava over placebo in the treatment of GAD, and also is shown to be safe, then this plant-medicine can be considered a 'first-line' therapy for GAD. Genomic and neuroimaging data may reveal clinical response patterns and provide more evidence of the neurobiological activity of the plant extract.

Trial Registration Information: ClinicalTrials.gov: NCT02219880 Date: 13 August 2014:

Keywords: Protocol, Anxiety, GAD, RCT, Kava, Kavalactones, GABA, Anxiolytic, Nutraceutical

Background

Generalised anxiety disorder (GAD) is defined through cognitive and somatic symptomatology; involving primarily excessive chronic worry and anticipatory anxiety [1], in addition to a constellation of presentations such as sleep disturbances, restlessness, edginess/irritability, fatigue, concentration difficulties, and muscular tension [2]. GAD is ubiquitous and persistent, usually with early onset and high prevalence of co-morbid affective disorders and substance misuse [3–6]. Unfortunately, available pharmacotherapies have a modest clinical effect [7, 8] and may elicit undesirable side effects [9–11], and while psychotherapeutic treatments are found to be efficacious [12, 13], they are of limited utility for some patients [14, 15]. As a result, many GAD sufferers do not receive adequate treatment [16–18], thereby providing an impetus to explore other therapeutic options.

One such option is kava (*Piper methysticum*), a plant native to the South Pacific, whose roots have been used in traditional medicine in the form of cold-water extractions (non-alcoholic) to treat a range of health conditions, including anxiety, stress, muscular spasms, pain, and menstrual disorders [19, 20]. The therapeutic effect of kava is based on the six major lipophilic kavalactones, of which kawain and dihydrokawain (see Fig. 1) have the strongest anxiolytic activity [21]. Limbic structures of the brain have previously been suggested as the principal site of kavalactone action [22]. Kavalactones exert their anxiolytic effect through an array of neurobiological activity, primarily from modulation of gamma-aminobutyric acid (GABA) receptors via blockade of voltage-gated sodium ion channels [23, 24], reduced excitatory neurotransmitter release via blockade of calcium ion channels [25, 26], and enhanced ligand binding to GABA type A receptors [27]. Other neurochemical effects include reversible inhibition of monoamine oxidase B [28], inhibition of cyclooxygenase [29], and reduced neuronal reuptake of dopamine [30] and prefrontal cortex noradrenalin [31]. This noradrenergic effect differentiates the central bio-behavioural effects of kava from those of alcohol and benzodiazepines [32], while the combination of GABA modulation and increased noradrenergic activation contributes to feelings of physical relaxation with increased hedonic tone, with no deleterious effects on cognition [33].

Background evidence

A Cochrane review and meta-analysis of seven randomised clinical trials (RCTs) using kava mono-preparations (60–280 mg kavalactones) for the treatment of generalised anxiety symptoms found a significant reduction of anxiety on the Hamilton Anxiety Rating Scale (HAM-A) for kava compared with placebo ($P=0.01$) [34]. Similarly, a more recent pooled analysis of six studies using kava versus placebo in the treatment of anxiety symptoms found a significant effect in favour of kava on the HAM-A, with an effect size (Cohen's d) of 1.1 [35]. Our previous three-week double-blind, placebo-controlled, crossover RCT ($n=60$), using a water-soluble rootstock extract of a noble kava cultivar in chronic generalised anxiety [36], involved a preparation standardised to a dose of 250 mg kavalactones. Following a placebo run-in phase there was a reduction of -9.9 points on the HAM-A in the first kava phase versus only a -0.8 point in the first placebo phase, and a reduction in the second crossover phase from kava of -10.3 points compared to a rise of +3.3 points for placebo (Cohen's $d=2.24$, $P<0.0001$).

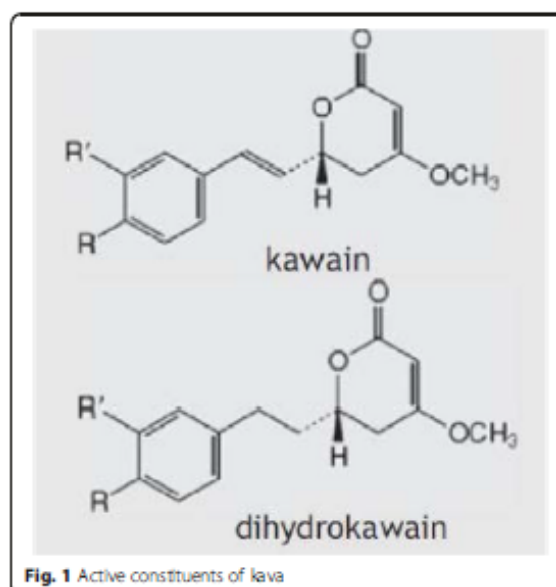


Fig. 1 Active constituents of kava

In a subsequent six-week parallel double-blind RCT [37] involving 75 participants with diagnosed GAD (58 randomised to 120 mg daily kavalactones titrated to 240 mg for non-response), a group $\times$ time interaction was found ($P = 0.046$) for a significant reduction in HAM-A scores in favour of kava over placebo. Further, kava significantly reduced participant anxiety by -4.2 , representing a moderate effect size (Cohen's $d = 0.63$). For participants with moderate-to-severe level anxiety (as assessed on the MINI Plus diagnostic interview), the treatment effect was more pronounced ($P = 0.020$), with a larger effect size ($d = 0.80$). The effects were still significant after controlling for baseline Montgomery-Åsberg Depression Rating Scale (MADRS) depression ($P = 0.01$), baseline Beck Anxiety Inventory (BAI) anxiety ($P = 0.05$), thyroid function ($P = 0.02$), and weekly caffeine use ($P = 0.03$). Further sub-analysis of participants with pure GAD and no other DSM-IV diagnosed co-morbid anxiety disorder (panic disorder, social phobia, post-traumatic stress disorder, obsessive-compulsive disorder) revealed a significant group $\times$ time interaction ($P = 0.020$; $d = 1.28$), with a reduction of -8.5 points for kava on the HAM-A compared to -2.3 points for placebo. As part of this trial we also examined five GABA transporter polymorphisms as potential pharmacogenetic markers of kava response. We observed a significant monotonic trend for two polymorphisms in which the number of rs2601126 T-alleles ($P = 0.021$) or of rs2697153 A-alleles ($P = 0.046$) was associated with significant reductions in HAM-A scores within the kava group.

Trial objectives

The primary objective of this trial is to confirm the effectiveness and safety of kava as a pharmacological approach to treating GAD, by conducting a longer term, multicentre RCT with a large sample using a standardised pharmaceutical-grade aqueous rootstock extract of a noble kava cultivar in participants with current diagnosed GAD. As secondary objectives, we also seek to: 1) replicate our previously observed association between genetic variation in the GABA transporter and kava treatment response, 2) elucidate the mechanisms underpinning the efficacy of kava within the GABA and glutaminergic pathways through functional and metabolic neuroimaging, and 3) examine the effects of kava on the expression of selected genes within the GABA, glutamate, dopamine, serotonin, and adrenergic pathways.

Hypotheses

Primary hypothesis

- 1) Kava will be superior to placebo in the treatment of GAD symptoms, assessed by change in Structured Interview Guide for the Hamilton

Anxiety Rating Scale (SIGH-A) [38] scores after the 16-week treatment phase.

Secondary hypotheses

- 2) Kava will be superior to placebo on a range of secondary affective disorder outcome scales (Beck Anxiety Inventory [39], Penn State Worry Questionnaire [40], and Montgomery-Åsberg Depression Rating Scale [41]).
- 3) Kava will be superior to placebo on health-related quality of life measured using The World Health Organization Quality of Life - BREF (WHOQOL-BREF) [42], the Social Re-adjustment Rating Scale [43], and the Kessler Psychological Distress Scale [44].
- 4) Overall sexual satisfaction and performance rated on the Arizona Sexual Experiences Scale [45] will be in favour of kava compared to placebo, in women but not men.
- 5) Personality trait factors including anxiety, extraversion, socialization, and aggression rated on the Swedish Universities Scales of Personalities (SSP) may be associated with GAD symptomatology and will be measured via this self-reporting measure.
- 6) Anterior cingulate cortex (ACC) activation and metabolite profiles will be differentially affected in the kava treatment group over placebo from baseline to week 8. Specifically, for ACC metabolite levels, there will be an increase in GABA and decrease in glutamate/glutamine ratio (Glu/Gln); ACC activation: reduction in activation during resting state; ACC activation: reduction in activation associated with anticipatory anxiety in a task-dependent manner. Further, individual differences in rostral ACC activation measured pre-treatment may predict GAD treatment outcomes for kava compared to placebo. Increased levels of activity in the ACC will also be associated with better clinical outcome in GAD participants.
- 7) Response to kava will be moderated by GABA transporter polymorphisms. Specifically, for every rs2601126-T allele or rs2697153-A allele an individual carries, the greater the reduction of anxiety that will be observed.
- 8) Individuals in the kava group will have distinct gene expression change profiles compared to the placebo group. Specifically, we hypothesise that expression of GABA pathway genes will be up-regulated following kava treatment and remain stable in the placebo group.

Methods/Design

Study design and plan

The design of the study is a phase III, multi-site, two-arm, 18-week, randomised, double-blind, placebo-controlled trial using a standardised pharmaceutical-grade, water-

soluble extract of kava (240 mg of kavalactones per day) or matching placebo (inert plant-based fibre) in 210 currently anxious participants with GAD. The trial sites are at the Centre for Human Psychopharmacology (Swinburne University of Technology), Hawthorn, Melbourne, Australia and the Academic Discipline of Psychiatry (The University of Queensland) based at the Royal Brisbane and Women's Hospital, Herston, Brisbane, Australia. Recruitment is scheduled to commence September 2015 and through to June 2017 (Fig. 2).

Participants will be required to attend seven visits at the study sites at week 0 (baseline), 2, 4, 8, 12, 16, and 18. At the baseline visit, participants will complete informed consent forms, screening assessments, and mood and anxiety questionnaires (self-reporting and interview-based). Brain imaging component visits (Melbourne site only) are to be scheduled within one week of baseline and week 8 visits.

The study employs an additional two-week single-blind placebo-controlled post-study observation period (that is, for a total trial length of 18 weeks - refer Fig. 3).

All eligible participants will be randomly allocated to a treatment arm, and corresponding treatment will be provided following clearance of blood tests taken at the baseline visit. All subsequent visits will follow the same outline of the baseline session excluding consent forms and screening assessments and including a safety assessment. In addition, participants will be required to provide a blood sample within several days of the baseline, and at week 2, 8, and 16 visits. For female participants not taking hormone-modulating contraception who are otherwise eligible to partake in the brain imaging components, the baseline visit is to be scheduled on days 1–5 of the menstrual cycle.

Ethics and trial registry

This study has ethical clearance (Alfred Hospital Research Ethics Committee 137/14, University of Queensland Research Ethics Committee 2014000876,

Swinburne University Human Research Ethics Committee 2014/204), and is registered on ClinicalTrials.gov (protocol number: NCT02219880).

Recruitment

Participants will be recruited through various advertisement sources:

- A dedicated study website
- Advertisements in local newspapers and radio
- A press release which may involve featured articles in newspapers and appearances by the chief investigator on radio and TV
- Online advertisements, including the Swinburne University 'Participate in Research' webpage (<http://www.swinburne.edu.au/lss/chp/projects/kava.html>) and the websites of local anxiety disorder organisations (for example, Anxiety Disorders Association of Victoria, Anxiety Recovery Centre Victoria), Google, and Facebook
- Posters and brochures displayed on campus and in the waiting rooms of local Healthscope pathology centres and medical clinics

Primary outcome measure

The primary outcome is symptoms of GAD measured by the Structured Interview Guide for the Hamilton Anxiety Rating Scale (SIGH-A), at baseline and subsequent visits up to 18 weeks following randomization. The SIGH-A is a widely used measure of anxious symptomatology in GAD research. It includes measures of persistent worry, anxiety, mood, and somatic symptoms. The SIGH-A has been used in previous studies of pharmacological and psychological treatments for people with GAD. It is reliable (intraclass correlation coefficients for inter-rater reliability of 0.98 and test-retest reliability of 0.88), has high convergent validity

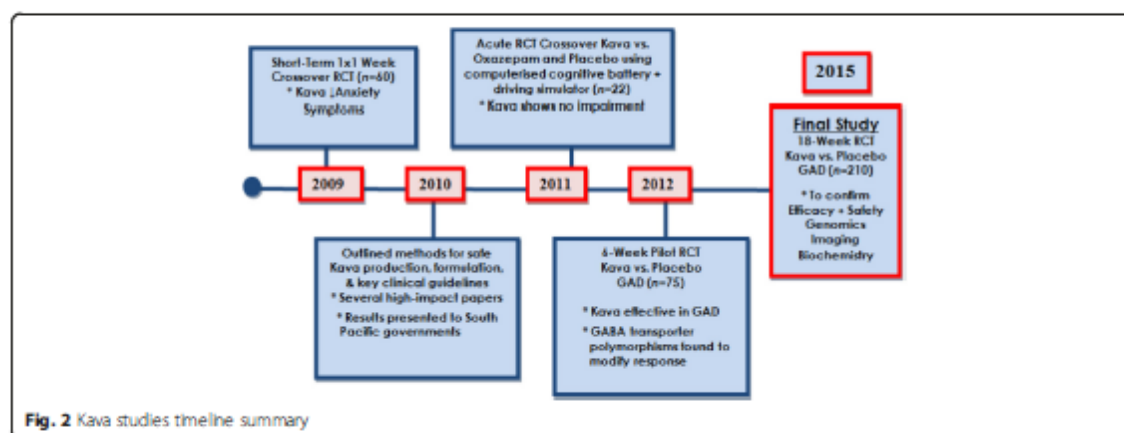
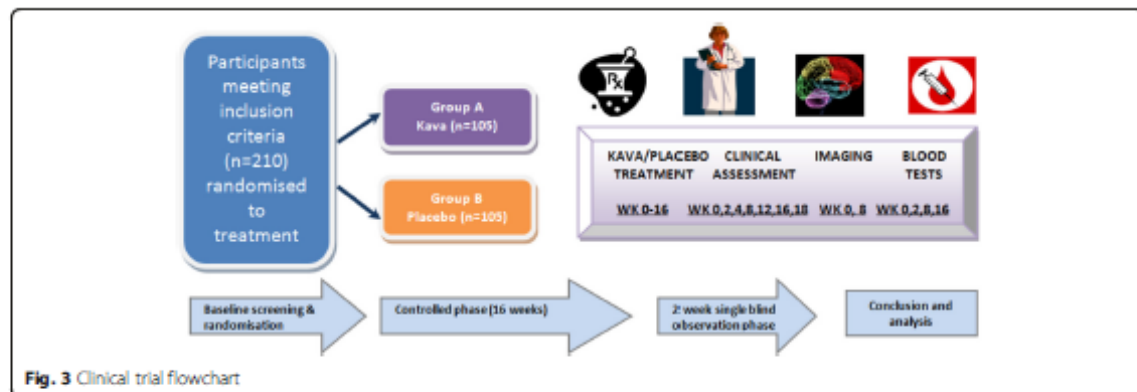


Fig. 2 Kava studies timeline summary



with structured clinical ratings of symptoms of GAD (for example, MINI PLUS 6.0 [46]), and is sensitive to change [47, 48].

Secondary outcome measures

- In addition, the Beck Anxiety Inventory (BAI) will be used as a participant self-report scale to assess anxiety levels. It consists of 21 items ranging from 0 (not present) to 3 (severe). These items are more somatically orientated than the SIGH-A and provide additional information on anxiety outcomes.
- The Penn State Worry Questionnaire (PSWQ) is a 16-item self-report measure of the intensity and excessiveness of worry. Examples of items are "Many situations make me worry" and "Once I start worrying, I cannot stop." Participants rate items on a 5-point Likert scale from 1 ("Not at all typical of me") to 5 ("Very typical of me"). Total scores range from 16 to 80, with higher scores indicating pathological worry. The PSWQ is widely used and has shown good to very good internal consistency, test-retest reliability, and discriminant validity for GAD versus other anxiety disorders [49].
- Mood will be assessed at each visit using the SIGMA (Structured Interview Guide for the Montgomery-Åsberg Depression Rating Scale (MADRS)), a diagnostic questionnaire which measures severity of depression symptoms. The SIGMA provides structured questions for each item to ensure standardisation of administration. Each item yields a score of 0 to 6, producing an overall score ranging between 0–60. A higher MADRS score indicates more severe depression. Inter-rater reliability with the SIGMA is reported to be excellent ($r = 0.93$) [50]. The use of this scale is important, as sufferers of GAD often have co-morbid depression, which may affect their response to treatment.
- Quality of life/life stressor measures will be administered at selected visits. The WHOQOL-BREF measure is a reduced version of the original instrument, designed for clinical trial use. It comprises 26 items, covering the domains of physical health, psychological health, social relationships, and environment. The Social Readjustment Rating Scale (SRRS) is a scale using 'Life Change Units' (LCUs) to quantify the number and severity of life stressors occurring in the preceding 12-month period. Both will be administered at baseline and at week 16. The Kessler Psychological Distress Scale (K10) is a 10-item questionnaire designed to obtain a global measure of perceived stress level, with questions pertaining to anxiety and depressive symptoms experienced in the past four-week period. For this study it will be administered at each visit.
- The Arizona Sexual Experiences Scale (ASEX) will be employed to assess the effects of kava on participants' sexual functioning. Specifically, the self-report scale explores effects on desire, arousal level, and physiological responses over the course of the past week. It consists of five self-report items, each rated on a 6-point scale. Total scores can range between 5–30, where higher scores indicate greater sexual dysfunction. The use of this scale is important, as conventional SSRI antidepressant treatments (used to treat GAD) commonly cause sexual side effects, and results of a previous study [37] showed a significant improvement in sex drive for women. If this finding is replicated, it will show a point of difference between kava and conventional treatments. Due to the sensitive nature of the items, completion of this scale is optional.
- The Swedish Universities Scales of Personalities (SSP) is a 91-item self-rated questionnaire based on the Karolinska Scales of Personality (KSP), a scale designed to measure stable personality traits related to psychopathology [51]. It assesses four general temperament dimensions: anxiety, extraversion, socialization, and aggression. This questionnaire will

be given to participants after their initial baseline visit to complete in their own time and return at the next visit.

- Genotyping of candidate and haplotype-tagging single nucleotide polymorphisms in monoamine, glutaminergic, and GABA transporter genes, measured via blood sample at week 1, will be achieved using a Sequenom Mass-Array.
- A custom RT² PCR Array (Qiagen) will be used to examine gene expression of candidate genes (Table 1) at weeks 1 and 8.
- Anterior cingulate cortical (ACC) region metabolite profiles, including N-acetyl-L-aspartate (NAA), creatine (Crn), glutamate/glutamine (Glu/Gln), and gamma-amino butyric acid (GABA), will be measured using single voxel magnetic resonance spectroscopy (MRS) at weeks 1 and 8.
- ACC region activity at rest and functional connectivity network (that is, default mode network) will be analysed using resting-state functional magnetic resonance imaging (fMRI) at weeks 1 and 8.
- ACC region task-dependent activation levels associated with anticipatory anxiety will be assessed using task fMRI (the International Affective Picture System (IAPS; [52]) at weeks 1 and 8.
- Acute anxiety levels related to undergoing scanner session at weeks 1 and 8 will be measured on the Spielberger State-Trait Anxiety Inventory — Trait and State components [53].

Inclusion criteria*

1. Aged between 18–70 years
2. Meets the DSM-IV and DSM-5 diagnostic criteria for generalised anxiety disorder (GAD) based on structured interview; Mini International Neuropsychiatric Interview-6.0 (MINI 6.0) [54], (note that while the MINI 6.0 uses the DSM-IV criteria, the same criteria are used in the DSM-5)
3. Presents with anxiety (SLGH-A ≥ 18) at the time of study entry
4. Fluent in spoken and written English
5. Provides a signed copy of the consent form

***MRI component** – Participant eligibility for the imaging component of the study will follow a listing of restrictions typical of imaging safety at the 3-T scanner. All participants will be right-handed, with no metallic implants, piercings, or residue. For females in the imaging component, there will be conditions of enrolment relating to menstrual cycle dates, and they must not be taking hormone-modulating contraception (such as oral contraceptive pills or hormonal implants).

Exclusion criteria

1. Primary diagnosis other than GAD as determined by the MINI 6.0
2. Presentation of moderate to severe depressive symptoms (MADRS ≥ 18) at time of study entry
3. Presentation of suicidal ideation (≥ 3 on MADRS suicidal thoughts domain) at time of study entry
4. Current diagnosis of a psychotic disorder (for example, schizophrenia) or bipolar I on structured interview (MINI 6.0)
5. Current substance/alcohol use disorder on structured interview (MINI 6.0)
6. Currently taking an antidepressant, mood stabiliser, antipsychotic, anticonvulsant, warfarin or thyroxine, or regularly using a benzodiazepine or opioid-based analgesic (more than two days per week)
7. Current use of St John's wort or contraindicated herbal medication
8. Previous intolerance to kava
9. Three or more failed trials of pharmacotherapy for the current GAD episode
10. Recently commenced psychotherapy (within four weeks of study entry)
11. Known or suspected clinically unstable systemic medical disorder
12. Diagnosed hepato-biliary disease/inflammation
13. Elevated liver enzymes at baseline blood test
14. Pregnancy or breastfeeding, or trying to conceive
15. Not using a medically approved form of contraception (including abstinence) if female and of childbearing age
16. Unable to participate in all scheduled visits, treatment plan, tests, and other trial procedures according to the protocol.

Treatment interventions, randomisation, and blinding

The active treatment (kava) and placebo product will be identical in colour, size, and shape, bottled and labelled with trial treatment numbers by an independent researcher. The kava is a high-quality noble *Borogu* cultivar acquired from Southern Pentecost Island, Vanuatu, and manufactured by MediHerb (Integria Healthcare (Australia) Pty. Ltd.). Each tablet is standardised to contain 60 mg of kavalactones with a higher level of kavain and dihydrokavain, and a lower relative level of dihydro-methysticin. To ensure replication of odour, a small porous sachet of kava powder is inserted into all treatment bottles. The trial products will be manufactured and stored in accordance with manufacturer instructions, following Pharmaceutical Good Manufacturing Practice.

Participants are assigned study identification numbers sequentially, and treatments are randomised via identification numbers using permuted 3×2 block randomisation,

Table 1 Gene expression list

Gene symbol	Gene name
GABBR1	GABA _B receptor 1
GABBR2	GABA _B receptor 2
GABRA1	GABA _A receptor, alpha 1
GABRA2	GABA _A receptor, alpha 2
GABRA4	GABA _A receptor, alpha 4
GABRA5	GABA _A receptor, alpha 5
GABRA6	GABA _A receptor, alpha 6
GABRB1	GABA _A receptor, beta 1
GABRB3	GABA _A receptor, beta 3
GABRD	GABA _A receptor, delta
GABRE	GABA _A receptor, epsilon
GABRG1	GABA _A receptor, gamma 1
GABRG2	GABA _A receptor, gamma 2
GABRG3	GABA _A receptor, gamma 3
GABRQ	GABA _A receptor, theta
GABRR1	GABA _A receptor, rho 1
GABRR2	GABA _A receptor, rho 2
ADCY7	Adenylate cyclase 7
ADORA1	Adenosine A1 receptor
ADORA2A	Adenosine A2a receptor
CACNA1A	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit
CACNA1B	Calcium channel, voltage-dependent, N type, alpha 1B subunit
GNAI1	G protein, alpha inhibiting activity polypeptide 1
GNAQ	G protein, q polypeptide
GPHN	Gephyrin
SNCA	Synuclein, alpha
NSF	N-ethylmaleimide-sensitive factor
P2RX7	Purinergic receptor P2X ₇ , ligand-gated ion channel, 7
SLC1A3	Glial high affinity glutamate transporter
SLC32A1	Solute carrier family 32 (GABA vesicular transporter), member 1
SLC38A1	Solute carrier family 38, member 1
SLC6A1	Solute carrier family 6 (neurotransmitter transporter), member 1
SLC6A11	Solute carrier family 6 (neurotransmitter transporter), member 11
SLC6A12	Solute carrier family 6 (neurotransmitter transporter), member 12
SLC6A13	Solute carrier family 6 (neurotransmitter transporter), member 13
ABAT	4-aminobutyrate aminotransferase
ALDH5A1	Aldehyde dehydrogenase 5 family, member A1
GAD1	Glutamate decarboxylase 1
GLS	Glutaminase
GLUL	Glutamate-ammonia ligase
PHGDH	Phosphoglycerate dehydrogenase
DRD1	Dopamine receptor D1
DRD2	Dopamine receptor D2
DRD3	Dopamine receptor D3

Table 1 Gene expression list (Continued)

DRD4	Dopamine receptor D4
DRD5	Dopamine receptor D5
COMT	Catechol-O-methyltransferase
DBH	Dopamine beta-hydroxylase
DDC	Dopa decarboxylase
MAOA	Monoamine oxidase A
TH	Tyrosine hydroxylase
SLC6A3	Solute carrier family 6 (dopamine transporter), member 3
SLC6A4	Solute carrier family 6 (serotonin transporter), member 4
MAOB	Monoamine oxidase B
HTR1A	Serotonin receptor 1A
GRIA1	Glutamate receptor, ionotropic, AMPA 1
GRIA2	Glutamate receptor, ionotropic, AMPA 2
GRIA3	Glutamate receptor, ionotropic, AMPA 3
GRIA4	Glutamate receptor, ionotropic, AMPA 4
GRK1	Glutamate receptor, ionotropic, kainate 1
GRK2	Glutamate receptor, ionotropic, kainate 2
GRK4	Glutamate receptor, ionotropic, kainate 4
GRK5	Glutamate receptor, ionotropic, kainate 5
GRIN1	Glutamate receptor, ionotropic, N-methyl D-aspartate 1
GRIN2A	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A
GRIN2B	Glutamate receptor, ionotropic, N-methyl D-aspartate 2B
GRIN2C	Glutamate receptor, ionotropic, N-methyl D-aspartate 2C
GRM1	Glutamate receptor, metabotropic 1
GRM2	Glutamate receptor, metabotropic 2
GRM3	Glutamate receptor, metabotropic 3
GRM4	Glutamate receptor, metabotropic 4
GRM5	Glutamate receptor, metabotropic 5
GRM6	Glutamate receptor, metabotropic 6
GRM7	Glutamate receptor, metabotropic 7
GRM8	Glutamate receptor, metabotropic 8
ADRA1A	Adrenoceptor alpha 1A
ADRA1D	Adrenoceptor alpha 1D
ADRA2A	Adrenoceptor alpha 2A
ADRB2	Adrenoceptor beta 2
ADRB3	Adrenoceptor beta 3
SLC18A2	Vesicular monoamine transporter
SLC6A2	Norepinephrine transporter
CYP3A4	Cytochrome P450 3A4
CYP2D6	Cytochrome P450 2D6
CYP2E1	Cytochrome P450 2E1
AR	Androgen receptor
NRG1	Neuregulin-1
BDNF	Brain-derived neurotrophic factor
ABCG2	Breast cancer resistance protein

Table 1 Gene expression list (Continued)

ABCB1	P-glycoprotein
GAPDH	Reference genes
SADH	Reference genes
B2M	Reference genes

for example, ABAAABABBBA, based on these numbers. Randomisation is performed by staff unrelated to the study. Treatment is allocated sequentially to enrolled participants by trial clinicians who are blinded to treatment coding (with no identifying details on the bottle of which group [A or B] each participant is assigned to, thus providing double-blinding). Participants will take two kava tablets twice per day (total of 240 mg of kavalactones per day) or matching placebo for 16 weeks.

Statistical analyses

Analysis of data will be conducted with blinding to group allocations. The primary efficacy analysis will assess average treatment group differences for the primary outcome measure (SIGH-A) over the entire study period using a likelihood based mixed-effects model, repeated measures approach (MMRM). Results from the analysis of dichotomous data (for example, demographics and genetic data) will be presented as proportions (for example, relative risks), with 95 % confidence interval, and Fisher's exact test *P* value where appropriate. Non-parametric statistics will be used when assumptions for parametric methods are violated. Cohen's *d* effect sizes will be calculated. All tests of treatment effects will be conducted using a two-sided alpha level of 0.05 and 95 % confidence intervals. Data will be analysed using Statistical Package for Social Sciences software (SPSS) [55].

Imaging analysis

Imaging data acquisition

A 3-tesla scanner (Siemens Tim Trio MR scanner) is utilised for the neuroimaging component of the study. **Structural MRI:** A high-resolution (1×1×1 mm) T1 weighted scan will be performed. **MRS:** To measure the level of metabolite concentrations within the region of interest (anterior cingulate cortex (ACC)), single voxel MRS will be applied. PRESS sequence (TE = 30, TR = 2000, Ave = 128, weak water suppression) is first conducted to measure the main metabolism, followed by MEGA-PRESS (TE = 68, TR = 2000, suppression frequency = 1.95 ppm, Ave = 64) [54, 56–58] sequence at the identical location to measure concentration of GABA. Finally a water-unsuppressed sequence (16 averages) will be conducted for quantification. **Functional MRI** Resting state fMRI (EPI sequence, TE/TR = 30/2500, 3×3×3 mm, 33 slices, 10-min scan acquisitions) will be conducted with the subject's eyes closed in addition to task-based cognitive activation

fMRI study utilising IAPS to elicit anxiety (EPI sequence, TR = 2000, 3×3×3 mm, 27 mins).

Image data analysis

MRS: LC-model will be used to fit and quantify the concentration of all metabolites (total N-acetyl-l-aspartate, creatine, total choline, myo-inositol, glutamate/glutamine, and gamma-aminobutyric acid (GABA)), after quality control (that is, CLRB < 20 %, SNR > 10). Further, partial volume effect will be corrected using high-resolution T1 structural image and in-house voxel co-registration script. Imaging data analysis will be conducted using Statistical Parametric Mapping (SPM) and MATLAB software [59]. **Functional MRI:** Pre-processing and statistical analysis will be conducted using SPM12 (Wellcome Department of Neurology) and associated toolboxes run on MATLAB [59]. Pre-processing will include slice timing correction, motion correction, co-registration of function and structural data, then non-linear warping into standardised stereotactic space (MNI) and spatial smoothing. Resting-state fMRI will be further band-pass filtered and de-trended, then subjected to correlational connectivity analysis using REST v1.8 (http://www.restfmri.net/forum/REST_V1.8). The pre-processed task-based fMRI data will be high-pass filter analysed using the general linear model approach in SPM12, from which contrast images of crucial interest will be generated and entered into second level group models. To test the longitudinal effect of treatment group, a flexible factorial design will be used with main effect of time and group, interaction of time × group and covariance. Significant clusters surviving multiple comparison corrections will be reported.

Power analysis

The study will recruit a sample size of 210 participants (105 participants per arm). The study is powered to detect a small-to-moderate difference between active treatment and placebo on efficacy outcomes. Data will be analysed using data with at least one post-baseline, and all data including baseline measurement, in an intention-to-treat analysis. As such, a conservative effect size *F* of 0.15 on the primary outcome measure (SIGH-A) for a two-tailed analysis (with alpha = 0.05), and the study powered at 80 % (*Z* beta = 0.80), with a correlation among repeated measures (analysis of variance (ANOVA) model) over six time points, will require 206 participants (critical *F* of 3.88). A sample size of 206 (rounded up to 210) will be sufficiently

powered to provide a statistical difference between active treatment and placebo groups (using the intention-to-treat analysis) with the data.

The sample size of the imaging component of this study is 40 per treatment arm ($n = 80$). Following 12-week supplement administration, a recent MRS study with a sample size of 24 (12 in each arm) detected significant changes in metabolites by 39 % [60]. Our sample size of 40 participants in each arm will provide higher statistical power compared to previous studies, whilst still maintaining sufficient power with a 10 % participant attrition rate, as well as attrition due to unreliable metabolite estimates in one or both time points.

Safety considerations

While kava is a Therapeutic Goods Association (TGA) listed, over-the-counter nutraceutical product in Australia and has not been shown to cause any confirmed serious adverse reactions in this country, concerns over hepatotoxicity have led to withdrawal or restriction in some other countries. As discussed in the literature [61–63], many reported cases have involved concomitant ingestion of other compounds with potential hepatotoxicity (such as other medications and/or alcohol). At the clinical level, a variety of case study data from patients with kava hepatotoxicity have been gathered, with probable causation reported in a few cases [63]. Previously reported hepatotoxicity issues identified with German kava products may also be due in part to the extraction (ethanol or acetone extraction) method; the use of non-water soluble chemical (ethanol or acetone) extraction techniques (the traditional solvent is water), and use of aerial parts of the plant and root and stem peelings, and poorly prepared, potentially contaminated raw material [63].

In response to safety concerns, the World Health Organization commissioned a report assessing the risk of kava products [64]. Recommendation 2.1.3 from the report suggested that products from water-based suspension preparations be preferentially used over acetonetic and ethanolic extracts. The current study addresses these safety concerns by using standardised aqueous formulations of kava from the peeled rootstock of a noble cultivar (such cultivars are higher in kawain and dihydrokavain and lower in dihydromethysticin). Note that in June 2014, the German Federal Institute for Drugs and Medical Devices (Bundesinstitut für Arzneimittel und Medizinprodukte) overturned the previous 2002 ban on the use of kava products. As a European Union regulatory and guideline body, such a change has positive ramifications for the potential reinstatement of kava products based on ongoing empirical evidence from clinical trial research.

Monitoring and treatment compliance

Study staff involved in all aspects of participant recruitment, assessment, and monitoring hold postgraduate psychology qualifications, undergo training including inter-rater reliability of interviewer scale measures, and are supervised by psychiatrists and medical staff. This study has comprehensive medical supervision. Regardless, the occurrence of an adverse event (AE) may come to the attention of study personnel during study visits and interviews of a study subject presenting for medical care, or upon review of subject data by a study monitor. Any medical condition that is present at the time that the subject is screened for inclusion will not be reported as an AE. However, if it deteriorates at any time during the study, it will be recorded as an AE. All AEs will be graded for severity and relationship to study product. Participants will also be required to complete the Systematic Assessment for Treatment of Emergent Effects (SAFTEE) [65] at each assessment session to assess for AEs. All AEs will be recorded in the adverse event log in the participant case report form (CRF), including the seriousness, severity, and relationship to study product, duration, and outcome. In all cases, researchers will maintain contact with participants who experience an AE until it has been resolved and symptoms disappear. They will also be asked to notify their general practitioner. If a worsening of a participant's mental state occurs, the study psychiatrist and/or clinical psychologist will be consulted and appropriate steps taken.

Liver function will be assessed pre-treatment, and participants with any abnormal results will be excluded as a safety precaution. In people with healthy liver function, it is not expected that any elevation of serum liver enzyme levels will occur (based on our previous studies using similar standardised pharmaceutical-grade extract of kava). Nevertheless, liver function will be closely monitored via blood samples at regular intervals throughout the study (weeks 2, 8, and 16).

Participants will be required to take the tablets as instructed during the 18 weeks of the study. At each assessment session they will be asked to return all unused tablets, which will be counted for compliance rate (tablet counts will be employed by an independent researcher).

Data and documentation

Source documents will be kept in the participant files, which will be stored in a locked cabinet in a locked room accessible only to the investigators. Data from study measures will be entered into a password-protected electronic database on a secure network drive and backed up onto a USB/external hard drive stored alongside the participant files in a locked cabinet. All electronic data will be verifiable against source documents. As per Good Clinical Practice guidelines, upon request of regulatory authorities, the principle investigator will make all requested trial-

related records, including source documents, available for direct access. The study files and all source data will be retained for 15 years from the date of publication of study results, in accordance with university policy. Study protocol and processes have been developed in line with Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines [66].

Discussion

As detailed above in the literature, and through research related to the current project, there is evidence for kava's anxiolytic effect in both subthreshold generalised anxiety and GAD. Due to WHO recommendations to test water-soluble extracts, and the importance of using high-quality rootstock extracts from noble cultivars of kava, further research is required. Although our pilot data are promising, confidence in the use of a water-soluble extract of kava for the treatment of GAD cannot be established without a confirmatory larger, longer term rigorous study. Kava for use in anxiety has many advantages including clinical evidence for efficacy and safety, a relatively low cost, and the general appeal of nutraceutical approaches in the provision of treatment options outside of the conventional medication armamentarium.

Further, examining the neurobiological actions that underpin the anxiolytic effects of kava may contribute to understanding anxiolytic pharmacodynamics within relevant neural pathways, in turn, better informing their use within anxiety disorders. SNPs account for the pharmacodynamics and pharmacokinetics of drug response, and the current study aims to examine the genetic profiles of study participants, and subsequent metabolic and substrate pathway functionality behind differential response to study treatment. Relevant SNPs involved in pathways such as GABA may serve as predictive markers for determining the effectiveness of kava via allelic group in the GAD sample.

The pharmacogenomic component of the study is to be integrated with an investigation of brain metabolites and neurobiological function underpinning response to kava administration.

The interface between the purported anxiolytic properties of kavalactones, the functional and metabolic properties of brain regions associated with the disorder, and the differential response in GAD symptomatology (guided by pharmacogenomic markers) is of particular interest. Several studies have examined kava modulation on neurotransmitters *in vitro* (for a review see [67]), yet to date no studies have examined *in vivo* mechanisms of action which may underlie cognitive and physiological effects of kava prescription. Changes to the properties of GABA metabolites as a product of the intervention may be measurable in limbic brain regions in the GAD sample via MRS [54, 60, 67–70] and could be the key to

understanding the neurobiological efficacy of kava administration.

The anterior cingulate cortex's (ACC's) connectivity from lower limbic and hippocampal regions to the prefrontal cortical regions implies a central role in the organisation of affective and cognitive information. The ACC is thought to mediate affective and cognitive aspects of phenomena such as sadness recall, selective attention, loss, error and reward, suppression of negative emotion or cognitions, and autonomic processes that underpin anxiety states [71–79]. As such, (dysfunction within) the ACC may be a principal region underpinning GAD symptomatology, and is therefore the specific region under investigation for changes related to kava ingestion in the current study.

Some limitations to the study design are recognised. First, the design does not include a positive control product such as an SSRI, which may on some measures outperform the active treatment. Second, the use of imaging and genomic testing will need to ensure statistical correction applied for multiple comparisons, and thus the sample size must be reached to ensure adequate power in order to confirm any findings. Third, consideration of the external generalisability of study findings must be given, as the study sample will be 'pure GAD', that is, GAD that is currently untreated without comorbid conditions such as major depression, and with no current symptom treatment regime, either pharmaceutical or psychotherapeutic.

In summary, if this traditional extract of kava is confirmed as safe and effective, it will provide a significant 'Level 1' treatment option which may help sufferers of anxiety and provide significant support to use in a clinical setting. It may also ease concerns about the potential re-institution to restricted markets, which would provide socioeconomic benefit to poorer Pacific Island nations.

Trial status

Recruitment to commence September 2015.

Abbreviations

ACC: anterior cingulate cortex; AE: adverse event; ANOVA: analysis of variance; ASEX: Arizona Sexual Experiences Scale; BA: Beck Anxiety Inventory; CRF: case report form; DSM-IV: Diagnostic and Statistical Manual of Mental Disorders - Fourth Edition; GABA: gamma-aminobutyric acid; GAD: generalised anxiety disorder; HAM-A: Hamilton Anxiety Rating Scale; IAPS: International Affective Picture System; MADRS: Montgomery-Åsberg Depression Rating Scale; MINI-6.0: Mini International Neuropsychiatric Interview Version 6.0; MMRM: Mixed-effects model, repeated measures; MRS: magnetic resonance spectroscopy; NAA: N-acetyl-H-aspartate; NHMRC: National Health Medical Research Council; PSWQ: Penn State Worry Questionnaire; RCT: randomised clinical trial; SAFTEE: Systematic Assessment for Treatment of Emergent Effects; SIGMA: Structured Interview Guide for the Hamilton Anxiety Rating Scale; SIGMA: Structured Interview Guide for the Montgomery-Åsberg Depression Rating Scale; SNP: single nucleotide polymorphism; SPSS: Statistical Package for Social Sciences software; SRRS: Social Readjustment Rating Scale; WHOQOL-BREF: World Health Organization Quality of Life - BREF.

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