Neurometabolites in anterior cingulate cortex in chronic fatigue syndrome: A magnetic resonance spectroscopy study at 7 Tesla

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Abstract

Background:

Chronic fatigue syndrome (CFS) is a disorder characterized by prolonged physical and mental fatigue that cannot be explained by another established medical diagnosis. The anterior cingulate cortex (ACC) and putamen are two regions involved in frontal-striatal neural circuitry, which may be related to the pathophysiology of CFS. The aim of this study was to investigate the concentrations of neurometabolites, including glutamate, gamma-aminobutyric acid (GABA) and glutathione, in the ACC and putamen, using magnetic resonance spectroscopy (MRS) at 7 Tesla (7T). In addition, this study also aimed to evaluate resting-state functional connectivity in CFS with functional magnetic resonance imaging (fMRI).

Methods:

This study involved 12 patients who met the Oxford criteria for CFS and 25 healthy controls. Participants rated themselves on the Chalder Fatigue Questionnaire (CFQ) and the Beck Depression Inventory (BDI). All participants had a single proton (^{1}H) MRS and resting-state fMRI scan with a 7T Siemens MAGNETOM scanner (Siemens, Erlangen, Germany) with a Nova Medical 32 channel receive array head coil. Spectra were measured from voxels in the ACC (20 × 20 × 20 mm), putamen (10 × 16 × 20 mm) and occipital cortex (20 × 20 × 20 mm). Spectra were analysed with LCModel to obtain absolute concentrations of the neurochemicals. Differences in functional connectivity between CFS and healthy participants were tested using multivariate exploratory linear optimized decomposition into independent components (MELODIC) and dual regression.

Results:

Concentrations of putamen glutamate and glutamate+glutamine (Glx) were increased in CFS while that of ACC GABA was decreased. Putamen Glx and ACC glutamine were negatively associated with the severity of self-reported fatigue. There were main effects of CFS diagnosis on glutathione (GSH) and total creatine, indicating decreases of these neurometabolites in all the regions studied in CFS patients. In addition, the CFS patients demonstrated elevated functional connectivity between the default mode network and right supracalcarine cortex, precuneus cortex and dorsolateral prefrontal cortex.

Conclusions:

The increased putamen glutamate, decreased ACC GABA and elevated resting state functional connectivity of the default mode network suggest a hyperactive brain status in CFS. The global decrease of GSH and total creatine also suggest that CFS patients may have an abnormal bioenergetic status with higher oxidative stress.

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Tables

Figures

Abbreviations

ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
ADP	Adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
B_0	Static magnetic field
BDI	Beck depression inventory
BOLD	Blood oxygenation level dependent
CBF	Cerebral blood flow
CBT	Cognitive behavioral therapy
CDC	Centers for Disease Control and Prevention
CFQ	Chalder fatigue questionnaire
CFS	Chronic fatigue syndrome
CMRO2	Cerebral metabolic rate of O2 consumption
COMPASS	The composite autonomic symptom scale
CRH	Corticotropin-releasing hormone
CRLB	Cramer-Rao lower bounds
CRP	C-reactive protein
DHEAS	Dehydroepiandrosterone sulfate
DLPFC	Dorsolateral prefrontal cortex
DMN	Default mode network
DSM	Diagnostic and statistical manual
fMRI	Functional magnetic resonance imaging
FSL	The FMRIB Software Library
GABA	γ-Aminobutyric acid
GET	Graded exercise therapy
Glx	Glutamate + Glutamine
GSH	Glutathione
HAM-D	Hamilton Depression Rating Scale
HPA axis	Hypothalamic-pituitary-adrenal axis
ICA	Independent component analysis
IFN-alpha	Interferon-alpha
LCModel	Linear Combination of Model Spectra

MELODIC	Multivariate Exploratory Linear Optimized Decomposition into Independent Components
MRS	Magnetic resonance spectroscopy
NAA	N-Acetylaspartate
NMDA	N-Methyl-D-Aspartate
OCC	Occipital cortex
PET	Positron emission tomography
RF pulse	Radiofrequency pulse
SEM	Standard error of mean
SNR	Signal-to-noise ratio
STEAM	Stimulated Echo Acquisition Mode
TCA	Tricarboxylic acid
tCho	Total choline
tCr	Total creatine
TFCE	Threshold-free cluster enhancement
TGF-beta	Transforming growth factor-beta

Chapter 1: Introduction

Chronic fatigue syndrome (CFS) is a disabling syndrome characterized by severe prolonged fatigue that cannot be explained by established medical conditions. In addition to fatigue, patients also experience a wide range of symptoms, including muscle pain, multiple joint pain, headache, sore throat, impaired attention, impaired memory, poor sleeping quality, anxiety and depression (Afari et al., 2003). According to different diagnosis criteria and ways of assessment, the prevalence reported by previous studies has a wide range from 0.2% to 6.41% (Nacul et al., 2011; Yiu et al., 2005). A meta-analysis (Johnston et al., 2013) that included 14 different epidemiological studies in CFS concluded that the pooled prevalence was 0.76% (95% CI: 0.23-1.29) when the diagnosis is based on clinical assessment. If the diagnosis is based on self-reported assessment, the pooled prevalence was 3.28% (95% CI: 2.24-4.33)

Currently, there is no unifying explanation for the underlying aetiology of CFS. Despite this, studies have found evidences of alterations in different organ systems, including the immune, neural, gastrointestinal and endocrine system (Blundell et al., 2015; Frémont et al., 2013; Milrad et al., 2017; Papadopoulos et al., 2012; Wyller et

al., 2009). In addition, a twin study showed that chronic fatigue syndrome–like illness had 55% and 19% concordance rates in monozygotic and dizygotic twins, respectively (Buchwald et al., 2001). Taken these findings together, CFS is thought to be a condition with complex pathophysiology, involving both hereditary and environmental factors.

Evidence from proton magnetic resonance spectroscopy (MRS) studies in CFS have shown several changes in the level of neurometabolites, such as choline, N-acetyl aspartate and lactate, in the central nervous system (Brooks et al., 2000; Chaudhuri et al., 2004; Mathew et al., 2009). However, detailed understanding about the neurochemical changes, especially the glutamatergic/GABAergic neurotransmitters, is still scant. In addition, some of the brain regions that are related to the symptoms of CFS, such as the basal ganglia, are not yet fully studied. The availability of imaging scanners with higher magnetic field (7 Tesla in this study) can enable clearly separated measurements of glutamate/glutamine/gamma-aminobutyric acid (GABA) levels and the detection of signal from subcortical region, such as putamen.

In this chapter, overviews of CFS, and the techniques used in this study, including MRS and resting-state functional magnetic resonance imaging (fMRI), will be carried

out. In addition, the findings of MRS and resting-state fMRI in CFS will be reviewed.

1.1 An Overview of Chronic Fatigue Syndrome

1.1.1 Diagnosis of Chronic Fatigue Syndrome

The current diagnosis of CFS is solely based on operational diagnostic criteria; there are no reliable biomarkers of the disorder. CFS has a variety of symptoms, including prolonged self-reported fatigue, post-exertional malaise, impaired memory, unrefreshing sleep, sore throat, tender cervical or axillary lymph nodes and muscle or joint pain (Collin et al., 2016). As for clinical signs, although some studies demonstrated dysregulated hypothalamic-pituitary-adrenal axis, heart rate variability and blood pressure variability in patients with CFS, no such alterations seem to be adequate for a diagnostic test due to the lack of consistency (Burton et al., 2010; Papadopoulos et al., 2012; Wyller et al., 2011). As a result, clinicians and investigators routinely utilize operational diagnostic criteria to identify patients with CFS.

There are several different case definitions for ‘chronic fatigue syndrome’. The first case definition was proposed by Holmes et al. in 1988 (Holmes et al., 1988). Since

then, various revised versions of case definitions have been introduced by different study groups. These include the CDC-1994 criteria (Fukuda et al., 1994) and the Oxford criteria (Sharpe et al., 1991), which are two of the most accepted and cited case definitions.

The difference between the CDC-1994 criteria and the Oxford criteria is mainly attributable to the importance of the accompanying symptoms. Both of the criteria define chronic fatigue as medically unexplained and persistent fatigue lasting more than 6 months. In the CDC-1994 criteria (Fukuda et al., 1994), patients with chronic fatigue must also meet at least four of the eight listed accompanying symptoms. In contrast, the Oxford criteria only lists several common accompanying symptoms without defining them as sufficient conditions for the diagnosis of CFS (Sharpe et al., 1991). As a result, patients with chronic fatigue but without sufficient numbers of accompanying symptoms will be diagnosed with CFS under the Oxford criteria while the CDC-1994 criteria will exclude them.

A meta-analysis suggested that the Oxford criteria are more inclusive than the CDC-1994 criteria, with the population prevalence being around 1.5% (range 0.4-3.7%; median 1.5%) and 1% (range 0.1-6.4%; median 1%) according to the Oxford criteria

and the CDC-1994 criteria, respectively (Brurberg et al., 2014). The inclusiveness of the Oxford criteria seems not to compromise the representativeness of CFS significantly. According to a clinical study which includes participants based on the Oxford criteria, nearly a third of the participants did not meet the CDC-1994 criteria (White et al., 2011). Nevertheless, treatment effectiveness is similar across participants' groups defined by different diagnostic criteria. This indirectly assures the reliability of the Oxford criteria.

1.1.2 Epidemiology of Chronic Fatigue Syndrome

As mentioned above, the prevalence of CFS reported by previous studies was strongly affected by the diagnostic criteria and the assessment approach adopted by the researchers.

The incidence of CFS was investigated by a research team in UK using the Oxford criteria for CFS (Lawrie et al., 1997). This study firstly randomly mailed 1 in every 10 people registered in local healthcare centre (N=1039). It then surveyed the 695 subjects who responded to the postal survey one year later and resurveyed them after another 18-22 months. During the survey and interview, demographic data, the existence of fatigue or chronic fatigue and other general health problem were

obtained. Results showed that the annual incidence of CFS was 370/100,000 (95% CI 40–1,330), with prevalence of 740/100,000 (200–1890). Although an epidemiological study with relatively small number of subjects, this survey has been one of the few to provide an estimate of the annual incidence of CFS in a sample of the UK population (Ranjith, 2005).

Larger population-based and community-based cross-sectional studies have identified epidemiological associations between CFS and other factors. Higher prevalence in women was reported by an UK population-based study that included 15,283 people (Pawlikowska et al., 1994). This studied showed that, compared to men, the relative risk of fatigue was 1.3 in women. Another community-based study in Chicago that included 18,675 people also showed higher prevalence in women compared to men, with rates of 522/100,000 in women and 291/100,000 in men (Jason et al., 1999). This study also found that middle-to-low socioeconomic status was associated with higher prevalence. This finding is similar to two surveys of fatigue in the UK and France, which also demonstrated that people with lower socioeconomic status have increased levels of fatigue (Cox et al., 1987; Fuhrer et al., 1995). A possible explanation for the socioeconomic differences in fatigue and CFS could be that people with lower socioeconomic status have more social adversity, which is itself a predictor of fatigue

(Ranjith, 2005).

Higher prevalence of psychiatric co-morbidities compared to the general population is also an important issue in CFS. Farmer et al. (1995) conducted a structured clinical assessment on 100 patients with CFS diagnosed by the CDC-1994 criteria and 50 gender and age-matched healthy controls in a general medical clinic. The results showed that, among the 100 CFS patients, 27 met the diagnosis of depressive disorder and 22 met the diagnostic criteria of anxiety disorder according to the DSM-III-R. These proportions were significantly higher in comparison to the healthy controls. Moreover, another study found that CFS patients diagnosed by stricter diagnostic criteria had a higher rate of psychiatric comorbidity than a CFS population diagnosed by looser diagnostic criteria, which required fewer symptoms (Skapinakis et al., 2003).

Another important issue is the socioeconomic impact of CFS. A study that included 535 Australian patients diagnosed with CFS found that only 12% and 28% of the patients had full-time and part-time jobs, respectively (Johnston et al., 2013). Over half of the patients were either unemployed (27%) or on disability pension (34%). Furthermore, the mean age of all patients was 46.4 years. These figures emphasis the

considerable socioeconomic impact caused by CFS.

1.1.3 Current Treatment for Chronic Fatigue Syndrome

Currently, there are two evidence-based treatments for CFS: cognitive behavioural therapy (CBT) and graded exercise treatment (Larun et al., 2016; Price et al., 2008). CBT for CFS is a psychological therapy based on the model of three perpetuating disease factors. These include disproportionate focusing on bodily symptoms, low physical activity and low sense of control (Prins et al., 2001). During CBT sessions, therapists explain these perpetuating factors to the patients and challenge patients' fatigue-related cognitions in order to encourage behavioural changes, such as increasing physical activity and rehabilitation for work (Scheeres et al., 2008). Several studies have demonstrated that CBT reduces fatigue more effectively than usual care. This effect is mediated by changing illness-related cognition among patients with CFS (Price et al., 2008; Wiborg et al., 2010).

Graded exercise treatment (GET) aims to improve CFS symptoms through regular exercise which gradually increases in both duration and intensity (Larun et al., 2016; Moss-Morris et al., 2005). Studies have found that the improvement in fatigue severity is mediated by reducing patients' focus on symptoms, but not by increasing

patient's physical fitness (Moss-Morris et al., 2005). In contrast to CBT, GET does not challenge patients' illness-related cognition directly. Instead, it seeks to convince and assure the patients that they are able to increase their activity without being harmed or exacerbating the symptoms.

CBT and GET have similar effectiveness in terms of decreasing fatigue levels with a response rate around 30%. In contrast, the response rate of usual care is around 15% (White et al., 2011). Although the effectiveness is proven and serious adverse reactions are uncommon in both CBT and GET, there is still a substantial room for improvement in the efficacy and response rate of current CFS treatment. Besides these current treatments, other therapeutic targets, including immune modulation and nutritional supplements, are under extensive study (Castro-Marrero et al., 2017).

1.1.4 Findings from MRS Studies in Chronic Fatigue Syndrome

Proton MRS enables the non-invasive assessment of the concentration of brain neurometabolites *in vivo* (MRS will be discussed in detailed in section 1.2). This method has been utilized in several studies in CFS. The categories of neurometabolites that can be detected by MRS are largely depend on the pulse sequences and strength of static magnetic field provided by the scanner. Previous

proton MRS studies in CFS will be reviewed in this section.

There are 3 studies using scanners with static magnetic field of 1.5 Tesla which will be reviewed here. Brooks et al. (2000) investigated the concentrations of N-acetylaspartate (NAA), creatine and choline in right hippocampus of 7 patients with CFS and 10 healthy controls. This study found that NAA, which is a marker of neuronal/glial metabolism, was decreased in patients, without a significant difference in hippocampal volume. It is notable that the patient sample in this study was composed mostly of men (5/2, men/women) while epidemiological studies showed that the prevalence of CFS in women is higher than that in men. There might therefore be a lack of representativeness of the general CFS population; in addition, the sample size was small.

Chaudhuri et al. (2003) and Puri et al. (2002) conducted proton MRS in left basal ganglia and occipital cortex, respectively. Both of the groups studied 8 patients using relative quantification by referencing the signal to creatine. This quantification method is based on the assumption of an equivalent brain creatine level between patients and controls. Increases in choline-to-creatine ratio in patients with CFS were reported by both groups. Since choline is a marker for lipid metabolism, Chaudhuri et

al. (2004) suggested that there might be an elevated membrane turnover rate, which could be induced by inflammatory cytokines or viral infection.

There are 6 studies using scanners with static magnetic field of 3 Tesla reviewed here. Puri et al. (2009) measured the concentration of an antioxidant, glutathione, in the non-dominant cerebral cortex in 26 subjects, finding no significant difference in glutathione level between patients and healthy controls. This study did not specify the exact location of the 20 x 20 x 20 mm^3 voxel used in this study in the non-dominant cerebral cortex. This study also did not specify how many subjects were patients, which makes the quality of the results unreliable.

A recent study investigated the gray matter volume and NAA/creatine ratio in left dorsolateral prefrontal cortex in 89 women with CFS and 26 age, gender and education-matched healthy controls (van der Schaaf et al., 2017). Although there were no differences in the value of gray matter volume or NAA/creatine ratio, this study demonstrated that gray matter volume or NAA/creatine ratio were both negatively associated with the severity of pain, which is a common symptom (87%) in patients with CFS (Collin et al., 2016). This study showed that structural and neurochemical changes in brain could be associated with certain symptoms of CFS.

A series of studies conducted by one research group have consistently demonstrated elevated lactate level in the ventricular cerebrospinal fluid from different CFS patient cohorts compared to healthy controls (Mathew et al., 2009; Murrough et al., 2010; Natelson et al., 2017; Shungu et al., 2012). In the first study, increased lactate in ventricular cerebrospinal fluid level in CFS was firstly reported, with no correlation with the severity of fatigue (Mathew et al., 2009). In a subsequent study, a MEGA-PRESS pulse sequence, which is able to detect GABA level and the combined concentration of glutamate + glutamine (Glx), was employed (Murrough et al., 2010). This study, as well as measuring ventricular lactate, also measured the concentrations of GABA and Glx in the anterior cingulate cortex (ACC) and occipital cortex (OCC). A negative correlation between ACC GABA concentration and reduced activity was found in patients with CFS, but no difference between the concentrations of all neurometabolites in all regions studied was found, except for ventricular lactate.

In the third and fourth studies, a pulse sequence with J-edited spin echo difference technique was used in both of the study to detect glutathione (GSH) levels in the OCC (Natelson et al., 2017; Shungu et al., 2012). In the third study, OCC GSH level was decreased in CFS. This study included not only 15 patients with CFS and 13 healthy controls, but also 15 patients with major depression disorder who also manifested

lower GSH levels (Shungu et al., 2012). A correlation analysis combining participants from all three groups found that OCC GSH concentration was negatively associated with general fatigue and poor sleeping quality. In the fourth study, 27 CFS patients without psychiatric comorbidity, 16 with psychiatric comorbidity and 17 healthy controls were included into the study (Natelson et al., 2017). OCC GSH levels were significantly decreased in patients with CFS, but there was no significant difference between CFS patients with and without psychiatric comorbidity. In addition, ventricular lactate also showed no difference between CFS patients with and without psychiatric comorbidity. Results from these 4 studies have demonstrated reproducible measurements of elevated ventricular cerebrospinal fluid lactate level and decreased OCC GSH in patients with CFS. These data also indicate the association between the severity of some symptoms and ACC GABA and OCC GSH concentrations.

In summary, in studies using scanners with 1.5 Tesla static magnetic field, neurometabolites measured were focused on NAA, choline and creatine. Increased choline/creatine ratio was found in the left basal ganglia and occipital cortex while NAA concentration was increased in right hippocampus among the patients. Studies conducted with 3 Tesla scanners, with the aid of higher static magnetic field and specialized or modified pulse sequences, investigated neurometabolites including

ventricular lactate and cortical GSH, GABA and Glx. The increased of ventricular lactate and decreased OCC GSH were founded and replicated. It is notable that no previous study has measured glutamate and glutamine level separately due to the limitation of the strength of magnetic field and pulse sequence.

1.1.5 Findings from Resting-state fMRI studies in Chronic Fatigue Syndrome

Resting-state fMRI enables the assessment of the connectivity of large-scale neural circuitries (this will be discussed in detailed in section 1.3). Resting-state fMRI, compared to task-dependent fMRI, measures functional connectivity, that is, the synchronization of neural activities between regions, without requesting the subject to carry out any specific task. In this section, 4 studies measuring resting-state functional connectivity will be reviewed.

Kim et al. (2015) studied the resting-state functional connectivity in 18 women with CFS and 18 gender and age-matched healthy controls. This study chose posterior cingulate cortex as the seed region for a seed-based analysis. The connectivity strength from the posterior cingulate cortex to the dorsal and rostral ACC was

significantly higher in CFS patients. The connectivity strength between the posterior cingulate cortex to the dorsal ACC was also associated with the severity of general fatigue. Global efficiency of the posterior cingulate cortex was also decreased in patients. These results together showed a more active but less efficient neural connectivity in CFS.

Boissoneault et al. (2016) utilized arterial spin labelling fMRI to study seed-based functional connectivity of 17 CFS patients and 17 healthy controls Multiple connectivity between several seeds to different regions showed different strength in CFS patients, including increased functional connectivity from the ACC to posterior cingulate gyrus and decreased functional connectivity from the ACC to right putamen and insula. These results have demonstrated alterations of functional connectivity covering regions involved in neurocognitive and affective-related functions in CFS.

Gay et al. (2016) used network-based analysis of resting-state functional connectivity in 19 patients with CFS and 17 healthy controls. There were five networks analysed in this study, including the default mode network, salience network, sensory motor network and the left and right frontoparietal network, also known as the central executive network. The analysis showed decreased intrinsic functional connectivity in

the left frontoparietal network, which is involved in cognitive functions such as working memory, attention and language. In addition, decreased functional connectivity of posterior cingulate cortex with the salience network in patients with CFS was found.

Wortinger et al. (2016) conducted a study that included adolescent with CFS and 18 age-matched heathy controls. Network-based analysis using three networks, including the default mode network, central executive network and salience network, was utilized to assess the resting-state functional connectivity. This study showed decreased functional connectivity of right posterior insula to the salience network. These results from a young population (mean age = 16 years-old) provide a valuable information of the less-studied adolescent CFS population.

In summary, these resting-state fMRI studies showed noteworthy changes in patients with CFS. The first two seed-based showed multiple foci with altered functional connectivity to other regions. Among these foci, the ACC and posterior cingulate were found to have changes in both studies. In the latter two network-based analyses in adult and adolescent CFS, dysconnectivity in the salience network were found in both studies while alterations in left central executive network were only found in adult

CFS. Taken together, these abnormalities in large-scale neural circuitry in patients with CFS suggest that defective resting-state functional connectivity could be involved in the pathophysiology of CFS.

1.1.6 Other Relevant Findings in Chronic Fatigue Syndrome

There are a variety of neurobiological findings shown in patients with CFS. This include changes in cognitive functions, emotion, hypothalamic-pituitary-adrenal (HPA) axis, immune system, autonomic nervous system and microbiota. Among these aspects, the ACC, which is a region investigated in the present study, is involved in cognitive functions, emotion, HPA axis and autonomic nervous system in the normal condition (Gasquoine, 2013). On the other hand, the immune system and gut microbiota are closely related with brain function, the latter described as the brain-gut axis (Konturek et al., 2016). These findings will be briefly reviewed in this section.

1.1.6.1 Changes in Cognitive Functions

Patients with CFS often report cognitive complaints such as poor word-finding ability, insufficient concentration and reduced memory for recent events (Barrows, 1995). Several studies have made efforts to clarify the cognitive dysfunction in patients with

CFS. A study showed that CFS patients were impaired on tasks of information processing, attention and memory compared to heathy controls (DeLuca et al., 1997). This study also compared the performance of CFS patients with psychiatric comorbidity and patients without psychiatric comorbidity but did not find any significant difference. The conclusion of this study is that the concurrent psychiatric conditions in patients with CFS cannot explain the impairment in cognitive function found in this study.

A review summarizing studies testing neuropsychological functioning in patients with CFS also concluded that the cognitive dysfunction in patients with CFS is prominent and cannot be explained by psychiatric comorbidity (Michiels et al., 2001). Another point made by this review is that the cognitive performance of CFS patients may not be simply related to the severity of subjectively experienced fatigue. These findings not only show the involvement of the brain in the pathophysiology of CFS but also highlight the possibility of integrating considerations of the impaired cognitive function into the treatment and management of patients with CFS (Michiels et al., 2001).

1.1.6.2 Changes in Mood and Emotional Functions

As mentioned in the previous sections, patients with CFS have increased rates of major depressive disorder and anxiety disorders (Farmer et al., 1995; Skapinakis et al., 2003). The cause of these concurrent psychiatric disorders has not been well explained. Interestingly, increased resting-state functional connectivity of the ACC was demonstrated in major depressive disorder (Greicius et al., 2007). Moreover, the OCC GSH level was decreased in depressed patients (Godlewska et al., 2014). These resting-state fMRI studies and MRS studies showed results similar to the results found in patient with CFS (Boissoneault et al., 2016; Kim et al., 2015; Natelson et al., 2017; Shungu et al., 2012), indicating a possible shared pathophysiology of CFS and major depressive disorder.

In addition to mood disorder, altered emotion processing has been found in patients with CFS. In adolescent CFS, patients showed difficulties in the ability to recognize, label, and describe emotions in the 20-item Toronto Alexithymia Scale (Van de Putte et al., 2007). However, the contribution of psychiatric comorbidity cannot be excluded due to the high prevalence of anxiety and depression in the studied population. In adult CFS, a study showed that, compared to healthy controls, patients with CFS scored higher in the Distress Tolerance Scale and the Silencing the Self Scale,

showing higher tendency of self-sacrifice and avoidance of affect (Hambrook et al., 2011). The difference in self-sacrifice subscale in the Silencing the Self Scale remained significant after controlling for differences in age, anxiety, and depression.

1.1.6.3 Hypothalamic-Pituitary-Adrenal Axis Dysfunction

The HPA axis is involved in the regulation of responses to stress through negative feedback loops with a chain of releasing factors and hormones. These include corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), cortisol and dehydroepiandrosterone sulfate (DHEAS) (Papadopoulos et al., 2012). When stimulated by stresses such as inflammation, hypoglycemia and hypotension, the HPA axis is activated with increased secretion of CRH secreted by parvocellular neurons. The release of cortisol is induced by CRH, mediated by ACTH secreted by the anterior pituitary. On the other hand, elevated cortisol level inhibits the secretion of CRH and ACTH, forming negative feedback control loops.

Studies using stimulation tests and suppression tests to investigate the HPA axis in patients with CFS have found a decreased activity of the HPA axis. In the stimulation tests, subjects were stimulated with stressors such as insulin and social stress (Gaab et al., 2004, 2005). In addition, wakening can be also regarded as a stimulus, and there

are also studies using this model to measure the responsiveness of the HPA axis (Heim et al., 2009; Nater et al., 2008; Roberts et al., 2004). Most of these studies demonstrated the response of HPA axis was blunted in patients with CFS.

The responsiveness of the negative feedback to cortisol is also an important regulatory factor in the HPA axis. Hyper-responsiveness to the negative feedback could cause increased suppression of the release of CRH and ACTH, eventually leading to hypo-activity in the HPA axis (Papadopoulos et al., 2012). Three studies using dexamethasone or prednisolone suppression test investigated the difference between patients with CFS and healthy controls in the response to these high potency cortisol analogues. The results showed that the decreases of salivary cortisol of CFS patients were significantly greater than that of healthy controls, indicating a hyper-responsiveness to the negative feedback of cortisol (Jerjes et al., 2007; Papadopoulos et al., 2009; Van Den Eede et al., 2008).

Although the evidences indicate decreased activity of the HPA axis in patients with CFS, treatment with cortisol in CFS is not of clear effectiveness and is not recommended due to the lack of reliable benefit and potential adverse effects (Reid et al., 2011). However, some studies showed that the activity of HPA axis can be a

predictive marker for the response to treatments such as CBT, with, for example, patients responding well to CBT having higher pretreatment urinary free cortisol levels (Jason et al., 2007; Roberts et al., 2009). According to these findings, Papadopoulos et al. (2012) suggest that the alterations in HPA axis could be an epiphenomenon and a consequence of CFS.

1.1.6.4 Immune System Alterations

Patients frequently report an episode of infection or flu-like illness before the onset of CFS, which suggests the possible involvement of the immune system in the pathogenesis of CFS. The immune system response to infection and inflammation is largely regulated by cytokines, which are a group of protein mediators. A recent systematic review included 38 studies that covered the measurements of 77 serum or plasma cytokines in patients with CFS. The results from the included studies are heterogeneous and inconsistent. The only cytokine that showed increased concentrations across more than half of the studies (63%) is the transforming growth factor-beta (TGF-beta, Bennett et al., 1997; Chao et al., 1991; Kennedy et al., 2004; White et al., 2004).

TGF-beta, depending on the context, has not only anti-inflammatory but also pro-

inflammatory effects (Sanjabi et al., 2009). The most studied function of TGF-beta is its role in peripheral tolerance, an immunological process of suppressing self-reactive immune cells in the periphery (Kronenberg et al., 2005). TGF-beta inhibits self-reacting $CD4^+$ and $CD8^+$ T cell from proliferating and differentiating to maintain peripheral tolerance (Li et al., 2006).

TGF-beta can also affect the brain. A study using transgenic mice overexpressing TGF-beta found that, compared to non-transgenic mice, cerebral blood perfusion of mice with overexpressed TGF-beta was significantly diminished (Gaertner et al., 2005). Interestingly, decreased cerebral blood perfusion has been demonstrated in the cerebral cortex, basal ganglia and brain stem in patients with CFS (Costa et al., 1995; Ichise et al., 1992). Whether brain hypoperfusion in CFS is associated with TGF-beta or other cytokines still needs further investigation.

1.1.6.5 Autonomic Nervous System Dysfunction

The autonomic nervous system (ANS) has a critical role in regulating physiologic homeostasis in human body. A clinical rating scale, The Composite Autonomic Symptom Scale (COMPASS), lists some of the important aspects of ANS function (Suarez et al., 1999). There are eight domains evaluated in the COMPASS, including

(i) Orthostatic Intolerance (generalized adrenergic); (ii) Vasomotor (peripheral adrenergic); (iii) Secretomotor (cholinergic); (iv) Gastrointestinal, including Autonomic Diarrhoea and Constipation; (v) Bladder; (vi) Pupil Responses; (vii) Sleep disorder; and (viii) Syncope. A study using the COMPASS assessed 40 patients with CFS and 40 age and gender-matched healthy controls. COMPASS scores were significantly higher in CFS patients, with a positive association between COMPASS score and the severity of fatigue (Newton et al., 2007). Using the mean + 2SD of the scores from healthy controls as the threshold for the diagnosis of ANS dysfunction, 75% of CFS patients had a COMPASS score suggesting dysfunction in ANS.

Other studies investigating heart rate variability during sleep, blood pressure variability and baroreflex in CFS also indicated ANS dysfunction (Burton et al., 2010; Wyller et al., 2011). These results suggest increased sympathetic activity and decreased parasympathetic activity. The ACC, especially the ventral ACC, is involved in the modulation of the ANS (Critchley et al., 2003; Matthews et al., 2004). Currently, there is no study investigating the role of the ACC in ANS dysfunction in patients with CFS. As a result, studies of the relationship between ACC and ANS dysfunction in CFS seem warranted.

1.1.6.7 Microbiota Alteration

There is a growing acceptance of the concept of gut-brain axis, which suggests an impact of gastrointestinal microbiota on brain function (MacQueen et al., 2017). An example of gut-brain axis interaction was demonstrated by the alterations of GABA receptor in mice receiving long-term *Lactobacillus* strain (Bravo et al., 2011). An analysis of the microbiome in CFS showed that the proportions of *Lactonifactor* and *Alistipes* were increased while the proportions of several Firmicutes populations were decreased in CFS patients (Frémont et al., 2013). Treatments targeting the microbiota, including probiotics and faecal microbiota transplantation in CFS were carried out by several groups (Borody et al., 2012; Rao et al., 2009; Sullivan et al., 2009). A randomized, double-blind, placebo-controlled trial using *Lactobacillus casei* strain Shirota as probiotic treatment showed decrease in anxiety symptoms in CFS patients (Rao et al., 2009). However, the actual effect of treatment targeting gut microbiome still need more clinical trials and evidences.

1.1.6.8 Summary of Findings in Chronic Fatigue Syndrome

In summary, changes in patients with CFS have been found in multiple biological systems, including cognitive function, emotion, HPA axis, immune system, autonomic

nervous system and microbiota. Interestingly, some of the altered function could be related to changes in the activity of the ACC, suggesting a potential role of the ACC in the pathophysiology of CFS.

1.1.7 Conclusion

In this section, the diagnosis, epidemiology and current treatments of CFS were briefly summarized. The devastating impacts on patients in terms of functional disability and relatively low response rate to current treatments raise the importance of improvements in the management of CFS. Findings reported by studies using MRS and resting-state fMRI were described, showing that the brain is likely to be involved in pathophysiology. In addition, findings from other relevant studies in CFS were also described. Altogether, these findings have shown the complexities and uncertainties in the current understanding in CFS, which requires further investigations.

1.2 Magnetic Resonance Spectroscopy

Magnetic Resonance Spectroscopy (MRS) is a technique enabling non-invasive measurements of neurometabolite concentrations in defined regions of the brain *in vivo*. This technique utilizes the physical property of the interaction between the electromagnetic radiation and matter under an external magnetic field. The resulting

magnetic resonance spectrum from the brain is mainly composed by signal of several neurometabolites including N-acetylaspartate (NAA), choline and creatine. Furthermore, neurotransmitters including glutamate and GABA can also be detected by MRS. Using this quantitative technique, researchers can investigate the levels of different neurometabolites, allowing the comparison between physiologic and pathologic conditions among different individuals and populations.

In this section, the basic principles of MRS will be briefly summarized, followed by discussion on the advantages of using high static magnetic field in MRS studies. The functions of several neurometabolites studied will also be described.

1.2.1 Basic Principles of MRS

Under the static magnetic field (abbreviated as B_0) generated by the superconducting electromagnets of the scanning devices, odd mass nuclei having half-integer spin quantum number, such as ^{1}H, ^{13}C and ^{31}P, will align to the B_0 in an either parallel or antiparallel fashion. The nuclei will also precess along the direction of B_0. This characteristic-spinning phenomenon is named as magnetic resonance. The angular frequency of the precession can be described by the Larmor equation as follows:

$$\omega_0 = \Upsilon B_0$$

where ω_0 is the precession angular frequency of nucleus, Υ the nucleus-specific gyromagnetic ratio (42.58 MHz/Tesla for ^1H) and B_0 the static magnetic field.

To detect the magnetic resonance signal, a second external magnetic field, the radiofrequency (RF) pulse, is introduced. The RF pulse can tip the spins off from the longitudinal direction, enabling the receiver coil to detect the current produced by electromagnetic induction. In addition, the RF pulse will only excite nuclei precessing with a similar frequency. This property makes localized excitation possible by generating additional gradient magnetic fields in three orthogonal directions (x, y and z). According to the Larmor equation, nuclei will hence have region-specific precession frequencies under the 3-dimensional magnetic field gradients. Localized excitation is therefore accomplished by matching the frequency of RF pulse and regional nucleus precession.

Within the molecular scope, nuclei also experience magnetic effects produced by shielding electrons in addition to the B_0. The electron shield can reduce the effective magnetic field that nucleus perceived, which can be represented as

$$B_{eff} = (1-\sigma)\, B_0$$

where B_{eff} is the effective magnetic field and σ is the chemical shielding constant.

Therefore, the Larmor equation can be represented with the consideration of chemical shielding effect as

$$\omega_0 = \Upsilon (1-\sigma) B_0$$

As the nuclei located in different molecules that have different chemical shielding constants, cause different precession frequencies, the signal detected can be Fourier transformed into a spectrum with the frequency as x-axis and the strength of signal as y-axis. The concentrations of different chemicals can therefore be quantified according to the frequency and strength of signal in the spectrum.

1.2.2 Advantages of High Field MRS

The strength of static magnetic field B_0 can influence the signal-to-noise (SNR) ratio and spectral dispersion in MRS (Lemke, 2015). The overall SNR is proportional to B_0 and the spectral dispersion increases when B_0 increases. These enable the separation of overlapping peaks under higher B_0. For example, compared to MRS with B_0 at 3 Tesla, MRS with B_0 at 7 Tesla has increased spectral resolution and approximately two-fold higher SNR (Mekle et al., 2009). Additional neurometabolites, such as glutamate and glutamine, can therefore be resolved and quantified separately.

There are also challenges in high B_0 MRS, including energy deposition and inhomogeneity of RF pulse and B_0 (Lemke, 2015). For energy deposition, careful

calculations of specific absorption rate (SAR) of tissue are needed in order to avoid increasing regional temperature in the tissue to an unacceptable degree. As for the inhomogeneity of RF pulse, equipment such as multiple transmit array coils and high dielectric materials can increase the homogeneity (Adriany et al., 2005; Haines et al., 2010). For small studied volumes, first and second-order shim system can sufficiently improve homogeneity of B_0 while larger studied volumes need further considerations (Tkáč et al., 2009).

1.2.3 Neurometabolites Measured by MRS

1.2.3.1 Glutamate

Glutamate is the major excitatory neurotransmitter and most abundant free amino acid in the central nervous system (Rae, 2014). There are four major subtypes of glutamate receptor, including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic (AMPA), Kainate and metabotropic (mGlu) receptors. The first three are ionotropic receptors while mGlu receptors act through second messengers. Glutamate is intensely studied in the pathophysiology of many psychiatric disorders (Godlewska et al., 2017; Hasler et al., 2007), yet the role of glutamate in CFS has not been extensively researched. Glutamate also has key roles in cellular metabolism

which can make distinguishing its neurotransmitter role with MRS challenging.

1.2.3.2 Glutamine

After glutamate is released by neurons, most of the glutamate is taken up by astrocytes and converted to glutamine in a reaction catalysed by glutamine synthetase (Rae, 2014). Glutamine is then transported back to glutamatergic neurons and can be converted to glutamate via a phosphate activated enzyme, glutaminase. Glutamine thus serves as a precursor and metabolite of glutamate. Glutamine also provides the carbon backbone for the synthesis of GABA (Peng et al., 1993).

1.2.3.3 Gamma-aminobutyric acid (GABA)

GABA is the major inhibitory neurotransmitter in the central nervous system, with about 20% of synapses expressing it (Douglas et al., 2007). In addition to its neurotransmitter role, GABA is also involved in cerebral metabolism (Jones, 1993). GABA is mainly synthesized in the GABAergic neuron in a reaction catalysed by glutamate decarboxylase. After the release of GABA into synapse, in contrast to glutamate, most of the GABA is reuptake by the GABAergic neuron while a small proportion of GABA is catabolised after entering astrocyte (Hertz et al., 2016).

1.2.3.4 Glutathione

Glutathione (GSH) is a tripeptide which plays a major role in reductive processes as an antioxidant. The level of GSH in brain is relatively high compared to the other parts of the body (Rae, 2014). GSH is synthesized by a two-step pathway. Firstly, gamma-glutamylcysteine synthetase converts glutamate and cysteine together into gamma-glutamylcysteine. Next, gamma-glutamylcysteine is combined with glycine via glutathione synthetase (Meister et al., 1983). These processes can be carried out in neurons or astrocytes, which both have active metabolism and high oxidative stress (Rae, 2014).

1.2.3.5 Creatine and Phosphocreatine

Creatine and phosphocreatine create a buffer system for the storage of high-energy phosphate bonds from ATP:

$$\text{Creatine} + \text{ATP} \leftrightharpoons \text{Phosphocreatine} + \text{ADP} + H^{+}$$

Compared with ATP, phosphocreatine is less negatively-charged and has higher diffusibility, making it favourable for the storage and distribution of cellular energy (Walliman et al., 1992; Yoshizaki et al., 1990). As a result, total creatine (the combination level of creatine and phosphocreatine) can be regarded as a marker for

cellular bioenergetics. Creatine can be synthesized by brain, liver and pancreas or derived from diet. In addition, creatine oral supplement can increase the creatine level significantly and reduces mental fatigue among the healthy population (Dechent et al., 1999; Watanabe et al., 2002).

1.2.3.6 N-acetylaspartate

N-acetylaspartate is one of the free amino acid with high concentrations in brain, contributing the most prominent peak in the 1H MRS-acquired spectra (Rae, 2014). The definite function of NAA currently remains uncertain. NAA is regarded as an osmolyte, contributing around 7% of neuronal osmolarity (Baslow, 2000). NAA could also be a marker for neuronal viability, with white matter diseases including hypoxic encephalopathy and multiple sclerosis showing decreased NAA levels (Rosen et al., 2007).

1.3 Resting-State Functional Magnetic Resonance Imaging

Detecting the blood oxygenation level dependent (BOLD) signal by functional magnetic resonance imaging (fMRI) enables the evaluations of real time human brain neural activity without any invasive procedure. Several aspects of brain function, such

as self-referencing, retrieving personal memory and planning one's future, are associated with the brain activity in the resting state. In this section, the concepts of fMRI and resting-state functional connectivity will be summarized. The three networks model for large-scale neural circuitries, which are related to resting-state functional connectivity, will also be introduced.

1.3.1 Basic Concepts of fMRI

fMRI can measure brain neural activity by detecting the changes in BOLD signal. When neurons fire, the cerebral metabolic rate of O_2 consumption ($CMRO_2$) increases by 5-25% (Chiew, 2017; Logothetis et al., 2004). The activation of neurons is accompanied by neuro-vascular coupling, which results in increased cerebral blood flow (CBF, 20-70%) in activated regions. Additional oxygen provided by the increased CBF is more than the increase of CMRO, resulting a net elevation in the proportion of oxygenated haemoglobin in the regional vein. The increase of oxygenated/de-oxygenated haemoglobin ratio further changes the magnetic susceptibility of blood and eventually leads to a decrease in B_0 inhomogeneity. The decay of signal caused by B_0 inhomogeneity will be reduced and finally the increased signal can be detected by fMRI. Researchers can hence measure the brain neural activity (Chiew, 2017).

1.3.2 Basic Concepts of Resting-State Functional Connectivity

With the recent advances in the measurement and analyses of functional interactions between brain regions, there is an increasing number of studies of functional connectivity, which assesses the temporal dependence of neural activity, in human brain (Van Den Heuvel et al., 2010). Several networks, which are composed of functionally connected regions, were identified and will be discussed in section 1.3.3 (Menon, 2011). Interestingly, some of the networks showed elevated activity during the resting state, which is a period when subjects rest quietly in the camera and are not requested to perform any specific task. These resting-state activities are related to several cognitive and emotional functions of brain (Kaiser et al., 2015; Waites et al., 2005). Furthermore, aberrant resting-state connectivity have been found in several psychiatric disorders, such as schizophrenia, major depressive order and bipolar disorder (Mamah et al., 2013; Sheline et al., 2010).

1.3.3 Triple Network Model

Menon proposed a triple network model suggesting that there is a dynamic interaction between three brain networks, including the default mode network, central executive network and salience network (Menon, 2011). The activities of networks can be

regulated by extrinsic stimuli or activated or inhibited by the activities of other networks. In this section, the three networks mentioned in this model and their interactions will be briefly summarized.

1.3.3.1 Default Mode Network

The default mode network is the first identified resting-state network (Greicius et al., 2003). The posterior cingulate cortex, medial prefrontal cortex (including ACC), medial temporal lobe and angular gyrus comprise the default mode network (Menon, 2011). These regions are involved in different aspects of self-referential mental processes, such as autobiographical memory retrieval, self-related processes and emotion regulation (Dastjerdi et al., 2011; Etkin et al., 2011; Spreng et al., 2009). Abnormal functional connectivity in the default mode network has been reported in many psychiatric and neurological disorders, such as schizophrenia, depression, autism, anxiety, epilepsy and dementia (Menon, 2011).

1.3.3.2 Central Executive Network

The central executive network is composed of the dorsolateral prefrontal cortex and lateral posterior parietal cortex. In contrast to the default mode network, the central

executive network is activated during tasks with cognitive demands, such as working memory, goal-directed decision making and rule-problem solving (Koechlin et al., 2007; Müller et al., 2006). Although studies have found abnormal changes in psychiatric disorders in regions within the central executive network, relatively few studies have investigated overall activity in the central executive network itself (Manoliu et al., 2013; Menon, 2011).

1.3.3.3 Salience Network

The salience network is anchored in the dorsal ACC and frontoinsular cortex, with some overlapping regions with the central executive network at the anterior insula, ACC and dorsolateral prefrontal cortex (Seeley et al., 2007). Two subcortical regions, the amygdala and substantia nigra, are also parts of the salience network. The salience network is involved in detecting and integrating interoceptive, autonomic and emotional information (Seeley et al., 2007). In anxiety, hyperactivity of the salience network, especially the anterior insula, has been consistently reported (Paulus et al., 2006; Stein et al., 2007). The salience network also plays a regulatory role between the default mode network and central executive network.

1.3.3.4 Interactions between Networks

The salience network, especially the anterior insula, is suggested to be the integral hub for the network dynamics in the triple network model. For example, after detecting error signals, the salience network could initiate network switching from the default mode network to the central executive network to enhance attention. An overreacting salience network could be associated with anxiety while an underactive salience network could be associated with autism (Uddin et al., 2009). Another suggestion made by the triple network model is that impairment in one network can impact other two networks. In depressed patients, rumination is associated with the hyperactivity in the default mode network with excessive accompanied activity of the salience network (Berman et al., 2011). The triple network model described in this section provides a framework for the activities found within and between networks. With this framework, some changes found in psychiatric disorders can be explained and interpreted by networks dynamics.

1.4 Aims of the Study

Chronic fatigue syndrome is a complex condition with abnormalities in neural, immune and endocrine systems. Effective treatments and managements for CFS can

be developed more effectively with the aid of a more comprehensive understanding of its pathophysiology. Multiple lines of evidence from both CFS and non-CFS studies indicate that changes of neurometabolites levels and resting-state functional connectivity could be involved in CFS. However, due to the limitations of MRS in lower static magnetic field, the separated measurements of glutamate and glutamine have not thus far been conducted in CFS patients. Furthermore, there is no study that integrates the neurometabolites levels measured by MRS and resting-state functional connectivity measured by fMRI together to explore the interactions between the neurochemical and functional changes in CFS.

The first aim of this pilot study is to investigate the neurometabolites, especially glutamatergic/GABAergic neurotransmitters, in the ACC, putamen and OCC. The second aim of this study is to measure resting-state functional connectivity and analyse the associations between the functional connectivity, CFS and changes in neurometabolites levels.

In this thesis, the methods used in this study, including MRS at 7 Tesla and resting-state fMRI, will be described in chapter 2. In chapter 3, the results from 12 CFS patients and 25 healthy controls will be demonstrated. In chapters 4 and 5, a

discussion and conclusion will focus on the neurochemical and functional changes found in this study, followed by suggestions for future investigations.

Chapter 2: Methods

2.1 Study Approvals

All experimental procedures in this study were approved by National Research Ethics Service Committee (NRES), South-Central – Oxford A and performed according to the Declaration of Helsinki. All of the participants were presented with the Patient Information Leaflet describing this study and gave full-informed, written consent.

2.2 Study Eligibility

CFS patients who met Oxford Criteria for CFS (Sharpe et al., 1991) aged between 18 to 60 years were recruited through a specialist clinic at the John Radcliffe Hospital and advertisements in local newspapers and a local CFS support group. Healthy participants with no previous or current psychiatric disorder on DSM-5 were selected to match enrolled participants with CFS in terms of age and gender.

Before enrolment, all participants were interviewed and screened with Structured Clinical Interview for DSM-5 Disorders (First et al., 2015). The clinical course of CFS symptomatology among patients was also obtained during the interview. Patients who met the diagnosis of schizophrenia, bipolar disorder, substance dependence or eating disorder and healthy participants who met any diagnosis of mental disorder defined by DSM-5 were excluded. Healthy participants who took medication or supplements that could alter, MRS neurochemicals, such as benzodiazepines, were also excluded.

Exclusion criteria for all participants included contraindications to MR imaging, history of problematic claustrophobia, pregnancy, breast feeding or participating in other research project within a month prior to inclusion.

2.3 Participants

12 patients (6 female) with CFS and 12 healthy (6 female) controls were included in this study. In addition, an MRS dataset of 13 healthy (all female) control participants of a previous study was also included. Demographic data, including gender, age, weight and height, family medical history, smoking habits, alcohol consumption and current medications were recorded at the screening visit.

On the scan day, the Chalder Fatigue Questionnaire (CFQ, Chalder et al., 1993) was completed to assess the severity of fatigue. The Hamilton Depression Rating Scale (HAM-D, M. Hamilton, 1960), Beck Depression Inventory (BDI, Beck et al., 1961), and the state measure of the State-Trait Anxiety Inventory (STAI, Spielberger, 1983) were also completed to assess the level of depression and anxiety of all participants, respectively.

2.4 Magnetic Resonance Imaging

All scans were performed on a 7T Siemens MAGNETOM scanner (Siemens, Erlangen, Germany) with a Nova Medical 32 channel receive array head coil at the Functional Magnetic Resonance Imaging of Brain (FMRIB) Centre in Oxford. There were three parts in the scan session, the structural MRI, MRS and resting-state fMRI.

Structural 3-dimensional T1-weighted MRI data was obtained with the magnetisation prepared rapid gradient-echo (MP-RAGE) sequence (Brant-Zawadzki et al., 1992) with slice thickness = 1 mm, FOV=192×192 mm^2, TR = 2.2 s, TE = 2.82 ms, TA = 3 min, non-selective inversion recovery pulse.

2.5 Magnetic Resonance Spectroscopy

2.5.1 Data Acquisition

Three voxels of interest were placed manually in the pre-genual ACC (20x20x20 mm), the right putamen (10x16x20 mm, oblique voxel) and occipital cortex close to the occipital pole (20x20x20 mm). Gradient-echo shimming was firstly used to adjust first- and second-order shims (Shah et al., 2009) followed by FASTMAP (Gruetter et al., 2000) for fine adjustments of first order shims.

Localised spectra were acquired using a stimulated echo acquisition mode (STEAM) pulse sequence (Frahm et al., 1987), TE=11 ms, TR = 5 s, NT = 64, with water suppression and outer volume saturation by variable power radiofrequency pulses with optimized relaxation delays (VAPOR, Emir et al., 2012). To conduct the correction of residual eddy current effects and the reconstruction of phased array spectra, unsuppressed water spectra were also acquired from the exact voxel.

2.5.2 Preprocessing and Quantifications

The quantification of metabolites was performed with LCModel (Provencher, 2001).

Based on previously reported coupling constants and chemical shifts (Govindaraju et al., 2000; Tkac, 2008), the model spectra of aspartate (Asp), ascorbate/vitamin C (Asc) , glycerophosphocholine (GPC), phosphocholine (PC), creatine (Cr), phosphocreatine (PCr), γ-amino-butyric acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glutathione (GSH), myo-inositol (myo-Ins) , N-acetylaspartate (NAA) , N-acetylaspartylglutamate (NAAG), phosphoethanolamine (PE), scyllo-inositol (scyllo-Ins) and taurine (Tau) were created by using GAMMA/PyGAMMA simulation library of VErsatile Simulation, Pulses and Analysis (VESPA, Soher, Semanchuk, Todd, Steinberg, & Young, 2011) for implementing the density matrix formalism. The RF pulses and sequence timings in this study were employed to perform the simulations. The model spectra also included a macromolecule spectrum acquired from the OCC, using an inversion recovery sequence (TR = 3 s, TE = 11 ms, inversion time TI = 0.685 s). The absolute concentrations of metabolites were calculated by taking unsuppressed water spectra acquired from the same voxel as references under the assumptions that 82% of the ACC and the OCC is water content and 78% for the putamen (Gelman et al., 2001).

To correct the concentrations of metabolite according to the cerebrospinal fluid (CSF) fraction, the T1-weighted structural images were segmented by FAST (FMRIB's Automated Segmentation Tool, part of the FSL, FMRIB Software Library), to determine the fractions of gray matter, white matter and CSF (Zhang et al., 2001). The corrections of metabolite concentrations were then performed with the following formula:

$$[Mcorr] = [M] \times (1/[1 - fCSF])$$

where [Mcorr] = corrected concentration and [M] = metabolite concentration from LCModel output.

Quantified metabolites with Cramer-Rao lower bounds (CRLB, the lowest possible standard deviations of unbiased parameter estimates) >35% were categorised as not detected. As a secondary filter to select reliable metabolite concentrations, only metabolites quantified with CRLB ≤35% in at least half of the spectra from a brain region were reported.

2.6 Resting-state Functional Magnetic Resonance Imaging

2.6.1 Data acquisition

The resting-state fMRIs were performed in the same session, after the MRS acquisition. A gradient echo - echo planar imaging (GE-EPI) multiband sequence provided by the University of Minnesota's Center for Magnetic Resonance Research (CMRR, Moeller et al., 2010) was used for data acquisition with 245 whole-brain volumes, slice thickness = 1.5 mm, FOV=192×192 mm^2, TR = 1472 ms, TE = 25 ms, TA = 6.5 min, multiband acceleration factor = 4, in-plane phase encoding acceleration factor (iPAT factor in Siemens terminology) = 2 and generalised autocalibration partial parallel imaging (GRAPPA) reconstruction (Griswold et al., 2002). A static field map was acquired after the GE-EPI sequences to correct the distortion caused by static field inhomogeneity. During the scan, the participants were asked to keep their eyes closed and to try not to fall asleep.

2.6.2 Preprocessing

The preprocessing of data employed tools from FMRIB Software Library (FSL Version 5.0) package. First, motion correction, brain extraction and spatial smoothing with a 3-mm full-width-half-maximum Gaussian kernel and high-pass temporal filter with a 100-second cutoff were performed on all individual data (Jenkinson et al., 2002; Smith, 2002). Each 4-dimensional dataset was then spatially aligned to the Montreal Neurological Institute avg152 (MNI152) T1 weighted 2-mm isotropic template through 12-parameter affine linear and nonlinear registration. Single subject independent component analysis (ICA) was carried out with automatically determined dimensionality using the Multivariate Exploratory Linear Optimized Decomposition into Independent Components software (MELODIC Version 3.15, part of FSL, Beckmann & Smith, 2004)**.** The components abstracted from the ICA were manually classified into signal and noise, such as physiological noise, motion-related noise, cerebrospinal fluid pulsation or MRI-related (multiband-related) noise, according to a guideline on hand classification of ICA noise components (Griffanti et al., 2016). Noise components were then filtered and regressed out from the 4-dimensional data.

2.6.3 Group-Level ICA

Denoised 4-dimesional resting-state data was analysed in group level using MELODIC (Beckmann et al., 2009). In brief, individual scans were temporally concatenated and decomposed into a set of spatially independent maps and a set of time-courses. Large-scale patterns of functional connectivity common to all subjects were identified to fit the spatial maps and time-courses. The maximum dimensionality was limited to 25 to acquire meaningful components according to the number of

subjects in this study and previous studies and guidelines (Abou-Elseoud et al., 2010; Smith et al., 2011).

2.6.4 Dual regression

Dual regressions were performed with FSL to analyse the differences in functional connectivity between patients and healthy controls. Subject-specific spatial maps and time series were generated from the set of 25 independent components from the group level ICA by dual regression (Beckmann et al., 2009). Spatial regression was firstly introduced to regress the unthresholded group-level independent components into each subject's 4-dimensional data set. This step generated a set of subject-specific time series of function for each independent component. Next, these time series were regressed into the corresponding resting-state fMRI 4-dimensional data set. This temporal regression generated a set of spatial maps for each component. These spatial maps, which indicate the coactivation of each component, were then utilized to test whether there was any difference between healthy control and CFS groups. The spatial maps were also used to test the correlations between the functional connectivity and the levels of neurometabolites in ACC, including glutamate and GABA. The statistical inference was performed by FSL's nonparametric (randomise) permutation-testing tool and stringent threshold free cluster enhancement (TFCE) analysis. Regions with a corrected $p < 0.05$ were visualised with FSLeyes (FSL image viewer, part of FSL.)

2.6.5 Seed-Based analysis

Seed-based analyses were also carried out on the resting-state fMRI data. Spatial

maps of the regions of interest (ROIs), including pre-genual ACC and right putamen, were produced according to the positions of VOIs in MRS sessions and the functional connectivity distribution of group-level ICA. These spatial maps of ROIs were then analysed identically as the independent components. Dual-regression, FSL's nonparametric (randomise) permutation-testing tool and TFCE analysis were introduced to test whether there was any correlation between the seed-based functional connectivities and the levels of glutamate and GABA level in the corresponding ROIs. The analysis of the correlations with Regions with a corrected $p < 0.05$ were visualised with FSLeyes.

2.7 Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics (version 24, IBM Corp, Armonk, NY). Demographic data and questionnaire scores were compared using chi-square tests and independent samples t-tests with Levene's test for equality of variances. Concentrations of neurochemicals acquired from MRS were analysed with repeated measures analysis of variance (ANOVA), with 'diagnosis' (CFS or control) as a between subject factor and 'region' (ACC, OCC or putamen) as a within-subject factor. Significant main effects on the ANOVA were followed up with unpaired two-tailed *t* tests.

Chapter 3: Results

3.1 Participants

There were 12 patients who met the Oxford criteria for CFS and 25 healthy controls included in this study. There were no significant differences between patients and controls with respect to age, gender and BMI. The mean CFQ score among the patient group was 24.3 ± 1.2 (±SEM; range 15-30). The BDI score in the patient group was significantly higher than that in the control group. In contrast, the rating of HAM-D and STAI did not differ significantly between patient group and control group. There was also no significant difference on the level of serum CRP (Table 3.1).

3.2 Magnetic Resonance Spectroscopy

Among all the MRS spectra, 3 spectra from the ACC and 1 from the putamen were excluded for the reasons of quality. There were therefore 34 spectra from the ACC (10 patients), 36 from the putamen (12 patients) and 37 from the OCC (12 patients) included in the final analysis. There were no significant differences with respect to signal-to-noise ratio (SNR), grey matter, white matter or CSF content between patients and controls in the ACC, putamen or OCC (Table 3.2).

3.3 Glutamate/GABA/Glutamine/Glx

The CRLB values of glutamate, GABA, glutamine and Glx have been reported in Table 3.3. There were no significant differences between patients and controls on CRLB values (all $p > 0.05$). The repeated measures ANOVA for glutamate showed an interactive effect of diagnosis with region ($F = 4.478$; df = 2,31; $p = 0.022$, Greenhouse-Geisser-corrected for sphericity) but no main effect of diagnosis ($F = 0.147$; df = 1,31; $p = 0.704$). The repeated measures ANOVA for GABA and Glx also showed an interactive effect of diagnosis with region ($F = 3.424$; df = 2,28; $p = 0.040$ and $F = 4.866$; df = 2,31; $p = 0.011$, respectively) but no main effect of diagnosis ($F = 0.002$; df = 1,27; $p = 0.965$ and $F = 0.151$; df = 1,31; $p = 0.700$, respectively). Follow-up pairwise comparisons of neurometabolite concentrations showed significant increases in the concentrations of glutamate and Glx in the putamen in patients. There was also a significant decrease in the concentration of GABA in the ACC in patients (Table 3.4). In contrast, glutamine concentrations showed no main or interactive effect of diagnosis ($F = 0.416$; df = 1,30; $p = 0.416$ and $F = 2.014$; df = 2,30; $p = 0.063$). There were no main or interactive effects of gender or gender with diagnosis on either glutamate, GABA, glutamine or Glx (all p values > 0.05).

To explore the correlations between clinical presentations and MRS measures of

glutamate, GABA, glutamine and Glx in ACC and putamen, bivariate Pearson correlation analyses were conducted on the concentrations of these neurometabolites and CFQ, BDI or STAI in the patients. The normality of distributions of the neurometabolite concentrations were determined by inspecting Q-Q plot manually. All of the measures were determined to be normally distributed. There was a significant negative correlation between ACC glutamine and CFQ (Pearson's r = -0.661, p = 0.037, Figure 3.1). There was also a significant negative correlation between putamen Glx and CFQ (Pearson's r = -0.609, p = 0.036, Figure 3.1). There were no significant correlations between other neurometabolite concentrations and CFQ, BDI, STAI or CRP.

3.4 Other Neurometabolites

The CRLB values of GSH, total choline (glycerophosphocholine + phosphocholine), total creatine (creatine + phosphocreatine) and total NAA have been reported in Table 3.5. There were no significant differences between patients and controls on CRLB values of these neurometabolites (all $p > 0.05$). The repeated measures ANOVA for GSH and total creatine showed main effects of diagnosis (F = 9.957; df = 1,29; p = 0.004 and F =5.756; df = 1,31; p = 0.023, respectively) but no interactive effect of diagnosis with region (F = 0.930; df = 2,29; p = 0.400 and F = 2.814; df = 2,31; p =

0.068, respectively) indicating reductions of GSH and total creatine in all three regions (Figure3.2). Follow-up pairwise comparisons showed significant decreases in the concentrations of GSH and total creatine in the ACC in patients (Table 3.6). Other neurometabolite concentrations showed no significant main effect of diagnosis or interactive effect of diagnosis with region. There were no main or interactive effects of gender or gender with diagnosis on either GSH, NAA, total choline or total creatine (all p values > 0.05).

Bivariate Pearson correlation analyses were also conducted on the concentrations of GSH, total choline, total creatine and total NAA in ACC and putamen and CFQ, BDI or STAI in patients. The normality of distributions of the neurometabolite concentrations were determined by inspecting Q-Q plot manually. All of the measures were determined to be normally distributed. There was a significant negative correlation between putamen NAA and CFQ (Pearson's r = -0.799, p = 0.002, Figure 3.3). There was also a significant positive correlation between ACC GSH and STAI (Pearson's r = 0.698, p = 0.036, Figure 3.3). There were no significant correlations between other neurometabolite concentrations and CFQ, BDI or STAI.

3.5 Resting-State Functional Magnetic Resonance Imaging

There were 12 CFS patients and 12 healthy controls in the analyses for resting-state fMRI. There were no significant differences between patients and controls with respect to age, gender and mean displacement for motion correction during the fMRI scan. (Table 3.7).

3.6 Independent Component Analysis-Based Resting-State Functional Connectivity

25 independent components (ICs) were produced from group MELODIC. 12 components, which are consistent with previous resting-state network studies, were shown here (Figure 3.4 and 3.5). These include the default mode network, salience network, central executive network, ventral stream network, sensory-motor network, lateral visual network, primary visual network, cerebellum-thalamus network, and auditory network. Dual regressions using these 25 ICs as spatial maps showed increased connectivity between the default mode network (Figure 3.4A) and right supracalcarine cortex, right precuneus cortex, left cuneal cortex and right dorsolateral prefrontal cortex (DLPFC) in patients (Table 3.8 and Figure 3.6).

3.7 Seed-Based Resting-State Functional Connectivity

Seed-based analyses were conducted in the ACC and putamen. The masks for the ACC and putamen were created according to the regions that were 100% overlapped by the voxels used in the MRS scan of every subject (Figure 3.7). Dual regressions using these masks as spatial maps showed no area of significantly differently functional connectivity with the ACC or putamen in healthy controls relative to CFS individuals.

3.8 Effect of Neurometabolites on Functional Connectivity

Levels of glutamate, GABA, glutamine and Glx in the ACC and putamen measured by MRS were incorporated into generalized linear models as covariates separately to test the positive effect and negative effect of these neurometabolite on the resting-state BOLD signal. ICA-based and seed-based analyses showed no significant effect between the concentration of these neurometabolites on the resting state BOLD signal in any area. Seed-based analyses on the ACC and putamen also showed that there was no significant interaction between these neurometabolite, diagnosis of CFS and resting-state BOLD signal.

Table 3.1: Participant characteristics. BMI, body mass index; CFQ, Chalder Fatigue Questionnaire; BDI, Beck Depression Inventory; HAM-D, 17-item Hamilton Depression Rating Scale; STAI, State-Trait Anxiety Inventory; CRP, C-Reactive Protein. Values represent mean ± SEM. *$p < 0.05$, **$p < 0.001$

	Controls (N=25)	Patients (N=12)	*p* value
Age, mean ± SEM (Range)	29.8 ± 1.5 (20-54)	32.2 ± 3.7 (20-61)	0.556
Sex (Female/Male)	19/6	6/6	0.114
BMI (Mean ± SEM)	23.5 ± 1.2	23.6 ± 0.9	0.94
CFQ (Mean ± SEM)	11.0 ± 0.2	24.3 ± 1.2	**<0.001****
BDI (Mean ± SEM)	0.5 ± 0.2	9.3 ± 2.9	**0.011***
HAM-D (Mean ± SEM)	1.4 ± 0.65	5.6 ± 2.1	0.081
STAI (Mean ± SEM)	25.8 ± 1.5	28.4 ± 2.8	0.42
CRP (Mean ± SEM)	1.1 ± 0.3	1.3 ± 0.5	0.693

Table 3.2: Voxel compositions and signal-to-noise ratio (SNR) for the anterior cingulate cortex (ACC), putamen and occipital cortex (OCC).

Parameter (Mean ± SEM)	Controls	Patients	*p* value
ACC grey matter, %	79.5 ± 1.1	82.1 ± 1.5	0.198
ACC white matter, %	11.3 ± 0.6	9.5 ± 0.6	0.078
ACC CSF, %	9.1 ± 0.9	8.3 ± 1.6	0.651
ACC SNR	32.5 ± 1.7	33.5 ± 2.8	0.762
Putamen grey matter, %	38.4 ± 2.7	42.9 ± 4.2	0.363
Putamen white matter, %	61.4 ± 2.7	57.1 ± 4.2	0.374
Putamen CSF, %	0.01 ± 0.01	0.00 ± 0.00	0.134
Putamen SNR	14.8 ± 0.7	13.8 ± 1.0	0.401
OCC grey matter, %	74.1 ± 0.9	75.2 ± 1.7	0.559
OCC white matter, %	16.6 ± 0.6	16.8 ± 0.9	0.831
OCC CSF, %	9.2 ± 0.8	7.8 ± 1.6	0.387
OCC SNR	42.3 ± 1.8	39.8 ± 2.3	0.411

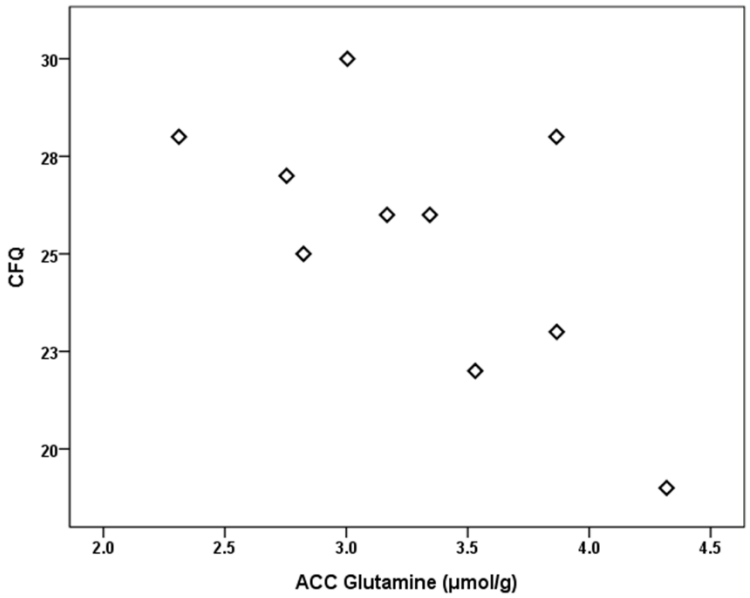
Table 3.3: Glutamate, γ-aminobutyric acid (GABA), Glutamine and combined measurement of glutamate and glutamine (Glx) Cramér–Rao lower bound (CRLB, mean ± SEM) values.

Controls	Glutamate CRLB	GABA CRLB	Glutamine CRLB	Glx CRLB
(Mean ± SEM)				
ACC	2.2 ± 0.1	10.0 ± 0.7	7.9 ± 0.5	2.3 ± 0.1
Putamen	4.2 ± 0.3	12.6 ± 0.6	15.6 ± 1.0	4.2 ± 0.2
OCC	2.5 ± 0.2	11.3 ± 0.8	8.6 ± 0.6	2.8 ± 0.2
Patients				
(Mean ± SEM)				
ACC	2.2 ± 0.2	9.8 ± 0.5	6.7 ± 0.7	2.2 ± 0.2
Putamen	4.1 ± 0.3	13.4 ± 1.0	14.8 ± 1.0	4.2 ± 0.3
OCC	2.6 ± 0.2	9.8 ± 0.9	9.0 ± 1.0	2.8 ± 0.3

Table 3.4: Metabolite concentrations of glutamate, γ-aminobutyric acid (GABA), Glutamine and combined measurement of glutamate and glutamine (Glx). There were significant differences in putamen glutamate and Glx between controls and patients. * $p < 0.05$

		Controls (Mean ± SEM)	Patients (Mean ± SEM)	*p* value
Glutamate	ACC	11.25 ± 0.25	10.56 ± 0.28	0.121
	Putamen	7.39 ± 0.17	7.99 ± 0.24	**0.048***
	OCC	9.39 ± 0.19	9.61 ± 0.22	0.478
GABA	ACC	2.17 ± 0.10	1.80 ± 0.11	**0.041***
	Putamen	1.87 ± 0.07	1.99 ± 0.09	0.343
	OCC	1.92 ± 0.10	2.09 ± 0.13	0.331
Glutamine	ACC	3.25 ± 0.12	3.30 ± 0.19	0.833
	Putamen	2.10 ± 0.1	2.47 ± 0.17	0.053
	OCC	2.76 ± 0.07	2.77 ± 0.13	0.919
Glx	ACC	14.50 ± 0.28	13.86 ± 0.39	0.214
	Putamen	9.45 ± 0.2	10.46 ± 0.32	**0.008***
	OCC	11.38 ± 0.13	11.71 ± 0.22	0.165

Figure 3.1: Correlations between concentrations of anterior cingulate cortex (ACC) glutamine (Pearson's r = -0.661, p = 0.037) and putamen combined measurement of glutamate and glutamine (Glx, Pearson's r = -0.609, p = 0.036) and the scores of Chalder Fatigue Questionnaire (CFQ) in the patients.



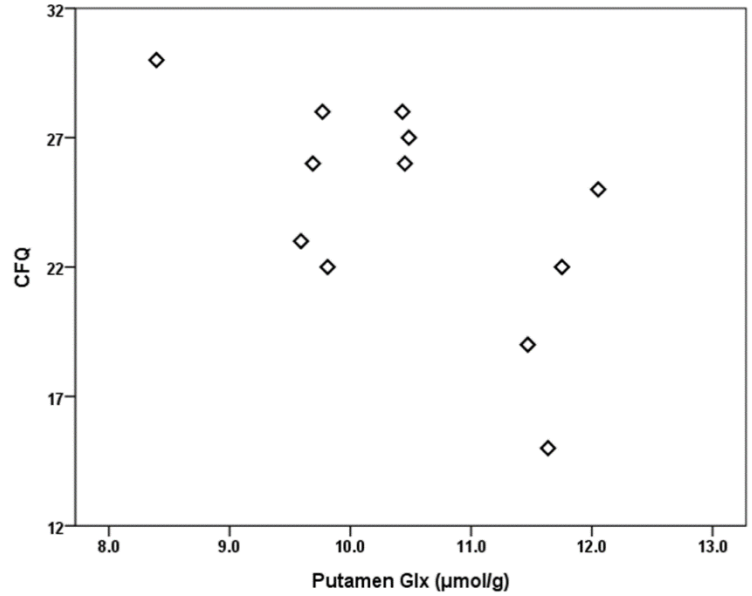


Table 3.5: Glutathione (GSH), total choline (tCho), total creatine (tCr) and total N-acetylaspartate (tNAA) Cramér–Rao lower bound (CRLB, mean ± SEM) values. *ACC* anterior cingulate cortex, *OCC* occipital cortex

Controls	GSH CRLB	tCho CRLB	tCr CRLB	tNAA CRLB
(Mean ± SEM)				
ACC	9.9 ± 0.6	4.3 ± 0.4	2.1 ± 0.1	1.4 ± 0.1
Putamen	16.0 ± 0.9	7.1 ± 0.4	2.7 ± 0.2	2.4 ± 0.1
OCC	13.5 ± 1.5	7.2 ± 0.5	2.0 ± 0.1	1.2 ± 0.1
Patients				
(Mean ± SEM)				
ACC	9.7 ± 0.9	4.4 ± 0.8	2.2 ± 0.2	1.5 ± 0.3
Putamen	18.3 ± 1.9	7.7 ± 0.7	3.0 ± 0.3	2.5 ± 0.2
OCC	17.8 ± 5.5	7.6 ± 0.7	2.2 ± 0.2	1.2 ± 0.2

Figure 3.2: Mean (SEM) glutathione (GSH) and total creatine (tCr) levels in controls (HC) and patients (CFS) in anterior cingulate cortex (ACC), occipital cortex (OCC) and putamen. There are main effects of diagnosis on GSH and tCr, independent of region (F = 9.957; df = 1,29; p = 0.004 and F =5.756; df = 1,31; p = 0.023, respectively)

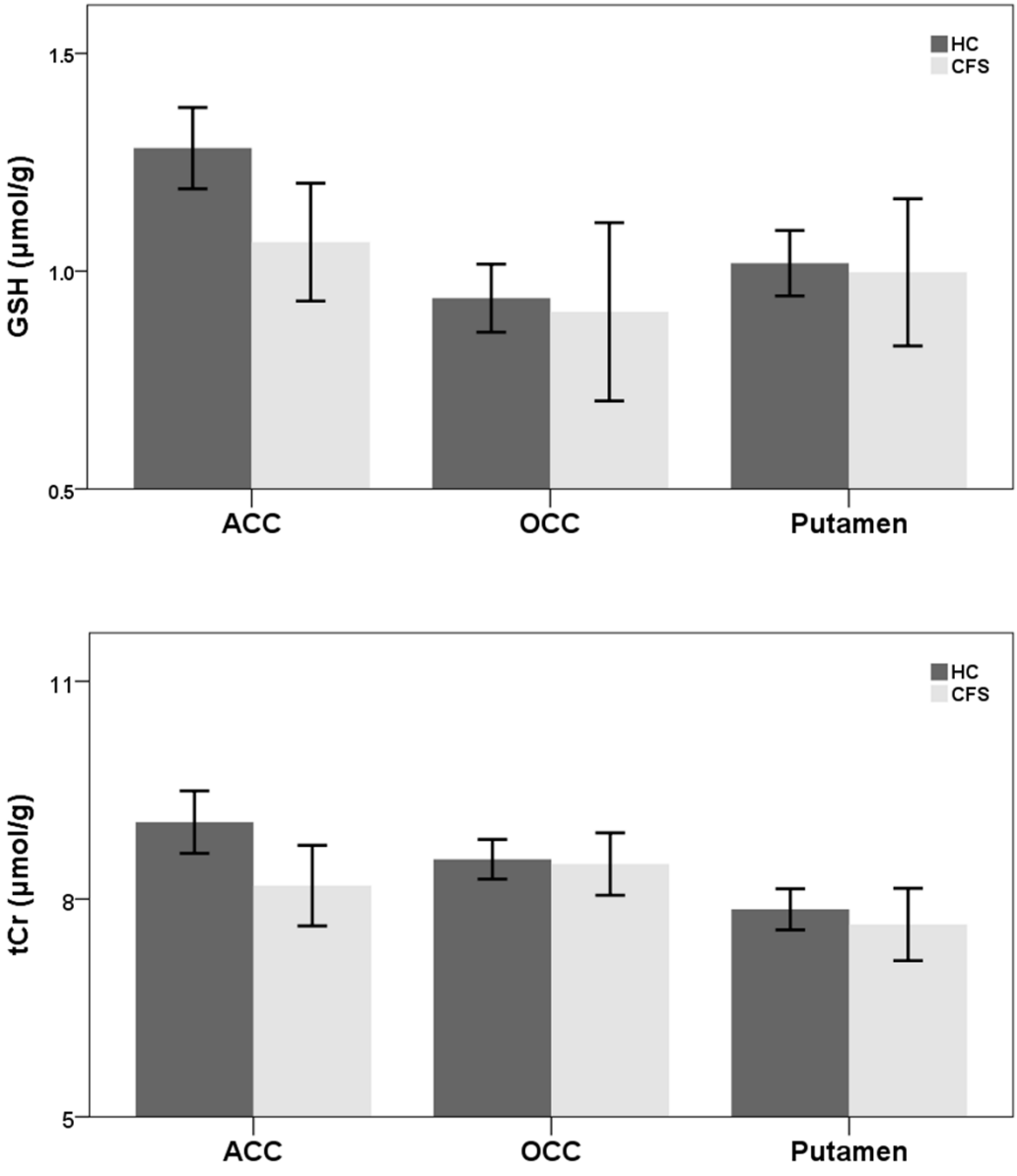
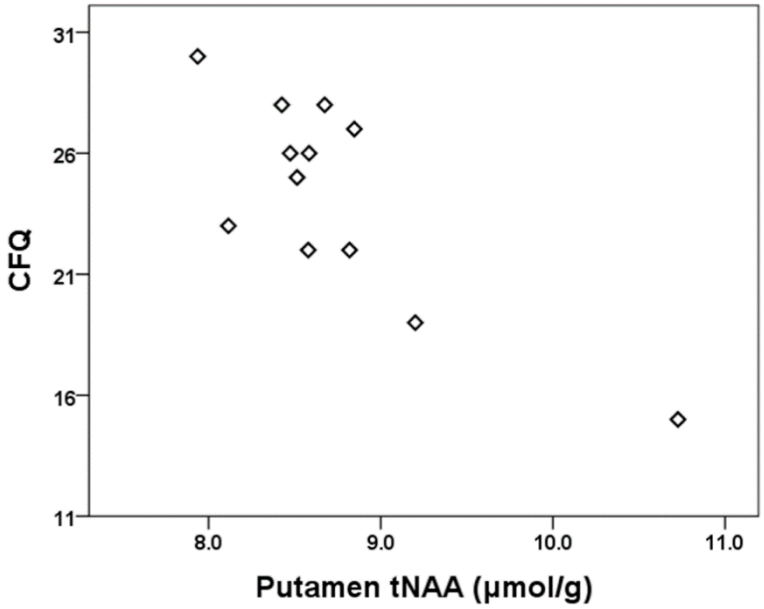


Table 3.6: Metabolite concentrations of GSH, total choline (tCho), total creatine (tCr) and total N-acetylaspartate (tNAA. There were significant differences in anterior cingulate cortex (ACC) GSH, tCr and tNAA between controls and patients. * $p <$ 0.05, *OCC* occipital cortex

		Controls (Mean ± SEM)	Patients (Mean ± SEM)	*p* value
GSH	ACC	1.28 ± 0.05	1.07 ± 0.06	**0.013***
	Putamen	1.02 ± 0.04	1.00 ± 0.08	0.776
	OCC	0.94 ± 0.04	0.91 ± 0.09	0.718
tCho	ACC	1.67 ± 0.06	1.53 ± 0.06	0.194
	Putamen	1.35 ± 0.05	1.36 ± 0.07	0.937
	OCC	0.89 ± 0.02	0.88 ± 0.05	0.777
tCr	ACC	9.06 ± 0.21	8.19 ± 0.25	**0.021***
	Putamen	7.86 ± 0.14	7.65 ± 0.23	0.411
	OCC	8.55 ± 0.13	8.48 ± 0.20	0.785
tNAA	ACC	11.63 ± 0.27	10.33 ± 0.34	**0.011***
	Putamen	8.60 ± 0.16	8.74 ± 0.20	0.598
	OCC	13.76 ± 0.38	13.66 ± 0.34	0.868

Figure 3.3: Correlation between concentrations of putamen total N-acetylaspartate (tNAA, Pearson's r = -0.799, p = 0.002) and the scores of Chalder Fatigue Questionnaire (CFQ) in the patients. Correlation between anterior cingulate cortex (ACC) glutathione (GSH) and the scores of State-Trait Anxiety Inventory (STAI, Pearson's r = 0.698, p = 0.036) in the patients.



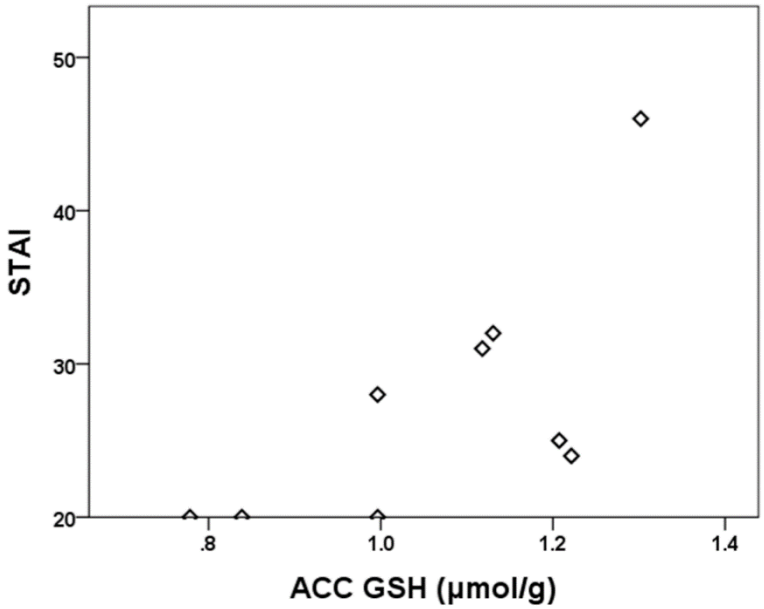


Table 3.7: The characteristics of participants in resting-state functional magnetic resonance imaging analyses.

	Controls (N=12)	Patients (N=12)	*p* value
Age, mean ± SEM (Range)	30.8 ± 2.8 (23-54)	32.2 ± 3.7 (20-61)	0.762
Sex (Female/Male)	6/6	6/6	>0.999
Mean displacement, absolute (mm, Mean ± SEM)	0.23 ± 0.04	0.28 ± 0.06	0.499
Mean displacement, relative (mm, Mean ± SEM)	0.12 ± 0.04	0.10 ± 0.04	0.275

Figure 3.4: Resting-state networks from group MELODIC. A. Default mode network (posterior); B. Default mode network (anterior); C. Salience network; D. Central executive network (right); E. Central executive network (left); F. Ventral stream network.

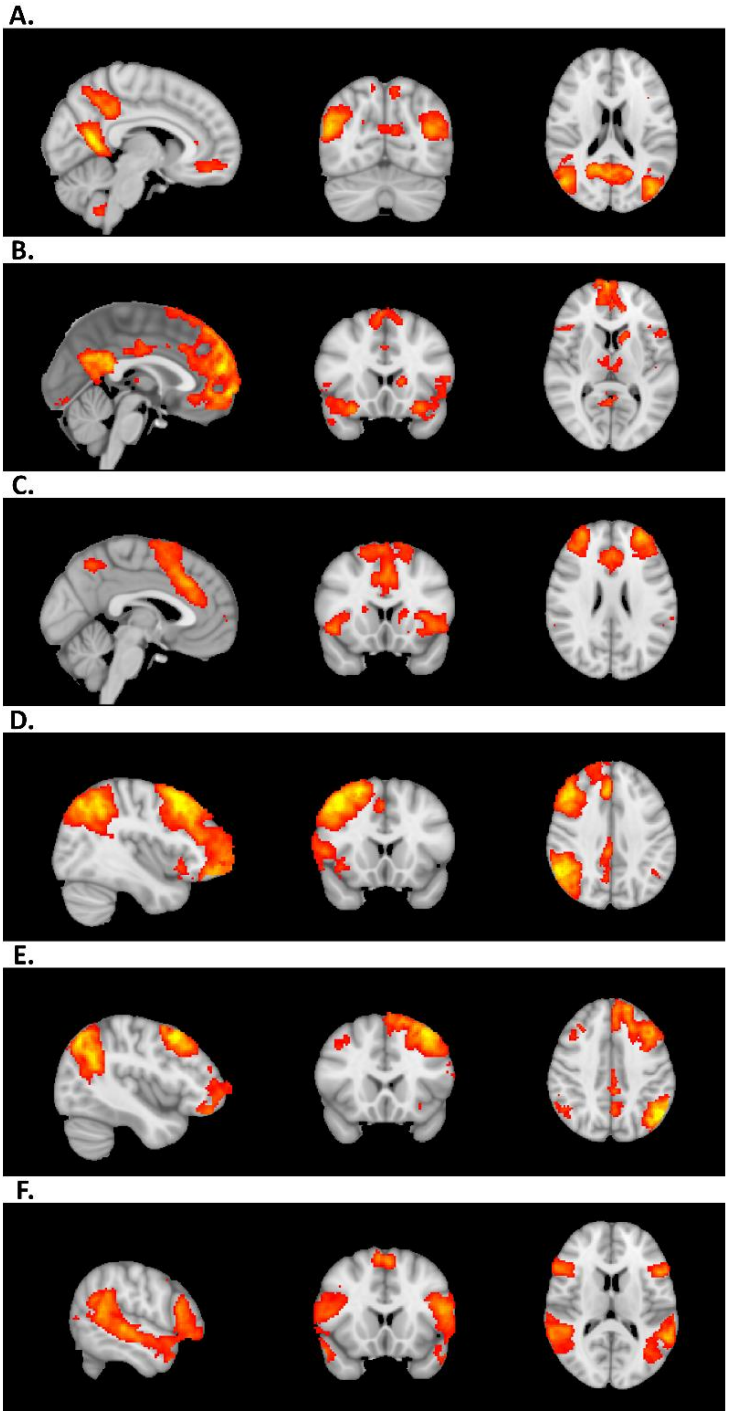


Figure 3.5: Resting-state networks from group MELODIC. A. Sensory-motor network (precentral); B. Sensory-motor network (postcentral); C. Lateral visual network; D. Primary visual network; E. Cerebellum-thalamus network; F. Auditory network.

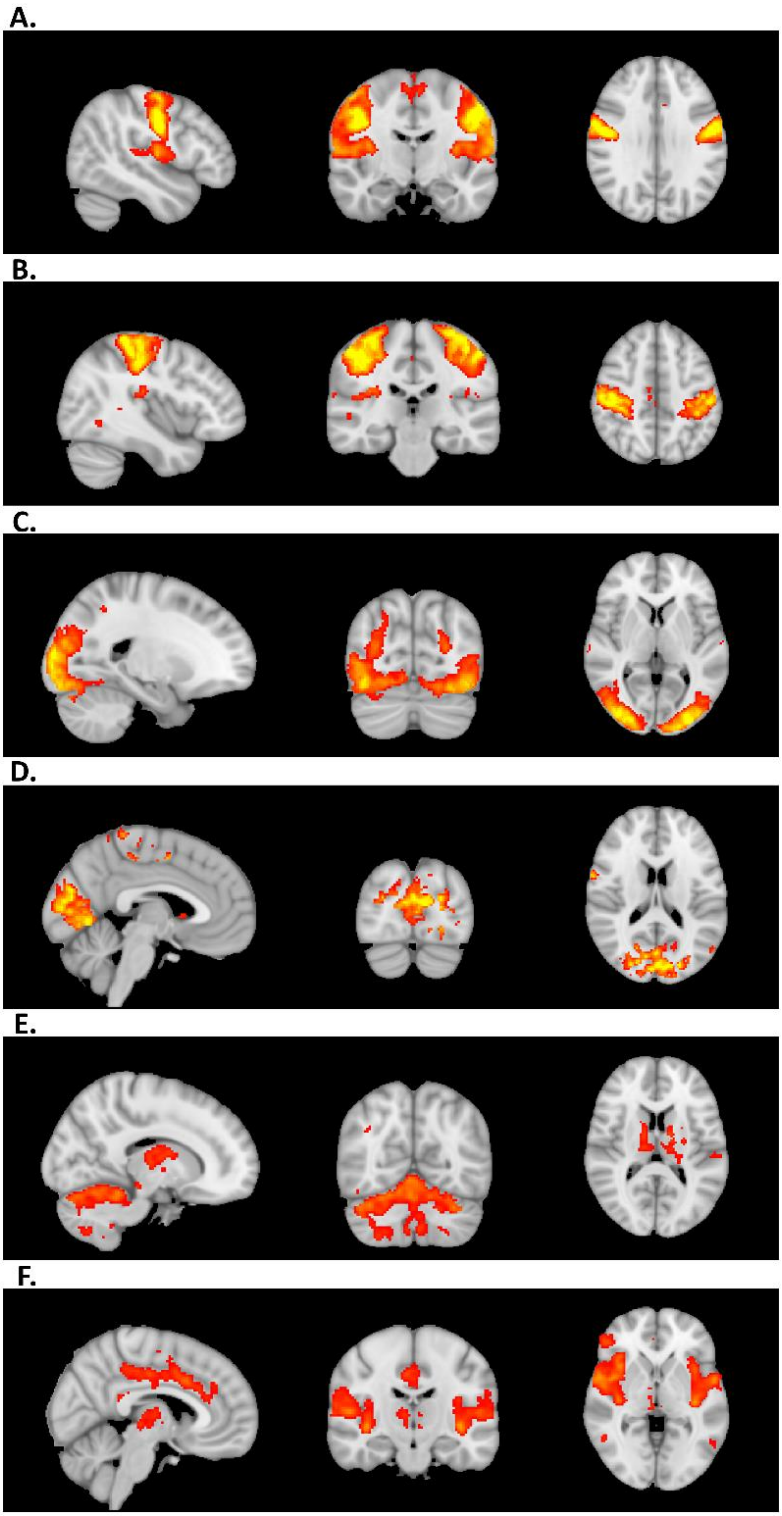


Table 3.8: Foci with increased functional connectivity with the default mode network in CFS patients compared to healthy controls. MNI, Montreal Neurological Institute 152 standard-space T1-weighted average structural template image; R right; L, left; TFCE, threshold-free cluster enhancement.

Brain region	Side	MNI			Number of voxels	Z-score	*p*-value, TFCE
		x	**y**	**z**			
Supracalcarine cortex	R	29	33	45	112	6.00	0.006
Precuneus cortex	R	42	32	46	68	4.72	0.017
Cuneal cortex	L	48	26	47	22	3.91	0.038
Dorsolateral prefrontal cortex	R	23	83	54	9	3.68	0.047
Dorsolateral prefrontal cortex	R	26	82	57	2	4.08	0.045
Intracalcarine cortex	L	51	22	44	1	4.98	0.045

Figure 3.6: Foci with increased functional connectivity (red, *p*-value < 0.05) with default mode network (green) in CFS patients compared to healthy control.

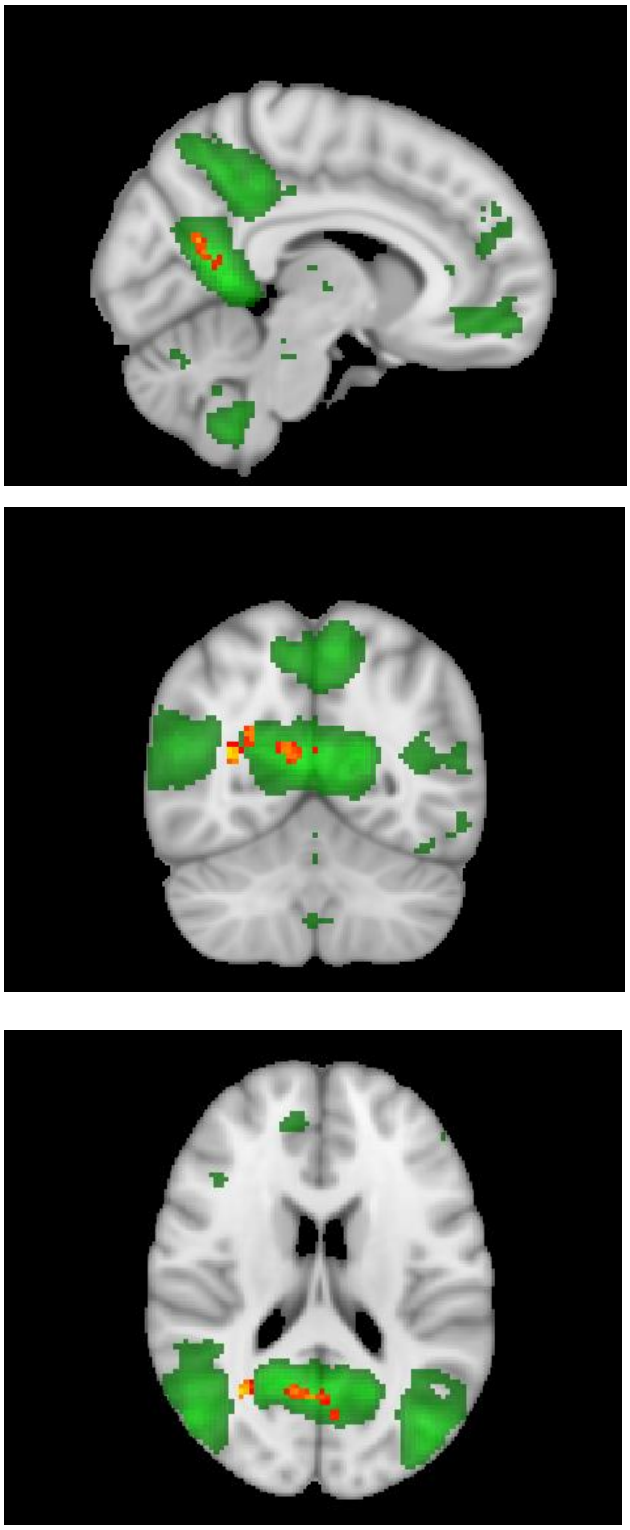
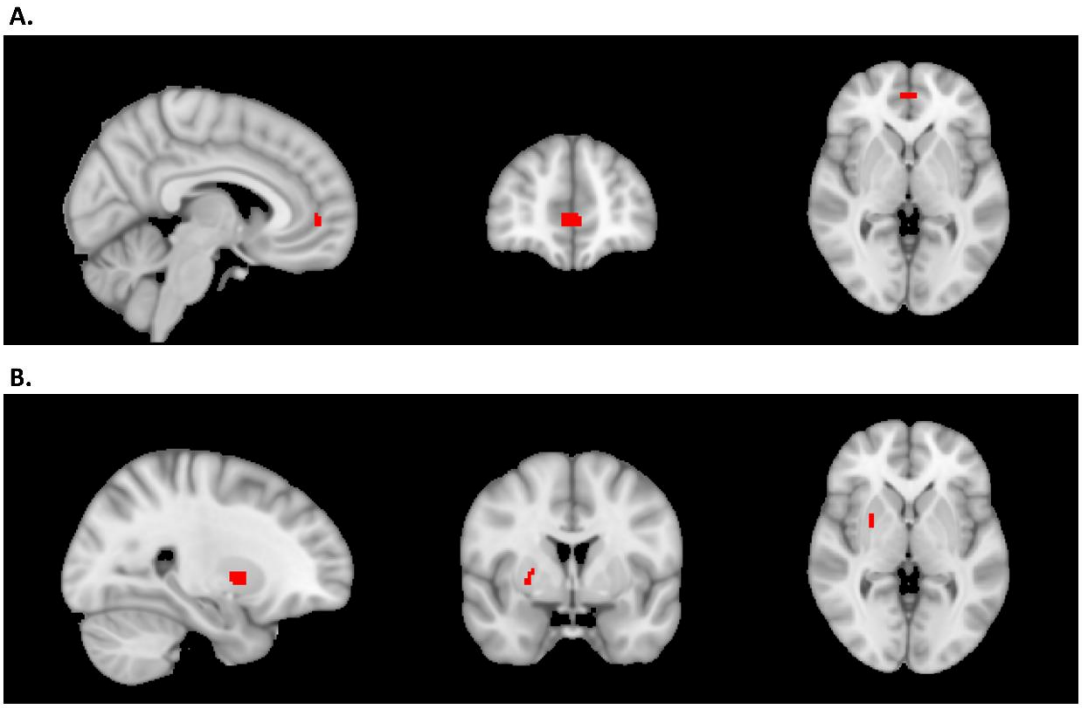


Figure 3.7: Masks for seed-based analyses. Masks for the anterior cingulate cortex (ACC) and putamen were created according to the regions that were 100% overlapped by the voxels used in the MRS scans of every subject. A. Seed for the ACC, (MNI: x=44, y=87, z=36); B. Seed for the putamen, (MNI: x=32, y=64, z=37).



Chapter 4: Discussion

4.1 Summary of Findings

The present study, employing MRS at 7 Tesla, demonstrated alterations in a number of brain neurometabolites, including glutamatergic/GABAergic neurotransmitter and cellular bioenergetic biomarkers, in patients with CFS. Among the patients, concentrations of right putamen glutamate and Glx were increased while that of ACC GABA was decreased. Negative correlations between self-report clinical fatigue severity and putamen Glx and ACC glutamine were also found. As for the cellular bioenergetic biomarkers, there were main effects of CFS diagnosis without interactive effects with region on GSH and total creatine, indicating global decreases of these neurometabolites in CFS patients. In addition, there was elevated functional connectivity between default mode network and right supracalcarine cortex, precuneus cortex and DLPFC, a finding supported by previous studies. However, no correlation between any MRS-measured ACC or putamen neurometabolite level and resting-state functional connectivity was found in either ICA-based or seed-based analysis.

4.2 Elevated Glutamate in Putamen

Putamen glutamate and Glx concentrations were significantly higher in patients with CFS compared with healthy controls. The putamen is part of the striatum in basal ganglia, receiving glutamatergic excitatory afferent projections from multiple regions, including the motor, premotor, dorsolateral prefrontal cortex, ACC, thalamus, amygdala and hippocampus (Dobryakova et al., 2013; Marchand et al., 2010). It not only processes motor functions but also has functions related to learning, emotion, cognition and reward (Chang et al., 2007; Delgado et al., 2003; Dobryakova et al., 2013; Graybiel, 2005)

The connection between the ACC and putamen is one aspect of the cortico-striatal circuitry that could contribute to mental fatigue. While the ACC has important cognitive functions, such as calculating the effort needed for a particular task, the putamen is associated with reward anticipation and reward-related decision making (Ballein et al., 2007; Haruno et al., 2006; Walton et al., 2003). An imbalanced perception of costs and reward in this circuitry could be a potential cause for mental fatigue. For example, rats having lesion in either the ACC or striatum lost the motivation to work for larger food rewards (Salamone et al., 2003; Walton et al., 2003).

In the present study, the elevation of putamen glutamate concentration in CFS might be a result of excessive glutamatergic projection from other cortical regions, such as the ACC, in which the level of GABA was decreased, indicating a potentially more active status. The decrease in ACC GABA level will be discussed in a later section.

There was no previous MRS study in CFS focused on this region although some other imaging studies have found several changes in putamen in CFS. There are studies demonstrating that both childhood- and adult-type CFS had decreased basal ganglia activation during reward-processing tasks (Miller et al., 2014; Mizuno et al., 2016). Moreover, the decrease of activation was positively correlated with severity of mental fatigue and reduced activity. These findings, however, seems to be contradicted by our finding of elevated glutamate, which is an excitatory neurotransmitter, in putamen. Such contradiction can perhaps be explained by the neurotoxicity of glutamate.

Apart from being the major excitatory neurotransmitter in the nervous system, glutamate can induce neurotoxicity, including degeneration and dysfunction, when present at an excessive level (Lau et al., 2010). The underlying mechanisms of glutamate-induced neurotoxicity are complex and not fully understood. Apoptosis-like and necrosis-like neuronal cell death following NMDAR-mediated calcium influx and

free radical production are believed to play critical roles in glutamate neurotoxicity (Bonfoco et al., 1995; Choi, 1987; Lafon-Cazal et al., 1993). Studies have found an association between glutamate-induced neurotoxicity and several neurological diseases, including multiple sclerosis, traumatic brain injury and ischemic brain injury (Lau et al., 2010; Pitt et al., 2003; Srinivasan et al., 2005). In the present study, increased putamen glutamate in patients with CFS could also lead to neuronal dysfunction and hence might explain the weakened activity demonstrated during reward-processing tasks reported by previous studies.

The elevation of putamen glutamate could also be caused by systemic inflammation, which is a possible factor in CFS aetiology (Blundell et al., 2015; Lorusso et al., 2009; Milrad et al., 2017). In previous studies, inflammation-related increases of glutamate in basal ganglia have been found in interferon (IFN)-alpha induced depression and major depression (Haroon et al., 2014, 2016). IFN-alpha is an inflammatory cytokine that is used for the treatment of hepatitis C. Depression and fatigue are common side effects of such treatment, and systemic inflammation is believed to be the cause of these clinical symptoms. An MRS study in patients with IFN-alpha injection showed a significant increase of glutamate in left putamen and caudate nucleus. There was also a significant correlation between the level of

glutamate and lowered motivation (Haroon et al., 2014). On the other hand, another study in patients with major depression showed a positive correlation of left basal ganglia glutamate and serum CRP, anhedonia and psychomotor slowing (Haroon et al., 2016). However, there was no correlation between CRP and glutamate in the present study.

Astrocyte activation could be one of the underlying mechanisms for glutamate elevation in systemic inflammation. Astrocytes take up the glutamate released from glutamatergic neurons with the glutamate transporter-1 (Glut-1) and GLutamate ASpartate Transporter (GLAST, Zhou et al., 2013). Among the glutamate accumulated in astrocytes, 25% is oxidatively degraded during the process of ammonia detoxification (Zielińska et al., 2014). The remaining glutamate is converted to glutamine by glutamine synthetase in the cytosol of astrocytes (Derouiche et al., 1991). Glutamine is than released from astrocytes through the sodium-dependent neutral amino acid (SNAT) family transporter (Mackenzie et al., 2004). After entering neurons, glutamine is converted to glutamate by glutaminase.

Inflammatory cytokines have been shown to block the reuptake and stimulate the release of glutamate by astrocytes through multiple pathways, such as downregulation

of the expression of glutamate transporters and increased production of quinolinic acid through the kynurenine pathway (Ida et al., 2008; Tavares et al., 2002; Tilleux et al., 2007). Astrocyte activation in CFS has been reported by positron emission tomography (PET) study using ^{11}C-(R)-PK11195 as a ligand (Nakatomi et al., 2014). ^{11}C-(R)-PK11195 is a translocator protein that is expressed by activated astrocytes. In this PET study, increased signal of ^{11}C-(R)-PK11195 was found in multiple brain region, including thalamus, cingulate cortex and amygdala. Although this study did not find significant differences in the putamen, it is still reasonable to conclude that astrocytes in CFS are abnormally activated in the central nervous system, which could potentially increase putamen glutamate concentration.

Some pathophysiological findings in CFS are comparable with major depressive disorder, such as elevated ACC activity and signs of systemic inflammation (Boissoneault et al., 2016; Connolly et al., 2013; Miller et al., 2016; Milrad et al., 2017). An MRS study in major depressive disorder showed elevated glutamine (but not glutamate) levels in the putamen (Godlewska et al., 2017), which has some resemblance to the findings in the present study in hinting at glutamatergic overactivity. In depression, however, it appears that the increased glutamate is successfully metabolised to glutamine in astrocytes whereas in CFS the glutamate is

not cleared and remains present at increased levels. This could suggest that the integrity of astrocytes in CFS is impaired. However, this hypothesis needs further study.

The increase in glutamate level could have a close relationship with the elevated lactate level in cerebrospinal fluid and spinal fluid (Mathew et al., 2009; Murrough et al., 2010; Natelson et al., 2017; Shungu et al., 2012). Apart from its role as a neurotransmitter, glutamate is part of the cellular metabolic cycle, such as the tricarboxylic acid (TCA) cycle. There is a bidirectional exchange between alpha-ketoglutarate and glutamate through a transamination-mediated process (Fitzpatrick et al., 1990). On the other hand, glucose enters the TCA cycle after being converted to pyruvate through glycolysis and oxidised to acetyl-CoA. In addition, malate can be oxidised to pyruvate by malate dehydrogenase and thus leaves the TCA cycle (Banaszak et al., 1975). Pyruvate can be promptly converted to lactate and vice versa (Hertz et al., 2016). Therefore, if there is an excessively high level of glutamate, it is possible that glutamate may enter the TCA cycle through alpha-ketoglutarate and thereby increase lactate level in the central nervous system. It might be also possible that the lactate level is abnormally raised by other factors, such as inflammation or infection, and this, in turn, eventually increases the glutamate level through the

pathway mentioned above. Still, this hypothesis also needs further study.

4.3 Negative Correlation between Putamen Glx and Fatigue Severity

Although putamen glutamate concentration was elevated in CFS in general, a negative correlation between putamen Glx concentration and self-report fatigue scale was demonstrated in CFS patients. This means that patients with higher putamen Glx concentration have lower level of fatigue. This suggests that increased Glx in putamen in CFS patients might be an adaptive compensatory effect to carry out enhanced neural activation to mitigate the greater effort needed to perform daily activities among the patients (De Lange et al., 2004). Generally, it also important to consider whether the neurobiological changes seen in CFS might be secondary to the decrease in activity that is central to the disorder. Thus, another possible cause of the changes in Glx detected here, could be that the sedentary lifestyle that characterises severe CFS, may lead to altered neurometabolite profile in the putamen and the other MRS changes detected in this patient group. In this case patients reporting more severe fatigue might be less active which could, for example, account for the correlation between increased Glx in putamen and scores on the CFQ.

4.4 Decreased GABA in Anterior Cingulate Cortex

The present study showed a decline in ACC GABA concentration among the patients. A previous MRS study in CFS found a negative correlation between ACC GABA concentration and reduced physical activity without a significant drop in concentration compared to healthy controls. In our study, however, no correlation was found between ACC GABA concentration and any clinical data, including the severity of fatigue, depressive mood or anxiety or serum CRP.

Prolonged stress could contribute to the decrease of ACC GABA concentration. Previous animal studies have shown that the number of GABAergic interneurons was decreased after exposure to chronic stress (Czéh et al., 2015). In addition, behavioural alterations after stress can be inhibited by administering benzodiazepines, a class of drug that enhance the activity of GABA on $GABA_A$ receptor (Ramirez et al., 2016). On the other hand, in patients with CFS, the uptake of acetylcarnitine, a precursor for the biosynthesis of GABA, has been shown to be reduced in several brain regions, including the ACC (Kuratsune et al., 2002). This evidence indirectly suggests that the production of ACC GABA might be decreased in CFS, which is in accordance with our finding.

In the healthy population, ACC GABA concentration has a negative correlation with aversiveness, that is, the BOLD signal induced by anticipation of an aversive shock is reduced in participants with higher ACC GABA concentration (Duncan et al., 2014; Levar et al., 2017). A similar finding was reported by a study using emotional stimuli, where BOLD responses in the amygdala were negatively correlated with ACC GABA level. Such GABA-related alterations of activity in ACC could be linked to ACC hyperactivity or hyperarousal. For instance, MRS studies on primary insomnia and panic disorder have shown decreased ACC GABA level (Ham et al., 2007; Plante et al., 2012). Furthermore, the total sleep time was positively correlated with ACC GABA level reference studies, which means more severe insomnia in patients with lower ACC GABA level.

Poor sleeping quality and higher prevalence of anxiety disorder are also important features of CFS, with prevalence of 95% and 42% respectively (Collin et al., 2016; Daniels et al., 2017). The current study analysed the relationship between ACC GABA level and anxiety and sleep using the STAI and sleep quality questions in the HAM-D, which are related to the initiation and maintenance of sleep. There was no significant correlation between ACC GABA level and the scores for these questions (data not shown). This could be due to the less detailed sleep-related questions in the

HAM-D, which is not specific for the investigation of sleeping disorders. Whether the decreased ACC GABA level plays a role in anxiety and sleep symptoms in CFS needs further investigation.

4.5 Decrease of Glutathione

Previous studies have shown increased oxidative stress markers in serum and muscle tissue in CFS (Fulle et al., 2000; Kennedy et al., 2005). The decrease in glutathione (GSH) observed in the present study could strengthen the potential mechanism of increased oxidative stress in CFS aetiology. In brain, GSH is one of the major antioxidants that detoxifies reactive oxygen species (ROS) produced during the oxidative metabolism (Dringen, 2000). Decreased expression of GSH in brain has been consistently found in different neurological diseases and psychiatric disorders, including Alzheimer's disease, bipolar disorder, major depressive disorder, and schizophrenia, indicating a pivotal role of GSH in brain physiology (Gawryluk et al., 2011; Rae et al., 2017).

In comparison to other types of cell in the central nervous system, astrocytes have rapid GSH turnover rate and high intracellular GSH concentration (Rae et al., 2017; Yudkoff et al., 1990). Astrocytes also release GSH into extracellular space through

different pathways, such as the transporter protein, MRP1, and gap junction hemichannel (Hirrlinger et al., 2002; Rana et al., 2007). An *in vitro* study has shown the neuroprotective function of astrocytes under elevated oxidative stress via a glutathione-dependent mechanism (Chen et al., 2001). The antioxidant production of astrocytes can be stimulated by inflammatory cytokines, however, the surge of the production and release of GSH returns to normal level under chronic or prolonged inflammatory condition (Sharma et al., 2007; Steele et al., 2013). This phenomenon of exhaustion could be an explanation for the decreased GSH level found in our study with abnormally activated astrocytes found in the PET study noted above (Nakatomi et al., 2014).

The decrease of GSH could also contribute to symptoms of CFS through altering normal mitochondrial function. Mitochondria are the main cellular organelle that produce ATP through oxidative phosphorylation (Hatefi, 1985). Impaired mitochondrial function can induce severe neurological-related symptoms, such as MELAS: myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (Goto et al., 1992). During oxidative phosphorylation, reactive oxidative species are generated by the electron transport chain located at the inner mitochondrial membrane (Liu et al., 2002; Modinos et al., 2017). The reactive oxidative species, including hydrogen

peroxide, superoxide, hydroxyl, peroxyl and hydroperoxyl radicals, can be reduced to stable molecules, such as water, by GSH and glutathione peroxidase (Bayr, 2005; Sies, 1997).

The subcellular distribution of GSH in neurons is localised in mitochondria (Huang et al., 1995), indicating a high demand of antioxidant to attenuate oxidative stress generated during the energy production. The decreased GSH in CFS could possibly interrupt this neuroprotective mechanism. Studies analysing mitochondria in peripheral blood have found abnormal mitochondrial function in patients with CFS (Booth et al., 2012; Myhill et al., 2009). The latter study demonstrated that decreased mitochondrial functions was associated with the severity of illness. These functions include the efficiency of oxidative phosphorylation, the efficiency of transferring ATP/ADP and the availability of ATP.

There were also mitochondrial abnormalities found in the muscle of patients with CFS. Weakened leg quadricep muscle and increased cardiovascular response to exercise were also shown in CFS (Fulcher et al., 2000). Furthermore, a ^{31}P MRS study measuring post-exertional phosphocreatine resynthesis rate, an indicator of mitochondrial efficiency, found the resynthesis rate was lower in patients with CFS

compared to healthy controls (McCully et al., 1996).

It is noticeable that a series of MRS studies consistently showed elevated lactate concentrations in ventricular cerebrospinal fluid of CFS patients from different cohorts (Mathew et al., 2009; Murrough et al., 2010; Shungu et al., 2012). A recent study in the spinal fluid of CFS patients further demonstrated elevated level of lactate and decreased level of GSH (Natelson et al., 2017). Lactate can be serve as indirect indicator of mitochondrial dysfunction since cells will increase the level of glycolysis, which is a lactate-producing mechanism, when they cannot generate enough energy through mitochondria-mediated oxidative phosphorylation (Fernie et al., 2004; Schurr et al., 1998).

Currently, there is no published study describing the use of glutathione or N-acetylcysteine, a GSH precursor, as supplements for the treatment for CFS. There were, however, several clinical studies testing the effect of a variety of antioxidants, including reduced form NADH, coenzyme Q10 and multivitamins in CFS (Castro-Marrero et al., 2016; Forsyth et al., 1999; Maric et al., 2014). Although the effects of these antioxidant are not well-established, several positive responses to supplement treatments, including decreases in fatigue and increases in sleep quality have been

reported. Important considerations of oral supplement treatment are the bioavailability of the active components and their ability to pass through blood-brain barrier. GSH has been shown to be transportable through blood-brain barrier by a carrier-mediated pathway (Kannan et al., 1990). As a result, reduced form GSH or N-acetylcysteine supplementation seems to be a potential candidate for supplement treatment for CFS. Whether antioxidant supplement can normalise the level of brain GSH and improve the clinical symptoms in CFS needs investigation by controlled clinical trials.

4.6 Decrease of Total Creatine

In addition to GSH, this study also found that total creatine levels were decreased in all three voxels studied. Creatine is mainly produced in liver and pancreas in human (Walker, 1979). It can also be produced endogenously in the brain by neurons, astrocytes and oligodendrocytes (Braissant et al., 2001; Dringen et al., 1998). Creatine plays an important role in cellular bioenergetics in brain through interacting with cytoplasmic creatine kinase (CK) and mitochondrial creatine kinase (MiCK). In mitochondria, creatine is phosphorylated by adenosine triphosphate (ATP) and then catalysed by MiCK to form phosphocreatine, which can be hydrolysed by CK and produce ATP in cytoplasm (Kottke et al., 1994; Walliman et al., 1992). The energy in the phosphate bond is therefore stored in phosphocreatine. As a less negatively

charged and smaller molecule compared to ATP, phosphocreatine is suitable for deposition by cells as a buffer for energy. Furthermore, its high diffusibility makes it a favourable vehicle to deliver high-energy phosphate bonds rapidly throughout the cell (Walliman et al., 1992; Yoshizaki et al., 1990).

The depletion of creatine in brain can cause serious neurological symptoms. For example, defects in SLC6A8, a creatine transporter, or AGAT, an enzyme needed for creatine biosynthesis, can all result in severe depletions of creatine in brain (Salomons et al., 2001; Stöckler-Ipsiroglu et al., 2001). The clinical manifestations of these deficiencies are similar, such as mental retardation, developmental delay and impaired cognitive function (Schulze, 2003). In contrast, oral supplement of creatine monohydrate can effectively raise brain creatine concentration among healthy population by up to 15% (Dechent et al., 1999). Studies have found that, in healthy participants, creatine supplement can reduce mental fatigue induced by repetitive tasks and improved cognitive functions such as working memory, auditory memory and intelligence tests (Hammett et al., 2010; Rae et al., 2003; Watanabe et al., 2002). Further studies using oral creatine supplement as an adjunctive therapy for psychiatric disorders, including major depression and bipolar depression, showed improvements in clinical outcome or cognitive function compared to control group (Lyoo et al.,

2012; Toniolo et al., 2016).

There is a recent study using oral supplement of guanidinoacetic acid, a precursor of creatine, as a treatment for CFS (Ostojic et al., 2016). Serum and muscular creatine level was effectively increased after the administration of guanidinoacetic acid. However, no effect on main clinical outcomes, including general fatigue score and muscle soreness, was detected. Nevertheless, increased activity, motivation, leg muscle power and decreased mental fatigue were reported. These improvements, combined with the decreased level of creatine shown in the present study, could indicate a possibility that moderate depletion of creatine could contribute to the symptomatology of CFS.

An important issue raised by the discovery of decreased creatine is that MRS studies in CFS should be cautious about the quantification method. In the present study, a water signal was acquired to serve as a reference in order to conduct absolute quantification. In contrast, some MRS studies assume cellular creatine concentration to be a constant and use it as the reference for relative quantification. A previous study has demonstrated in healthy participants that creatine-referencing relative quantification could generate more variability than it prevents (Li et al., 2003). There

are also studies that found alterations in creatine levels in different psychiatric disorders when using absolute quantification, including schizophrenia, bipolar disorder obsessive-compulsive disorder (Frye et al., 2007; Mirza et al., 2006; Öngür et al., 2009). These findings further challenge the assumption of constant cellular creatine level.

As an alteration of creatine was shown in this study, future studies should avoid creatine-referencing relative quantification on data from patients with CFS. Instead, using water as a reference could be the correct option for the calculation of absolute concentration. Several previous CFS MRS studies have used creatine as the reference (Chaudhuri et al., 2003; Puri et al., 2002; van der Schaaf et al., 2017). Whether the alteration of creatine in CFS could bias the results reported by these studies is uncertain; however, caution is clearly needed when interpreting creatine-referenced MRS data.

4.7 Hyperactivity in the Default Mode Network

In this study, hyperactive functional connectivity was found between the default mode network and several regions around posterior cingulate cortex, including right supracalcarine cortex and precuneus cortex. The default mode network is a resting-

state network which is related to self-referential thinking and stimulus-independent thought (Whitfield-Gabrieli et al., 2012). Examples of tasks related to the default mode network are judging one's own character, retrieving personal memory and planning one's future (D'argembeau et al., 2005; Moran et al., 2006; Whitfield-Gabrieli et al., 2012). Increased activities of the default mode network have been shown to be associated with multiple psychiatric disorders, such as major depressive disorder, obsessive-compulsive disorder and schizophrenia, in which excessive rumination or self-referential thought are common clinical symptoms.

Among the patients with CFS, catastrophising and ruminative thinking were frequently seen and were related to the severity of symptoms (Nijs et al., 2008). A resting-state fMRI study on CFS patients has found that the functional connectivity from the posterior cingulate cortex to ACC, which both are components in the default mode network, was significantly increased (Kim et al., 2015). This study also demonstrated that the clinical fatigue severity was positively correlated with the strength of functional connectivity between the posterior cingulate cortex and ACC.

Posterior cingulate cortex is part of the inferior parietal lobe. Structural studies using tractography and graph-theoretic analyses have shown high structural connectivity of

posterior cingulate cortex, with the connections originating from medial temporal lobes and ACC (Beckmann et al., 2009). Functionally, the posterior cingulate cortex is connected with several different networks, including the default mode network, fronto-parietal network, salience network and sensorimotor networks (Leech et al., 2012).

Confirming the results found in a previous seed-based study (Kim et al., 2015), hyperactivity in similar regions around the posterior cingulate cortex were found in the present ICA-based study. Furthermore, this study showed the hyperactivity is functionally connected with the default mode network. One of the advantages of ICA-based analysis is that, when investigating a certain brain region involved in multiple networks, it can distinguish the interactions between this region and different networks while seed-based analysis cannot (Joel et al., 2011). The results in this study hence add to the strength of evidence of hyperactivity within the default mode network in regions around posterior cingulate cortex, including precuneus and supracalcarine cortex in payients with CFS.

The activity of default mode network is suppressed during tasks that require attention for external focus (Fox et al., 2005). In contrast, rest or tasks that require internally

directed attention, such as planning for the future or daydreaming, can increase the activity of default mode network (Spreng, 2012). The close association between default mode network and attention has been further demonstrated in patients with traumatic brain injury (Bonnelle et al., 2011). Impaired abilities to conduct and maintain goal-directed behaviour are commonly seen symptoms in traumatic brain injury (Robertson et al., 1997). A multimodal neuroimaging study in traumatic brain injury found that impaired attention is associated with hyperactivity in precuneus and posterior cingulate cortex (Bonnelle et al., 2011). Furthermore, this study also showed that structural alterations within default mode network were associated with impaired attention.

On the other hand, patients with CFS have been found to have impaired executive attention, speed of information processing and planning capacity. (Hou et al., 2014; Joyce et al., 1996; Togo et al., 2015) However, currently there is no study correlating alteration of cognitive function with the activity of default mode network and clinical symptoms. In the present study, although there was a significant group difference between patients and healthy controls, there was no correlation between the BOLD signal and clinical symptom scales used in this study, including CFQ and BDI, within patients.

The failure to find an association between resting-state BOLD signal could be a result of the subjective natures of the CFQ and BDI. Both of the assessments are self-report questionnaires. There are some assessments utilised in other CFS studies that could be more objective, such as 6-minute walk distance (White et al., 2011). Six-minute walk distance is an assessment that is mainly used for the measurement of patients with cardiovascular or respiratory diseases, such as heart failure and chronic obstructive pulmonary disease (Enright, 2003). Patients will be asked to "walk as far as you can during 6-minutes," and the total distance walked within 6 minutes has been shown a good indicator of patients' general physical condition (Miyamoto et al., 2000). The 6-minute walk distance has also been shown to be a reliable and reproducible way to measure fatigue in multiple sclerosis (Goldman et al., 2008). Having some subjective assessments, such as the 6-minute walk distance, could provide more covariates in the analysis and should be considered.

Another possible reason for the lack of association between connectivity and clinical measures could be the small number of patients studied. When analysing the group difference, data from 12 patients and 12 healthy controls (total 24 participants) were incorporated to the generalized linear model and dual regression. However, heathy controls were excluded from the correlation analysis between BOLD signal and

severity of fatigue since most of the heathy controls obtained exactly same score in CFQ (11, which means that the participant had the common condition of experiencing fatigue in the last week). Minimal differences between subjects on the CFQ score made the analysis on healthy control inapplicable. As a result, only data from 12 patients were analysed for the effects of clinical symptoms severity on brain activity in resting-state. Increasing the number of patients would give the analysis stronger statistical power, possibly enabling it to clarify the association between fatigue severity and resting-state brain activity.

Foci located in right dorsolateral prefrontal cortex also showed elevated functional connectivity with default mode network in patients with chronic fatigue syndrome. The dorsolateral prefrontal cortex includes parts of superior and middle frontal gyrus. It is connected with the lateral parietal cortex, frontal eye fields and premotor areas, receiving projections from most of the sensory cortices (Barbas, 2000). The dorsolateral prefrontal cortex is mainly involved in cognitive and executive tasks (Koenigs et al., 2009). It has also been shown to be related to the active control of pain perception via modulating the activity between midbrain and medial thalamus (Lorenz et al., 2003).

Among the large-scale neural circuitry, the dorsolateral prefrontal cortex is one of the components of the central executive network, or so-called frontal-parietal network (Menon, 2011). This network also includes the posterior parietal cortex. Unlike the default mode network, the central executive network is often activated by stimulus-driven tasks that have the cognitive requirement, such as a memory-guided spatial delayed-response task (Rowe et al., 2000).

There is a strong anticorrelation between the default mode network and central executive network, that is, while one of these networks is activated, the other one will be strongly deactivated (Menon, 2011). The switching of activation between the default mode network and central executive network is mediated by the salience network, another large-scale neural network (Goulden et al., 2014; Sridharan et al., 2008). The salience network is composed of the dorsal ACC and frontoinsular cortex (Menon, 2011).

In the group ICA in the present study, the default mode network, central executive network and salience network were extracted successfully from the data of all participants. Group differences were shown in the default mode network but not the other two networks. Interestingly, the right dorsolateral prefrontal cortex, which is the

hub of the central executive network (Buckner et al., 2009) and should be deactivated during the activation of default mode network, was found to have elevated functional connectivity with the default mode network in CFS patients compared to healthy controls.

This finding could indicate an impaired switching between networks, which has also been found in several psychiatric disorders, such as post-traumatic stress disorder, major depressive disorder and schizophrenia (Daniels et al., 2010; Hamilton et al., 2011; Manoliu et al., 2013). In schizophrenia, for example, the deactivation of posterior cingulate cortex is impaired during a target detection task (Hasenkamp et al., 2011). Furthermore, the functional connectivities between the salience network and the default mode network and central-executive network had negative correlations with the severity of hallucination (Manoliu et al., 2013).

Altered correlations between different network can also be found in patients with major depressive disorder. During the external-focus task, which is designed to activate the central executive network and deactivate the default mode network, patients with major depressive disorder show higher activity in default mode network compared to healthy controls (Belleau et al., 2014). Another study comparing non-

emotionally blunted and emotionally blunted depressed patients with an N-Back task found that emotionally blunted depressed patients showed a lower negative correlation between the activities of default mode network and central executive network compared to non-emotionally blunted patients. The strength of such negative correlation between the two networks was further shown to be negatively associated with the severity of clinical symptoms in emotionally blunted depressed patients.

Although currently there are no studies demonstrating temporal network dynamic changes in CFS, results from the current study and other resting-state studies in general have shown that functional connectivity is increased in the default mode network and decreased in central executive network and salience network in CFS (Boissoneault et al., 2016; Gay et al., 2016; Kim et al., 2015; Wortinger et al., 2016). These findings indicate a dominant default mode network, which fits the triple network model since the activations of other two networks are more synchronised. Although there was no attention-required or attention switching task in the present study and the temporal dynamics of the networks during resting-state were not analysed, the paradoxical increase in functional connectivity between dorsolateral frontal cortex and default mode network in patients with CFS suggest possible alterations of network dynamics.

4.8 Seed-Based Resting-State Analysis in the Anterior Cingulate Cortex and Putamen

The seed-based resting analysis in this study showed no significant difference between patients and healthy controls. Instead of using masks created according to brain atlas, the masks used in the analyses were created based on the voxel placements in the MRS study. The final seed masks were the regions that were 100% overlapped by voxels of all MRS scans. The size of the seeds was surprisingly small, showing potential inter-subject variation on voxel placements during MRS study. This could be also caused by the stringent threshold used in this study. The size of the seeds increases to a comparable volume to the voxel volume in the MRS study if the threshold is decreased to 95% or 90%. This indicates that differently placed voxels happened only to a few subjects while most participants have similar placements for the voxels in the ACC and putamen.

There are several limitations that make the standardised voxel placement not practicable. The anatomical differences between each subject, including the size of the brain and vasculature, could sometimes hinder the voxel from being placed at the optimal location. In addition, some technical differences, such as the degree of neck

extension of the subjects during the scan, could increase the difficulties on choosing a location for the voxel precisely and consistently.

The ACC has been shown to be structurally and functionally highly connected with multiple brain regions, including the posterior cingulate cortex, midcingulate, prefrontal cortex, and putamen (Gasquoine, 2013; Margulies et al., 2007; Szekely et al., 2017). The seed mask of this study was placed at the pre-genual ACC, and it successfully showed functional connectivity between the seed and most of the regions mentioned above, except the putamen (data not shown).

On the other hand, the putamen is functionally connected with the ACC, posterior cingulate cortex, precentral cortex, postcentral cortex, inferior parietal lobule and superior temporal gyrus (Di Martino et al., 2008). In addition, bilateral putamens are highly interconnected (Cao et al., 2009). The putamen seed mask of this study is placed at right dorsal rostral putamen, and it showed only the functional connectivity between bilateral putamens (data not shown). There was no significant functional connectivity with other regions mentioned in the previous studies.

These results in some degree have validated the methods used in the seed-based

analyses in this study. Regions that were functionally connected with the seed have all been reported by other studies (Cao et al., 2009; Di Martino et al., 2008; Szekely et al., 2017). There were, however, limited regions showed to be functionally connected with the seed in putamen. This may be due to weaker signal detected from subcortical region. Increasing the numbers of subjects to increase the power of the analysis sensitive could be an effective solution for this situation.

4.9 Neurometabolites and Functional Connectivity

This study attempted to clarify the associations of neurometabolites, especially glutamatergic and GABAergic neurotransmitters, with brain activity in resting-state. Analyses incorporating the concentrations of neurometabolites measured by MRS as covariates into the generalized linear model and dual regression of both seed-based and ICA-based analysis.

Multimodal fMRI-MRS study design could be a powerful way to understand neural physiology. The integration of these two modalities enables more comprehensive interpretations of the results compared to studies using fMRI or MRS alone. Among the neurometabolites that can be measured in proton MRS, glutamate and GABA have been particularly extensively studied since both of these neurotransmitters are closely

related to the excitation-inhibition balance (Duncan et al., 2014). Excitation-inhibition balance has been shown to be an important factor in neural activity, with glutamate and GABA being able to predict the neural signal output such as value comparison (Jocham et al., 2012).

There have been several multi-modal task-based studies investigating the ACC. For example, the study mentioned above measured the baseline levels of glutamate and GABA in the ventro-medial prefrontal cortex, which includes the ACC, before the task, hypothesising that the excitation-inhibition balance status in the individuals would affect their responses in the value-guided choice task (Jocham et al., 2012). The ACC BOLD signal in this task was elevated at the first 4 seconds, followed by a suppression. This study found that the initial surge and the later depression was positively correlated with the concentrations of glutamate and GABA, respectively.

Another example, a study using emotional picture viewing task, which can cause task-induced negative BOLD responses in the ACC, has demonstrated a positive correlation between the ACC regional GABA concentration and the degree of task-induced deactivation (Northoff et al., 2007). This study also conducted a task that can induce positive BOLD responses in ACC. However, there was no correlation between

GABA concentration and the degree of activation during this task.

These two studies showed that data from MRS can be utilised in the analysis of task-performing fMRI, particularly the concentrations of glutamate and GABA. As for the investigation of resting-state fMRI and MRS, a study has shown that the concentrations of glutamate and GABA in posterior cingulate cortex were associated with functional connectivity in the default mode network in the resting-state (Kapogiannis et al., 2013). The posterior cingulate cortex is the hub for the default mode network, which is a dominant resting-state network (Menon, 2011). Kapogiannis et al. (2013) found that the intrinsic functional connectivity of the default mode network was positively and negatively associated with the levels of glutamate and GABA in the posterior cingulate cortex, respectively. In addition, this study also used glutamate/GABA ratio as an indicator for the status of the excitation-inhibition balance. Such glutamate/GABA ratio was also positively associated with the intrinsic functional connectivity of the default mode network.

There are two recent fMRI-MRS studies in populations with abnormal personality, including borderline personality disorder and people with high schizotypy (Modinos et al., 2017; Wang et al., 2017). Both of the studies focus on the ACC, with fMRI measuring task-dependent functional connectivity or BOLD response. In the

borderline personality disorder, the levels of ACC GABA were positively associated with the activation in putamen, caudate and frontal regions in patients but not in healthy controls (Wang et al., 2017). Meanwhile, the study on people with high schizotypy found that there was a negative correlation between ACC glutamate level and striatal activation in healthy controls but no in people with high schizotypy (Modinos et al., 2017). According these results, the appearance or disappearance of correlations between neurometabolites and brain activity could be related to the pathophysiology of psychiatric disorders.

In the present study, the concentrations of glutamate, glutamine, Glx or GABA in the ACC or putamen showed no significant association with the resting-state functional connectivity in both ICA-based and seed-based analysis. A similar protocol for analysis was conducted on the dataset of 89 subjects from another study in major depressive disorder. This analysis successfully demonstrated positive correlations between putamen glutamate concentration and the resting-state functional connectivity from left putamen to bilateral insular and temporal cortex (unpublished data). These results suggest that the current protocol for analysis in this study should be appropriate. As a result, the failure to detect the correlations between neurometabolites and resting-state functional connectivities could result from the

small number of subjects in this study.

A review on multimodal imaging studies has calculated the average effect size of correlations between GABA concentrations and task-dependent BOLD response of 35 multimodal studies (Duncan et al., 2014). It concluded that, to detect such association without adding covariates, the minimum sample size is 16. In the present study, there were 12 patients and 25 healthy controls. Among the healthy controls, 13 of them did not have resting-state fMRI data and hence were excluded in the MRS-fMRI analyses. Therefore, the numbers of patients or healthy controls alone may not be sufficient to detect the association between neurometabolites and resting-state functional connectivities within a single group. On the other hand, combining patients and healthy controls to run the analysis will raise the concern of potentially different pattern of associations between heathy controls and patients. In addition, the minimum sample size of 16 was calculated according to results from studies using tasks-based fMRI (Duncan et al., 2014). Whether this number is comparable in that of resting-state fMRI study remain unknown.

4.10 Limitations

There are several limitations in this study. Firstly, the patients involved in this study

might not be representative of the whole CFS population. Previous studies reported that about 25% of patients with CFS were regularly bedridden (Komaroff et al., 1991), which would prevent them from participating in studies of this nature. Whether patients with these more severe symptoms have consistent changes in neurometabolites and resting-state functional connectivity still needs to be studied. More generally the small sample size gives rise to the possibility of chance findings and lack of reproducibility. The latter are current major concerns in biological psychiatry studies.

Secondly, the concentrations of neurometabolites, such as glutamate and GABA, measured by MRS are not totally contributed by the neurometabolites that act as synaptically but also those act extrasynaptically (De Graaf et al., 1990; Kauppinen et al., 1991; Stagg et al., 2011). Therefore, caution is needed when interpreting MRS data of glutamate and GABA. Another MRS-related limitation is that studies have shown the effect of menstrual cycle on glutamate/creatine level in the ACC of a population with premenstrual dysphoric disorder (Batra et al., 2008). Although not significantly different, the proportion of women in healthy control is relatively higher (76% compared to 50% in patients). If the effect of menstrual cycle on glutamate also present in the studied population, it could be a confounding factor. More generally

gender differences in MRS glutamate levels have been reported in some studies and there could presumably also be gender-related differences in other metabolites measured. These were not detected in the repeated measures ANOVA (data not shown) but future studies need to control better for gender.

Lastly, due to the nature of cross-sectional study, the causal relationship between the findings and the aetiology of CFS is unable to be clarified in the present study. The changes in brain could also be resulted from the sedentary lifestyle that patients tend to lead (Voss et al., 2014). Hence, having a group of controls with sedentary lifestyle should be considered in the future study.

Chapter 5: Conclusion and Future Works

5.1 Suggestions for Future Works

This study aimed to evaluate alterations in neurometabolites and resting-state functional connectivity in patients with CFS by MRS and fMRI at 7 Tesla. Several neurochemical changes were demonstrated, highlighting potential targets for treatments for CFS. Generally, the sample size needs to be significantly enlarged to confirm and extend the findings obtained here. In addition, patients and controls

should be better matched for gender. The addition of a sedentary control group would also be valuable. Studying a group of recovered patients with CFS might also help establish if any of the abnormalities found here are 'trait' changes which might represent markers of vulnerability.

Some of the findings in the present study might have implications for treatment. For example, since the concentration of ACC GABA was decreased in patients, it might be worthwhile evaluating the potential treatment effect of benzodiazepines on CFS. Benzodiazepines are a type of medication that can enhance the activity of GABA on $GABA_A$ receptor, inducing sleep-inducing and anxiolytic effects (Ramirez et al., 2016). An informal online survey of 174 responders conducted in an online CFS patient supporting forum showed that around 60% of the responders found that clonazepam, a type of benzodiazepine, was 'very helpful' on in aiding sleep and decreasing anxiety (Johnson, 2012). However, there was a proportion (13%) of responders suggested that clonazepam was "very harmful" to them with side effects such as tolerance and dependence Currently, the potential clinical benefit of benzodiazepines has not been carefully studied. Therefore, future studies on this issue could be valuable. However, benzodiazepines have many disadvantages, principally dependence and tolerance. Therefore, other agents facilitating GABA

neurotransmission might be more clinically useful. For example, the neurosteroid, brexanolone, which is a positive allosteric modulator of GABA-A receptors has recently been shown to be effective in post-partum depression (Kanes et al., 2017).

Another alteration in neurotransmitter level found in this study is elevated putamen glutamate concentration. Hence agents that lower glutamate availability might be useful agents in some CFS patients. A possible treatment would be riluzole, currently licensed for the management of motor neurone disease, which in animal studies lowers the availability of glutamate. However, it should be noted that in the current study, elevated glutamate correlated inversely with symptomatic fatigue. Hence lowering glutamate activity might not be therapeutically helpful.

The global decrease of GSH and total creatine level found in this study also indicates the possible beneficial treatment effects of GSH or creatine supplements in patients with CFS. There is a study using guanidinoacetic acid, a precursor of creatine, showing treatment effect in a limited aspect of symptomatology among CFS patients (Ostojic et al., 2016). Otherwise, there are no studies in the effects of supplements such as creatine monohydrate, GSH or its precursor N-acetylcysteine on patients with CFS. The safety and dosage of these supplements are relatively well-established in the

healthy population. Further studies on these supplements could be helpful for current understanding of CFS and its management.

There are some limitations in MRS studies, including the unspecific localisation in cellular and subcellular level and the voxel-defined regions studied. Additional neuroimaging studies could provide further biological and pharmacological information about CFS. For example, a PET study using radiolabelled ^{11}C-flumazenil can demonstrate the specific binding affinity of $GABA_A$ receptor on a whole-brain level (Malizia et al., 1998), which could further explore GABA-related alteration in CFS

5.2 Conclusion

In summary, the results of this study demonstrate an increase in glutamate in the putamen and decrease in GABA in the ACC, indicating an alteration of glutamatergic/GABAergic excitation-inhibition balance in patients with CFS. The elevated resting-state functional connectivities of the precuneus and dorsolateral prefrontal cortex in the default mode network also suggest hyperactive and aberrant brain activity. In addition, the global decreases of GSH and total creatine also suggest that CFS patients may have an abnormal bioenergetic status with higher oxidative

stress. These findings highlight the potential roles of neurometabolites in the pathophysiology of CFS and provide several possible treatment targets for future CFS studies.

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