Variation in *in vivo* prefrontal GABA, glutamate and glutamine – effects of reproductive factors, cortisol and major depressive disorder

by

Paramjit Bhardwaj

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Department of Psychiatry
University of Alberta

Abstract

Variations in glutamate and γ-aminobutyric acid (GABA), excitatory and inhibitory amino acid neurotransmitters in the brain, have been linked with cyclical changes across the menstrual cycle and in stress and stress-related mental disorders.

We used 3.0 Tesla proton magnetic resonance spectroscopy (^{1}H-MRS) to investigate $GABA^{+}$ levels (GABA + macromolecules) in the anterior cingulate/prefrontal cortex (ACC) in relation to menstrual cycle phase and the use of hormonal birth control and in a case-control comparison of patients with major depressive disorder (MDD) and healthy matched controls. We measured ACC $GABA^{+}$ using a double quantum filter with selective dual band refocusing, a method that is more selective for $GABA^{+}$ than the commonly used *J*-difference editing method.

We did not confirm the hypothesis that ACC $GABA^{+}$ would decrease between the follicular (1.12 ± .30 mmol/L) and late luteal phase (1.05 ± .31 mmol/L) of the menstrual cycle (n = 20; paired t = 1.28; df = 19; p = 0.22). ACC $GABA^{+}$ levels did not differ between women in the follicular phase of the menstrual cycle (n = 16; 1.11 ± .27 mmol/L) and those in the first week of pill-use of hormonal birth control (n = 14; 1.08 ± .19 mmol/L; t = 0.39; df = 28; p = 0.70).

We did not confirm the hypothesis that ACC $GABA^{+}$ would be lower in patients with MDD (n = 35; 1.04 ± 0.31 mmol/L) than in healthy controls (n = 42; 1.11 ± 0.22; t = 1.24; df = 75; 1-tailed p = 0.11). In a *post hoc* analysis, MDD patients with the most severe depressive anhedonia showed lower ACC $GABA^{+}$ levels (0.98 ± 0.05 mmol/L; $F_{1,58}$; p = 0.04) than controls. Strengths of the GABA studies include improved measurement selectivity,

quantification of ACC $GABA^+$ with respect to brain water and the largest sample sizes for these investigations to date. The main limitation was that the quantification method did not provide estimates of fitting precision.

^{1}H-MRS was also used to study the acute (within 30 minutes) and delayed (48 hours) effects of the administration of cortisol (40-100 mg) on glutamate and glutamine in healthy volunteers, in a double-blind, randomized, placebo-controlled design. We measured glutamate and glutamine separately, using spectrally-selective refocusing acquisitions. The administration of cortisol 40 mg (n = 6) or 100 mg (n = 8) did not lead to significant acute or delayed changes in glutamate or glutamine levels compared with placebo (n = 5). Strengths of the cortisol study were the selective measurements of glutamate and glutamine and their quantification with respect to brain water, using LCModel analysis. A major weakness was that the sample size was under-powered to test hypotheses definitively.

The selective measurement of $GABA^+$ and glutamine using ^{1}H-MRS is technically challenging, particularly for the ACC region that was studied. The current study of MDD adds to evidence that differences in ACC $GABA^+$ may only be large enough to be detectable in more severely ill patients.

Preface

This thesis is an original work by Paramjit Bhardwaj. The research projects, of which this thesis is a part, received research ethics approval from the University of Alberta Health Research Ethics Board, Biomedical Panel A.

"Magnetic resonance study in depression and social phobia", No. 6961, 23 December 2008.

"Cortisol modulation of prefrontal glutamate-glutamine activity in controls and PTSD. Pilot and healthy control studies", 9 Jan 2009.

The research for this thesis is part of collaborative research at the University of Alberta, led by Dr Nick Coupland. I was responsible for the data collection, statistical analysis, discussion and conclusions in chapters 6-8, as well as the literature review, under the supervision of Dr Nick Coupland.

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^{1}H-MRS – Proton Magnetic Resonance Spectroscopy

11β-HSD – Hydroxysteroid Dehydrogenase

17β-HSD – 17β-hydroxysteroid Dehydrogenase

ACC – Anterior Cingulate Cortex

ACTH – Adrenocorticotropic Hormone

ADIS-IV-L – Anxiety Disorders Interview Schedule Lifetime Version

AHP – After Hyperpolarization

AMPA – 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid

ANCOVA – Analysis of Covariance

ANOVA – Analysis of Variance

ATP – Adenosine Triphosphate

AVP – Arginine Vasopressin

BDNF – Brain Derived Neuroptrophic Factor

BLA – Basolateral Amygdala

BSA – Bovine Serum Albumin

Cho – Choline

Cr – Creatine

cAMP – Cyclic Adenosine Monophosphate

CBG – Corticosteroid Binding Globulin

CRH – Corticotropin-Releasing Hormone

CNS – Central Nervous System

CRLB – Cramer-Rao Lower Bounds

CSF – Cerebrospinal Fluid

CTQ – Childhood Trauma Questionnaire

DAG – Diacylglycerol

DST – Dexamethasone Suppression Test

DEX-CRH – Combined Dexamethasone/Corticotropin-Releasing Hormone Test

DQF – Double Quantum Filter

DQF-S – Double Quantum Filter with Selective Refocusing

E1 – Estrone

E2 – Estradiol

E3 – Estriol

EAAT – Excitatory Amino Acid Transporter

ELISA – Enzyme-linked Immunosorbent Assay

ER – Estrogen Receptor

ERK 1/2 – Extracellular Signal-Rgulated Kinase 1/2

FSH – Follicle Stimulating Hormone

Gln – Glutamine

Glu – Glumate

Glx – Glutamate + Glutamine

GnRH – Gonadotropin-Releasing Hormone

GABA – Gamma Aminobutyric Acid

GABA+ – GABA + Macromolecules

GABA-T – GABA Transaminase

GAD – L-glutamic acid Decarboxylase

GM – Gray Matter

GMF – Gray Matter Fraction

GPR 30 – G-Protein Coupled Estrogen Receptor 30

GR – Glucocorticoid Receptor

HCG – Human Chorionic Gonadotropin

HDRS – Hamiltion Depression Rating Scale

HPA – Hypothalamic-Pituitary-Adrenal

HPG – Hypothalamic-Pituitary-Gonadal

IP_3 – Inositol Triphosphate

IPSC – Inhibitory Post-Synaptic Current

IPSP -Inhibitory Post-Synaptic Potential

IV – Intravenous

LH – Lutenizing Hormone

LTD – Long Term Depression

LTP – Long Term Potentiation

MASQ – Mood and Anxiety Symptoms Questionnaire

MC – Menstrual Cycle

MDD – Major Depressive Disorder

MDE – Major Depressive Episode

mEPSC – Miniature Excitatory Post-Synaptic Current

mGluR – Metabotropic Glutamate Receptor

MM – Macromolecules

mPFC – Medial Prefrontal Cortex

MQC – Multiple Quantum Coherence

MR – Mineralocorticoid Receptor

NAA – N-Acetylaspartate

NART-R – National Adult Reading Test-Revised

NMDA – N-Methyl-D-Aspartate

OCC – Occipital Cortex

PCP - Phenylcyclidine

PFC – Prefrontal Cortex

PMDD – Premenstrual Dysphoric Disorder

PPD – Post-Partum Depression

PPM – Parts Per Million

PST – Prednisolone Suppression Test

PTSD – Post-Traumatic Stress Disorder

PVN – Paraventricular Nucleus

RF – Radiofrequency

SNR – Signal-to-Noise Ratio

T – Tesla

TCA – Tricarboxylic Acid

TE – Echo Time

VGLUT – Vesicular Glutamate Transporter

WM – White Matter

ZAN-BPD – The Zanarian Rating Scale for Borderlin Personality Disorder

INTRODUCTION

Knowledge of the mechanisms of neurotransmitter function and dysfunction is critical for our understanding of normal neurophysiology and the pathophysiology of psychiatric disorders. However, it is a challenge to implicate specific neurotransmitters in discrete pathophysiologies for specific disorders. In major depressive disorder (MDD), numerous pharmacotherapies have been developed over six decades that target monoamine neurotransmission, specifically the dopamine, serotonin and noradrenaline systems (Stahl, 2013). However, in recent years there has been a massive increase in interest in the amino acid neurotransmitters, glutamate and GABA, the most abundant excitatory and inhibitory neurotransmitters in the brain, respectively. This has been driven by several areas of research, in particular by robust evidence for the rapid antidepressant effect of the N-methyl-D-aspartate (NMDA) receptor antagonist, ketamine (Krystal et al, 2013; Papadimitropoulou et al, 2017), but also evidence from postmortem studies of MDD for reductions in glial cells and GABAegic neurons in multiple brain regions, including those involved in affective regulation (Sanacora and Banasr, 2013; Northoff and Sibille, 2014) and *in vivo* studies of glutamate and GABA using proton magnetic resonance spectroscopy (^{1}H-MRS) (Arnone et al, 2015; Schür et al, 2016).

The focus of this thesis is on ^{1}H-MRS studies of GABA and glutamate, in relation to reproductive factors in healthy premenopausal women, in MDD patients who were in major depressive episodes (MDEs) and in relation to the acute effects of exogenous administration of the stress hormone, cortisol. In chapter 1, I will summarize the basic physiology of the glutamatergic and GABAergic systems. In chapter 2, I will review how acute stress and glucocorticoids interact with glutamatergic neurotransmission, via both rapid non-genomic mechanisms and delayed genomic mechanisms. In chapter 3, I will outline how dysregulation of

the hypothalamic-pituitary-adrenal (HPA) axis in MDD leads to increased circulating glucocorticoids via a loss of feedback regulation. In chapter 4, I will describe the use of ^{1}H-MRS to measure amino acid neurotransmitters, with a focus on spectral editing methods used to measure metabolites with *J*-coupled spins, including GABA, glutamate and glutamine. In chapter 5, I will describe the normal menstrual cycle and mechanisms of how ovarian steroids and neurosteroids may influence GABA and glutamate neurotransmission. In the following three chapters, I will report original research studies that used ^{1}H-MRS to investigate GABA and glutamate *in vivo*.

Chapter 6 describes a study of normal variation in anterior cingulate/prefrontal cortex (ACC) GABA levels during the follicular and luteal phases of the menstrual cycle. The goal was to determine whether a phase difference previously reported for the occipital cortex (follicular GABA > luteal GABA) was also present in ACC in healthy premenopausal women. Secondary goals were to determine whether ACC GABA was influenced by age in premenopausal women and to determine whether ACC GABA levels differ in women using hormonal birth control.

Chapter 7 describes a case-control study of ACC GABA in MDD patients, who were in a MDE, compared with healthy controls, matched for age, sex and education. The hypothesis was that ACC GABA levels would be lower in patients with MDD.

Chapter 8 describes the acute (30 minutes) and delayed (24-48 hour) effects of cortisol (hydrocortisone) administration on ACC glutamate and glutamine in healthy volunteers. The hypothesis was that cortisol would decrease glutamine levels acutely and increase them after a delay.

Chapter 9 describes my conclusions from the above research.

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CHAPTER 1. GLUTAMATE AND GAMMA-AMINOBUTYRIC ACID (GABA)

1.1. Glutamate

1.1.1. Glutamate synthesis

Glutamate synthesis occurs primarily in two ways: by way of transamination of α-ketoglutarate from the tricarboxylic acid cycle (TCA cycle, also Kreb's Cycle) or from neuronal conversion of glutamine to glutamate by way of the enzyme glutaminase (see Figure 1) (Stahl, 2013).

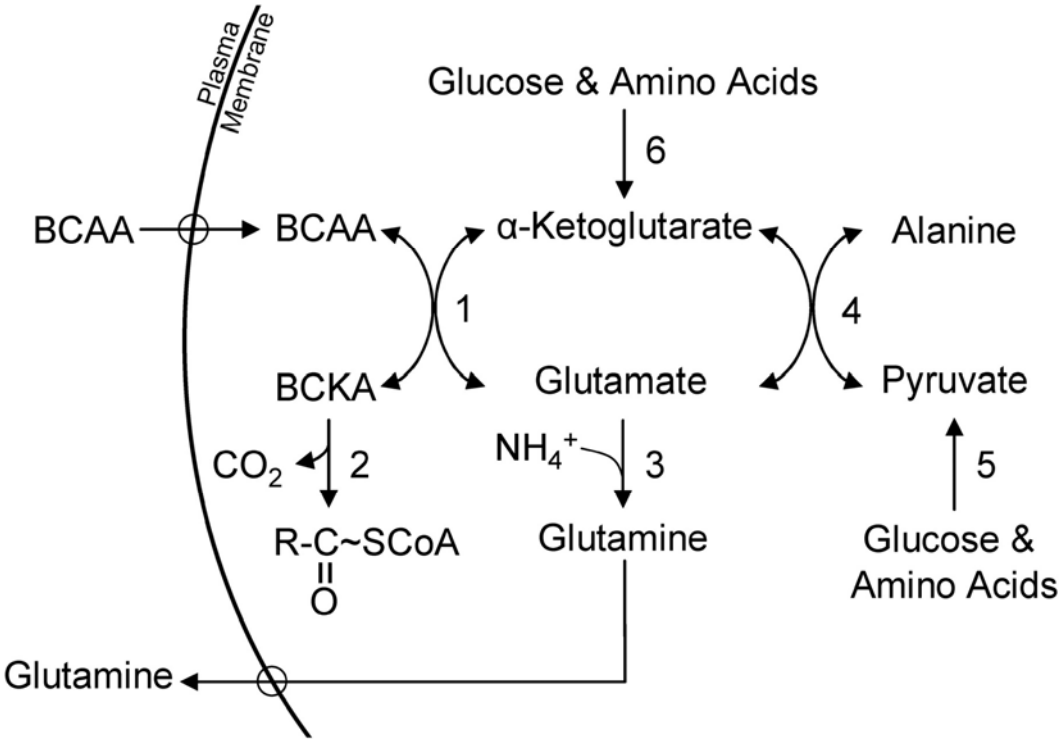

Figure 1.1. Glutamate synthesis from α-ketoglutarate (adapted from Self et al., 2004). BCAA = branched-chain amino acids; BCKA = branched-chain alpha-keto acids; R-C~ScoA = acyl coenzyme A.

In glutamatergic and GABAergic neurons, glutamine is converted to glutamate via the enzyme glutaminase. When glutamate acts as a neurotransmitter, replenishment of glutamate comes largely from glutamine supplied by glia, which is converted to glutamate by the mitochondrial enzyme glutaminase. Thus, a major role of glia is to take up glutamate from the synaptic cleft for conversion to glutamine by the enzyme glutamate synthetase. Glutamine is released from astrocytes (a type of glial cell) and taken up by neurons for conversion to glutamate (see Figure 2). This metabolic cycle is critical for glutamate neurotransmission (Stahl, 2013) and for ensuring that the TCA cycle continues to function optimally. The process of pyruvate reacting with CO_2 forms the TCA intermediary malate, which is a precursor of oxaloacetate, through pyruvate carboxylation. This reaction supports the glutamate-glutamine metabolic cycle – specifically the export of glutamine from astrocytes for uptake into neurons and conversion into glutamate (Hassel & Dingledine, 2006). This process ensures that the TCA cycle will continue to function by preventing excessive depletion of α-ketoglutarate.

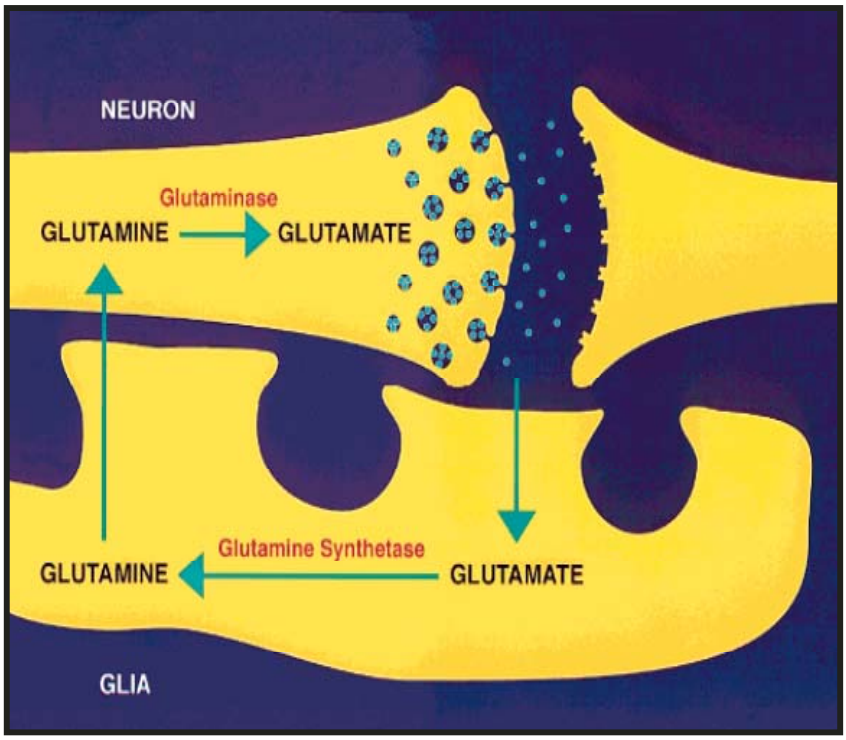

Figure 1.2. Glutamate metabolic cycle

1.1.2. Glutamate Metabolism

The metabolic cycle of glutamate and glutamine is one of several pathways of glutamate metabolism. Another of these pathways involves the conversion of glutamate to GABA, which is the principal inhibitory neurotransmitter in the brain. This pathway, known as the GABA shunt, uses the enzyme L-glutamic acid decarboxylase (GAD), as well as the co-factor pyridoxal phosphate. GABAergic systems play a key role in regulating central excitability (Deutch & Roth, 1999). Glutamate is also converted to glutathione, which plays a key neuroprotective role by reducing cell death during times of stress and acting as an anti-oxidant to protect against free radicals (Persson, 2006).

1.1.3. Glutamate Uptake and Release

Vesicular glutamate transporters (VGLUTs) package glutamate into vesicles in preparation for synaptic release. Once synaptic release is completed, Excitatory Amino Acid Transporters (EAATs) perform the task of clearing the neural synapse through reuptake by way of membrane-bound pumps. EAATs are responsible for terminating the excitatory signal, thereby preventing excitoxicity (Hassel & Dingledine, 2006).

1.1.4. Glutamate Receptors

Glutamate can bind to both ionotropic and metabotropic receptor sites. Ionotropic receptor sites are characterized by cation channels that open once glutamate binds to their receptor, while the key feature of the metabotropic receptor sites is the activation of intracellular second messengers through G-protein coupling. The ionotropic glutamate receptors (NMDA,

AMPA, and kainate) and metabotropic ($mGluR_{1-8}$) receptors are all composed of multiple subunits.

1.1.4.1. Ionotropic Receptors – N-methyl D-aspartate (NMDA) receptor

The NMDA receptor site is a complex that houses six distinct binding sites and has high Ca^{2+} permeability. Subunits of the NMDA receptor are grouped based on gene homology. NR1, encoded by a single gene, is present throughout the brain. NR2A, NR2B, NR2C, and NR2D are encoded by four genes, with differing concentrations throughout the forebrain, midbrain, hindbrain, and cerebellum. NR3A and NR3B are encoded by two genes and are present in high concentrations in the spinal cord and cortex, and the pons and medulla respectively (Hassel & Dingledine, 2006).

The NMDA receptor has binding sites for the agonists, glutamate and glycine. The receptor is activated by the binding of both co-agonists. Although the glutamate binding site can also bind aspartate, it does not activate the receptor to the same degree. Further, D-serine can also act as a co-agonist in place of glycine, with a higher binding affinity. Binding of glutamate and glycine leads to membrane depolarization and an expulsion of Mg^{2+}. Extracellular Mg^{2+} binds to a site on the NMDA receptor and acts as a voltage-dependent block of the open ion channel. A Zn^{2+} site also acts as an NMDA blocker, but in a voltage-independent manner. Thus, both Mg^{2+} and Zn^{2+} act as NMDA receptor modulators (Deutch & Roth, 1999).

The NMDA receptor also houses antagonist binding sites that act as voltage-dependent blockers. There is a binding site for phencyclidine (PCP), as well as for the non-competitive antagonist MK-801, which is a powerful anticonvulsant. Ketamine is another NMDA receptor antagonist, used for anesthesia and for its antidepressant action. There is also a polyamine

regulatory site that facilitates NMDA transmission. However, when this site is occupied at high concentrations, a modulatory voltage-dependent block is produced. Because this site can produce this voltage-dependent block, bound polyamines may inhibit or potentiate responses that are mediated by glutamate (Hassel & Dingledine, 2006).

The NMDA receptor is also sensitive to changes in acidity. Hydrogen ions can act as allosteric inhibitors and thus, by definition, will reduce NMDA receptor activity, specifically at the NR2B subunit, reducing the frequency of channel opening at this subunit (Hassel & Dingledine, 2006).

1.1.4.2. Ionotropic Receptors – AMPA and Kainate

The AMPA and kainate receptors have low permeability to Ca^{2+} ions, but will be active when there is an influx of Na^{+} ions. They both serve as receptors for rapid excitatory synaptic transmission that is glutamate modulated, but the binding profiles of these receptors are opposite to one another, as AMPA receptors will preferentially bind AMPA > glutamate > kainite, whereas kainate receptors will preferentially bind kainite > glutamate > AMPA. Once bound, rapid receptor desensitization occurs in milliseconds. The receptor subunits associated with AMPA receptors are $GluR_{1\text{-}4}$, while those associated with kainate receptors are $GluR_{5\text{-}7}$, KA1, and KA2 (Kandel & Siegelbaum, 2000).

1.1.4.3. Metabotropic Glutamate Receptors

Metabotropic glutamate receptor (mGluR) subtypes are categorized into three distinct classes based on genetic homology. Subunits $mGluR_1$ and $mGluR_5$ are categorized in Class I. Their primary function is the stimulation of phospholipase C and release of Ca^{2+}, which in turn

promotes the formation of inositol triphosphate (IP_3). Phospholipase C also promotes the formation of diacylglycerol (DAG) which activates protein kinase C. IP_3 and DAG are important in signal transduction (Stahl, 2013).

Subunits $mGluR_2$ and $mGluR_3$ belong to Class II, while $mGluR_{4,6,7\ and\ 8}$ belong to Class III. Both of these classes inhibit the production of adenylate cyclase, thereby reducing the activity of cyclic adenosine monophosphate (cAMP). Formed from adenosine triphosphate (ATP), cAMP is a second messenger that is primarily responsible for intracellular signal transduction (Kandel & Siegelbaum, 2000).

1.2. GABA

GABA is the most abundant inhibitory neurotransmitter in the central nervous system (CNS). It is present in high concentrations in the brain and spinal cord, and in low concentrations peripherally. It does not cross the blood-brain barrier. The binding of GABA to the receptor opens the chloride channel, allowing negatively charged chloride (Cl-) ions to flow into the cell and positively charged potassium ions (K+) to flow out of the cell, leading to hyperpolarization. An exception is during foetal development, when GABA can also transiently be excitatory, leading to depolarization (Luhman et al, 2014). Further, GABA can also have an indirect excitatory effect on glutamate via chandelier cells – types of GABAergic relay or interneuron cells present in the cortex. Chandelier cells have been shown to depolarize pyramidal cells, resulting in signal excitation (Szabadics et al, 2006).

1.2.1. GABA synthesis and metabolism

GABA is synthesized in the brain from glutamate via the enzyme glutamic acid decarboxylase (GAD) along with the co-factor pyridoxal phosphate. It can subsequently be converted back to glutamate via the GABA shunt pathway (Schousboe et al, 2013).

Prior to glutamate conversion to GABA, the first step of the shunt pathway is the conversion of α-ketoglutarate, from the Kreb's cycle, to glutamate via α-oxoglutarate transaminase. Glutamate is then converted neuronally to GABA through the actions of GAD via decarboxylation. GAD expression is restricted to GABA neurons and not glia. Cytoplasmic GABA is then packaged into transport vesicles for delivery to the presynaptic terminal and subsequent release into the synaptic cleft. GABA is removed from the synaptic cleft by GABA transporters. At this point, GABA is either returned to the terminal where it is repackaged for release, or taken up by glial cells. In glia, GABA is converted to succinic semialdehyde by GABA transaminase (GABA-T). Succinic semialdehyde is converted to succinic acid by succinic semialdehyde dehydrogenase, and this compound is eventually returned to the TCA cycle (Schousboe et al, 2013).

1.2.2. GABA receptors

GABA can bind to two general classes of receptors: $GABA_A$ and $GABA_B$. The ligand-gated ionotropic receptor $GABA_A$ has a GABA binding site that is coupled with a Cl^- channel. There are 15 distinct $GABA_A$ subunits. Bicuculline, a strong convulsant, is a competitive antagonist at the GABA binding site. Along with the GABA binding site, $GABA_A$ receptors have several other binding sites, including benzodiazepines, such as diazepam. The benzodiazepine binding site acts as an allosteric modulatory site as, when bound, benzodiazepines increase the

action of GABA to open the chloride channel. $GABA_A$ receptors also have a binding site for barbiturates, which can open the chloride channel. Both the benzodiazepine and barbiturate sites increase GABA receptor activation and prolong the time that the Cl^- channel remains open. Similarly, ethanol will also augment GABA receptor functioning, while penicillin tends to block this effect. Picrotoxin is a non-competitive antagonist that can result in stimulant and convulsant actions. Furthermore, the $GABA_A$ receptor houses a binding site for neuroactive steroids, such as allopregnanolone, which is also a positive allosteric modulator ((Backstrom et al., 2014). Typically, anxiolytic or anti-convulsant medications that do not act as allosteric modulators will have effects on the enzymes that promote GABA synthesis or limit its degradation. For example, vigabatrin, an anti-convulsant, acts by irreversibly inhibiting the GABA-T enzyme responsible for GABA conversion to succinic semialdehyde (Todd and Baker, 2008).

The $GABA_B$ receptors are metabotropic and belong to the super family of G-protein coupled receptors. These receptors are not affected by bicuculline. They can be activated by the muscle relaxant baclofen, a competitive agonist, and inhibited by phaclofen, a competitive antagonist (Kerr et al., 1987). $GABA_B$ receptors are expressed both pre- and post-synaptically and act to open K^+ channels. $GABA_B$ receptors produce longer lasting inhibition than $GABA_A$ receptors, but they also require stronger stimulation to generate an inhibitory post-synaptic potential (IPSP). Presynaptically, $GABA_B$ receptors function as autoreceptors and inhibit further GABA release from the presynaptic terminal. Interestingly, $GABA_B$ autoreceptors can also inhibit the release of other neurotransmitters such as noradrenaline, dopamine, serotonin and substance P, by inhibiting Ca^{2+} channels and decreasing the Ca^{2+} influx that occurs when an action potential is generated (Nestler et al, 2015).

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CHAPTER 2. GLUCOCORTICOIDS, STRESS & EXCITATORY NEUROTRANSMISSION

Glucorticoids are a unique type of steroid hormone synthesized in the adrenal cortex. Along with mineralocorticoids, glucocorticoids are a type of corticosteroid. While mineralocorticoids serve a critical function of balancing electrolytes through kidney regulation, glucocorticoids are the key in stress responses, regulating inflammation and lipid and protein metabolism.

2.1. Glucocorticoids and the brain

Central effects of corticosteroids depend on their ability to cross the blood-brain barrier, which separates circulating capillary blood from the extracellular fluid in the brain. Larger molecules may be restricted from the brain, whereas smaller molecules can diffuse across the blood-brain barrier. Although corticosteroids can diffuse across the blood-brain barrier, some, such as dexamethasone and cortisol, are restricted from the brain via P-glycoproteins, which remove foreign substances. Thus, in cases of corticosteroid depletion, dexamethasone treatment is not a viable treatment option due to poor diffusion through the blood-brain barrier (De Kloet, 1997).

Once cortisol has crossed through the blood-brain barrier, it is acted upon by 11β-hydroxysteroid dehydrogenase (HSD), which exists in Type 1 and Type 2 isoforms, with the Type 1 isoform being more prevalent in the brain. The Type 2 isoform exists in greater numbers in the kidneys where it serves to inactivate glucocorticoids, thereby allowing the mineralocorticoid aldosterone to bind and control salt and water balance in the body. The Type 1 isoform is more present in brain regions, as its primary actions involve converting the inactive

dehydroxycorticosterone and cortisone to the active corticosterone and cortisol (Molinari et al, 2013).

During basal glucocorticoid release, mineralocorticoid receptors (MRs) are generally more active than glucocorticoid receptors (GRs) as MRs have a much higher affinity for glucocorticoids than mineralocorticoids (approximately tenfold higher). However, during bursts of high cortisol secretion, MRs become saturated and GRs are more activated than at basal cortisol levels. GR occupancy is therefore higher during the peak of the diurnal variation of cortisol levels (mornings for those with regular sleep-wake cycles), or when cortisol levels are elevated during stress. The increased GR activation at the end of the sleep, or the inactive period, anticipates increased metabolic demands during the day, or during the active period (Joëls and de Kloet, 1992). The diurnal cycle is controlled by the suprachiasmatic nucleus of the hypothalamus (Kalsbeek & Buijs, 2002).

Glucocorticoids and mineralocorticoids exert their classical effects through genomic actions. They bind to intracellular cytosolic MRs and GRs, which then interact with chaperone proteins and are transported to the cell nucleus, where they regulate DNA transcription. Such genomic effects have a relatively slow onset (hours to days). However, glucocorticoids are also able to exert rapid effects occurring within seconds or minutes that do not involve gene transcription (Joëls et al, 2012).

2.2. Glucocorticoids and glutamate neurotransmission

2.2.1. Glucocorticoid dependency of glutamate response to stress

Stress and glucocorticoid release influence glutamate neurotransmission. Pioneering work used *in vivo* microdialysis to investigate extracellular glutamate levels in CA3 hippocampus, in rats that were either adrenal-intact or adrenalectomized (Lowy et al, 1993). Physical restraint stress led to a very rapid elevation (76% over baseline) of extracellular glutamate in the CA3 hippocampal region in adrenal-intact rats that was significantly attenuated in the adrenalectomized animals.

This link was further explored in the medial prefrontal cortex (PFC), striatum, nucleus accumbens and hippocampus (Mogghadam et al, 1994). In this study, three groups of rats were examined: adrenalectomized rats with a placebo pellet implanted, adrenalectomized rats that had a corticosterone pellet implanted, and adrenal-intact rats. Restraint stress was applied for 20 minutes, with dialysate being collected for 2 hours. The glutamate response in all brain regions was attenuated in the rats that were adrenalectomized and had an implanted placebo pellet. However, there was no significant difference in the glutamate levels of the rats that had their adrenal glands intact and the rats that had been adrenalectomized, but implanted with a corticosterone pellet. The latter two groups had elevated glutamate levels in all of the brain regions examined. This study demonstrated that corticosterone availability affects extracellular glutamate and that the attenuated response due to adrenalectomy can be abolished with corticosterone replacement.

Subsequent research examined the relationship between corticosterone and extracellular glutamate in a paradigm that included temporal dynamics (Bagley and Moghaddam, 1997).

Hippocampal and PFC glutamate was measured with microdialysis in response to three consecutive tail-pinch stressors at 2.5 h intervals. Extracellular glutamate concentrations increased rapidly in the PFC in response to the stressor, but changes in glutamate were not as profound with each subsequent stressor, suggesting habituation. Results from the dorsal hippocampus showed similar, but less profound, increases in extracellular glutamate than the PFC. Furthermore, habituation did not occur with repeated tail-pinches, suggesting that the PFC may play a stronger adaptive response to repeated stressors than the hippocampus.

The involvement of corticosteroids in glutamate release was further examined using microdialysis to measure extracellular glutamate and GABA in response to direct dexamethasone infusion into the hippocampus, with striatal infusions as a regional control (Abraham et al, 1996). Glutamate was selectively increased 280% in the hippocampus and not striatum by dexamethasone, with no response in either region to control infusions of the steroid precursor, cholesterol. Extracellular GABA was unaffected.

2.2.2. Non-genomic glutamate response to glucocortioids

The rapid elevation in extracellular glutamate indicated a non-genomic mechanism of action of dexamethasone infusion. In a subsequent study, corticosterone was infused either peripherally, or directly into the hippocampus of sham-operated or adrenalectomized rats. Corticisterone led to increases in extracellular glutamate within 15 minutes that were not prevented by the administration of the protein synthesis inhibitor, anisomycin, confirming a non-genomic pathway (Venero & Borrell, 1999).

2.2.3. Mechanisms of rapid non-genomic effects of corticosteroids

Rapid excitatory effects of corticosteroids that are not due to translational or transcriptional changes, or protein synthesis, have now been well documented in several brain regions. These effects appear to be mediated by glutamate via the AMPA and NMDA receptors.

Glucocorticoids have a higher affinity to cytosolic MRs and lower affinity to cytosolic GRs, so that increases in glucocorticoids will classically lead to increases in binding to GRs at concentrations above which MRs are already saturated. However, it has been shown subsequently that the rapid, non-genomic effects of glucocorticoids may be mediated by the actions of MRs (Karst et al., 2005). In this study, corticosterone administration increased glutamate release, as evidenced by miniature excitatory post-synaptic currents (mEPSCs) in the CA1 region of hippocampal slices within 10 minutes, even after pre-treatment with cycloheximide, which inhibits protein synthesis. This effect occurred at glucocorticoid concentrations above those that would saturate classical MRs, showing a 10-fold lower receptor affinity. Furthermore, it was not antagonized by a selective GR antagonist and could be reproduced in hippocampal slices from GR knockout mice. In contrast, it was not reproduced by a selective GR agonist with low affinity for MR, could be blocked by pre-treatment with a MR antagonist and was abolished in hippocampal slices from MR knockout mice. Furthermore, when corticosterone was conjugated with bovine-serum-albumin (BSA), which reduces the ability of corticosterone to enter the cell cytoplasm (Daufeldt et al, 2003), the corticosterone-BSA conjugate still resulted in a strong mEPSCs. These data suggested that these non-genomic effects were mediated by membrane MRs with lower affinity than classical cytosolic MRs that respond rapidly to stress hormone release to modulate excitatory neurotransmission during stress.

A subsequent extension of this research examined the onset and offset of effect in the amygdala in intact animals after an initial restraint stress. Once corticosterone was brought back to baseline, mEPSCs also returned to pre-treatment levels, indicating this is a rapidly reversible phenomenon contingent upon corticosterone. A second elevation of corticosterone (1 hour later) resulted in a mEPSC very similar to the first – an effect that was not altered with the introduction of cycloheximide, thereby indicating this relationship was not dependent on a genomic pathway (Karst et al, 2010).

Further evidence of a rapid non-genomic response came from studies of the involvement of the presynaptic extracellular signal-regulated kinase 1/2 (ERK 1/2) (Olijslagers et al., 2008). This pathway is thought to play a role in learning (Kushner, 2005) as well as aid in synaptic plasticity (Sweatt, 2004). Blockade of the ERK 1/2 phosphorylation pathway by inhibitors U0126 and PD098059 in CA1 hippocampus resulted in a diminished MR-mediated non-genomic response, indicating that rapid non-genomic effects increase glutamate release pre-synaptically, as well as membrane activation post-synaptically.

In addition to hippocampus, rapid glucocorticoid effects have been observed in other brain regions, including the basolateral amygdala (BLA), in which the increase in mEPSC frequency remains persistent even after the clearance of corticosterone (Karst et al, 2010). Glucocorticoids can influence glutamate transmission in PFC (and other brain regions) via interactions with the endocannabinoid system, leading to increased endocannabinoid synthesis and the inhibition of glutamate release via CB1 receptors (Hill et al, 2009). Further evidence of the acute effects of stress on prefrontal glutamate release has been derived from studies using synaptosomal preparations, which show increased presynaptic glutamate release in rats exposed

to acute footshock stress, which appears to involve regulation of the binding of synaptic vesicles to neuronal membranes (Musazzi et al, 2010).

2.3. Delayed effects of glucocorticoids on glutamate neurotransmission.

Glucocorticoids easily cross the cell membrane into the cytoplasm, where they bind to receptors and chaperone proteins, creating ligand-receptor complexes. These complexes are transported to the cell nucleus, where they bind to regulatory sites on DNA that increase or decrease gene transcription. Cytosolic MRs and GRs classically act as transcription factors and their effects occur over hours to days (Joëls & de Kloet, 1992). However, whereas there have been several studies of the acute effects of stress and glucocorticoids on glutamate release, their chronic effects have received very limited study, except to show that chronic stress may lead to more prolonged glutamate release in CA3 hippocampus in response to a subsequent acute stressor (Yamamoto and Reagan, 2006).

The rat ventral hippocampus, which is involved in affective memory and regulation, may respond differently to corticosterone than the dorsal hippocampus, which is involved in visuospatial memory. In the ventral hippocampus, corticosterone lowered the threshold potential to generate action potentials, making the cells more excitable (Maggio & Segal, 2009). Glucocorticoids have also been shown to increase the amplitude of calcium currents (Karst et al, 1994). This effect persisted for hours after the removal of glucocorticoids and involved the formation of glucocorticoid chaperone dimers and increased expression of calcium channel subunits, indicating a genomic response (Joëls et al, 2003). When depolarization of CA1 pyramidal neurons occurs, there is a resulting action potential. However, a slow calcium-dependent potassium current is also initiated, so the frequency of the action potential will slowly

accommodate during depolarization. Neural accommodation (or, ramp depolarization) is defined as depolarization of a neuron by a slowly rising current. Unlike sudden depolarization, the slowly rising depolarization can mediate activation or inactivation, as well as potassium channel permeability, while never evoking an action potential (Liebmann et al., 2008). When termination of depolarization occurs, the calcium-dependent potassium channel is slowly deactivated, but there is a resulting after-hyperpolarization (AHP). High stress levels of corticosterone enhance the amplitude of the calcium-dependent potassium currents, as well as AHP in CA1 pyramidal neurons, persisting for 1-4 hours after the initial corticosterone spike. It has been suggested that the AHP may serve to dampen excitatory responses following their initial augmention by corticosterone (Liebmann et al., 2008).

When examining what occurs at a single synapse in the region after a corticosterone spike, the observed effect is similar to what is seen during long-term potentiation (Karst & Joëls, 2005). At CA1 hippocampal neurons, corticosteroids enhanced the amplitude, but not frequency of mEPSCs, with a delayed response after 60 minutes that peaked at 150-200 minutes after exposure. Furthermore, inhibitory post-synaptic current (IPSC) amplitude was enhanced in the dorsal hippocampus via GRs, but reduced in the ventral hippocampus via activation of MRs, indicating that corticosteroids may have opposing, or balancing/regulatory delayed effects in different hippocampal subregions (Maggio & Segal, 2009).

Research examining corticosteroid effects on the basolateral amygdala (BLA) show a delayed and prolonged ramp depolarization and reduced IPSPs. This effect was not caused by a corticosteroid-induced GABA reduction, as only GABA-A receptor associated IPSPs changed, and not GABA-B receptor associated IPSPs, suggesting a postsynaptic effect (Duvarci & Pare, 2007).

2.4. Glucocorticoids and memory

2.4.1. Glucocorticoids and synaptic plasticity

Given that GRs are plentiful in the hippocampus and PFC in humans, regions that are critical for memory, it is important to note that acute administration of cortisol can exert rapid effects on human memory. Given this premise, there has been much research done on glucocorticoids and memory modulation, as well as specific mechanisms of actions concerning enhancing and impairing memories. Memory enhancement and impairment involve long-term potentiation (LTP) or long-term depression (LTD) which are both examples of synaptic plasticity.

LTP is accomplished by a process that involves co-activation of neurons that results in synaptic strengthening and, ultimately, memory encoding, whereas LTD leads to a long-lasting decrease in synaptic efficiency (Cooke & Bliss, 2006). The underlying mechanism of action of LTP is modulated by the hippocampus, glutamatergic neurotransmission, and the NMDA receptor. NMDA receptor antagonists impair hippocampal-dependent memory tasks in rodents, for example, spatial memory performance in the Morris water maze (Morris et al, 1986). Hippocampal excitatory neurotransmission is a critical component in this type of memory, but glucocorticoids also play a role in this process. Glucocorticoids can affect both memory consolidation and memory retrieval. Glucocorticoid administration immediately after a training experience has been shown to enhance memory consolidation, suggesting that the endogenous release of cortisol after a traumatic or impactful event could also facilitate the storage of that memory (Roozendaal, 2002). There is further evidence of HPA axis involvement, in that memory consolidation is impaired in adrenalectomized animals, but restored with corticosterone

replacement, thus suggesting that glucocorticoid availability mediates memory consolidation (Roozendaal et al, 1996). In order to adequately study memory consolidation, there needs to be a long interval between training and drug administration to retrieval (24-48h) to ensure that consolidation has occurred, because circulating glucocorticoids impair memory retrieval shortly after training and prior to consolidation (Roozendaal, 2002).

2.4.2. Glucocorticoids and human memory

A meta-analysis of acute cortisol administration and subsequent effects in humans reported heterogeneity in the findings (Het et al, 2005). The meta-analysis classified studies into two distinct categories. First, in line with Roozendaal and colleagues' review, the research in which glucocorticoid administration occurred prior to a memory retrieval task predominantly showed impaired retrieval in placebo controlled trials (Roozendaal et al, 1996). In the second category, the meta-analysis findings indicated there was no overall effect when glucocorticoid administration occurred prior to training. However, there was some individual study variance. One study found that the administration of cortisone (25mg) in humans, one hour before a delayed retention test and 24 hours after word presentation, resulted in significant impairment in the number of freely recalled words compared to placebo. However, when cortisone was administered one hour before or immediately after the word was presented, there was no observable impairment. This research suggested that high glucocorticoid levels could result in retrieval impairment in stressful settings (de Quervain et al, 2000). However, another study found that cortisol administration had a beneficial effect on declarative memory. This beneficial effect was not affected by whether the memory was emotional or neutral. Two doses of cortisol were used, one thought to facilitate memory (20mg) and the other thought to impair it (40mg), to mimic mild stress to severe stress responses. This is also due to the fact that previous research

has demonstrated an inverted U-shaped relationship between memory and glucocorticoids. Fewer recall errors were observed in the cortisol groups compared with the placebo group, with greater memory facilitation in the 20mg group, compared with the 40mg group (Abercrombie et al, 2003). In an event-related potential (ERP) study, hydrocortisone administration resulted in impairment in facial recognition following a 30 minute delay, with no change in object recognition tasks (Monk and Nelson, 2002).

Research involving glucocorticoids and their relationship with memory may be limited by many inherent confounds. When reviewing the literature (see above), it becomes apparent that more research is required with respect to specific memory types (e.g., working memory, declarative memory, etc.), timing of the study due to the diurnal variations of cortisol, and also timing with memory type taken into consideration.

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CHAPTER 3. THE HYPOTHALAMIC-PITUITARY-ADRENAL (HPA) AXIS

Physiological and psychological stress can have very profound effects on neurological functioning. Stress responses are primarily mediated by the HPA axis, which is regulated by positive and negative feedback. Dysregulation of the HPA may contribute to several mental disorders, including posttraumatic stress disorder (PTSD) (Heim & Nemeroff, 2009) and MDD (Pariante & Miller, 2001).

3.1. The acute stress response and glucocorticoid feedback

An acute stressor results in activation of the paraventricular nucleus (PVN) of the hypothalamus, which releases corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) into the portal circulation. CRH and AVP co-activate adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary into the general circulation. ACTH acutely stimulates the release of cortisol and chronically promotes growth of the adrenal cortex. In healthy humans, increased circulating cortisol exerts negative feedback on this release cascade, inhibiting CRH and ACTH release by activating GRs at the levels of the PVN and pituitary gland. In addition to its actions at the levels of the hypothalamus and pituitary, cortisol also activates GRs and MRs in regions that include the amygdala, hippocampus and prefrontal cortex that have regulatory inputs to the PVN. Whereas activation of GR in the hippocampus and prefrontal cortex exerts an inhibitory downstream effect on the PVN, consistent with a long negative feedback loop, in the amygdala it exerts positive downstream effects on the PVN (Herman et al, 2016).

Feedback inhibition influences circulating cortisol concentrations and different mechanisms of feedback operate over different time intervals (Herman et al., 2016). For

example, fast feedback operates over a period of several minutes and has been shown to involve a non-genomic effect of glucocorticoids on cannabinoid transmission in the hypothalamus (Di et al, 2003). However, chronic elevations of cortisol may act over hours and this delayed feedback has been assessed clinically, using dexamethasone suppression, in which next day cortisol levels are measured after a night-time dose of dexamethasone (Miller et al., 1992).

3.2. Tests of HPA axis function

3.2.1. The Dexamethasone Suppression Test (DST)

It has been well established that individuals with MDD have dysregulated HPA axis function. Specifically, hyperactivity of the HPA axis leads to increased circulating cortisol (Gibbons, 1964). This finding has been replicated many times over the years and has been supported by findings using the DST (Ribeiro et al., 1993). The DST involves administering a nighttime dose of dexamethasone, typically 1mg, and then measuring plasma cortisol the following afternoon. Elevated cortisol levels on the following afternoon, above a normal laboratory threshold, indicate escape from negative feedback by dexamethasone. Previous research indicates that non-suppression is prevalent in only 6-8% of the healthy population (Zimmerman & Coryell, 1987). An early meta-analysis conducted by Ribeiro et al. (1993) showed that non-suppression of cortisol in individuals diagnosed with MDD is observed in around 50% of patients and is linked with treatment outcomes. Specifically, cortisol non-suppression after DST administration was correlated with more frequent early relapse after hospital discharge. In contrast to MDD, in PTSD Yehuda et al. (1993) used a lower dose of dexamethasone to demonstrate that PTSD patients showed enhanced cortisol suppression compared to healthy controls.

3.2.2. The Dexamethasone-Corticotropin-Releasing Hormone test

Although dexamethasone exerts negative feedback at the level of the pituitary, its brain entry is limited by P-glycoproteins, such that it may reduce glucocorticoid feedback within the brain and lead to activation of the PVN. This leads to uncertainty whether the escape from cortisol suppression is due to higher stimulatory drive to the pituitary, or decreased negative feedback at the pituitary. The combined dexamethasone-CRH (DEX-CRH) test was devised so that negative feedback is assessed in the presence of a standardized stimulatory drive, by measuring the ACTH response to CRH on the afternoon following the administration of a nighttime dose of dexamethasone. The DEX-CRH test increases the sensitivity to detect reduced negative feedback in MDD to over 80% of subjects, compared with approximately 50% for the standard DST (Holsboer, 2001).

3.2.3. The Prednisolone Suppression Test

Further, Pariante and colleagues (2002) examined the limitations of the DST and developed the prednisolone suppression test (PST) to assess impaired negative feedback. By using a non-selective synthetic corticosteroid, they proposed that both impairment and enhancement of the negative feedback loop could be reliably investigated, since dexamethasone is selective to GRs, but not MRs, whereas cortisol and prednisolone bind to the corticosteroid binding globulin (CBG) and to both GRs and MRs, which are involved in glucocorticoid feedback (Pariante et al, 2002). Glucocorticoids have a higher affinity to classical (non-membrane-associated) MRs and will bind further to GRs at concentrations at which MRs are saturated. MRs serve more regulatory functions, such as the regulation of diurnal secretion patterns, whereas GRs are involved in stress responses (de Kloet et al., 1998). In their

comparison of the DST and PST, Pariante and colleagues (2002) examined the MR and GR responses to dexamethasone and prednisolone in patients with treatment-resistant depression and a history of childhood abuse, in comparison with healthy controls. The patient sample showed increased escape from dexamethasone suppression, compared with controls, but normal prednisolone suppression, suggesting that there may be differences in negative feedback above the level of pituitary in MDD.

The findings of HPA axis dysregulation in MDD and PTSD together with evidence for interactions between glucocorticoids and neurotransmission, discussed in chapter 2, suggest that the effects of altered circulating glucocorticoids on neurotransmission could play a role in the pathophysiology of these disorders.

3.3. References

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CHAPTER 4.THE MENSTRUAL CYCLE

4.1. Menstrual cycle phases

4.1.1. The ovarian cycle.

The menstrual cycle (MC) occurs in mammalian females during the reproductive phase of their lives. It consists of both the ovarian and endometrial cycles, driven primarily by the hypothalamic-pituitary-gonadal (HPG) axis. The ovarian cycle consists of the follicular phase and the luteal phase. The follicular phase begins with the start of menstruation and lasts around 14 days. In this phase, folliculogenesis occurs, which is the development of ovarian follicles under the drive of follicle stimulating hormone (FSH), released from the anterior pituitary. During this phase, granulosa cells of the follicles increase their estradiol production, which in turn increases lutenizing hormone (LH), released from the anterior pituitary. Release of both LH and FSH are regulated by the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. The surge in LH at mid-cycle results in ovulation, when a follicle ruptures and releases a fully developed oocyte, ready for fertilization. In the luteal phase, the follicle turns into the corpus luteum. Luteal cells secrete progesterone and estrogen, but in the absence of conception, towards the end of this phase, the corpus luteum diminishes sex steroid production, resulting in the degeneration of the uterine lining and menstruation (Jones and Lopez, 2014).

4.1.2. The endometrial cycle

The endometrial cycle itself has three phases: proliferative, secretory, and menstrual. In the proliferative phase (overlapping with the follicular phase of the ovarian cycle), repair and endometrial growth occur. The secretory phase begins once ovulation is complete and coincides

with the luteal phase of the ovarian cycle. It ends once progesterone production falls and the corpus luteum atrophies and menstruation begins (Jones and Lopez, 2014).

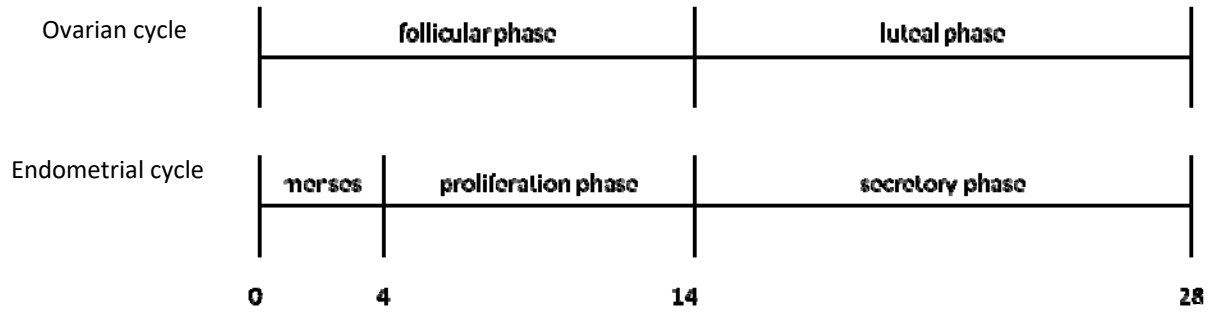

Figure 4.1. Phases of the menstrual cycle.

4.2. Ovarian Steroids

Ovarian steroids can have both slow genomic mechanisms of action, mediated primarily by intracellular receptors that regulate gene transcription, or rapid non-genomic actions through membrane receptors and activation of second messenger systems (Thakur & Paramanik, 2009). The primary ovarian steroids are estrogen and progesterone.

4.2.1. Estrogens

Estrogens are a group of sex steroids that constitute the primary female sex hormone. These steroids can easily diffuse across cell membranes where they bind intracellularly to estrogen receptors (ER). The estrogen/ER complex binds to regulatory sites on DNA to activate gene transcription. The estrogen/ER complex can also bind to and activate G-Protein Coupled Estrogen Receptor-30 (GPR30), resulting in rapid signaling (Prossnitz and Barton, 2014). There

are three main types of estrogens. Estrone (E1), which predominates during menopause but is present throughout the lifetime, can be converted to the more powerful estradiol (E2) through the actions of the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD). Estradiol is present in high concentrations in serum once the female is capable of reproduction. Through the actions of the liver, both estradiol and estrone can be converted into the weaker estriol (E3), which is present in higher concentrations during pregnancy (Jones and Lopez, 2014). Commonly, the term 'estrogen' is used to describe the most abundant female sex hormone, estradiol. Estrogens are synthesized from cholesterol, via androstenedione and testosterone as intermediate steps.

4.2.2. Progesterone

Progesterone is a steroid hormone of the progestogen class, synthesized by the corpus luteum, but it is also produced by the adrenal glands. Progesterone is synthesized from cholesterol, via pregnenolone as an intermediate step. Progesterone is also a precursor of mineralocorticoids, as well as androstenedione, which can be converted into estrogens. During the different phases of the menstrual cycle, there are changes in progesterone levels. Before ovulation, progesterone levels are relatively low. However, they increase after ovulation and remain high during the luteal phase. If conception occurs, human chorionic gonadotropin (hCG) is released to maintain the corpus luteum, and thereby progesterone production. Although progesterone is a precursor for mineralocorticoids, it is also a potent MR antagonist (Rupprecht et al., 1993). Progesterone acts both intracellularly at the progesterone receptor, but also has non-genomic actions via a membrane receptor (Jang & Yi, 2005).

4.3. Ovarian Steroids and Mood

Classical ER and PR, GPER30 and PGRMC1 are distributed within brain regions involved in emotion regulation, including the amygdala, hippocampus and cortex (Hazell et al, 2009; Intlekofer et al, 2011; Wharton et al, 2012). Ovarian steroids have modulatory effects on monoamine, glutamate and GABA neurotransmission (Toffoletto et al, 2014). For example, monoamine oxidase-A and catechol-O-methyl transferase expression are regulated by estradiol and progesterone (Gundlah et al, 2002); estradiol enhances NMDA receptor-mediated glutamate transmission (Smith et al, 2009) and both estradiol and progesterone increase brain-derived neurotrophic factor (BDNF) expression in the hippocampus (Aguirre and Baudry, 2009). In addition to being derived from the metabolism of circulating progesterone, a variety of steroids, including allopregnanolone, can also be synthesized *de novo* in the brain, such that they are classed as neurosteroids, which can exert positive or negative modulatory effects on GABA-A receptor function (Backstrom et al, 2013; Mackenzie et al, 2007). An altered sensitivity to the increase in circulating neurosteroids during the luteal phase of the MC has been proposed to mediate mood symptoms in women with premenstrual dysphoric disorder, since patients also show abnormal responses to exogenously administered neurosteroids and other GABA-A receptor modulators (Backstrom et al, 2013). Although a specific relationship with ovarian steroids has not established, variations in brain GABA levels, as measured using ^{1}H-MRS, have also been demonstrated in some studies of the MC in healthy women and women with premenstrual dysphoric disorder (Epperson et al, 2002; Liu et al, 2015).

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CHAPTER 5. PROTON MAGNETIC RESONANCE SPECTROSCOPY (^{1}H-MRS) OF GABA, GLUTAMATE AND GLUTAMINE.

5.1. ^{1}H-MRS

5.1.1. Basics of ^{1}H-MRS

In vivo proton Magnetic Resonance Spectroscopy (^{1}H-MRS) has become a useful tool for non-invasively measuring the brain chemistry of neurotransmitters and neurometabolites in living individuals. When placed in a static magnetic field, nucleii with an odd number of nucleons precess at a frequency proportional to the magnetic field strength and an element dependent constant known as the gyromagnetic ratio. In addition they align with the magnetic field with some parallel, and some anti-parallel, and with a slightly higher population parallel. If an RF pulse is applied at the frequency of precession, the nucleii are efficiently rotated from this equilibrium parallel to the main field, and with sufficient RF lie perpendicular. The small difference in the parallel / anti-parallel population can be measured while it is precessing perpendicularly to the main magnetic field since it induces an electromagnetic field (EMF) in the RF coil, known as the NMR signal. This signal decays over time and this decay is referred to as the transverse relaxation time or T2. Return of the nuclei to their equilibrium state aligned with the main field follows another relaxation process known as longitudinal relaxation or T1. The electrons in the chemical bonds within metabolites influence spins, by shielding protons from the magnetic field, which changes the frequency of the RF signal, such that the signal peaks in the different chemical environments of different metabolites are spread along the chemical shift axis (figure 5.1), in parts per million (ppm). This is independent of the field strength of the scanner, measured in Tesla (T). The area of metabolite peaks is related to the concentration of the

metabolites. The linewidth is related to the T2 relaxation time and the homogeneity of the magnetic field. Metabolites may have protons located in different molecular groups within the molecule, producing more than one signal in the chemical shift axis. Some metabolite signals have singlet peaks (shaded in figure 5.1); in the brain, the major singlet peaks include N-acetylaspartate (NAA), choline (Cho) and creatine (Cr), which have been measured in numerous studies of psychiatric disorders.

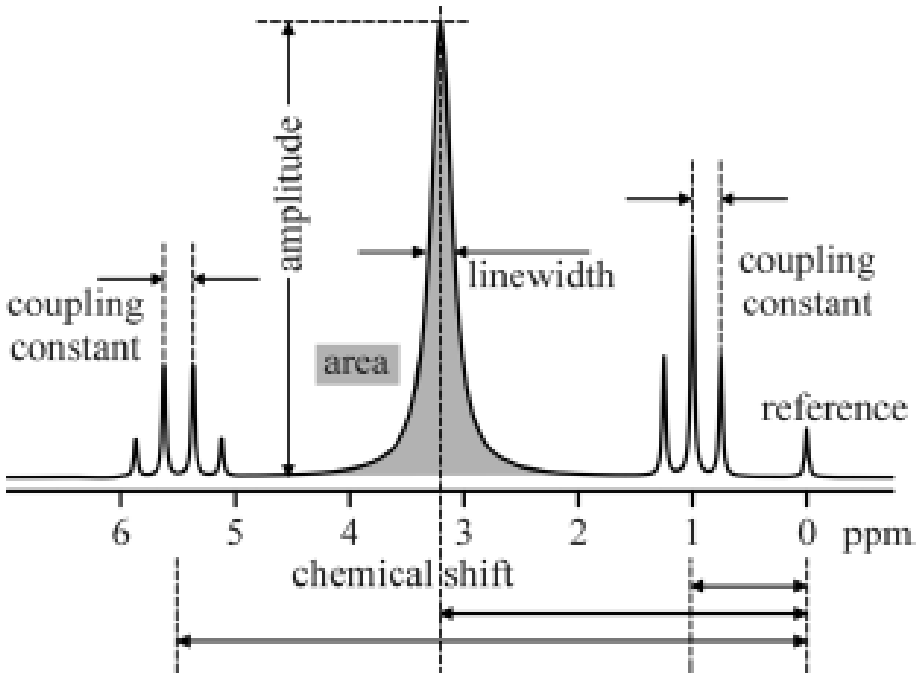

Figure 5.1. Illustrative magnetic resonance spectrum and terminology

5.1.2. Coupled spins

Other metabolite signals are split in multiplets due to scalar (*J*) coupling, resulting from interactions of adjacent nuclear spins that have a different electronic environment. These interactions split the signal by a coupling constant (*J* Hz) and the number and amplitude of the

multiplet peaks depends on the number of coupling partners (Thompson and Allen, 1998). Since the signal is spread along the chemical shift axis and reduced in amplitude, concentrations of metabolites with coupled spins may be harder to measure. In the brain, GABA, glutamate and glutamine are examples of metabolites with coupled spins (Harris et al, 2017).

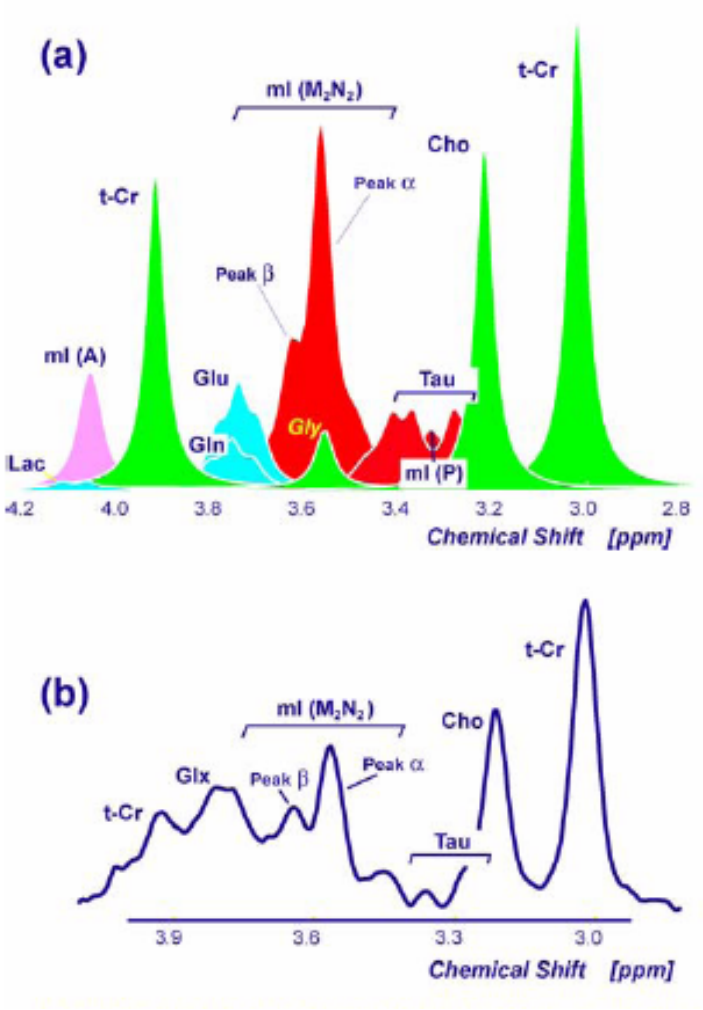

Figure 5.2 Chemical shift axis. (a) individual metabolites and (b) *in vivo* spectrum, showing the spectral overlap of glutamate (Glu) and glutamine (Gln), resulting in an additive Glu + Gln (Glx) peak and a broad macromolecule baseline that is seen in short echo time, TE spectra. (Courtesy of PS Allen).

5.2. Spectral editing

The brain contains multiple metabolites with often overlapping signals, such that it may be difficult to identify and quantify the signals of metabolites of interest in the crowded chemical shift spectra acquired. For example, in Figure 5.2b, the glutamate and glutamine peaks fall at the same frequency in the spectrum and their signals produce a composite peak, labeled Glx. In addition, the *in vivo* spectrum does not have a flat baseline, which is due to the presence of substantial but broad resonances from macromolecules (MM).

For this reason, two different types of methods may be applied to improve the detection of signals that are obscured by overlapping "contaminant" peaks. One method is to spread the signals over two dimensions, where signals from uncoupled spins appear on the diagonal and those from coupled spins produce cross peaks off the diagonal. The second method is to spectrally edit one dimensional spectra, by exploiting differences between metabolites with and without *J*-coupled spins. Although many types of spectral editing have been developed, I will describe the methods that have been most commonly applied to the measurement of GABA and glutamate *in vivo*.

5.2.1. Echo Time Averaging

Whereas the amplitude of singlet peaks decreases with TE, the lineshape of *J*-coupled spins also changes. For example, for a triplet peak, the two outer peaks can acquire equal, but opposite, phases at different TE, whereas the centre resonance loses amplitude, but does not change phase. If spectra are acquired at multiple TE and then averaged, the centre resonance is retained, but the outer peaks are averaged out. This principle has been applied in TE-averaged

point-resolved spectroscopy (PRESS) for glutamate, where the signal from the C4 glutamate protons at 2.35 ppm can be retained, whilst the overlapping signals from glutamine and the N-acetyl signals from NAA are averaged out. This method also reduces MM contamination, because the MM signals decays rapidly with increasing TE, due to their short T2 relaxation times (Hurd et al, 2004).

5.2.2. *J*-difference editing

This method uses frequency selective RF editing pulses to acquire two sets of spectra. In one set, the "On" acquisition, an editing pulse is applied that refocuses the evolution of a *J*-coupled metabolite of interest, whereas in the second set, the "Off" acquisition the editing pulse is not applied. When the difference between the sets of spectra is obtained by subtraction, the resulting *J*-difference spectrum should contain only the information from the metabolites affected by the editing pulse. This method is the most frequently used spectral editing method to measure GABA, for which an editing pulse is applied at 1.9 ppm that, at the appropriately selected TE, leads to a maximum inversion of the two outer peaks of the GABA triplet resonance at 3.0 ppm. When the spectra are subtracted the GABA signal is retained, but the much larger overlapping creatine peak is removed (Rothman et al, 1993). The most frequently used *J*-difference method for GABA, MEGA-PRESS, uses a 1.9 ppm editing pulse in the "On" sequence and a 7.45 ppm editing pulse in the "Off" sequence, which does not affect GABA (Mescher et al, 1998).

A limitation of the method is that editing pulses are not perfectly selective at a specific frequency and the 1.9 ppm pulse also affects a MM resonance at 1.7 ppm that leads to co-editing of GABA and MM at 3.0 ppm, resulting in approximately 45% of the signal being derived from

MM (Harris et al, 2015). The sequence also co-edits glutamate and glutamine, which produces an additional peak at 3.75 ppm that has been used to measure Glx in GABA *J*-difference editing studies. As an alternative to the MEGA-PRESS method, it has been shown that GABA can be edited with lower MM contamination when the editing pulses in the "On" and "Off" sequences are applied symmetrically around the 1.7 ppm MM resonance, at 1.9 and 1.5 ppm respectively (Henry et al, 2001).

Another limitation of *J*-difference editing methods is that they are highly vulnerable to frequency drift and subject movement, which lead to unwanted differences between the "On" and "Off", such that contaminant signal remain present after subtraction. This leads to residual unedited signal from the creatine 3.0 ppm peak overlapping the GABA peak. One indicator of this can be the presence of a residual choline signal at 3.2 ppm in the edited spectra (Evans et al, 2013). One common cause of frequency drift is when ^{1}H-MRS experiments are performed after functional imaging, because the high duty cycle of imaging experiments leads to heating and then subsequent cooling during ^{1}H-MRS can lead to substantial frequency drift (Harris et al, 2014).

Due to the impact of subject motion and frequency drift on *J*-difference editing, methods have been developed to reduce their impact, such as updating the shim with each acquisition using MEGA within SPECIAL localization and including Echoplanar Imaging navigators to correct for motion (Saleh et al, 2016).

5.2.3. Multiple Quantum Filtering

Multiple Quantum Filtering exploits the fact that multiple quantum coherence (MQC) can only exist for J-coupled spins and not uncoupled spins. The read-out of a signal that is derived

from a MQC will therefore have all of the signal from uncoupled spins removed. A Double Quantum Filter (DQF) method has been applied to measure the GABA 3.0 ppm resonance, removing the overlying creatine singlet, although MM and glutathione co-editing can still occur (McLean et al., 2002). The major advantage of the DQF is that the unwanted signal is removed in a single acquisition, using gradients or phase-cycling, and it is much less sensitive to subject movement or scanner instability than *J*-difference editing. The disadvantages are that only the signal from the metabolite of interest is retained, so an unedited spectrum has to be collected to have a reference for quantification and the signal amplitude is reduced by 75%, leading to a poor signal-to-noise ratio (SNR) (Harris et al, 2017).

5.2.4. Spectrally selective refocusing

5.2.4.1. GABA

These methods use coherence transfer in *J*-coupled spins, by which excitation is applied to one spin to detect it on the coupled spin. This has been applied to GABA editing by incorporating a dual band selective refocusing pulse, tuned to the GABA 3.0 and 1.89 ppm resonances into the first echo period of a DQF (Shen et al, 2002; Choi et al, 2005). The DQF with selective refocusing (DQF-S) method increases the amplitude of the GABA signal compared with DQF alone and reduces contamination by MM, glutathione and aspartate, due to the spectrally selective refocusing pulse. The DQF eliminates the creatine signal. We have shown that MM contamination using the DQF-S method is reduced to 9% of the signal, comparing favourably with standard MEGA-PRESS (Choi et al, 2007; Harris et al, 2015). The incorporation of a frequency-selective pulse makes the method more sensitive to frequency drift

than a simple DQF, which can be monitored by interleaving the acquisitions with an unedited spectrum.

5.2.4.2. Glutamate and glutamine

Spectrally selective refocusing has also been used to measure signals from the C4 protons of glutamate and glutamine, which resonate at slightly different frequencies (a difference of 13 Hz at 3T). The methods use 180° triple-resonance-selective editing pulses, tuned either to refocus the glutamate resonance at 2.35 ppm and the creatine resonances at 3.0 and 3.92 ppm, or to refocus the glutamine 2.39 ppm resonance and the creatine resonances (Choi et al, 2006). The method also utilizes the different evolution of the lineshapes of glutamate, glutamine and contaminants with TE, to select echo times at which any overlap from the partly co-edited signals from glutamate, glutamine, glutathione and NAA is minimized (Choi et al, 2006). The major advantages of the method are that glutamate and glutamine can be quantified independently at 3T, that MM contamination is minimized by the relatively long TE. The major disadvantages are loss of SNR due to the long TE and that the method is sensitive to frequency drift, due to the frequency selectivity of the refocusing pulses. However, the creatine signals can be used to monitor the frequency drift.

5.3. Metabolite Quantification

5.3.1. Internal reference

Data quantification is achieved by referencing the peak area of the metabolite of interest to that of a reference of known concentration in the brain, with the most commonly used references being the creatine signal in unedited spectra or the water signal. For unedited spectra, using a creatine reference does not require any additional data acquisition, but it relies on the

assumption that creatine is stable in disease and similar between subjects, which may not always be true. Furthermore, the measurement error in estimating the creatine value is added to that for estimating the metabolites of interest (Connelly et al, 1994; Jansen et al, 2006; Lundbom et al, 2001). The water signal can be acquired by collecting additional spectra with the water suppression sequence turned off, which is brief because the water signal is 10,000 times larger than the metabolite signals (Jansen et al, 2006). Brain water is more constant between individuals and across disease, although changes have been reported within multiple sclerosis lesions or with cerebral oedema (Grasso et al, 2002; Laule et al, 2004). Since many metabolites are present in much lower concentrations in cerebrospinal fluid (CSF) than in brain tissue, quantification should be adjusted to account for the proportion of CSF in the volume of interest, using structural image analysis, or a double inversion recovery method (Redpath and Smith, 1994). In order to move closer to metabolite concentrations using brain water, as opposed to ratios expressed as institutional units, other data need to be incorporated into the calculations, including the proportions of grey matter (GM) or white matter (WM) in the voxel, the relative concentrations of the metabolite in GM and WM, the concentration of water in GM and WM, the relaxation times for the metabolite of interest and water in GM and WM and the MR visibility of water (Ernst et al, 1993; Harris et al, 2015). However, not all of the above values may be known.

5.3.2. Spectral fitting

Whereas it is relatively simple for ^{1}H-MRS data from experiments with single metabolites to be quantified using the peak area of the metabolite signal, for *in vivo* data there are multiple metabolites in the chemical shift spectrum. Spectral fitting has been developed as a tool to quantify the peak areas of multiple metabolites simultaneously. This method of data quantification involves the development of initial estimates of resonance frequency, intensity,

and line width, derived either from phantom experiments or experimental simulations. *In vivo* spectra are then fitted to this estimate using an algorithm, so as to maximize the fit between the two. The fitting estimates may be constrained by parameters that limit the possible fitting solution to physiologically feasible metabolite concentration ranges (Jansen et al, 2006). A variety of different software packages have been developed for spectral fitting of ^{1}H-MRS data. Examples include LCModel (Provencher, 1993), which uses *a priori* knowledge of the metabolite basis spectra in the frequency domain; AMARES (Vanhamme et al, 1997), which uses user-defined *a priori* knowledge in the time domain and Gannet (Edden et al, 2014), which was developed for *J*-difference edited GABA and uses simple Gaussian models. A recent comparison of these methods showed somewhat lower reliability for quantification using Gannet than the other two methods (Saleh et al, 2016).

5.4. References

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CHAPTER 6. GABA AND THE MENSTRUAL CYCLE IN HEALTHY WOMEN.

6.1. Background and rationale

6.1.1. GABA and the female reproductive cycle

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain, and the development of ^{1}H-MRS methods to study GABA in the human brain *in vivo* has led to an increasing number of studies examining the relationship of GABA levels to physiological functions and to neurological and mental disorders. One source of physiological variations in brain GABA levels may be related to hormonal fluctuations of the MC, or other stages of the female reproductive cycle, such as pregnancy, the postpartum period and menopause. An early study indicated that occipital cortex (OCC) GABA levels are influenced by menstrual phase (Epperson et al, 2002) and a few studies to date have implicated alterations in brain GABA in mental disorders associated with the female reproductive cycle, including premenstrual dysphoric disorder (PMDD) (Epperson et al, 2002; Liu et al, 2015) and postpartum major depressive disorder (postpartum depression: PPD) (Epperson et al, 2006). Other evidence implicating changes in the function of the GABA system in relation to reproductive factors and associated disorders include changes in plasma GABA levels (Halbreich et al, 1996), differences in response to pharmacologic challenges with drugs that act at the GABA-A receptor, such as the benzodiazepine agonist, diazepam (Sundström et al, 1997), the benzodiazepine antagonist flumazenil (LeMellédo et al, 2000) and the neurosteroid GABA-A receptor modulator allopregnanolone (Timby et al, 2016). Indirect evidence for changes in GABA function includes differences in cortical inhibition, as assessed using transcranial magnetic stimulation (Smith et al, 2002).

Table 6.1. ^{1}H-MRS studies of GABA and reproductive factors

Study 1st author	Treatment n	Age, y m ± sd	Meds % Washout	^{1}H-MRS	Brain Region	GABA Results
Epperson 2002	9 PMDD 14 Con*	35 ± 5	0 (5 yr)	2.1 T J-editing	OCC	F: PMDD < Con M: PMDD = Con L: PMDD = Con MDD: F < M = L Con: F > M > L
Epperson 2005	13 Con*	31 ± 6	0	2.1 T J-editing	OCC	Con: F > M > L
Epperson 2006	9 PPD 14 PPC 10 FCon*	30 ± 5	0 (9 mo)	2.1 T J-editing	OCC	LCon > PPD = PPC
Harada 2011	7 Con	22 ± ns	ns	3T ME-PR	ACC (L) LFrontal LBG	F = L F > L F > L
Liu 2015	20 LPMDD 20 LCon	23 ± 2	0 (6 mo)	3T ME-PR	ACC LBG	L: PMDD < Con L: PMDD < Con
De Bondt 2015	11 Con 21 HBC	23 ± 3	0 (ns)	3T ME-PR	ACC	M > F = L HBC : on = off

Key: Meds % washout = percent of patients taking psychotropic medication and minimum medication washout period; n = sample size; ns = not stated; (L)PMDD = (luteal phase only) premenstrual dysphoric disorder; PPD = postpartum depression; PPC = postpartum control; (F/L) Con = (follicular/luteal phase) healthy control; HBC = hormonal birth control; mo = months; ME-PR MEGA-PRESS; OCC = occipital cortex; ACC (L) = bilateral anterior cingulate cortex (L, left only); LFrontal = left frontal cortex; LBG = basal ganglia; F = follicular; M = mid-cycle/ovulatory phase; L = luteal phase. * all three control groups were from the Epperson et al (2002) sample.

6.1.2. ^{1}H-MRS studies of GABA and the female reproductive cycle

In addition to their contribution to disorders associated with the female reproductive cycle, it is important to consider the potential impact of variations in brain GABA levels in association with the MC, use of hormonal contraception, or other agents that influence hormonal fluctuations, when designing case-control studies of disorders, or correlational studies of the relationship between GABA levels and other physiological variables. Failure to control for such factors could lead to spurious findings or reduce study power by adding to the variance of GABA measurements. To date, six studies have reported ^{1}H-MRS measurements of GABA in relation to phases of the MC (table 6.1.). However, two of these studies included a subsample of the healthy controls in the first study in the field (Epperson et al, 2005, 2006). Two studies only reported data for a single phase of the MC (Epperson et al, 2006; Liu et al, 2015). The early studies of brain GABA levels examined the OCC, in which it is technically simpler to measure GABA, due to a lack of air-filled sinuses in proximity, better magnetic field homogeneity and lower head motion (Epperson et al, 2002). In the earliest study (Epperson et al, 2002), follicular phase GABA levels were reported to be higher than mid-cycle GABA, in turn higher than late luteal phase (last 7 days of MC) GABA in healthy controls. More recently, two studies have reported GABA levels measured in voxels nominally positioned in the ACC, although the tissue encompassed extended anteriorly into PFC, due to the voxel volume required to obtain adequate GABA measurements. These studies did not report differences in ACC GABA between the follicular and late luteal phase of the MC (Harada et al, 2011; De Bondt et al, 2015). However, one of the studies reported follicular-luteal phase differences in the left frontal cortex and left basal ganglia (Harada et al, 2011) and the other reported a significant increase in GABA levels in the mid-cycle ovulatory phase (De Bondt et al, 2015). The latter study did not find differences in

GABA between the follicular and luteal phases of the MC or the pill-use and pill-free phases of hormonal birth control.

6.2. Goals and hypotheses

The first aim of the current studies was to determine whether there are differences in ACC GABA in healthy women between the follicular and late luteal phase of the MC. A second aim was to compare ACC GABA in younger (≤ 30 years) and older (40-50 years) subgroups, to assess whether age differences are related to ACC GABA levels in premenopausal adult women. The third aim was to determine whether there are differences in ACC GABA between the first week of pill-use in participants using hormonal birth control, compared with the follicular phase of the MC in women with natural MCs. The ACC was chosen for GABA measurements because it shows functional and structural abnormalities in depressive and anxiety disorders (Bora et al, 2012; Hasler and Northoff, 2011), which are a focus of investigation for our research group and it was important to plan how to control for MC phase and the use of hormonal birth control in such studies. The study measured GABA using a double quantum filter with selective refocusing (DQF-S) sequence, which is less susceptible to macromolecule (MM) contamination than the *J*-difference editing methods used in previous studies (Choi et al, 2007; Harris et al, 2015). The hypotheses were that GABA levels would be lower in the luteal than follicular phase, and in older than younger participants. There was no prediction for the use of birth control.

6.3. Methods and Materials

6.3.1. Study participants

The study was approved by the University of Alberta Human Research Ethics Board (project 6961). We recruited participants through posters advertising the research throughout the University of Alberta, as well as locations in close proximity. We recruited premenopausal females, who were between the ages of 18-50 years. All participants were pre-screened over the telephone, reviewed a written information sheet and had a detailed explanation of the study prior to written consent being obtained.

6.3.1.1. MC phase

For the MC phase section of the study, participants were divided into a young group, age ≤ 30 years and an older group, age 40-50 years, to determine whether GABA levels are related to aging in premenopausal women. We included healthy participants who reported a regular MC. We excluded those who reported marked premenstrual changes in mood, anxiety, irritability or physical symptoms, but participants with minor premenstrual symptoms were included. Participants who had used hormonal birth control, or who were pregnant or lactating in the last 12 months were excluded. Other exclusion criteria included participants with a lifetime history of psychiatric disorders, substance abuse or dependence, use of substances within the past three months, reported regular alcohol intake > 7 drinks per week or alcohol intake within 48 hours prior to scans, medical conditions that might affect brain structure or function, and metallic foreign objects or devices according to a standard MR safety checklist, including intra-uterine devices.

6.3.1.2. Hormonal birth control

For the hormonal birth control section of the study, we compared healthy premenopausal female participants who either (a) reported regular natural MCs, who were scanned during day 3-8 following the onset of menses and (b) those who were taking hormonal birth control medication for at least six cycles, who were scanned in the first week of pill use after starting a new cycle of medication. Participants were assessed as for the first section of the study, including the ADIS-IV (Brown et al, 2004) and the exclusion criteria were the same, except that we did not screen all participants specifically for marked premenstrual symptoms.

6.3.2. Procedures

6.3.2.1. Assessment

Participants were screened for psychiatric and a range of medical disorders using the Anxiety Disorder Interview Schedule IV – ADIS-IV (Brown et al, 2004), which also includes a medical history checklist. The ADIS-IV does not include a screen for premenstrual symptoms, and the screening question from the Mini International Neuropsychological Interview (Lecrubier et al, 1997) was added. Participants who answered positively to the initial screening question were excluded (*Y1. During the past year, were most of your menstrual periods preceded by a period lasting about one week when your mood changed significantly?*). Height, weight, blood pressure and heart rate were recorded and the participants were also seen by the study physician to review the findings and exclude any significant medical history.

6.3.2.2. Scan session timing

Participants were included in the data analyses if they were scanned twice – once in the follicular phase of the menstrual cycle and once in the luteal phase. The timing of the luteal phase scan was estimated from usual cycle length, and participants were booked within 7 days prior to the expected onset of menses, with the actual date of onset being recorded later. The follicular phase scan was scheduled within 3-8 days after the onset of menses. Participants were booked for scans depending on the time of the initial assessment within their cycle, the availability of the scanner and of the participant, in no specific order of menstrual phase. Scans were conducted in the same 3.0T magnet in the University of Alberta's Peter S. Allen MR Research Centre. Serum samples for estradiol and progesterone were obtained on the day of each scan, stored at -80°C and assayed by Enzyme-Linked Immunosorbent Assays (ELISA) in Dr. Hegadoren's laboratory. Hormonal samples were not obtained for analysis for the participants in the hormonal birth control section of the study and although scans were booked in the first week of pill use, the number of days of use was not recorded in the study file.

6.3.2.3. ^{1}H-MRS data acquisition

^{1}H-MRS data were acquired using a 3.0T magnet (Magnetic Scientific PCL) interfaced to a Surrey Medical Imaging System (SMIS) console. A 28cm diameter quadrature birdcage resonator coil was used for radiofrequency transmission and reception. A multi-slice gradient echo image (TR = 500ms, TE = 22ms, slice thickness = 5mm, 11 slices, resolution = 256 x 256), in the transverse, sagittal, and coronal planes, was used to visualize placement of the ^{1}H-MRS voxel. The voxel (30 x 25 x 30 mm^3) was positioned across the midline in bilateral rostral anterior cingulate and medial prefrontal cortex, above the callosal plane to avoid ventral PFC,

which has the highest magnetic field inhomogeneities. Voxel positioning was reviewed after the GABA acquisition to exclude shifts in position.

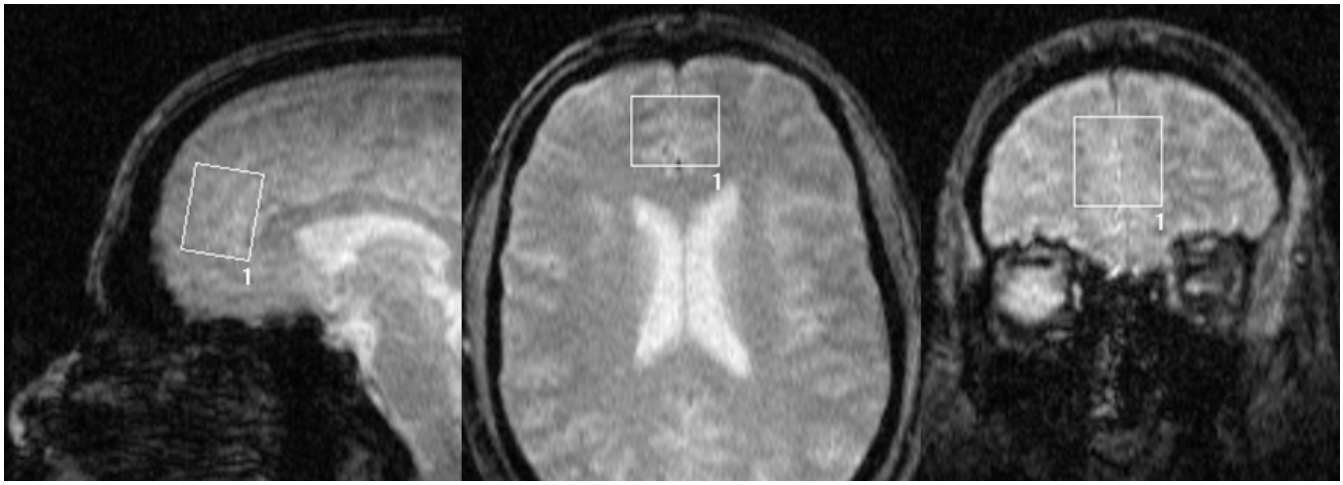

Figure 6.1. Voxel placement for GABA acquisitions (30 x 25 x 30 mm^3)

The voxel was shimmed using FASTMAP (Gruetter, 1993) followed by automated refinement of the linear shims using an in-house routine. GABA concentrations at 3.01ppm were measured using the DQF-S editing method (Choi et al., 2005). The acquisition protocol has two echo times (TE_1 = 49.4 ms and TE_2 = 32.6 ms; TE_{total} = 82.0 ms). TE_1 includes two slice-selective 3.1 ms long 90º excitation pulses and a 28.6 ms 180º dual-band selective refocusing pulse that is tuned to prepare the antiphase coherences of GABA at 3.01ppm and 1.89 ppm, which are converted to double quantum coherence (DQC) by the second 3.1 ms long 90º pulse. The duration and bandwidth of the dual-band 180º pulse were optimized to reduce preparation of the antiphase coherences of the MM resonance at 1.70 ppm (Choi et al, 2007). The DQC is phase encoded by the gradient G_{enc} and converted to antiphase coherence by a single-band 90º pulse tuned to 1.89 ppm. During the second echo period, TE2, the target doublet signal of GABA at

3.01 ppm is brought about by the decoding gradient G_{dec}. The maximum available GABA signal is achieved at an optimized TE_1 = 49.4 ms. The final 180º pulse sequence resets the resonances for re-excitation. Thus, the total time for each single shot GABA sequence was 87 ms (TE_1 = 49.4 ms, TM = 9 ms, TE_2 = 32.6 ms). Other acquisition parameters were recycling time TR = 2.4 s; number of DQF-S excitations = 512; spectral width = 5 kHz; acquisition time = 820 ms; phase cycling = 512 steps. The acquisition was divided into eight blocks, each with 64 DQF-S and four PRESS acquisitions, TE = 82 ms. B_0 frequency drift during the scans was reduced by adjusting the frequency offset based on the NAA signal from the PRESS acquisitions. The GABA DQF-S sequence is outlined in figure 2. A variable-flip-angle water suppression sequence with four 28 ms long Gaussian RF pulses separated by spoilers was applied prior to the DQF-S sequence.

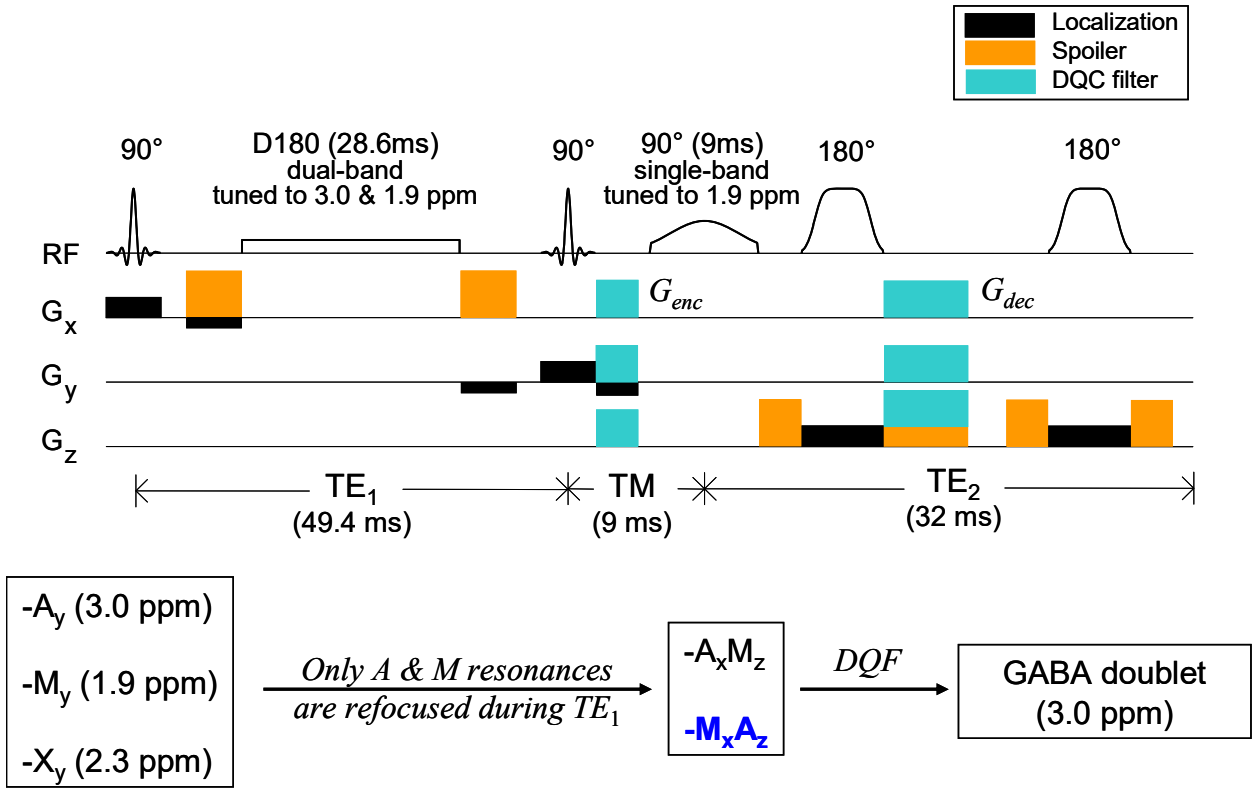

Figure 6.2: DQF-S sequence.

Tissue segmentation data were acquired using 1D imaging of the spatially localized STEAM water signal (Choi et al, 2006). Double inversion recovery was used to discriminate the water signals between the grey matter (GM), white matter (WM) and CSF compartments (Redpath & Smith, 1994). The GM and WM profiles at zero TE were obtained using published water values (Wansapura et al, 1999). The tissue water magnetization, without the CSF component, was then used as a reference in the estimation of GABA concentrations.

6.3.2.3. ^{1}H-MRS data analysis.

Data from the DQF-S sequence, the water signal and the voxel tissue composition measurement were used for metabolite quantification. The metabolite free induction decays were filtered with a 3Hz exponential and corrected for eddy currents before Fourier transformation. The brain water signal is derived from water in all the tissue compartments in the voxel (GM, WM and CSF water), but the proportion of the GABA signal from CSF water is negligible, because GABA is concentrated in the intracellular compartment. GABA concentrations were therefore measured with respect to brain tissue water (i.e., GM and WM water), excluding the CSF component, with adjustment for the difference in water concentration between GM (0.81 x 55.6 M) and WM (0.71 x 55.6 M; Choi et al., 2007). These calculations also included adjustments for signal loss due to relaxation effects, using T_1 and T_2 relaxation time estimates (water T_1 = 1.5 s, water T_2 = 100 ms; Wansapura et al, 1999; GABA T_1 = 1.5 s, GABA T_2 = 180 ms; Choi et al., 2007). It is noted that relaxation times have been obtained experimentally for GABA, T_1 = 1.3 s, T_2 = 88 ms (Puts et al 2013; Edden et al, 2012), and that the T_2 relaxation time in these studies was substantially shorter than the estimate used here. However, it is not clear whether the short T_2 relaxation time may in part have resulted from the co-editing of the 1.7 ppm MM resonance, which has a short T_2 relaxation time compared with most metabolites. Since

we did not obtain T_2 relaxation times for individual participants in the study and any adjustments to the data would have been applied uniformly across all the participant data, the different estimates do not contribute to any statistical differences in the results. The means and SDs in the present data set could be adjusted to match those that would be obtained using the other T_1 and T_2 relaxation times by applying a multiplication factor of 1.34 to all of the data. GABA T_1 and T_2 relaxation times are not available separately for GM and WM in the literature and therefore separate adjustments for relaxation effects in GM and WM have not been applied. Potential effects of differences in GM and WM composition between groups were examined in the statistical analyses using the grey matter fraction, GM/(GM+WM), where necessary. The peak area of the GABA resonance was integrated with respect to the peak area of the adjusted water signal. Since the edited GABA signal from the DQF-S sequence includes contributions from homcarnosine (a GABA-histidine dimer) and MM (Choi et al, 2007), it will be referred to as $GABA^+$ in the remainder of the paper.

Quality controls for the scans included (1) the technician monitoring the participants and scoring movement on a 3 point scale (bad, acceptable, good) with bad scans excluded; (2) the voxel position had not shifted significantly during the scan; (3) frequency drift < 2Hz; (4) line-width of the $GABA^+$ peak was 44 Hz.

Previous studies by Dr Choi and colleagues have provided evidence for the reliability and validity of the DQF-S method (Ogilvie, 2005; Choi et al, 2005). For subjects in whom the acquisition was obtained from the same voxel location twice within the same scanning session, while subject remained in the scanner, the intra-subject coefficient of variation for ACC $GABA^+$ was 8.0% (n = 14). For subjects who had two acquisitions at the same period of the day within 48 hours, requiring repositioning in the scanner, the intra-subject coefficient of variation for

ACC $GABA^+$ was 14.4% (n = 6) (Ogilvie, 2005). Validity was assessed by measuring $GABA^+$ levels before and 24 hours after the administration of a single dose of the GABA transaminase inhibitor, vigabatrin 50 $mg.kg^{-1}$. Post-vigabatrin $GABA^+$ levels were increased in every individual subject, with the mean increase being 45% of baseline (Choi et al, 2005), consistent with a previous study showing a 41% increase in OCC $GABA^+$, using the same vigabatrin protocol and an optimized STEAM sequence (Hanstock et al, 2002).

6.3.3. Statistical analysis

Hormone concentrations, tissue segmentation data and GABA levels were compared in the MC section of the study using paired or Student's t-tests, or repeated measures analysis of variance (RMANOVA, within group: menstrual phase; between groups: age group or phase order), with the inclusion of covariates of interest when significant. Welch's adjustment to the degrees of freedom was used in the case of unequal variance. Relationships between hormone concentrations and $GABA^+$ levels were tested using Pearson correlations. Data for the hormonal birth control section were analyzed using Student's t-tests or ANOVA. Analyses were conducted in IBM Statistics (SPSS 20) or Stata 14 and statistical significance was set at two-tailed $\alpha = 0.05$.

6.4. Results

6.4.1. Menstrual phase study

6.4.1.1. Participants

One participant dropped out after the first scan due to discomfort. One participant could not keep still in two sessions. One had a cough in one of the two sessions and could not return for a third. One had severe movement during their first scan, but was able to complete two

subsequent scans without issues. Two participants had poor quality spectra, but completed acceptable scans during another cycle. In total, 20 participants had follicular and luteal scans that could be included in the analyses (< 30 years, n = 10, age = 23.1 ± 2.9 years; 40-50 years, n = 10, age = 44.6 ± 3.4 years).

6.4.1.2. Hormone and ^{1}H-MRS results.

In the total sample, estradiol and progesterone levels were significantly higher in the luteal than the menstrual phase of the cycle (table 6.2.).

The STEAM water linewidth was 7.6 ± 0.9 Hz (range: 5.4 – 9.0 Hz). CSF and GMF tissue composition did not differ between the follicular and luteal phase (table 6.2.), suggesting that there were no marked differences in voxel placement on the two occasions.

$GABA^{+}$ concentrations were unrelated to the order in which scans were obtained ($F_{1,36}$ = 0.17; p = 0.69). Follicular $GABA^{+}$ (follicular/luteal = 1.10 ± 0.28; luteal/follicular = 1.16 ± 0.34; t = -0.44; df = 18; p 0.66). Luteal $GABA^{+}$ (follicular/luteal = 1.04 ± 0.11, luteal/follicular = 1.06 ± 0.38; t = -0.14; df = 18; p = 0.89). Order effects were therefore not examined in subsequent analyses.

$GABA^{+}$ levels did not differ significantly between the follicular and luteal phases. One participant had unusually high $GABA^{+}$ levels during both the follicular (1.84 mmol/L) and luteal (2.18 mmol/L) phases of the cycle, but although exclusion of this participant from the analysis reduced the variance, differences in $GABA^{+}$ between menstrual cycle phase remained non-significant (follicular: 1.08 ± .24; luteal: 0.99 ± .16; t = 1.61; df = 18; p = 0.12). One participant

did not have luteal phase hormonal data. Exclusion of this subject from the analyses also did not alter the findings (all t <1.5; all p > 0.1).

$GABA^+$ levels in the follicular and luteal phases were correlated (r = 0.65; p = 0.002), but $GABA^+$ levels were not significantly correlated with estradiol or progesterone concentrations at either phase of the cycle, all p > 0.07 (Bonferroni corrected α = 0.013).

Table 6.2. Hormone concentrations and magnetic resonance data for the two phases of the MC study.

	Follicular	Luteal	*t*	*df*	*p*
Day of cycle	5.5 ± 1.6 (range 3 to 8)	-3.8 ± -1.6 (range -7 to -1)			
Estradiol, pg/ml	78.2 ± 25.0	95.1 ± 30.5	2.2	18	0.04
Progesterone, pg/ml	0.6 ± 0.5	2.6 ± 1.2	6.5	18	< .001
GM, %	63.1 ± 5.8	62.3 ± 6.8	0.78	19	0.44
WM, %	22.6 ± 3.3	24.0 ± 5.0	1.74	19	0.10
CSF, %	14.3 ± 6.5	13.7 ± 5.3	0.99	19	0.34
GMF,%	73.6 ± 3.6	73.1 ± 72.1	1.50	19	0.15
$GABA^+$, mmol/L	1.12 ± .30	1.05 ± .31	1.28	19	0.22

Key: GM = grey matter; WM = white matter; CSF = cerebrospinal fluid; GMF = grey matter fraction.

6.4.1.3. Age group comparisons

Comparing the younger with the older participants, there were no significant differences in hormone levels, tissue composition or $GABA^{+}$ levels at either phase of the menstrual cycle (table 6.3.).

Table 6.3. Hormone concentrations and ^{1}H-MRS data for the two age groups and phases of the menstrual cycle study.

		< 30 years	40-50 years	*t*	*df*	*p*
Day of cycle	Follicular	5.1 ± 1.2 (range 3 to 7)	5.9 ± 2.0 (range 3 to 8)	1.10	16.2	0.29
	Luteal	-3.9 ± 2.3 (range -7 to -1)	-3.7 ± 1.9 (range -6 to -1)	0.21	18	0.83
Estradiol, pg/ml	Follicular	78.8 ± 25.7	77.7 ± 24.1	0.10	18	0.92
	Luteal	93.9 ± 37.7	95.5 ± 22.2	2.2	16.1	0.85
Progesterone, pg/ml	Follicular	0.43 ± 0.26	0.75 ± 0.65	1.5	12.4	0.17
	Luteal	2.3 ± 1.3	2.9 ± 1.2	1.1	17	0.29
CSF, %	Follicular	12.5 ± 7.2	16.0 ± 5.4	1.22	18	0.24
	Luteal	12.2 ± 5.3	15.1 ± 5.2	1.23	18	0.23
GMF,%	Follicular	74.0 ± 2.9	73.2 ± 4.3	1.50	19	0.15
	Luteal	74.2 ± 3.8	70.1 ± 7.1	1.6	14.7	0.13
$GABA^{+}$, mmol/L	Follicular	1.13 ± .31	1.12 ± .29	1.25	19	0.90
	Luteal	1.11 ± .41	1.00 ± .16	0.80	12.3	0.44

Key: CSF = cerebrospinal fluid; GMF = grey matter fraction.

6.4.2. Hormonal birth control study

6.4.2.1. Participants

The follicular phase and hormonal birth control groups were matched for participant characteristics, including age, sex, height weight and BMI (table 6.4).

Table 6.4. Participant characteristics and ^{1}H-MRS resonance data for the hormonal birth control study.

	Natural Cycle n = 16	Birth Control n = 14	*t*	*df*	*p*
Age, y	26.3 ± 7.6	27.7 ± 7.0	0.53	28	0.60
Education, y	14.4 ± 1.22	15.1 ± 2.0	1.02	28	0.32
Height, m	1.64 ± 0.07	1.64 ± 0.08	0.33	28	0.74
Weight, kg	60.6 ± 13.4	63.2 ± 7.8	0.63	28	0.54
BMI	22.3 ± 4.2	23.6 ± 2.8	1.00	28	0.34
Grey, %	64.1 ± 5.6	60.3 ± 5.0	1.98	28	0.06
White, %	22.4 ± 2.6	26.9 ± 4.5	3.25	25.5	0.003
CSF, %	13.3 ± 6.4	13.0 ± 5.5	0.13	28	0.90
GMF,%	74.0 ± 2.8	69.2 ± 4.5	3.60	26.9	0.002
$GABA^{+}$, mmol/L	1.11 ± .27	1.08 ± .19	0.39	28	0.70

Key: BMI = Body Mass index; GM = grey matter; WM = white matter; CSF = cerebrospinal fluid; GMF = grey matter fraction.

6.4.2.2. ^{1}H-MRS results

$GABA^{+}$ levels did not differ significantly between participants with natural cycles and those using hormonal contraception. However, although there were no differences in CSF content of the voxel, there were significant differences in the grey and white matter content. The $GABA^{+}$ data were therefore also analyzed using ANCOVA with group (natural cycle/birth control) as between-subject variable and GMF as a covariate. Although there was a trend for GMF to covary with $GABA^{+}$ ($F_{1,26} = 3.61$; $p = 0.07$), $GABA^{+}$ levels were not significantly different between groups ($F_{1,26} = 2.66$; $p = 0.11$) after covarying for GMF.

6.5. Discussion

6.5.1. Menstrual phase study

The main findings of the present study were that there were no significant differences in ACC $GABA^{+}$ between the follicular and late luteal phase of the MC, no differences in ACC $GABA^{+}$ between the follicular phase of the MC and the first week of pill-use for hormonal birth control and no differences in ACC $GABA^{+}$ between younger and older participants within the premenopausal adult age range. These findings for MC phase are consistent with those of the two previous studies that measured ACC $GABA^{+}$ in the follicular and late luteal phases of the MC, which also reported no significant differences (Harada et al, 2011; De Bondt et al, 2015). The findings for ACC $GABA^{+}$ differ from those in some other brain regions, in that follicular $GABA^{+}$ levels have been reported to be higher than in the late luteal phase in the OCC, left basal ganglia and left frontal cortex (Epperson et al, 2002; Harada et al., 2011). It is unclear whether

this is because of genuine regional differences in $GABA^+$ variations across the MC, or because $GABA^+$ levels are less affected by measurement error in the OCC, basal ganglia and frontal cortex because of higher $GABA^+$ levels, higher signal-to-noise ratios (SNR) and lower variance in these regions. The greater variance of ACC $GABA^+$ measures may be a factor, because in the present study, although follicular phase $GABA^+$ levels were 6.3% higher than the late luteal phase, this is lower than the intra-subject coefficients of variation (8.0-14.2%) for repeat GABA ^{1}H-MRS scans using the DQF-S method (Ogilvie, 2005). One of the other studies reported 10% higher levels in the follicular phase, but the sample size was only 11 participants (Harada et al, 2011). The other study did not detect any difference in the means, although the sample size was again only 11 participants (De Bondt et al, 2015).

6.5.2. Hormonal birth control study

With regard to the use of hormonal birth control, the present findings are similar to the one prior study, which did not find a significant difference between follicular phase ACC $GABA^+$ and the first week of pill use (De Bondt et al, 2015). The 3% difference in the present study was well below the intra-subject coefficient of variation. The present study did not find differences between the younger and older participants, within the premenopausal age range. An age-dependent reduction in medial frontal and medial parietal $GABA^+$ ratios to creatine and NAA levels has been reported in participants with a wider age range (20-76 years) than the present study (Gao et al, 2013). The present study differs in the smaller age range studied and in methodology, using DQF-S as opposed to MEGA-PRESS editing and quantification with respect to brain water as opposed to metabolite ratios.

6.5.3. Strengths of the studies

ACC $GABA^+$ measurements were obtained in 20 healthy participants in two age groups in two phases of the MC. The total sample for the MC study was larger than the two published studies, which both included 11 participants, or than in the OCC study, which included 14 participants (Epperson et al, 2002). The study used the DQF-S method, rather than *J*-difference editing, which is much less confounded by MM contamination (9% vs 45%; Choi et al, 2007; Harris et al, 2015). The data were also quantified using the water signal as a reference, adjusted for the CSF fraction, rather than the creatine signal (Epperson et al, 2002; De Bondt et al, 2015).

6.5.4. Limitations of the studies

$GABA^+$ and hormonal measures were obtained only in the follicular phase and luteal phases of the MC, whereas prior studies have reported differences in $GABA^+$ in the mid-cycle, although the direction of differences was not consistent in the ACC (mid-cycle highest) and OCC (follicular highest). A more detailed time course would have clarified whether $GABA^+$ levels are related to specific MC phases. From the point of view of the design of case-control studies in female participants, it appears that testing them within day 3-8 of the follicular phase, or the last week of the MC, or the first week of pill-use may not substantially compromise the results of a study.

We did not confirm that participants had ovulatory cycles: anovulatory cycles may occur in 3-7% of cycles in young healthy women, but were present in up to 37% of cycles in large community sample of older women (mean age, 42 years), based on a luteal phase progesterone threshold (Prior et al, 2015). The participants in our study were highly screened to exclude physical and mental disorders that might reduce ovulation rates. As a result of not testing

in the ovulatory phase, we were not able to confirm whether this phase is associated with increased or decreased $GABA^{+}$ levels compared with the follicular phase, as previously reported (Epperson et al, 2002; De Bondt et al, 2015). Although participants were screened to exclude a history of marked premenstrual symptoms, prospective symptom ratings were not obtained during the cycles in which they were examined. The findings may not generalize to women with more prominent premenstrual symptoms. For the comparison of participants with natural MCs and those using hormonal birth control, the participants were not specifically screened for premenstrual symptoms and hormone levels at the time of scanning were not assessed.

6.6. Conclusions

Although differences between follicular and luteal GABA have been reported for other brain regions and differences in ACC GABA have been reported in other phases of the MC, we did not find differences in ACC GABA between the follicular and luteal phases of the menstrual cycle, consistent with previous studies of this region. Hormonal birth control and age were not associated with differences in GABA within the limited age range of the participants included.

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CHAPTER 7. PREFRONTAL GAMMA-AMINOBUTYRIC ACID (GABA) AND MAJOR DEPRESSIVE DISORDER

7.1. Background and rationale

7.1.1. GABA and major depressive disorder (MDD)

Several lines of evidence have linked MDD with alterations in the γ-aminobutyric acid (GABA) system (Hasler and Northoff, 2011). Postmortem studies of MDD have shown reductions in glutamic acid decarboxylase- (GAD_{67}-), calbindin- and somatostatin-positive GABAergic interneurons in several cortical and subcortical regions, including prefrontal, ACC, auditory and occipital cortex and amygdala (Lin and Sibille, 2013; Maciag et al, 2010; Smiley et al., 2016; Torrey et al, 2005). Studies have also repeatedly reported reduced concentrations of GABA in plasma and cerebrospinal fluid (CSF) in MDD (Petty, 1995; Mann et al, 2014).

7.1.2. ^{1}H-MRS studies of GABA and MDD

More recently, ^{1}H-MRS has been used as a non-invasive method to study brain GABA in MDD and other disorders. To date, eight studies have reported GABA levels in the occipital cortex (OCC), with an additional two studies reporting subgroups of the same patient samples (Bhagwagar et al, 2007 and 2008; Price et al, 2009 and Abdallah, et al 2014). Five studies reported reduced OCC GABA levels in MDD groups compared with healthy controls (see table 7.1.), of which four included patients in a current major depressive episode (MDE). In one of the negative studies, OCC GABA was not different in postpartum MDD patients from postpartum controls, but levels were lower than in a historical comparison group of non-postpartum women in the follicular phase of the menstrual cycle (Epperson et al, 2006).

Table 7.1. ^{1}H-MRS studies of GABA in major depressive disorder, occipital and other regions.

Study (First author)	Sample	Age, y m±sd	Female %	Meds % Washout	^{1}H-MRS	Brain Region	GABA Results
Sanacora 1999	14MDD 18 Con	40 ± 10	41	0 (2 wk)	2.1 T J-editing	OCC	MDD < Con.
Kugaya 2003	6 MDD 12 Con	40 ± 10	0	0 (10 d)	2.1 T J-editing	OCC	MDD < Con.
Sanacora 2004	33 MDD 38 Con	39 ± 11	48	0 (2 wk)	2.1 T J-editing	OCC	MDD < Con
Epperson 2006	9 MDD* 14 Con* 10 F	31 ± 4	100	0 (9 mo)	2.1 T J-editing	OCC	MDD = Con < F
Bhagwagar 2008	12 rMDD 11 Con	38 ± 4	52	0 (6 mo)	3T ME-PR	OCC	rMDD < Con
Shaw 2013	19 rMDD 18 Con	22 ± 2	100	0 (ns)	3T ME-PR	OCC	rMDD = Con
Price 2009	33 MDD 24 Con	40 ± 13	48	0 (2 wk)	3T ME-PR	OCC	MDD < Con
Godlewska 2015	39 MDD 31 Con	30 ± 11	58	0 (3 mo)	3T SPECIAL	OCC	MDD = Con
Shaw 2013	19 rMDD 18 Con	22 ± 2	100	0 (ns)	3T ME-PR	L PFC	rMDD = Con
Shaw 2013	19 rMDD Con	22 ± 2	100	0 (ns)	3T ME-PR	SubC	rMDD = Con

Key: Meds % Washout = percent of patients taking psychotropic medication and minimum medication washout; ns = not stated; MDD = major depressive disorder (r, remitted; *post-partum); Con = controls; ns = not specified; ME-PR = MEGA-PRESS; OCC = occipital cortex; L PFC = left prefrontal cortex; SubC = subcortical; F = follicular phase of menstrual cycle.

Eight studies have reported GABA levels in the ACC, with ten comparisons, because two studies examined both a dorsally located and a ventrally located voxel (Hasler et al, 2005, 2007).

Although the volumes of interest examined are nominally described as ACC, they extended anteriorly into the medial PFC, due to the relatively large voxel volumes required to obtain GABA data. Four of these ten comparisons reported reduced ACC GABA in MDD, including three of seven comparisons in patients who were in a MDE at the time of the scan.

Table 7.2. ^{1}H-MRS studies of GABA in major depressive disorder, anterior cingulate cortex.

Study (First author)	Sample	Age, y m±sd	Female %	Meds % Washout	^{1}H-MRS	Brain Region	GABA Results
Hasler 2005	16 rMDD 15 Con	41 ± 12	77	0 (3 mo)	3T J-editing	dACC vACC	rMDD = Con rMDD = Con
Hasler 2007	20 MDD 20 Con	38 ± 12	65	0 (1 mo)	3T J-editing	dACC vACC	MDD < Con MDD = Con
Bhagwagar 2008	12 rMDD 11 Con	38 ± 4	52	0 (6 mo)	3T ME-PR	ACC	rMDD < Con
Price 2009	33 MDD 24 Con	40 ± 13	48	0 (2 wk)	3T ME-PR	ACC	MDD = Con
Walter 2009	19 MDD 24 Con	37 ± ns	67	0 (1 wk)	3T J-PRESS	ACC	MDD = Con
Gabbay 2012	20 MDD 10 Con	40 ± 2	66	0 (3 mo)	3T ME-PR	ACC	MDD < Con
Wang 2016	19 MDD 13 Con	54 ± 3	100	0 (6 mo)	3T ME-PR	ACC	MDD < Con
Zhang 2016	11 MDD 11 Con	34 ± 9	100	55 (ns)	3T ME-PR	ACC	MDD = Con

Key: Meds % Washout = percent of patients taking psychotropic medication and minimum medication washout; ns = not stated' MDD = major depressive disorder (r, remitted); Con = controls; ME-PR = MEGA-PRESS; ACC = anterior cingulate/medial prefrontal cortex (d, dorsal; v, ventral).

7.1.3. ^{1}H-MRS studies of GABA and MDD treatment

Other studies have examined the impact of treatments for depression on ^{1}H-MRS measures of GABA in MDD, with some reporting increased GABA following treatment, although none of these positive findings have been replicated (table 7.3.). If medications can alter brain GABA, it is important that studies should include unmedicated patients, with adequate washout periods for medications. In one of the negative studies of ACC GABA (table 7.2), 55% of the patients were taking antidepressants at the time of scanning (Zhang et al, 2016) and in two of the other negative studies, the washout period was ≤ 2 weeks (Price et al, 2009; Walter et al, 2009).

The majority of GABA studies to date have employed *J*-difference editing methods, with most groups using MEGA-PRESS (Mescher et al, 1998). In addition, all of the MEGA-PRESS studies acquired data with editing pulses tuned to 1.9 ppm in the 'On' sequence and 7.46 ppm in the 'Off' sequence. The data from the 'Off' spectra are subtracted from the 'On' spectra to remove the overlying resonances from the GABA signal at 3.0 ppm. This approach is quite vulnerable to subtraction errors, due to factors such as frequency drift and subject motion that lead to mismatches between the 'On' and 'Off' spectra (Evans et al, 2013). In addition, the 1.9 ppm editing pulse also co-edits a macromolecule (MM) resonance at 1.7 ppm, such that a substantial portion of the resulting GABA signal results from MM. MM contamination of the GABA signal is estimated to be 45% at 3T (Harris et al, 2015). MM contamination can be reduced by using editing pulses that are symmetrically tuned at 1.9 ppm and 1.5 ppm, around the 1.7 ppm MM resonance (Henry et al 2001; Harris et al 2015), but this approach has not yet been applied to MDD studies, due to its vulnerability to motion and frequency drift. The GABA signal

is therefore often referred to as $GABA^+$ to denote that it is not pure GABA and includes contaminant signals. We will use the term $GABA^+$ where appropriate in the rest of the paper.

Table 7.3. Treatment effects on ^{1}H-MRS measures of GABA in major depressive disorder.

Study (First author)	Treatment n	Age, y m±sd	Female, %	Meds % Washout	^{1}H-MRS	Brain Region	GABA Results
Sanacora 2002	SSRI 2 mo 11	39 ± 9	36	0 (10 d)	2.1 T J-editing	OCC	Post > Pre
Godlewska 2015	SSRI 6 wk 27	30 ± 11	58	0 (4 mo)	3T SPECIAL TE=8.5ms	OCC	Post = Pre
Licata 2014	Zolpidem 14	30 ± 5	71	100 SSRI	4T ME-PR	ACC Thal	Post > Pre Post > Pre
Sanacora 2003	ECT 10	46 ± 5	38	0 (2 wk)	2.1 T J-editing	OCC	Post > Pre
Dubin 2016	rTMS 5 wk 23	42 ± 16	70	89 (ns)	3T ME-PR	ACC	Post > Pre
Sanacora 2005	CBT 8	ns	ns	0 (24 mo)	2.1 T J-editing	OCC	Post = Pre
Abdallah 2014	CBT 30	42 ± 11	63	0 (ns)	4 T J-editing	ACC	Post = Pre
Salvadore 2012	Ketamine 14	50 ± 10	36	0 (2 wk)	2.1 T J-editing	ACC	Post = Pre
Milak 2016	Ketamine 8	38 ± 13	73	0 (3 mo)	3T ME-PR	ACC	During > Pre

Key: Meds % Washout = percent of patients taking psychotropic medication and minimum medication washout; n = sample size; ns = not stated; SSRI = selective serotonin reuptake inhibitor; ECT = electroconvulsive therapy; rTMS = repetitive transcranial magnetic stimulation; CBT = cognitive behavioural therapy; ME-PR = MEGA-PRESS; OCC = occipital cortex; ACC

= anterior cingulate cortex; Thal = thalamus; Pre, Post = pre- or post-treatment measurement. During = data acquired during ketamine infusion.

7.2. Goals and hypothesis

The aim of the present study was to compare ACC $GABA^+$ in MDD patients, in a current MDE and not receiving recent treatment (< 3months) and healthy controls using an alternative GABA-editing sequence, a double quantum filter with selective refocusing (DQF-S) (Choi et al, 2005). The DQF-S is a 'single-shot' technique that does not require the subtraction of difference spectra and which reduces the co-editing of MM compared with standard MEGA-PRESS, with co-edited MM calculated to contribute 9% to the $GABA^+$ signal (Choi et al, 2007). An ACC volume of interest, encompassing rostral ACC anterior to the genu of the corpus callosum, extending anteriorly into the medial PFC, was chosen as the volume of interest, given prior ^{1}H-MRS studies and evidence of structural and functional changes in this brain region in MDD (Bora et al, 2012; Northoff and Sibille, 2014). The hypothesis was that GABA levels would be lower in MDD patients than in healthy controls.

7.3. Methods and Materials

7.3.1. Study design

We obtained approval from the Heath Research Ethics Borad – Biomedical Panel at the University of Alberta for this research (Project 6961). Participants were informed in detail about all of the study components, including potential risks, prior to obtaining their written consent to

participate. The study design was a cross-sectional, case control investigation of MDD patients compared with healthy controls, group-matched for age, sex and education.

7.3.2. Participants

7.3.2.1. Inclusion criteria

The initial sample included 45 MDD participants (of whom 10 were not included in the fnal analysis) and 54 healthy controls (of whom 12 were not included), who all met the inclusion/exclusion criteria and had adequate ^{1}H-MRS data for analysis (see 7.4.1. for reasons why participants were not included). The inclusion criteria were for healthy male or premenopausal female participants, age 18-50 years. MDD participants met DSM-IV criteria for a current MDE, determined in a structured clinical interview using the Anxiety Disorders Interview Schedule IV-Lifetime Version (ADIS-IV-L; DiNardo et al, 1994). They also had to have a current MDE of at least moderate severity, with a severity rating ≥4), as assessed by the ADIS-IV-L and a 17-item Hamilton Depression Rating Scale (HDRS) score ≥18. Participants with MDD could be included with or without a comorbid lifetime diagnosis of panic, social or generalized anxiety disorder.

7.3.2.2. Exclusion criteria

Potential MDD participants were excluded if they had a lifetime history of schizophrenia, bipolar disorder, substance or alcohol abuse/dependence, eating disorders (i.e., anorexia nervosa or bulimia), posttraumatic stress disorder, obsessive compulsive disorder, antisocial or borderline personality disorder. MDD patients who met criteria for the atypical features specifier were excluded (mood reactivity and 2 of hypersomnia; increased appetite/weight; “leaden” anergy;

hysteroid dysphoria). For the healthy controls, participants were excluded if they had any lifetime history of axis I psychiatric disorders, as assessed using the ADIS-IV-L. The washout period for clinically prescribed treatments, including antidepressants, anxiolytics/hypnotics and atypical antipsychotics was 3 months. Use of alcohol and non-prescribed psychotropic drugs was assessed using the ADIS-IV-L. Participants were excluded if they had a history of alcohol dependence, or abuse within the last 12 months, or if their current reported alcohol intake was ≥ 14 (male) or ≥ 7 (female) standard alcoholic beverages per week. With respect to non-prescribed drugs, subjects with: 1) frequent and/or repeated use for a period of ≥ 1 year over their lifetime; 2) use within the last three months; and/or 3) substance use that led to an acute medical events (e.g., emergency room visit for severe intoxication) were excluded. Cigarette smoking was documented and heavy (≥ 1 pack per day) smokers were excluded. Excessive caffeine intake (≥ than 4 cups of coffee/day) was excluded. All participants using medications with known central nervous system effects were required to have stopped at least 4 weeks prior to participation. Clinically relevant medical conditions such as: 1) significant neurological disorders (e.g., seizures, brain injury); 2) the presence of major risks for cerebrovascular disease (e.g., uncontrolled hypertension, ischemic heart disease, diabetes); or 3) uncontrolled endocrine disorders, were excluded. Pregnant, lactating or post menopausal females were excluded. Participants who had metallic foreign bodies or implanted devices were excluded according to a standard MR safety checklist, including females with an intrauterine contraceptive device (IUD).

7.3.2.3. Assessment

Participants were recruited via primary care referrals, public notice boards (in the psychiatric clinic, hospital and university), local media, and internet advertisements. The assessment included an initial screening and clinical interview conducted over the phone,

followed by a psychiatric assessment by a licensed psychiatrist with training and experience in research assessments. The assessment included structured diagnostic psychiatric history; personal, medical and treatment histories; mental state and physical examination; and laboratory tests to exclude clinically relevant medical conditions. The 17-item Hamilton Depression Rating Scale was administered to assess current depressive symptom severity. A standard checklist was administered to exclude potential contraindications to magnetic resonance scans. Participants also completed self report scales, as detailed below.

Anxiety Disorders Interview Schedule IV-Lifetime Version (ADIS-IV-L)

Diagnosis of MDD and other axis I diagnoses were made using the ADIS-IV-L (DiNardo, Brown & Barlow, 1994), a structured interview designed for the differential diagnosis of anxiety disorders, mood disorders, psychosis and substance use disorders according to multiaxial DSM-IV criteria (Summerfeldt & Antony, 2002). Relative to the Structured Clinical Interview for Diagnosis (SCID) for Axis I disorders, the ADIS-IV-L provides more detail about comorbid anxiety disorders and asks about life stressors, medical disorders and the use of substances associated with the time of disorder onset (Summerfeldt & Antony, 2002). In addition to the systematic assessment of Axis I anxiety disorders, the ADIS-IV-L includes sections on mood disorders (major depressive disorder, dysthymic disorder, hypomanic and manic episodes), substance use disorders and screening assessments for psychotic symptoms. It provides assessments of episode severity in a life chart, which can be used to estimate age of onset, recurrence and duration. A checklist for common and clinically important medical information is also supplied.

The ADIS-IV-L generally has good-excellent reliability in current (Cohen kappa=0.67-0.86) and lifetime (Cohen kappa=0.58-0.83) diagnoses of MDD, anxiety disorders and substance use disorders (Brown et al., 2001). Clinical administration in MDD participants took on the order of 45-75 minutes. Screening of healthy controls was relatively brief, given that follow up answers were not required when answers to stem questions were negative.

The Zanarini Rating Scale for Borderline Personality Disorder (ZAN-BPD)

The ZAN-BPD was used to obtain dimensional ratings for the criterion symptoms of borderline personality disorder, which include affect disturbance, cognitive disturbance, impulsivity, self harm, and disturbed relationships. The ZAN-BPD has high internal consistency (α =0.85) and excellent inter-rater (ICC=0.96) and test-retest (ICC=0.93) reliability (Zanarini et al., 2003). The ZAN-BPD was used to corroborate clinical interview in the judgment that participants had met the exclusion criterion for borderline personality disorder.

Hamilton Depression Rating Scale (HDRS)

Severity of current depressive symptoms in MDD participants was assessed using the 17-item HDRS (Hamilton, 1967). The HDRS is a highly reliable and standard rating scale for severity of major depressive episodes (Iannuzzo et al., 2006).

Mood and Anxiety Symptom Questionnaire (MASQ)

The MASQ- Short Form is a self-report measure used to measure depressive anhedonia, general distress and somatic anxiety (Watson et al., 1995a, 1995b). These subscales are based on the tripartite model of anxiety and depression and on factor analyses of symptoms in non-clinical

and clinical populations. Anhedonic depression reflects a lack of positive affect, more specific to depression; general distress is common to both depressive and anxiety disorders; and somatic anxiety largely reflects physiological hyperarousal, more specific to anxiety disorders. Symptoms are rated on 5-point Likert-type scale.

The MASQ has good-excellent reliability for each of the three factors: Anhedonic Depression (α=0.95), General Distress (α=0.95), and Somatic Anxiety (α=0.88; Keogh & Reidy, 2000). The MASQ has good convergent and concurrent validity; however, its discriminant validity is inconsistent, varying as a function of the subscale examined (Watson et al., 1995b). The MASQ retains the same factor structure regardless of application to patient or healthy populations (Keogh & Reidy, 2000; Watson et al., 1995a).

Childhood Trauma Questionnaire (CTQ)

The CTQ (Bernstein & Fink, 1998) is a 28-item self-report measure used to assess childhood abuse and neglect. The CTQ queries five aspects of childhood maltreatment. These are physical abuse, emotional abuse, sexual abuse, emotional neglect and physical neglect, each based on five items. The CTQ also includes a 3-item minimization/denial scale designed to screen for false-negative trauma accounts. All items are rated on a 5-point Likert scale, ranging from 1 ‘Never True’ to 5 ‘Very Often True’. Scores are quantified by summating the subscale and total scores and comparing those values to cut scores that have been validated against other measures of past abuse and/or neglect severity (Bernstein & Fink, 1998). The CTQ subscales are stable (test-retest reliability = 0.79-0.86) and internally consistent (α =0.66-0.92; Bernstein & Fink, 1998; Scher et al., 2001).

National Adult Reading Test-Revised (NART-R)

The NART-R (Blair & Spreen, 1989) was used to estimate premorbid intelligence. Composed of 61 phonetically irregular words that do not follow normal pronunciation rules, the NART-R forces subjects to rely on familiarity (opposed to correct guessing) when completing the assessment. The NART-R has test-retest reliability ranging from 0.67-0.98 depending on the interval between tests and inter-rater reliability ≥ 0.88. The NART-R was not obtained for all participants, but was used to corroborate matching on education, since depressive episodes may interfere with MDD patients continuing their education.

7.3.3. ^{1}H-MRS data acquisition and analysis

The acquisition and quantification of GABA in the present study followed the same protocol as for the menstrual cycle study, (chapter 6.3.2.). Females were scanned within day 3-8 of the menstrual cycle or within the first seven days of pill use of hormonal birth control.

7.3.4. Statistical Analysis

Demographic data were analyzed using Chi-squared and Student's t-tests, with Welch's adjustment for unequal variance where indicated. ^{1}H-MRS data were analyzed using skew and kurtosis test, Student's t-tests, analysis of variance and covariance (ANOVA and ANCOVA). Relationships between variables were analyzed using Pearson correlations. Analyses were conducted in IBM Statistics (SPSS 20) and Stata 14. The α level was set at two-tailed $\alpha = 0.05$, except for $GABA^{+}$, which was set at one-tailed $\alpha = 0.05$, given that individual studies and meta-analysis have not reported increased $GABA^{+}$ levels in MDD (Schür et al, 2016).

7.4. Results

7.4.1. Participant Characteristics

In the MDD sample, one patient was disqualified due to HDRS < 18, one due to past substance abuse and one due to neurological disease. Two patients who were eligible after assessment dropped out before their scans. In the healthy control sample, two had a history of anorexia nervosa, one substance abuse, one an anxiety disorder and one dropped out. Data from seven healthy controls and five MDD participants had to be excluded from analysis due to poor quality spectra. One MDD patient was able to repeat a poor quality scan with good data.

In the final sample, healthy control participants were similar in age and other characteristics to the MDD patients, although they had more years of education (table 7.4.). NART-R scores were not significantly different between the groups. Since NART-R scores were not obtained for every participant, we also compared education in only those participants who also had NART-R scores (controls = 15.2 ± 1.3; MDD = 14.4 ± 1.92; $t = 1.66$; $df = 45$; $p = 0.11$).

Table 7.4. Participant characteristics.

	Healthy Controls n = 42	MDD n = 35	Chi^2		*p*
Sex, F/M	24/18	21/14	0.06		0.80
			t	*df*	*p*
Age,y	32.8 ± 9.3	33.4 ± 8.9	0.31	75	0.76
Education, y	15.5 ± 1.25	14.71 ± 1.84	2.08	75	0.04
NART-R	108.3 ± 11.1	109.1 ± 12.2	0.22	44	0.83
Height, m	1.69 ± 0.10	1.72 ± 0.11	1.00	75	0.32
Weight, kg	68.5 ± 12.9	74.0 ± 14.0	1.80	75	0.08
BMI	23.8 ± 3.2	25.1 ± 4.4	1.48	75	0.14
MASQ	97.5 ± 14.2	189.2 ± 25.7	18.8	52.1	<0.001
CTQ	35.7 ± 12.9	52.2 ± 16.5	4.3	55.0	<0.001
HDRS		21.6 ± 3.6			
Age onset, y		25.0 ± 8.6			
1st episode, yes/no		12/23			
Medication naïve, yes/no		18/17			
Washout, months Median (range)		24 (3-168)			

Key: F = female; M = male; y = years; NART-R = National Adult Reading Test-Revised; BMI = body mass index; MASQ = mood and anxiety disorders symptom questionnaire; CTQ =

childhood trauma questionnaire; HDRS = Hamilton depression rating scale; MDD = major depressive disorder.

7.4.2. ^{1}H-MRS results

For the ^{1}H-MRS data, the CSF content of the voxel did not differ between MDD and controls, but there were significant differences in the GM and WM composition of the voxel. MDD patients had lower GM and higher WM content, leading to a lower GMF.

Table 7.5. ^{1}H-MRS data in MDD patients and healthy controls.

	Healthy Controls n = 42	MDD n = 35	*t*	*df*	*p*
GM, %	60.9 ± 6.2	57.7 ± 6.8	2.19	75	0.03
WM, %	24.9 ± 4.2	29.3 ± 5.5	3.90	75	<0.001
CSF, %	14.3 ± 5.5	12.6 ± 3.6	1.52	75	0.13
GMF	70.9 ± 4.9	66.6 ± 6.2	3.44	75	0.001
$GABA^{+}$, mmol/L	1.11 ± 0.22	1.04 ± 0.31	1.24	75	$0.11^{\dagger}$

Key: MDD = major depressive disorder; GM = grey matter; WM = white matter; CSF = cerebrospinal fluid; GMF = grey matter fraction; all p values 2-tailed, except †1-tailed

Analysis of $GABA^{+}$ levels was conducted using ANCOVA, with group (MDD/Control) as a between-subject variable and GMF as a covariate. Education was also included as a covariate, due to the difference between groups. However, neither GMF ($F_{1,73} = 1.18$; $p = 0.28$), nor education ($F_{1,73} = 0.09$; $p = 0.77$) were significant covariates and there were no significant differences in $GABA^{+}$ concentrations after adjusting for these variable (Fs < 1.6; 1-tailed p >

0.1). $GABA^{+}$ levels were therefore compared using Student's t-tests and showed no group differences (table 7.5.). Sample spectra are shown in figure 7.1. and the individual data points in figure 7.2.

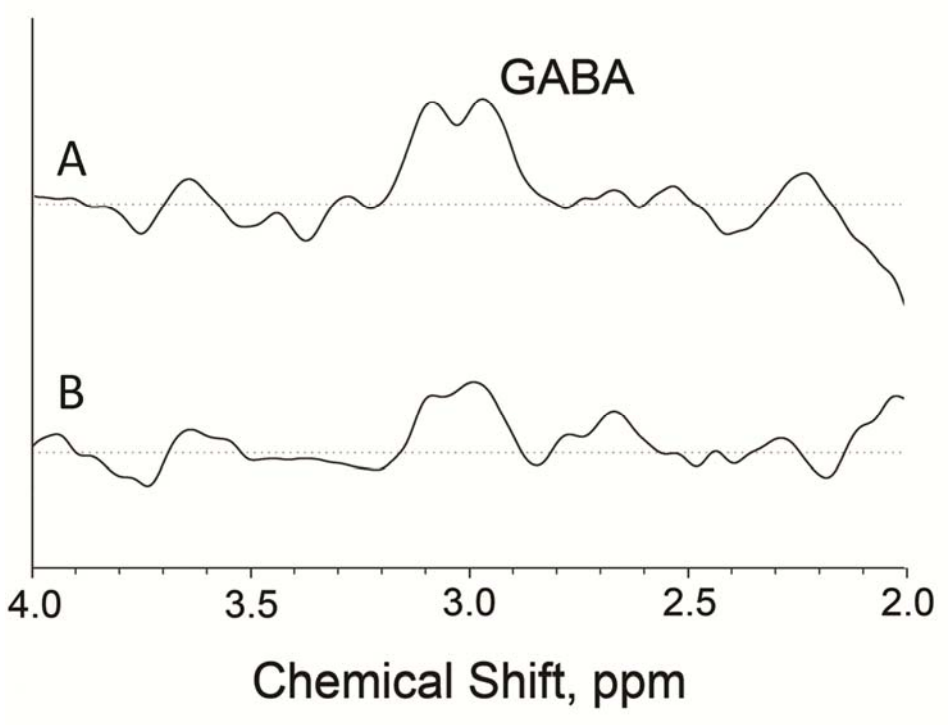

Figure 7.1. GABA spectra from A. healthy control and B. MDD patient

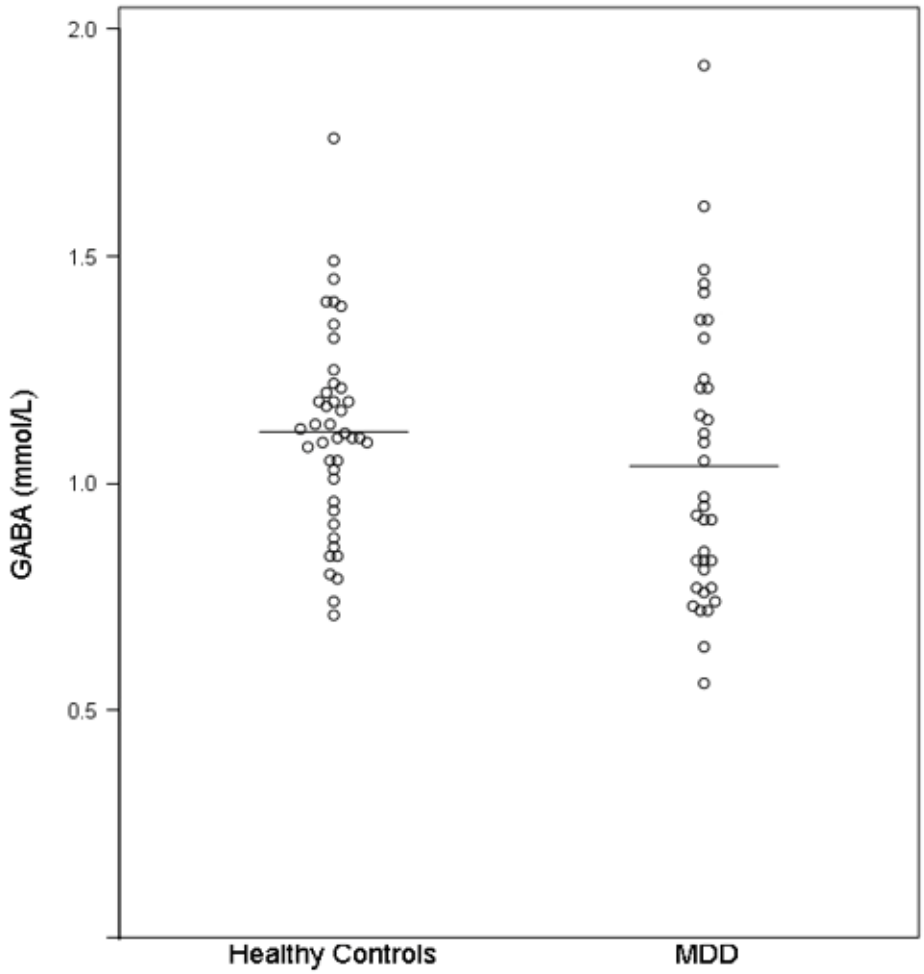

Figure 7.2. Individual data and means for GABA levels in healthy controls (n = 42) and MDD (n = 35).

A number of exploratory subgroup analyses were also conducted. We examined whether there might be sex-related differences in GABA between MDD and controls, using a 2-way ANOVA, but there was no main effect of sex ($F_{1,73}$ = 1.24; p = 0.27) or sex by diagnosis interaction ($F_{1,73}$ = 0.51; p = 0.48). There were also no differences between MDD with or without comorbid social anxiety disorder (t = 0.61; df = 33; p = 0.55), or generalized anxiety disorder (t = 0.29; df = 33; p = 0.78), or between first episode (n = 12) and recurrent MDD (n = 23)(t = 0.42;

df = 33; p = 0.68), or between medication naïve or previously treated MDD (t= 0.68; df = 33; p = 0.50).

Previous studies have reported that melancholia and depressive anhedonia are associated with lower $GABA^+$ levels in the OCC (Sanacora et al, 2004) or in the ACC (Gabbay et al, 2012). 32/35 MDD patients had depressive anhedonia ratings >80, as measured by the MASQ, which is 3 standard deviations above the mean of the depressive anhedonia ratings of the healthy controls. We subdivided MDD patients using a median split on the depressive anhedonia scores. $GABA^+$ levels in the higher anhedonia subgroup did not have lower GABA levels than those in the lower anhedonia group, after covarying for lower GMF ($GABA^+$: lower anhedonia, 1.10 ± 0.10; higher anhedonia, 0.98 ± 0.05; $F_{1,31}$ = 0.58; 2-tailed p = 0.23). However, the MDD patients in the highest anhedonia group had lower $GABA^+$ levels than healthy controls, covarying for GMF ($F_{1,58}$ = 3.30; 1-tailed p = 0.04).

Within the whole sample, $GABA^+$ concentrations were correlated negatively with CTQ scores (r = -0.28; p = 0.04) and specifically with childhood emotional abuse (r = -0.31; p = 0.02) and neglect (r = -0.37; p = 0.005). These correlations appeared to be driven by the MDD participants, emotional abuse (r = -0.34; p = 0.052) and neglect (r = -0.44; p = 0.01), since they were not present in the healthy controls, when analyzed separately ($r < 0.2$; $p > 0.4$). These results were not controlled for multiple comparisons and should be considered exploratory.

7.5. Discussion

7.5.1. Main findings

In contrast with some prior reports, this study did not find significant differences between ACC $GABA^+$ levels in MDD patients and healthy controls. $GABA^+$ levels in MDD were 6% lower than in healthy controls, but this difference is less than the intra-subject coefficient of variation for the $GABA^+$ measurement (8.0% for subjects scanned twice in a single session, or 14.2% over two sessions: Ogilvie, 2005). Similar to a prior report (Gabbay et al 2012), in one of a number of subgroup analyses, $GABA^+$ levels were lower in MDD patients with higher depressive anhedonia than in healthy controls (Gabbay et al 2012), based on a median split of the MASQ depressive anhedonia ratings. This finding should be treated cautiously, for several reasons. First, by design, we excluded MDD patients with atypical features from the sample, because this patient subgroup has been reported to be less likely to show differences in $GABA^+$ levels (Sanacora et al, 2004). Second, the continuous rating of depressive anhedonia did not correlate significantly with $GABA^+$, although the relationship was negative. Third, the α level for the subgroup analyses was not adjusted for multiple comparisons. Fourth, other prior studies that have not found differences in $GABA^+$ that were related to the severity of anhedonia (Walter et al, 2009). In our study, nearly all of the MDD patients reported marked depressive anhedonia (over 3 standard deviations > healthy control mean) and the median split separated a subgroup ~4 standard deviations > healthy control mean. On the basis of previous evidence that severely depressed in-patients show the lowest OCC $GABA^+$ levels (Sanacora et al, 2004), it is possible that differences in ACC $GABA^+$ are too small to be detected by current methods, except in the most severely affected patients. Although our patients had at least moderate MDEs and did not

have MDD with atypical features, they were out-patients and medication-free for at least 3 months.

7.5.2. Comparison with prior studies

Although there are four reports of reduced ACC $GABA^+$ in MDD patients, there have been ten comparisons published, of which six were negative (table 7.2). Several factors may have contributed to differences between studies, including sample characteristics and ^{1}H-MRS methodology. The present study included only 18-50 year old male and premenopausal out-patients, whereas other prior studies have included treatment-resistant subgroups (Price et al, 2009), only female first episode patients (Wang et al, 2016), adolescent patients (Gabbay et al, 2012), or older or postmenopausal patients (Price et al, 2009; Bhagwagar et al, 2008; Wang et al, 2016). There have also been differences in the inclusion of medicated patients and the length of medication washout (see tables 7.1. and 7.2.), with short washout periods potentially associated with withdrawal and uncertain effects on GABA. Most studies included recurrent MDE patients, but one included only first episode females (Wang et al, 2016). Some studies included patients with comorbid lifetime anxiety disorders (Gabbay et al, 2012; Price et al, 2009; Hasler et al, 2007), similar to the present study, whereas others excluded axis I comorbidity (Wang et al, 2016), or did not employ a structured diagnostic interview (Zhang et al, 2016).

7.5.3. Strengths and limitations

The present study is the first to report ACC $GABA^+$ data for MDD acquired using the DQF-S method, in contrast to nearly all of the other studies that have used *J*-difference editing (Table 7.2.). It is also the largest study of ACC $GABA^+$ in the field, with only one other study including more than 20 MDD patients (Table 7.2.). There are both strengths and weaknesses of

the current methods. The strengths are that DQF-S is a 'single shot' method that is less affected by factors that lead to subtraction errors in *J*-difference editing (Evans et al, 2013) and that the method markedly reduces the co-editing of MM signals (9% of signal) compared with *J*-difference editing (45% at 3T) (Choi et al, 2007; Harris et al, 2015). As examples, three of the eight reports of ACC $GABA^+$ to date show sample spectra in which there are residual choline signals at 3.2 ppm (Bhagwagar et al, 2008; Wang et al, 2016; Zhang et al, 2016), which is an indication of subtraction errors (Evans et al, 2013). Although two studies reported GABA levels, as opposed to $GABA^+$, this was based solely on performing an across-the-board subtraction of a fixed percentage of the signal as an estimated MM contribution and the results are not otherwise different other *J*-difference editing studies, in accounting for MM contamination (Hasler et al, 2005, 2007). Since MM contamination contributes a large proportion of the $GABA^+$ signal in *J*-difference editing compared with DQF-S, the signal-to-noise ratio (SNR) for $GABA^+$ will be apparently greater, which may mean that tests with low $GABA^+$ levels are less likely to be rejected from the analysis due to poor SNR. This could reduce the possibility of showing reduced $GABA^+$ levels with the DQF-S method, if scans with low SNR are more frequently rejected in the MDD sample. However, this would not seem to explain the current findings, since a similar number of scans were rejected in both samples.

Another difference between studies is that the present study quantified ACC $GABA^+$ with respect to brain water, controlling for the CSF fraction and GMF fraction, where appropriate, whereas four of the prior studies (including six brain region comparisons) used creatine as a reference (Bhagwagar at el, 2008; Hasler et al, 2005, 2007; Walter, et al 2009). One study did not specify the reference for quantification (Wang et al, 2016).

An advantage of the *J*-difference methods is that since they are the most commonly used, they have been implemented on a number of scanner platforms and in several analytical software packages, such as Gannet or LCModel (Edden et al, 2013; Provencher, 2003), whereas the current study data were analyzed by peak area integration. The use of spectral fitting methods has the advantages of being operator-independent and allowing for fitting quality to be assessed in terms of Cramér-Rao Lower Bounds (CRLBs). A caution is that since the CRLBs are expressed as a percentage of the signal, they may be artificially lowered by the contribution of MM to the $GABA^+$ peak.

7.6. Conclusions

In the present study, we did not show significant differences in ACC $GABA^+$ between MDD patients and healthy controls, or any robust differences in subgroups of MDD patients that would have withstood control for multiple comparisons. The strengths of the study were the largest sample size to date for ACC $GABA^+$ studies, the use of a DQF-S method to acquire $GABA^+$ data that is less vulnerable to hardware fluctuations and subject motion than *J*-difference editing and less affected by MM contamination, and quantification with respect to brain water, controlling for the tissue composition of the voxel. The main limitation was the lack of an operator-dependent method of data quantification. The findings cannot be generalized to depressive subgroups, such as more severely ill in-patients, or treatment-resistant patients.

7.7. References

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CHAPTER 8. THE ACUTE AND DELAYED EFFECTS OF HYDROCORTISONE ON PREFRONTAL GLUTAMATE AND GLUTAMINE IN HEALTHY VOLUNTEERS.

8.1. Background and rationale

8.1.1. Preclinical studies

Stress increases the adrenal secretion of glucocorticoids (corticosterone in rodents and cortisol in humans) which modify gene transcription via nuclear GRs and MRs (de Kloet et al, 2008). The effects of chronic stress and glucocorticoids on neuroplasticity and neurogenesis in part depend on glutamate and can be blocked by N-methyl-D-aspartate (NMDA) receptor antagonists (Cerqueira et al 2005a,b; McEwen 2005; Radley and Morrison 2005; Mirescu et al, 2006; Joëls et al, 2004; Nacher and McEwen 2006). In acute stress, increases in prefrontal and hippocampal glutamate release depend on glucocorticoid availability (Moghaddam 2002) and the direct systemic and local administration of glucocorticoids increases hippocampal extracellular glutamate within minutes (Venero and Borrell 1999). *In vitro* studies have also shown rapid effects of glucocorticoids on synaptic glutamate release (Karst et al, 2005; Olijslagers et al, 2008). These actions appear to be non-genomic, due to their speed of onset, resistance to transcriptional or protein synthesis inhibitors and initiation by cell membrane receptors (Losel et al, 2003; Moore and Evans 1999; Karst et al, 2005). In humans, rapid effects of glucocorticoids on cognition have been observed in as little as 15-30 minutes (Lupien et al, 1999; 2002).

Glutamate and glutamine are highly compartmentalized in glutamatergic neurons and glia respectively, with a small proportion localized to GABA neurons (Hyder et al, 2006; Ottersen et al 1992). Neuronal glutamate utilized in neurotransmission is rapidly replenished by the supply of glutamine and by anaplerotic synthesis (Bacci et al, 2002; Hyder et al, 2006; Waniewski and

Martin 1986) and it has been demonstrated that the activation of excitatory transmission leads to the depletion of glutamine from hippocampal neurons, whereas neuronal glutamate levels are maintained (Jenstad et al, 2009). An effect of glucocorticoids on excitatory neurotransmisson might therefore decrease glutamine.

8.1.2. Rapid effects of cortisol on glutamine measured by ^{1}H-MRS

In a previous study, we administered hydrocortisone, 35 mg intravenously to 12 healthy volunteers and measured prefrontal glutamate and glutamine levels before and after the injection while the participants were in the MR scanner. We found that glutamine levels were reduced within 30 minutes following hydrocortisone, but not placebo, whereas glutamate levels were unchanged (Bhardwaj, 2009). A change in glutamine might indicate increased glutamatergic neurotransmission, although the pools of glutamate and glutamine measured by ^{1}H-MRS are the total within a volume of tissue and are not related in a straightforward fashion to neurotransmission. Limitations of the study were that only a single dose of hydrocortisone was examined, only a single baseline acquisition was obtained for glutamine and only 128 excitations were used per acquisition, which limits the signal-to-noise ratio (SNR). The participants in the study were all experienced in having ^{1}H-MRS scans and the protocol might not be as robust in participants who are less compliant in remaining still. One aim of this study was to acquire multiple longer acquisitions (256 excitations) for glutamine to increase SNR. Two studies have examined the effects of acute stress on ACC Glx (glutamate+glutamine), using ^{1}H-MRS. One study found that stress associated with the threat of shock did not alter Glx, but did not measure cortisol levels (Hasler et al, 2010). The other found that cholecystokinin-4 injection increased panic and anxiety symptoms, cortisol and Glx, transiently (Zwanzgwer et al, 2013). Both of these studies used creatine as a reference.

8.1.3. Potential delayed effects of cortisol

In addition to having non-classical, rapid, non-genomic effects (onset <30 minutes) cortisol has classical effects via slower onset regulation of gene transcription (typically in hours to days). An established finding is that corticosteroids increase astrocytic glutamate transporter and glutamine synthetase (GS) transcription and GS enzyme activity, in primary glial cell cultures and in rat brain (Vardimon et al 1999; Rauen et al 2000), which could be predicted to increase brain glutamine. We therefore measured glutamate and glutamine during and after the repeated administration of hydrocortisone over 48 hours. Since there have been no comparable studies, the study was a pilot to determine if there is any change in metabolites over this period. Although chronic glucocorticoid administration has been used in animals to study the effects of chronic glucocorticoid hypersecretion during chronic stress (Cerqueira et al, 2005), this would not be ethical in human research due to deleterious health effects. A meta-analysis of ^{1}H-MRS studies of major depressive disorder (MDD) showed that levels of Glx (glutamate + glutamine), but not glutamate, were reduced, when referenced to internal water, suggesting that glutamine may be reduced in MDD (Arnone et al, 2015).

8.1.4. Brain region

In the previous and current study, glutamate and glutamine were measured in PFC, because this region is important for corticosteroid effects on human cognition (Lupien et al 1999; Putman and Roelfs, 2011), whereas most preclinical studies of rapid glucocorticoid effects on neurotransmission have been performed on hippocampus or amygdala (Joëls et al, 2013). Prefrontal cortex is more suited to the current ^{1}H-MRS method than the hippocampus or amygdala, by virtue of its large contiguous grey matter volume and better magnetic field

homogeneity during scanning. In contrast to rodents, human prefrontal cortex expresses high levels of GRs and MRs (Watzka et al, 2000; Webster et al, 2002). Prefrontal cortex is a site of stress-induced, corticosteroid-dependent glutamate release (Popoli et al, 2012) and is vulnerable to corticosteroid effects on structural remodeling in rodents (Cerqueira et al, 2005a,b; Radley and Morrison 2005) and possibly also in humans (Gold et al, 2005; MacLullich et al, 2006).

8.1.5. Dose-response

Studies of major depressive disorder (MDD) have consistently shown reduced negative feedback in the HPA axis, using the dexamethasone-suppression test (DST) (Ribeiro et al., 1993), or the combined corticotrophin-releasing hormone-dexamethasone test (Holsboer, 2001), (see chapter 3). However, dexamethasone enters the brain poorly and this reduction in negative feedback is probably observed at the level of the pituitary (de Kloet, 1997; Pariante et al, 2002). A consistent change in glutamine or glutamate in reponse to cortisol might be useful as a method to determine whether patients with MDD have a reduced sensitivity to cortisol at the level of the brain. For this reason, we wanted to determine whether there was a higher response to cortisol at a supraphysiological dose (hydrocortisone 100mg).

8.1.6. Spectral editing

Selective and reliable measurement of glutamate and glutamine by ^{1}H-MRS is not straightforward, because of their spectral overlap at 2.3~2.5 ppm and their strong *J*-coupling, which leads to complex evolution of their resonance line-shapes and rapid signal decay with increasing echo time (TE) (Thompson and Allen 1999). Furthermore, discrimination is required from overlapping resonances, particularly those of NAA, glutathione and MMs. Short TE methods are often employed in ^{1}H-MRS, but these enhance signal intensity rather than

metabolite discrimination, and contamination with macromolecules is greater at short TE. Our studies have therefore taken the alternative approach of using spectral editing to simplify the chemical spectra by removing overlapping resonances (Harris et al, 2017). The study acquisitions used spectrally-selective refocusing pulses designed to discriminate glutamate and glutamine at 3.0 T, whilst suppressing background contamination (Choi et al, 2006).

8.2. Goals and hypotheses

The goal of the study was to assess the acute and delayed onset effects of cortisol on brain glutamate and glutamine in healthy volunteers by measuring these metabolites within 30 minutes following the intravenous injection of hydrocortisone and then for 48 hours during and after the repeated administration of oral hydrocortisone at two dose levels (40 mg and 100 mg). Most studies of cortisol effects on cognition have examined single oral doses in the 20-50 mg range and it has been recommended to examine dose-response relationships (Lupien et al, 1999; Putman and Roelfs, 2011), in this case in the range of increasing membrane MR and GR activation (Karts et al, 2005). The lower dose was adjusted from the prior study, from 35 mg to 40 mg, to match the availability of the oral formulation (20 mg tablets). The hypothesis was that the acute effect of hydrocortisone was to decrease glutamine levels, as in the previous study, whereas the delayed effect would increase glutamine levels.

8.3. Methods and Materials

8.3.1. Design.

Participants provided written informed consent and the study was approved by the University of Alberta Human Research Ethics Board. The study was a double-blind, placebo-controlled comparison of the effects of hydrocortisone 40 mg or 100 mg on brain glutamate and glutamine.

8.3.2. Participants

8.3.2.1. Inclusion and exclusion criteria

The study included healthy males or females, aged 18- 35 years. Females were scanned within day 3-8 of the MC or were using hormonal birth control. Exclusion criteria were: 1. Lifetime psychiatric disorders. 2. Significant neurological disease, e.g. head injury with loss of consciousness, stroke, seizures, etc. 3. Other significant medical conditions, e.g. hypertension, inflammatory disease, hepatic/renal insufficiency. 4. Corticosteroid treatment within the last three months. 5. Shift work or other factors that would affect diurnal cortisol rhythm. 6. Any use of psychotropics (prescribed, OTC or illicit) within the last three months and any history of frequent use of psychotropics. 7. Regular intake of >14 standard alcoholic drinks per week (males) or 7 per week (females). 8. Alcohol intake within 48 hours of scans. 9. Use of any medications with potential CNS effects within one week or five half lives prior to scans. 10. Pregnancy, lactation. 11. Magnetic resonance exclusion criteria, e.g. the presence of metallic foreign objects or devices.

8.3.2.2. Assessment

Participants were recruited through poster advertisements across the University of Alberta campus. In total, of all screened respondents, 21 participants were suitable candidates for the study. The assessment included a structured interview using The Anxiety Disorder Interview Schedule IV – ADIS-IV (Brown et al, 2001) to exclude those with a lifetime history of psychiatric disorders. The ADIS-IV also includes a medical history checklist and participants were interviewed by the study physician and had height, weight and blood pressure recorded. A Magnetic Resonance safety checklist was administered.

8.3.3. Procedures

8.3.3.1. Testing schedule and preparation

Once included, participants were scheduled for three MR scans on three consecutive days, with each scan starting in the mid to late afternoon, when basal cortisol levels would normally be low due in their diurnal cycle. On each visit, participants had a brief health assessment to exclude intercurrent illness, and to check medication side effects and blood pressure.

Sixty minutes prior to scans, intravenous (IV) cannulae were inserted in both arms, one to collect blood samples for serum cortisol measurements and the other line to administer the study medication. The participants rested for 45 minutes post-cannulation before a cortisol sample was obtained and they then started the scanning protocol. Participants wore earplugs and headphones in the scanner to decrease mechanical noise. After the pre-injection, ^{1}H-MRS data were collected, and medications were administered over 60 seconds intravenously within the scanner. The intravenous dose was given as hydrocortisone (hydrocortisone sodium succinate,

Novopharm) 40 mg or 100 mg, or saline placebo. A sample for peak plasma cortisol was collected 5 minutes after the injection. Post-injection ^{1}H-MRS data were then collected. Following the scan, participants were instructed on oral medication dosing. Medication was taken on the mornings of day 2 and 3 of the study at 7am. The oral doses were given as placebo, or 2x or 5x 20 mg hydrocortisone tablets (Cortef, Pfizer) in two white gel caps. The mean plasma elimination half-life for hydrocortisone is 2.1 h (Hamitouche et al, 2017), so the afternoon data were acquired at approximately 3-4 elimination half-lives, when any acute effects that depend on the continued elevation of plasma cortisol would be expected to have diminished. The participant, research assistant and data analyst were blind to medication status.

8.3.3.2. ^{1}H-MRS data acquisition

The timeline of the procedures for the injection day is outlined in figure 8.1.

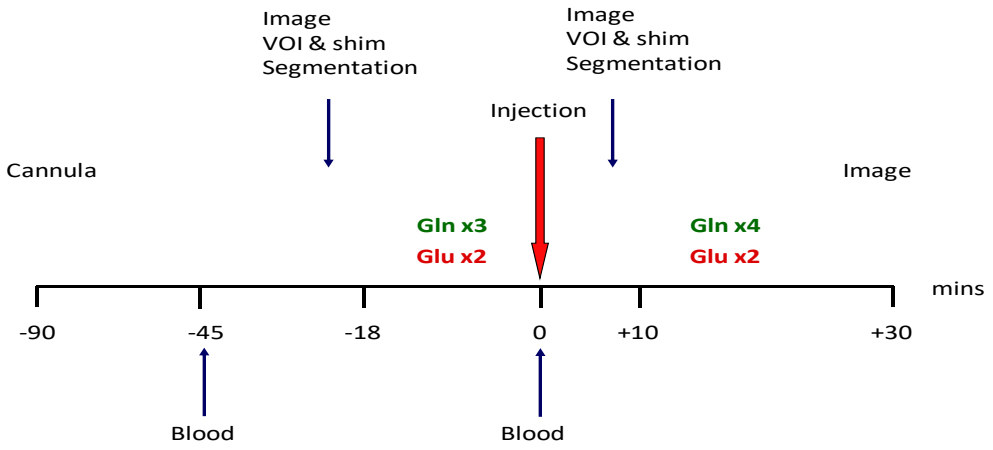

Figure 8.1. Glutamate and glutamine acquisition timings. Key: Glu = glutamate; Gln = glutamine; VOI = voxel of interest.

Data were acquired with a 3.0T magnet (Magnetic Scientific PCL) interfaced to a Surrey Medical Imaging System (SMIS) console. A 28cm diameter quadrature birdcage resonator coil was used for radiofrequency transmission and reception. A multi-slice gradient echo image (TR = 500ms, TE = 22ms, slice thickness = 5mm, 11 slices, resolution = 256 x 256), in the transverse, sagittal, and coronal planes, was used to place of the ^{1}H-MRS voxel. The voxel (30 x 25 x 30 mm^3) was positioned across the midline in bilateral rostral anterior cingulate cortex and medial PFC, above the callosal plane (between the lower borders of the genu and splenium of the corpus callosum, see figure 8.2). This location maximized the inclusion of grey matter and avoided the most ventral part of the PFC, which has the highest magnetic field inhomogeneities.

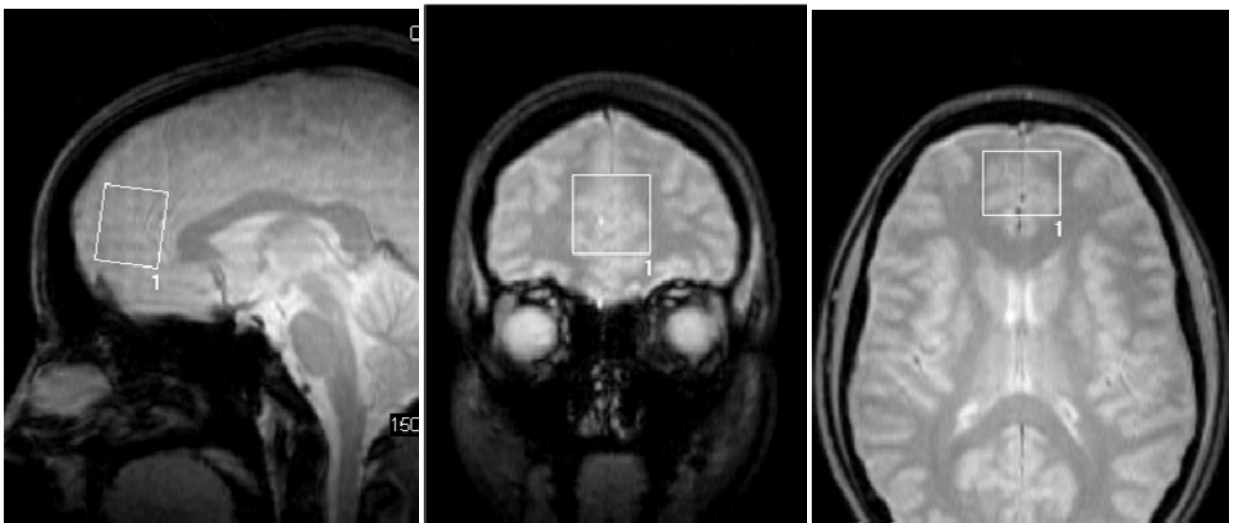

Figure 8.2. Voxel placement for glutamate and glutamine acquisitions (30 x 25 x 30 mm^3)

Shimming was accomplished using fast, automatic shimming technique by mapping along projections (FASTMAP) (Gruetter et al, 1993) and the shim fine-tune was performed using an automated in-house routine. Tissue segmentation data were acquired using a double-inversion recovery PRESS sequence to measure the grey matter, white matter, and CSF composition of the

voxel (Choi et al, 2006). Voxel placement, shimming and segmentation were repeated post-injection, prior to the metabolite sequences.

Metabolite data were acquired using published spectrally selective refocusing sequences within a PRESS localization scheme (Choi et al, 2006). They exploit the small chemical shift difference between the glutamate and glutamine C4 protons (~13 Hz at 3.0 T). Two different spectrally-selective triple-band 180° radiofrequency (T180) pulses, with excitation bandwidth of ~12 Hz, are implemented within PRESS localization. The glutamate filter includes an 81.9-ms long T180 with a single Gaussian radiofrequency waveform (truncated at 20%), with phase variations designed for refocusing at 2.35, 3.02, and 3.92 ppm (Geen et al, 1989). The glutamine filter has a 90.5-ms long T180, designed for refocusing at 2.51, 3.02 and 3.92 ppm. Both filters generate Cr singlets at 3.02 and, in inverted phase, at 3.92 ppm, that were used for metabolite quantification, phase correction and to monitor frequency drift during the acquisition. Glutamate and glutamine target multiplets are generated at 2.35 ppm and 2.39 ppm respectively (figure 8.3).

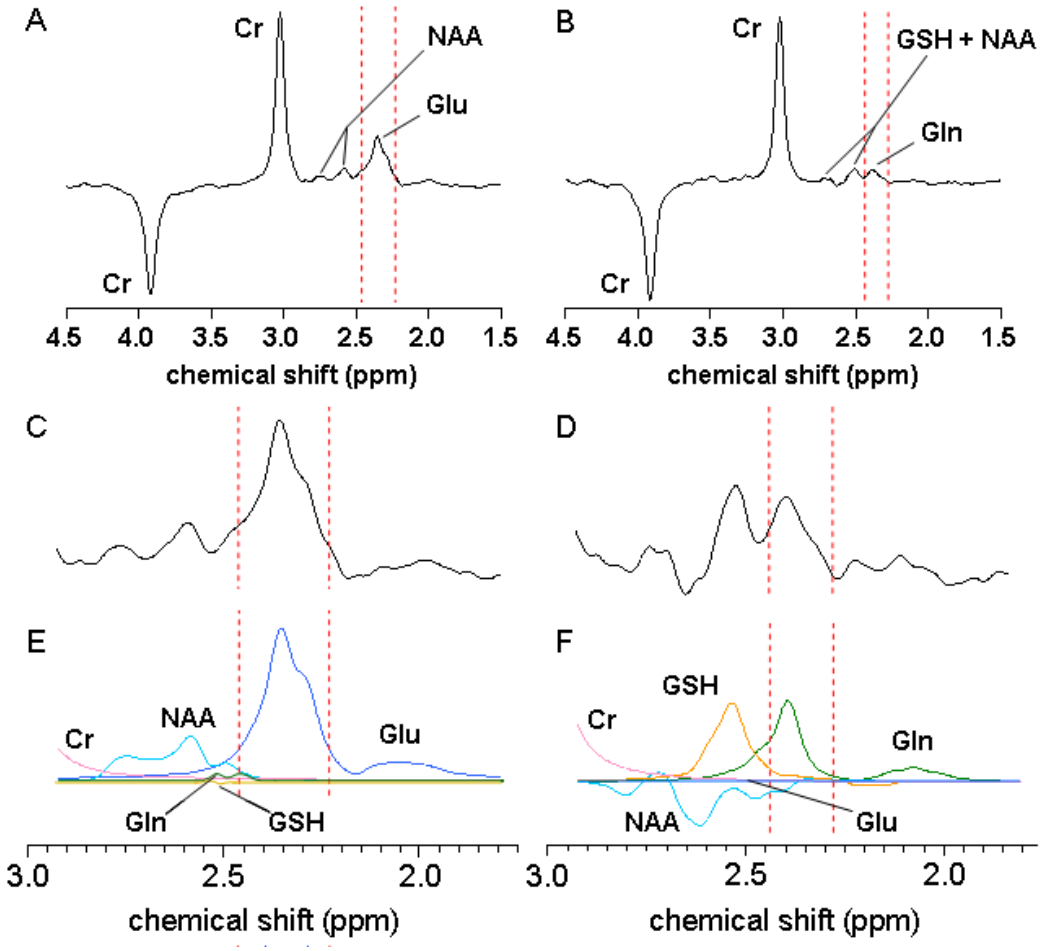

Figure 8.3. Glutamate and glutamine spectra acquired using selective refocusing.

Key: *In vivo* spectra for glutamate (Glu: A, C at larger scale) and glutamine (Gln: B, D) with fitted basis spectra (E and F). The Glu and Gln peaks show low overlap with contaminants and each other. Cr = Creatine; NAA = N-acetylaspartate; GSH = glutathione. Spectra were filtered with a 2-Hz exponential and a 4-Hz Gaussian function for illustration.

TEs were optimized for each filter (glutamate TE = 128 ms, glutamine TE = 158 ms) to maximise the target signal yield and minimize background contamination from GSH (2.56 ppm) and NAA (2.48 ppm). GABA, succinate and pyruvate contamination can be neglected due to their low refocusing ratios and low brain concentrations. Macromolecule contamination is

reduced by the selective refocusing and long TE. The other spectral parameters were: carrier frequency = 3.0 ppm; TE = 128 ms (glutamate) and 158 ms (glutamine); TR = 2.4 s; acquisition time, at = 820 ms; dwell time, Δt = 400 μs; number of complex points = 2048; number of excitations = 64 (glutamate) and 256 (glutamine); 64 and 256 step phase rotation. Variable-flip-angle water suppression was applied prior to the editing sequences. Eddy current effects were removed by correcting the phase factor of individual data points of the free induction decay, based on the phase factor of the water signal acquired with the 180º RF pulses tuned to the water resonance (Choi et al, 2006).

Segmentation of grey matter (GM), white matter (WM) and cerebrospinal fluid (CSF) within the voxel was carried out from 1-D imaging of the spatially-localized water signal following a Stimulated-Echo Acquisition Mode (STEAM) sequence, and extrapolation of the recorded 1-D profiles to zero TE (Choi et al., 2007), using published water T2 values (Wansapura et al., 1999). Double inversion recovery was used to discriminate the water signals between the three compartments (Redpath and Smith, 1994). The grey matter fraction (grey / grey + white matter) was calculated. Data were adjusted for different tissue water concentrations (Wansapura et al, 1999).

8.3.3.3. ^{1}H-MRS data analysis

Data from the glutamate and glutamine sequences (glutamate, glutamine, creatine) were quantified using LCModel analysis with respect to tissue water adjusted for the voxel tissue composition (Provencher, 1993) using a synthetic basis set derived from density matrix simulation of the metabolite responses to the selective refocusing sequences (Thompson and Allen, 2001). Metabolites included in the basis set for glutamate were glutamate, NAA and

creatine. Metabolites included in the basis set for glutamine were glutamine, NAA, creatine and GSH. For the glutamate and glutamine analyses, data bins of 16 acquisitions were checked for frequency drift and those exceeding a drift threshold of 2.4 Hz were excluded from the analysis. Metabolites with Cramér-Rao Lower Bounds (CRLBs) >20% (Provencher, 1993) were excluded from the statistical analysis. For single glutamine acquisitions, 77% of the data had Cramér-Rao Lower Bounds (CRLBs) <20%, which was increased to 92% when two acquisitions were combined. In order to maximize signal-to-noise, particularly for the glutamine acquisitions, data from the multiple glutamate and glutamine acquisitions at each time point were combined. Two glutamate acquisitions were combined for each time point (pre- and post-injection, day 2-3) and either three glutamine acquisitions (pre-injection) and four at the other time points (post-injection, day 2-3). Since the focus of the study was on within-subject changes in metabolites, data were not adjusted for relaxation effects and the values reported from LC Model analysis are in institutional units (IU). Ratio values of glutamate (glutamate/Cr) and glutamine (glutamine/Cr) to creatine were also calculated and analyzed.

8.3.3.4. Cortisol analysis

Blood samples were collected into chilled EDTA tubes, placed on ice and centrifuged after each scan session. Samples were stored at -80°C until analysis. Plasma cortisol concentrations were measured using a specific enzyme-linked immuno-absorbent assay (ELISA, Alpco Diagnostics, Salem). Due to high serum cortisol at the peak following hydrocortisone administration, serial dilutions were performed to bring the concentrations of the samples within the linear range of the assay.

8.3.4. Statistical Analysis

Descriptive data are reported as means ± standard deviations. Data were analyzed using repeated measures Analysis of Variance (RMANOVA), with Mauchley's Test for Sphericity used to test for within-subject correlation and Greenhouse-Geisser correction applied where indicated. The analyses included study drug (placebo, hydrocortisone 40 or 100 mg) as a between-subject factor and session (pre- or post-injection; pre-injection, day 2, day 3) as a within-subject factor for the metabolite analyses. *Post hoc* comparisons were made using Student's t-tests, with Welch's adjustment to the degrees of freedom for unequal variance.

8.4. Results

8.4.1. Participants

In total, 19 subjects (9 female, 10 male; 25 ± 2.6 years) from the 21 who completed the three days of scans were included in the analysis, because 2 subjects had several glutamine acquisitions that did not meet the criterion of Cramér-Rao Lower Bound (CRLB) <20% (Provencher, 1993). Both subjects were tested during a period where there was a systematic problem with quality control, with scans with poor line-widths and high levels of noise, that was resolved after removing a hairclip from the scanner bed. Although the acquisitions for glutamate for these participants were within the CRLB <20% criterion, these spectra were also of poor quality and the final metabolite concentration values were as low as 35-50% of the normal average. Both of these participants were in the placebo group and the final number of participants per group was placebo, n = 5; hydrocortisone 40 mg, n = 6 and hydrocortisone 100mg, n = 8.

8.4.2. Plasma Cortisol

Plasma cortisol increased following the hydrocortisone infusion, but there was no evidence of a concentration-response in the comparison of the 40 mg and 100 mg doses of hydrocortisone (Table 8.1.).

Table 8.1. Plasma cortisol levels before and after hydrocortisone and placebo administration.

	Dose	Pre-injection	Post-injection	Day 2	Day 3	*F*	*df*	*p*
Cortisol (µg/dl)	Placebo	13.0 ± 2.2	24.8 ± 3.9	10.1 ± 3.4	10.6 ± 3.9			
	40 mg	7.9 ± 2.5	236.4 ± 109.5†	11.6 ± 13.3	12.9 ± 16.9	9.4	6,44	.0011*
	100 mg	12.9 ± 3.8	200.9 ± 82.8†	21.8 ± 15.0	24.1 ± 20.0			

*Greenhouse-Geisser-adjusted. $^{\dagger}p < 0.002$ vs placebo.

8.4.3. ^{1}H-MRS Voxel Composition

The voxel composition did not differ in grey matter, white matter, CSF or grey matter fraction (GMF) between the four sets of acquisitions (see Table 8.2.). The analyses of metabolite values therefore did not use tissue composition values as covariates.

Table 8.2. Tissue composition of the ^{1}H-MRS voxel over the four sets of data acquisitions.

	Pre-injection	Post-injection	Day 2	Day 3	*F*	*df*	*p*
Grey, %	61.5 ± 4.1	60.8 ± 4.1	61.4 ± 3.5	60.3 ± 4.4	1.29	3,54	0.29
White, %	29.0 ± 2.7	28.9 ± 3.1	29.5 ± 2.6	29.7 ± 2.6	0.18	3,54	0.38
CSF, %	9.4 ± 3.0	10.3 ± 2.8	9.3 ± 3.1	10.1 ± 3.7	1.38	3,54	0.25
GMF, %	67.9 ± 3.2	67.8 ± 3.6	67.5 ± 2.9	67.0 ± 3.2	1.06	3,54	0.38

Key: CSF = cerebrospinal fluid; GMF = grey matter fraction (grey x 100/grey + white)

8.4.4. Pre- to post-injection changes in metabolites

8.4.4.1. Glutamate

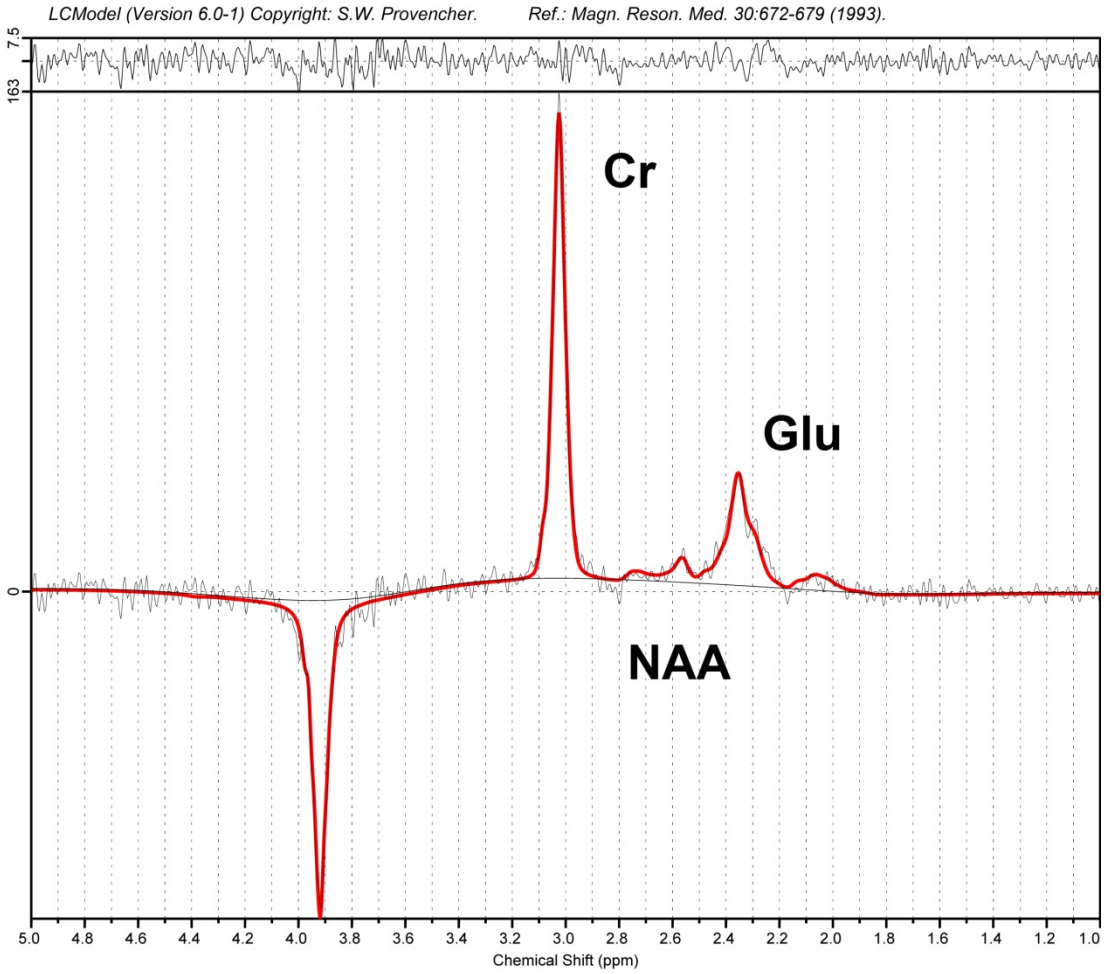

Figure 8.4. Glutamate spectrum with LCModel fit and residuals. Key: Gln = glutamine; Cr = creatine; NAA = N-acetyl-aspartate

Data quality (see figure 8.4. for example) was similar for the different sets of acquisitions There were no differences ($F_{2,26} = 0.42$; $p = 0.67$) between sets of acquisitions in the mean ± SD CRLB values, which ranged from 4.1 ± 0.8% to 5.2 ± 1.5%. Similarly, there were no differences ($F_{2,26} = 0.48$; $p = 0.63$) in the signal to noise ratio, SNR, which ranged from 17.0 ± 2.7 to 20. 5 ± 4.1.

Table 8.3. Glutamate before and after intravenous injection of study drug.

	Hydrocortisone dose	Pre-injection	Post-injection	*F*	*df*	*p*
Glutamate, IU	Placebo	1036 ± 222	1064 ± 206			
	40 mg	1132 ± 156	1145 ± 201	0.02	2,16	0.98
	100 mg	1137 ± 184	1162 ± 172			
Creatine (Cr), IU	Placebo	755 ± 106	754 ± 106			
	40 mg	807 ± 81	833 ± 101	0.17	2,16	0.85
	100 mg	841 ± 117	845 ± 100			
Glutamate/Cr	Placebo	1.36 ± .16	1.40 ± .12			
	40 mg	1.40 ± .13	1.37 ± .13	0.62	2,16	0.55
	100 mg	1.35 ± .07	1.38 ± .14			

Key: IU = institutional units.

There were no significant effects of study drug compared with placebo on glutamate, creatine, or the glutamate/Cr ratio (table 3).

8.4.4.2. Glutamine

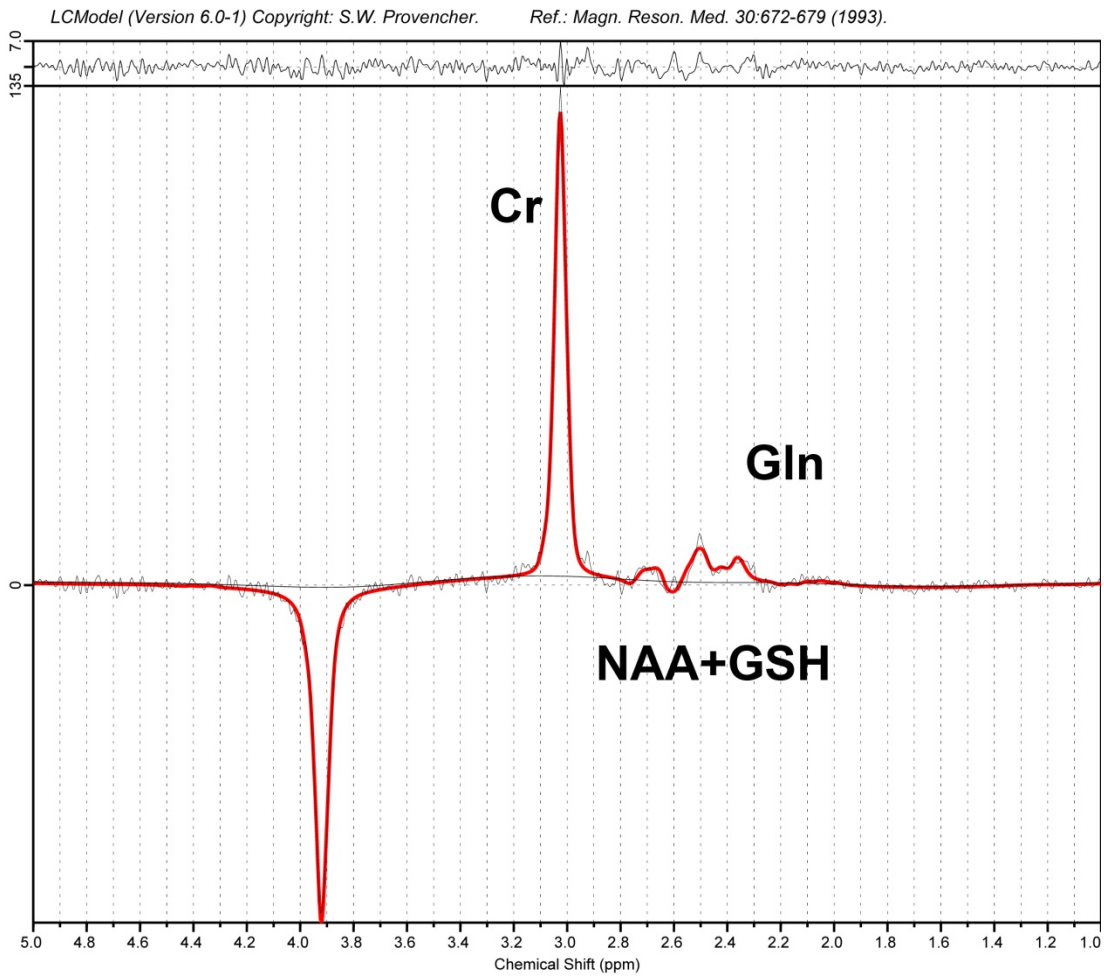

Figure 8.5. Glutamine spectrum with LCModel fit and residuals.

Key: Gln = glutamine; Cr = creatine; NAA = N-acetylaspartate; GSH = glutathione

There did not appear to be any systematic differences in data quality (see figure 8.5. for example) between the different sets of glutamine acquisitions. There were no differences ($F_{2,16}$ = 1.28; p = 0.22 between sets of acquisitions in the mean ± SD CRLB values, which ranged from

9.6 ± 2.1% to 11.6 ± 2.6%. Similarly, there were no differences ($F_{2,16}$ = 0.51; p = 0.61) in the SNR, which ranged from 22.6 ± 4.6 to 29. 8 ± 5.7.

Table 8.4. Glutamine before and after intravenous injection of study drug.

	Hydrocortisone dose	Pre-injection	Post-injection	*F*	*df*	*p*
Glutamine, IU	Placebo	275 ± 43	282 ± 44			
	40 mg	299 ± 80	256 ± 30	1.80	2,16	0.20
	100 mg	254 ± 49	279 ± 67			
Creatine (Cr), IU	Placebo	591 ± 46	574 ± 92			
	40 mg	620 ± 108	657 ± 73	0.01	2,16	0.99
	100 mg	720 ± 108	699 ± 95			
Glutamine/Cr	Placebo	.47 ± .06	.50 ± .09			
	40 mg	.51 ± .22	.40 ± .10	2.49	2,16	0.11
	100 mg	.36 ± .11	.40 ± .10			

Key: IU = institutional units

There were no significant effects of the study drug on glutamine, creatine or glutamine/Cr ratios (table 8.4.). Although post-injection glutamine and glutamine/Cr ratios were lower than pre-injection levels at the hydrocortisone 40 mg dose, the effect of this dose was not significantly different from placebo, when these data were examined separately from the 100 mg dose (glutamine: $F_{1,9} = 1.14$; $p = 0.31$; glutamine/Cr: $F_{1,9} = 1.94$; $p = 0.20$). A *post-hoc* power calculation showed that a sample size of 13 subjects would have been required for 80% power to show this reduction in glutamine after hydrocortisone 40 mg, compared with placebo, at one-tailed $\alpha = 0.05$.

8.4.5. Differences in metabolites across the three study days

8.4.5.1. Glutamate

Table 8.5. Glutamate levels on the three study days

	Drug	Day 1	Day 2	Day 3	*F*	*df*	*p*
	Placebo	1036 ± 222	984 ± 140	1022 ± 313			
Glutamate, IU	40 mg	1132 ± 156	1172 ± 159	1214 ± 161	0.53	4,32	0.71
	100 mg	1137 ± 184	1230 ± 105	1208 ± 145			
	Placebo	755 ± 106	760 ± 106	773 ± 82			
Creatine (Cr), IU	40 mg	807 ± 81	841 ± 101	865 ± 80	0.20	4,32	0.94
	100 mg	841 ± 117	886 ± 95	881± 124			
	Placebo	1.36 ± .16	1.29 ± .08	1.40 ± .20			
Glutamate/Cr	40 mg	1.40 ± .13	1.40 ± .13	1.40 ± .09	0.65	4,32	0.63
	100 mg	1.35 ± .07	1.39 ± .10	1.38 ± .15			

Key = IU = institutional units

Data quality was similar for the different sets of acquisitions. There were no differences ($F_{4,32}$ = 1.87; p = 0.14) between sets of acquisitions in the mean ± SD CRLB values, which ranged from 4.1 ± 0.8% to 5.2 ± 0.8% or in SNR ($F_{4,32}$ = 0.70; p = 0.60), which ranged from 17.0 ± 2.7 to 22. 0 ± 2.2. There were no significant changes in glutamate, creatine or glutamate/Cr ratios across the three study days (table 8.5.).

8.4.5.2. Glutamine

Table 8.6. Glutamine levels on the three study days

	Drug	Day 1	Day 2	Day 3	*F*	*df*	*p*
	Placebo	275 ± 43	275 ± 57	272 ± 60			
Glutamine, IU	40 mg	299 ± 80	296 ± 56	256 ± 30	1.87	4,32	0.14
	100 mg	254 ± 49	264 ± 66	291 ± 46			
	Placebo	591 ± 46	636 ± 82	617 ± 85			
Creatine (Cr), IU	40 mg	620 ± 108	680 ± 79	694 ± 56	0.80	4,32	0.54
	100 mg	720 ± 108	711 ± 70	724 ± 82			
	Placebo	.47 ± .06	.44 ± .10	.45 ± .13			
Glutamine/Cr	40 mg	.51 ± .22	.44 ± .10	.37 ± .04	1.56	4,32	0.21
	100 mg	.36 ± .11	.38 ± .10	.41 ± .09			

Key: IU = institutional units.

Data quality was similar for the different sets of acquisitions. There were no differences ($F_{4,32}$ = 0.53; p = 0.71) between sets of acquisitions in the mean ± SD CRLB values, which ranged from 10.7 ± 2.8% to 11.6 ± 2.6% or in SNR ($F_{4,32}$ = 0.75; p = 0.56), which ranged from

23.6 ± 4.6 to 31.3 ± 2.9. There were no significant changes in glutamine, creatine or glutamate/Cr ratios across the three study days (table 8.6.).

8.5. Discussion

8.5.1. Acute effects

Contrary to the study predictions, we did not show acute or delayed effects of hydrocortisone on prefrontal levels of glutamate or glutamine in the present study. However, although the study did not replicate the previous finding of an acute effect of the lower dose of hydrocortisone (35 mg) on glutamine levels, it did not refute the finding either, since there was an acute reduction in glutamine levels after the hydrocortisone 40 mg dose. Although this was not statistically significant, this could be due to the low power of the sample size, with only 5 participants in the placebo group and 6 in the hydrocortisone 40 mg group. *Post hoc* calculation suggested that a sample size of at least 13 participants would be needed for 80% power to show a significant reduction in glutamine using a paired t-test. It was not anticipated that there would be no effect of hydrocortisone 100mg at the post-injection time point, despite high plasma concentrations that would affect both GRs and membrane MRs (Karst et al, 2005). It is possible that there could be a non-linear dose-response effect of cortisol on receptors mediating its rapid effects, in the dose range of hydrocortisone that was studied. The previous study also differed from the present study in that it included only participants who had previous experience of ^{1}H-MRS scans, who may be more compliant with the procedures.

8.5.2. Delayed effects

No significant differences in glutamate or glutamine concentrations were shown over the 48 hours of hydrocortisone administration and we were therefore unable to provide any evidence for delayed effects of cortisol over this time period. It is possible that the time period is too short for such effects, although the hypothesis was based on genomic effects of glucocorticoids on glutamine synthetase gene expression and enzyme activity, which occur within several hours of glucocorticoid exposure (Vardimon et al, 1999). There were small increases in mean glutamate levels, but a sample size >100 would have been necessary to demonstrate significant changes and, furthermore, there were no differences over time in glutamate/Cr ratios. It is possible that there could be greater changes over longer intervals, but chronic administration of glucocorticoids to healthy participants raises ethical questions about the impact of glucocorticoids on health.

8.5.3. Plasma cortisol concentrations

An unexpected finding in the study was that although hydrocortisone was administered at both 40 mg and 100 mg doses, there was no evidence of a difference in plasma concentrations between the two doses. This may be because the plasma concentrations were only obtained at peak at a single time point, when cortisol is still undergoing rapid redistribution (Hamitouche et al, 2017). We did not wish to obtain repeated samples, since this might disturb participants within the scanner during the metabolite acquisitions. For this reason we administered study drug and collected a single sample, then repeated the imaging, shimming and segmentation between the two sets of acquisitions. In contrast to placebo, cortisol levels were somewhat higher on day

2 and day 3 after hydrocortisone than at baseline, with the highest levels after hydrocortisone 100mg, suggestive of a dose-response, although the differences were not statistically significant.

8.5.4. Limitations and strengths

In addition to the limitation of the small study sample for each dose, which was not powered for definitive tests of hypotheses, there were other weaknesses and some strengths. The main strength was that glutamine levels were measured using a selective ^{1}H-MRS sequence, rather than employing Glx as a combined measure of the glutamate/glutamine signals. Glutamine levels could be measured at 3T from a single acquisition within CRLBs < 20% for 74% of acquisitions, which is probably an underestimate, given the specific hardware problems in scanning two subjects. When two acquisitions were combined, 92% of scans fell within acceptable CRLBs. The methods of the present study therefore have the advantage that glutamine and glutamate concentrations can be quantified independently. It should be acknowledged that the voxel size also influences SNR and that this was relatively large in comparison to the voxel sizes typically used to measures Glx. The acquisitions for glutamate are relatively brief at this voxel size: similar SNRs could be maintained for smaller voxels by increasing the number of excitations.

There are other methods to reduce the co-editing of glutamine with glutamate, including echo-time averaging (Hurd et al, 2004) and sequence optimization (Thompson and Allen, 2001). To our knowledge, these have not been compared directly. The study did not measure absolute concentrations of glutamate and glutamine, which would require the inclusion of relaxation times into the LCModel analysis. Although our group has calculated T2 relaxation times for glutamate using experimental data (Choi et al, 2007), this requires multiple glutamate acquisitions at

different TE to allow values to be calculated on an individual basis. Using group averages to adjust the relaxation times would have affected all of participant data similarly and would not have altered the findings of the study.

8.6. Conclusions

In this study of the administration of cortisol to healthy volunteers, we were able to demonstrate the routine acquisition of selective glutamine measurements with CRLBs <20, using 256 averages, but preferably 512 averages. However, the current study design did not demonstrate significant rapid effects of cortisol on prefrontal glutamate or glutamine, or delayed effects at 48 hours. The lack of effects in the acute study could be exaplained by a lack of power at the low dose, or a non-linear dose-response. Animal models of the effects of chronic stress and glucocorticoids have used longer periods of exposure, on the order of two weeks. Effects of chronic effects of glucocorticoid administration might be examined in patients receiving corticosteroid therapy.

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Chapter 9. Conclusions

^{1}H-MRS provides a method with which the amino acid neurotransmitters GABA glutamate and their intermediate, glutamine, can be measured in the living human brain. However, it is technically challenging to measure GABA and glutamine selectively at 3.0T, because of their *J*-coupled spin systems and spectral overlap with other metabolites that are present in higher concentrations. This challenge is increased when obtaining measurements from the anterior cingulate/prefrontal cortices (ACC), which have lower magnetic field homogeneity than some other cortical regions because of their proximity to the frontal sinuses. In this research, we used three selective ^{1}H-MRS acquisition sequences for the measurement of GABA, glutamate and glutamine for clinical investigation.

In the study of variations in GABA across the MC, the hypothesis that ACC GABA would decrease between the follicular to luteal phase, as shown for the occipital cortex, was not supported (Epperson et al, 2002). This is consistent with two recent studies that reported similar ACC GABA levels in the follicular and luteal phase (De Bondt et al, 2016; Harada et al, 2011), but the current study tested the largest sample to date and was the first to use a DQF-S acquisition, which results in less MM contamination of the GABA signal than *J*-difference editing. We showed no significant difference in ACC GABA between women in the follicular phase of the MC and women in the first week of hormonal birth control pill-use. This is only the second study in this population, and the results of the two studies are consistent (De Bondt et al, 2015). We did not find an effect of age group in premenopausal women. In addition to providing normative data, these data studies should be of assistance in planning controls for menstrual phase and pill-use in clinical research studies.

The major study in this thesis compared unmedicated patients with MDD, in a MDE of at least moderate severity, with healthy controls matched for age and sex. We did not confirm the hypothesis that MDD patients would have lower ACC GABA levels than controls. This was the largest study of ACC GABA in the field to date, with only one other study having a sample size >20 MDD patients. Our study used a method with lower MM contamination than previously reported studies. A *post hoc* analysis suggested that the most severely anhedonic MDD patients may have lower GABA levels than controls, but this needs prospective confirmation.

In the final study, we used spectrally-selective dual band refocusing sequences to measure glutamate and glutamine. We demonstrated that glutamine can be quantified with acceptable Cramér-Rao Lower Bounds at 3.0T, using LCModel analysis. We did not confirm the hypotheses that the administration of cortisol (hydrocortisone 40 or 100 mg) would acutely decrease ACC glutamine, or increase ACC glutamine after 48 hours of repeat administration. No effects were demonstrated on glutamate. However, the study was not adequately powered for definitive tests of the hypotheses.

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