"The future belongs to those who believe in the beauty of their dreams"

~Eleanor Roosevelt

University of Alberta

Glutamate Levels in the Medial Prefrontal Cortex of Healthy Women during Pregnancy and the Postpartum

by

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Dedication

I would like to dedicate this thesis to my amazingly loving family.

To my dad, Warren McEwen, for your endless support, encouragement and pride in me, it has pushed me to achieve so much and overcome countless obstacles as I achieve success in life. I am forever grateful for all the love you have given me.

To my mom, Susan McEwen, for the infinite number of phone calls and "brain chats" over the years that have kept me interested and loving what I'm doing. Your support and love have kept me going through the tough times and I would not be where I am now if it wasn't for you.

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Abstract

The substantial female hormone fluctuations associated with pregnancy and the postpartum have been linked to a greater risk of developing depressive symptoms. Glutamate (Glu) has been implicated in the pathophysiology of major depression. The objective of this thesis was to investigate MPFC Glu levels from late pregnancy up to 7 weeks postpartum in women at a high risk for developing depressive symptoms and to compare MPFC Glu levels during late pregnancy in healthy pregnant and non-pregnant women.

Using *in vivo* proton magnetic resonance spectroscopy (^{1}H-MRS) we acquired single-voxel spectra from the MPFC of women. We found fluctuations of MPFC Glu levels from pregnancy up to 7 weeks postpartum in high risk women (HRW) compared to healthy controls (HCs). As well as decreased %GM during pregnancy and the early postpartum in both HRW and HCs with a progressive normalization as the postpartum progressed.

Findings may implicate Glu alterations and hormone fluctuations in the pathophysiology of PPD.

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Abbreviations

^{1}H	Proton or Hydrogen
^{1}H-MRS	Proton Magnetic Resonance Spectroscopy
5-HT	5-Hydroxytryptamine = Serotonin
α	Alpha
γ	Gamma
σ	Sigma
AMPA	α-Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid
Bo	External Magnetic Field
CHESS	Chemical Shift Selective Saturation
Cho	Choline
Cr	Creatine
CSF	Cerebrospinal Fluid
DHEA	Dehydroepiandrosterone
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision
EAAT	Excitatory Amino Acid Transporter
EPDS	Edinburgh Postnatal Depression Scale

FASTMAP	Fast Automatic Shimming Technique by Mapping Along Projections
fMRI	Functional Magnetic Resonance Imaging
FPHC	Follicular Phase Healthy Controls
GABA	γ-Aminobutyric Acid
Gln	Glutamine
Glu	Glutamate
Glx	Glutamix = Glutamate + Glutamine
GM	Grey Matter
HC	Healthy Women with No Risk Factors for Postpartum Depression
HRW	High Risk Women
KA	Kainate
mGluR	Metabotropic Glutamate Receptor
MD	Major Depression
MPFC	Medial Prefrontal Cortex
MRI	Magnetic Resonance Imaging
MRS	Magnetic Resonance Spectroscopy

NAA	N-Acetyl Aspartate
NAS	Neuroactive Steroids
NMDA	N-Methyl-D-Aspartate
PET	Positron Emission Tomography
pHC	Pregnant Healthy Controls
PMDD	Premenstrual Dysphoric Disorder
PPD	Postpartum Depression
PRESS	Point Resolved Spectroscopy
RF	Radio Frequency
ROI	Region of Interest ≡ Voxel
STEAM	Stimulated Echo Acquisition Mode
t-Cho	glycerophosphorylcholine plus phosphorylcholine
t-Cr	Cr and phosphocreatine
T	Tesla
TE	Echo Time
TR	Repetition Time
VGLUT	Vesicular Glutamate Transporter
WM	White Matter

I. Introduction

The purpose of the research described in this thesis was to study brain metabolites in women during pregnancy and the postpartum period in an attempt to determine the relationship to the development of mood disorders during this time. Magnetic resonance spectroscopy (MRS) is the only *in vivo*, non-invasive methods that allows for quantification of brain metabolites. The medial prefrontal cortex (MPFC) is the region of interest due to its role in mood regulation and emotions and its sensitivity to female hormone changes. As glutamate (Glu) has been implicated as a neurotransmitter involved in the development of mood disorders, it was the main metabolite of focus for this research.

I.1 Pregnancy

I.1.1 *Peripheral Alterations*

Pregnancy is associated with numerous physical changes necessary for supporting the developing fetus (Mattison et al., 1991). Aside from noticeable changes in outward appearance, almost all internal organ systems are affected by pregnancy. The developing fetus requires many resources which it obtains from the mother's body. As a result, the cardiovascular, haematological, respiratory, renal and gastrointestinal tract systems as well as the endocrine system of the mother undergo several changes to support the demands of the growing fetus (Girling, 2004).

The cardiovascular system is essential in providing the developing fetus with adequate blood flow and preventing fetal heat loss and in preparing the mammary gland for lactation (Mattison et al., 1991). Cardiac output increases 30-50% in pregnant women beginning in the first trimester and lasting throughout pregnancy. Cardiac output is a measure of both heart rate as well as stroke volume, which is the amount of blood pumped with each beat. Heart rate increases from an average of 75 beats per minute to 90 beats per minute in pregnant women, whereas stroke volume increases by 35% (Mattison et al., 1991). The increases in cardiac output allow for increased blood flow to the uterus. Not only are there cardiac output changes but changes in blood itself. Maternal blood volume increases by 50% (Mattison et al., 1991), with increases in white blood cell count, red blood cell mass and platelet production (Heidemann, 2005). An artificial decrease in haemoglobin levels in the third trimester is also observed as plasma content in blood rises by 40%, exceeding the increase in red blood cell mass. The increase in blood volume offers protection from haemorrhage for both the mother and baby during delivery. By approximately 2 weeks postpartum haematological changes, brought on by pregnancy, return to normal pre-pregnant levels (Heidemann, 2005). Oxygen levels within the blood also change during pregnancy, with the most significant changes observed at term when oxygen consumption and carbon dioxide output increase by 60% (Heidemann, 2005). Respiratory rate does not increase, but the volume of oxygen with each breath increases, supplying the developing fetus with oxygen necessary for development. The increasing size of the fetus by the third trimester causes a

decrease in lung residual volume and as such an increase in alveolar size. The increase in alveolar size allows for increased transfer of oxygen and carbon dioxide between the alveoli and the blood (Girling, 2004). Demands of the fetus by the third trimester cause an observed decrease in maternal blood oxygen levels as the increase in oxygen consumption can no longer be compensated by the increase in cardiac output, red blood cell mass and plasma levels (Heidemann, 2005).

The cardiovascular and haematological systems are also implicated in the renal changes that occur during pregnancy. There is an increase in renal blood flow and glomerular filtration rate, resulting in an increase in overall kidney size (Mattison et al., 1991) as well as in greater urea, creatinine and urate clearance (Heidemann, 2005). Pregnancy also causes the release of renin, an enzyme that mediates extracellular volume, causing sodium retention in the kidneys and thus an increase in total body water (Heidemann, 2005). This increase in total body water contributes to edema experienced by pregnant women. Increased abdominal pressure caused by the growing fetus contributes to the gastrointestinal changes observed during pregnancy as well. Up to 80% of pregnant women will experience oesophageal reflux at term, more commonly known as heartburn (Girling, 2004). This is brought on by a displacement of the gastric axis and reduction in oesophageal sphincter tone. Gastrointestinal changes are not long lasting into the postpartum period and return to normal by 24-48 hours postpartum (Heidemann, 2005).

Choline (Cho) is essential to fetal development, specifically the brain, and as such maternal Cho levels have been shown to be decreased during normal human pregnancy due to the demands of the developing fetus (Caudill, 2010). The placenta during the second half of pregnancy has been shown to contain 50 times the Cho levels that are found in maternal blood (van der Aa et al., 1994) and human neonates are born with three times the circulating blood Cho levels of their mothers (Caudill, 2010).

The endocrine system also undergoes several changes in relation to the developing fetus. Most importantly, the placenta takes over production of female hormones (estrogen and progesterone) in order to maintain pregnancy and stimulate childbirth (Smith, 2001). These endocrine changes are thought to play a role in some of the cerebral alterations observed during pregnancy and will be covered in more detail later on in the introduction.

I.1.2 *Cerebral Alterations*

Although the peripheral alterations of human pregnancy have been readily studied, less is known about the cerebral changes that occur during normal pregnancy. Approximately two thirds of pregnant women report symptoms of memory loss and cognitive decline during pregnancy, growing increasingly worse as the pregnancy progresses. This is more commonly known as “baby brain” or “pregnesia”. Attempts to validate an actual psychometric measurement associated with these complaints have been varied. Only one study to date in humans has

shown a potential underlying biological mechanism associated with the development of "pregnesia". An MRI study by Oatridge et al. (2002) compared nine healthy controls, before pregnancy, during pregnancy and within the year following pregnancy to a group of women with preeclampsia. The main finding was an overall decrease in brain size during pregnancy and a corresponding increase in ventricular size, maximal at term and both returning to pre-pregnant size by six months postpartum in all pregnant women. Although criticized for the small sample size and the implications of such a finding remaining speculative at this point, it does suggest cerebral changes associated with the development of "baby brain" during normal pregnancy. This finding was further supported by a study by Kim et al. (2010) observing changes in brain composition during the postpartum period. MRI scans showed an increase in grey matter (GM) in postpartum women from 2-4 weeks postpartum to 3-4 months postpartum. Although a pregnancy time point was not measured in this study, one might infer from the changes observed across the postpartum that the initial decrease in GM may have begun during the pregnancy phase. An increase in GM would contribute to an overall increase in brain size and a return to pre-pregnant levels as was seen by six months postpartum in the Oatridge et al. (2002) study. Certain cerebral alterations occurring during both pregnancy and the postpartum period may place women at risk for developing mood disturbances.

I.1.3 *Major Depression during Pregnancy*

Women are 2-3 times more likely to develop major depression (MD) than men (Goldman et al., 1999), with up to 20% of women presenting with MD during pregnancy (Bowen et al., 2006). A review by Bennett and colleagues found prevalence rates to be 7.4%, 12.8% and 12.0% for the first, second and third trimesters respectively (Bennett et al., 2004). Not only does depression during pregnancy have effects on the mother, it has also been shown that MD during pregnancy has been associated with increasing the risk of preterm birth and low birth weight, which are predictors of infant and child mortality (Grote et al., 2010).

In 2003, it was reported that 13% of pregnant women had taken some form of antidepressant during pregnancy, which had doubled the rate observed in 1999 (Cooper et al., 2007). MD relapse rates were found to be higher in women who went off antidepressant treatment during pregnancy (68%) compared to those who chose to continue antidepressant treatment during pregnancy (26%), indicating some merit to remaining on antidepressants during pregnancy (Cohen et al., 2006). However, it has also been found that antidepressant use during pregnancy increases the likelihood of miscarriage from 8.7% in unexposed patients to 12.4% in those on antidepressants (Hemels et al., 2005). All of these factors taken together make it difficult for both physicians and pregnant women to decide whether the benefits of treatment for MD during pregnancy outweigh the risks.

Considerable controversy also exists regarding treatment options for MD during pregnancy as fears ascend over the use of antidepressants and the effects they may have on the developing fetus. In the past five years many studies have taken a look at the increased risk for fetal heart defects associated with the use of antidepressants, with some finding an increase in fetal heart defects (Kallen & Otterblad Olausson , 2007; Cole et al., 2007) and others finding no difference in risk (Einarson et al., 2008). Many of these studies were based on the timing of antidepressant administration with more increased risks of heart defects associated with antidepressant use during the first trimester; however, in approximately one out of every one hundred births a baby will be born with a heart defect, making it extremely difficult to interpret the findings associated with antidepressant use (Lorenzo et al., 2011). With rates on the rise for antidepressant use during pregnancy and studies on fetal outcomes producing inconsistent findings it is important to gain a clear understanding of MD during this time with the hope of minimizing adverse birth outcomes.

MD during pregnancy has also been classified as one of the risk factors associated with the development of postpartum mood disturbances, with 17-51% of women who went on to develop postpartum depression (PPD) having an onset of depressive symptoms during pregnancy (Stowe et al., 2005**;** Josefsson et al., 2001). Understanding the cerebral changes occurring during pregnancy and the association with developing MD will be beneficial in furthering our knowledge of PPD.

I.2 Postpartum

I.2.1 Postpartum Depression

PPD is prevalent in 10-20% of the female population and is defined in the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition Text Revision* (DSM-IV-TR) as a major depressive episode with onset during the first four weeks postpartum (American Psychiatric Association, 2000). More recent literature has expanded this time frame of postpartum onset to be within one year following delivery (Perfetti et al., 2004). A more commonly accepted time frame for PPD onset is within the first 3 months postpartum, with a prevalence rate of 9.8% at 12 weeks postpartum for first time mothers (Leahy-Warren et al., 2011). PPD can be divided into early and late onset PPD, with early onset PPD occurring within the first 6-8 weeks postpartum and late onset PPD occurring after. Risk factors for early onset PPD differ from those for late onset PPD (Bloch et al., 2006). Early onset PPD risk factors include a traumatic birth experience as well as depression or increased stress during the pregnancy (Tronick and Reck, 2009). The "Baby Blues", which are a mild form of mood disturbance that occurs in up to 80% of mothers within the first few days following delivery and lasts up to 10 days postpartum, put mothers at up to a four times higher risk for developing PPD than mothers who do not experience baby blues (Sacher et al., 2010). The baby blues have been described as a prodromal stage for PPD, with greater severity of baby blues being associated with greater risk of experiencing PPD (Sacher et al., 2010). Other risk factors for early onset PPD include a history of depression or premenstrual dysphoric disorder (PMDD) as well as a past history of PPD.

Women with a history of PPD are up to 65% more likely to develop PPD in subsequent pregnancies compared to those without a history of PPD (Gaynes et al., 2005). Late onset risk factors tend to be more socioeconomic in nature and include a lack of support as well as low economic status and low level of education; a meta-analysis by Grote et al. (2010) found that poor urban women from minority backgrounds were twice as likely as middle class women to develop late onset PPD.

PPD is characterized by numerous symptoms (Table I-1), many similar to those required for a diagnosis of MD; however, symptoms unique to PPD include a loss of interest in the baby or excessive worry about the baby. PPD not only affects the mother but can have a lasting impact on the infant as well. The lack of mother-infant relationship can lead to hindered cognitive and social development in the child (Tronick and Reck, 2009). Children of postpartum depressed mothers are more likely to develop depression themselves; 41.5% of children of mothers who experienced PPD developed depression by the age of 16 compared to 12.5% of children of mothers who did not suffer from PPD (Murray et al., 2011). It was postulated that low infant attachment influenced adolescent depression via lower childhood resilience and family adversity (Murray et al., 2011). A gender difference has been observed in children of PPD mothers. Male babies tend to be more sensitive to mothers experiencing PPD as they are less able to control their own emotions than females. As such male babies require more positive reinforcement from their mothers, which they do not receive from mothers experiencing PPD (Tronick and Reck, 2009). This is further supported by Grace

et al. (2003) who found that the odds for an insecure attachment are 3.6 times higher in males than females of a mother experiencing PPD. This may lead to impeded social competence and foster behavioral problems in male children of PPD mothers by the age of five. Children's abilities to deal with stress and interact with peers in early school years are also affected by mothers experiencing PPD (Kersten-Alvarez et al., 2012). Children of mothers experiencing PPD have also been reported to develop disturbances in their sleeping patterns by twelve months of age (Pinheiro et al., 2011).

Table I-1: Symptoms of Postpartum Depression (American Psychiatric Association, 2000)

Symptoms of Postpartum Depression
• A depressed mood • Loss of interest in usual activities • Feelings of worthlessness • Excessive guilt • Appetite or sleep disturbances • Physical agitation • Extreme fatigue • Decreased concentration • Loss of interest in the baby • Excessive worry about the baby • Suicidal thoughts

I.3 Glutamate

Glu is the major excitatory neurotransmitter in the brain, found widespread throughout the brain and accounting for at least 60% of the synapses in the central nervous system (Pittenger et al., 2007; Javitt, 2004). Over the past several years Glu has gained a lot of interest as a contributing factor to the pathophysiology of MD (Mitchell & Baker, 2010). The relationship between Glu dysregulation and the development of MD during pregnancy and PPD is yet to be determined.

I.3.1 Glutamate Synthesis and Transport

Glu can be synthesized in two separate ways, either by glutaminase (Glut) converting Gln to Glu in the glutamate/glutamine cycle or from α-ketoglutarate (α-KG) via the TCA cycle. Figure I-1 shows a summary of Glu synthesis (Gao & Bao, 2011). Glu is transported to vesicles in the presynaptic neuron by three types of vesicular glutamate transporters (VGLUT1, VGLUT2 and VGLUT3). The main purpose of VGLUTs is to ensure that Glu is transported to and stored in synaptic vesicles until released into the synaptic cleft, preventing degradation of the Glu (Herzog et al. 2004). Once in the synaptic cleft, Glu targets receptors located mainly on the postsynaptic neuron. Remaining Glu is removed from the extracellular space via excitatory amino acid transporters (EAAT1 and EAAT2) returning the majority of Glu to astrocytes with some Glu returning to presynaptic neurons. Astrocytes then convert Glu back into Gln via glutamine synthetase (GS) (Gao & Bao, 2011). Gln is transported back to neurons where Glu cycling

resumes. It is crucial that Glu be quickly removed from the synaptic cleft so as to prevent excitotoxicity from occurring.

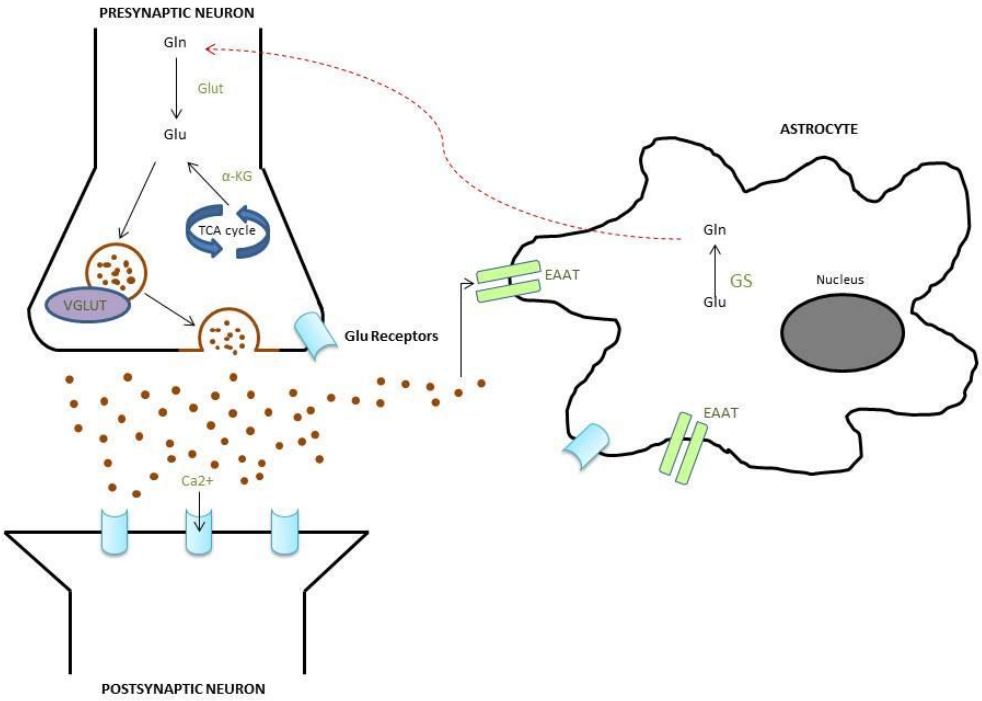

Figure I-1: Summary of Glu synthesis, release and reuptake (adapted from Gao & Bao, 2011).

I.3.2 Glutamate Receptors

Glu targets two different types of receptors in the brain, ionotropic receptors and the metabotropic receptors. There are three types of glutamatergic ionotropic receptors, the N-methyl-D-aspartate receptor (NMDA), the kainate receptor (KA) and the α-amino-5-methyl-3-hydroxy-4-isoxazole propionic acid (AMPA) receptor. The ionotropic receptors become activated when Glu and

glycine (or D-serine) bind to the receptor sites. This allows an influx and efflux of ions, specifically calcium, potassium and sodium (Belsham, 2001). Metabotropic receptors require G-proteins and work through second messenger cascades. There are eight glutamatergic metabotropic receptors (mGluR) that can be divided into 3 main groups. Group 1 consists of mGluR1 and mGluR5 and work to activate Phospholipase C cascades. Group 2 consists of mGluR2 and mGluR3 while Group 3 consists of mGluR4, mGluR6, mGluR7 and mGluR8, both of which are involved in cAMP cascades (Gao & Bao, 2011). Metabotropic and ionotropic receptors have been shown to interact. The stimulation of Group 1 mGluRs has been shown to potentiate ionotropic responses, specifically NMDA responses (Fitzjohn et al., 1996).

I.3.3 Glutamate Dysregulation in Major Depression

Increasing evidence shows dysregulation of Glu playing a significant role in the pathophysiology of MD. Glu synthesis, transport, reuptake and receptors have all been implicated in this role. It is important to note that the majority of MRS studies looking at Glu dysregulation have been observing glutamix (Glx), which is the combined signal of Glu and Gln together and may not fully represent Glu dysregulations. However, fluctuations in Glx do imply dysregulation within the glutamatergic system, contributing to the pathophysiology of MD.

An MRS study by Hasler et al. (2007) showed decreases in Glx in the ventromedial and dorsomedial prefrontal cortex of patients suffering from MD

compared to healthy controls. Two additional studies observing the anterior cingulate cortex (ACC) supported the findings of decreased Glx levels in MD patients compared to healthy controls (Auer et al., 2000; Pfleiderer et al., 2003). Additionally, one MRS study focusing on the hippocampus found decreased Glx in 18 unmedicated unipolar depressed patients compared to 10 healthy controls (Block et al., 2009). Studies specifically observing Glu as opposed to Glx have shown conflicting findings. An MRS study by Sanacora et al. (2004) showed an increase in mean Glu levels in the occipital cortex of 33 patients suffering from MD compared to 38 healthy controls. This was further supported by a postpmortem study showing increased Glu levels in the frontal cortex of patients suffering with MD compared to healthy controls (Hashimoto et al., 2007). Merkl et al. (2011) found decreased Glu levels in the ACC of patients suffering from MD compared to healthy controls. Given that various brain regions are being addressed in the above findings, one might conclude that glutamatergic dysregulation varies given the region of interest (ROI), which might help to explain the conflicting findings above.

A significant amount of attention has been focused on dysregulations in Glu receptors in the pathophysiology of MD and potential treatment options. In a study by Chourbaji et al. (2008) the main subunit of the ionotropic receptor AMPA was removed in knockout mice. These knockout mice showed symptoms characteristic of MD, indicating the importance of AMPA receptors in the pathophysiology of MD. Other studies looking at the role of AMPA receptors in MD found that AMPA potentiators increase the functioning of AMPA receptors

and show antidepressant-like effects in rodent models of MD (Alt et al., 2006). Not only have the AMPA receptors been indicated in MD but so have the NMDA receptors. NMDA receptor agonists have been shown to increase depressive symptoms whereas NMDA receptor antagonists have been shown to have antidepressant- like qualities (Trullas et al., 1990). AMPA receptors are often found in the brain with NMDA receptors at mature synapses and work with NMDA receptors in the transmission of Glu. AMPA receptor activation permits the inward flow of sodium, causing depolarization of the membrane which in turn causes the release of a Mg^{2+} plug from the NMDA receptor allowing for an influx of Ca^{2+} (Maeng and Zarate, 2007). This process becomes important in treatment associated with MD.

Ketamine is a high-affinity NMDA receptor antagonist and as such has gained a lot of interest as a treatment option for MD. Berman et al. (2000) showed that ketamine works to relieve depressive symptoms in patients suffering from treatment-resistant major depression within hours of intravenous ketamine administration. This is an extremely fast-acting and efficacious form of treatment compared to typical monoamine antidepressants such as serotonin reuptake inhibitors (SSRIs), which often take more than 2-3 weeks to produce any antidepressant effects, and full effects not seen until up to 6 weeks (Taylor et al, 2006). A study conducted by Maeng and Zarate (2007) further supported these findings, by administering ketamine intravenously to treatment resistant MD patients and observing an improvement in MD symptoms within hours of ketamine administration.

Ketamine is thought to exert its antidepressant effects by blocking the NMDA receptor. It has also been shown that ketamine acts to disinhibit GABAergic inputs to glutamatergic neurons. This increases the amount of glutamatergic neuronal firing and the amount of Glu being released into the synapse by the presynaptic neuron (Moghaddam et al., 1997). As a result of NMDA and AMPA receptors working together in mature synapses in Glu neurotransmission, the combination of increased Glu release and the blockade of NMDA receptors by ketamine, results in increased AMPA receptor activity. It is thought that this increase in AMPA receptor activity compared to the decrease in NMDA receptor activity is essential to the antidepressant actions of ketamine (Sanacora et al., 2008). Furthermore, animal studies have suggested that ketamine may have rapid onset antidepressant properties by increasing synaptogenesis, through increased brain derived neurotrophic factor (BDNF) production, in brain regions associated with mood and emotions (Li et al., 2010; Li et al., 2011, Autry et al., 2011). Not only is ketamine fast acting but often a single dose is enough for sustained effects for up to 7 days. Research is being conducted to try and develop novel methods of combination therapy to increase the length of ketamine's effect longer than 1 week. A pilot study by Mathew et al. (2010) attempted to administer riluzole, a common anticonvulsant known to increase Glu reuptake, to reduce relapse in patients being treated with I.V. ketamine. The study was stopped prematurely as initial results were not promising, with relapse rates being equal between patients receiving placebo and those receiving riluzole. Ongoing

investigations are underway to try and improve the remission rates of ketamine treatment.

Metabotropic receptors have also been implicated in the pathophysiology of MD. Group 1 mGluRs are especially important in regulating glutamatergic excitability through modulation of ion channel activity (Pisani et al., 2001). Antagonists of group 1 mGluRs have been shown to elicit antidepressant-like properties. It is believed these antidepressant effects result from the interaction between NMDA receptors and mGluRs. The Group 1 mGluR5 receptor antagonist 2-methyl-6-[phenylethynyl]-pyridine (MPEP) has shown promising antidepressant-like results in rat models of MD, but the mechanism of antidepressant action remains to be determined (Palucha & Pilc, 2002). It has been postulated that inhibiting the mGluR5 receptors may in turn inhibit NMDA receptor function due to the potentiation seen between metabotropic and ionotropic receptors (Pilc et al., 2008). Administering MPEP as opposed to ketamine or other direct NMDA antagonists may show potential for reducing side effects but still maintaining the antidepressant properties necessary. Interestingly, studies observing a monoamine antidepressant effect on group 1 mGluRs showed that continuous exposure to antidepressants decreased the sensitivity of group 1 mGluRs in the brain (Pilc et al., 2008). Group 2 mGluRs are involved in the regulation of release of neurotransmitters associated with mood, specifically serotonin and to a lesser extent norepinephrine. Group 2 mGluR antagonists (MGS0039 and LY341495) have been shown to increase the levels of serotonin in

synapses in rodent models (Kawashima et al. 2005), further implicating the glutamatergic system and its role in the pathophysiology of MD.

Given the role of the glutamatergic system in the development of MD, one might hypothesize that it is also playing a role in the development of PPD. A previous study by our research group showed increased Glu levels in the MPFC of 12 women suffering from PPD compared to postpartum-time matched healthy controls, implicating the glutamatergic system in the development of PPD (McEwen et al., 2012). An additional study has taken a look at the role of neurotransmitters in the development of PPD and found no change in GABA levels in the occipital cortex in women suffering from PPD compared to healthy controls (Epperson et al., 2006). As GABA is the major inhibitory neurotransmitter found in the brain and is counterbalanced by the action of Glu, this finding has potential to be important to understanding the involvement of Glu in the pathophysiology of PPD. However, changes in the occipital cortex do not necessarily imply changes in all brain areas. Understanding the peripheral changes and cerebral changes that occur during normal pregnancy and the postpartum, such as alterations in the endocrine system, and the relationship to Glu are vital to the pathophysiology of PPD.

I.4 Hormones

I.4.1 Estrogen and Progesterone

Pregnancy and the postpartum period are associated with fluctuations in the female hormones estrogens (estradiol, estriol and estrone) and progesterone. The placenta is responsible for the majority of production of estrogen and progesterone during pregnancy (Smith, 2001). Figure I-2 shows the pathway of production for progesterone and estrogen. During pregnancy estrogen and progesterone gradually rise, with progesterone production increasing from 20mg/day prior to pregnancy (during the luteal phase of the menstrual cycle) to 300mg/day during the third trimester at term and estrogen levels increasing 1000 fold, reaching an apex during the third trimester prior to delivery (Figure I-3) (Tulchinsky et al., 1975; Lin et al., 1972; Ahmed et al., 1972). Following parturition, both estrogens and progesterone levels drop drastically (Bloch et al., 2003; Buckwalter et al., 1999).

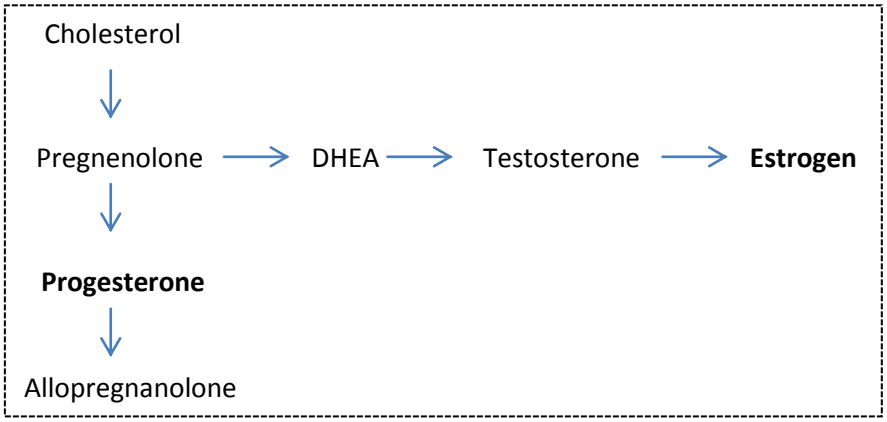

Figure I-2: The pathways of estrogen and progesterone formation starting from cholesterol (adapted from Mitchell et al., 2011).

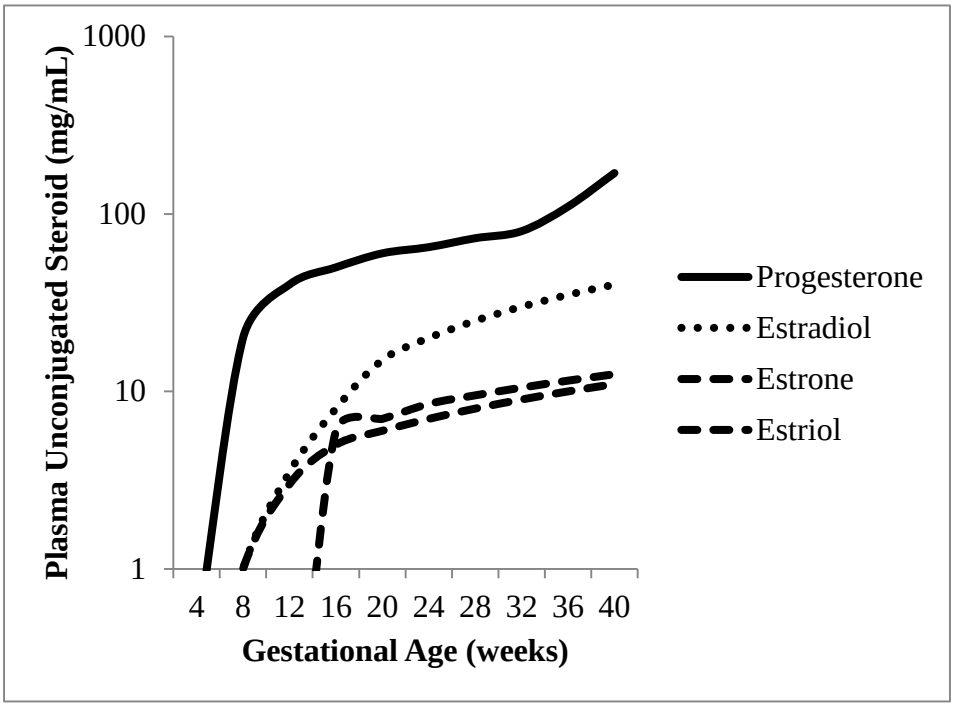

Figure I-3: Circulating concentrations of progesterone and estrogen during human pregnancy (adapted from Yen, 1991).

Estrogen and progesterone have been implicated in acting on the kidney to cause the release of renin, which is involved in the retention of sodium and increase in total body water volume seen during pregnancy and edema formation (Heidemann, 2005). Not only do the female hormones estrogen and progesterone play a role in changes during pregnancy but so do additional steroid hormones.

I.4.2 Neuroactive Steroids

Steroid hormones have two mechanisms of action. The first mechanism of action involves steroid hormones acting as transcription factors in the regulation of gene expression (Evans, 1988). The term neuroactive steroid (NAS) comes from additional evidence that indicates the second mechanism of action by which steroid hormones interact with neurotransmitter receptors in the central nervous system, altering neuronal excitability (Paul and Purdy, 1992). The ability to modulate neuronal excitability through the second mechanism of action can occur very rapidly, often within milliseconds to seconds as non-genomic activity is involved, whereas this process can be delayed when genomic activity is involved as occurs in the first mechanism of action (McEwen, 1991). Steroid hormones, including the female hormones estrogen and progesterone, can easily cross the blood-brain barrier (BBB) and act to modulate neuronal excitability. However, NASs can also be produced within the brain itself from cholesterol and are often referred to as neurosteroids (Baulieu 1998). NASs have been shown to modulate Glu receptors (AMPA, NMDA, KA) as well as additional receptors including GABA receptors, serotonin (5-HT) receptors, acetylcholine receptors, glycine receptors and sigma receptors (σ) (Rupprecht, 2003).

NASs have been implicated in the development of MD, although with numerous conflicting findings making it difficult to definitively conclude how NASs contribute to the development of MD (Mitchell et al., 2012). NASs of interest include pregnenolone, allopregnanolone, dehydroepiandrosterone (DHEA), progesterone, estrogen as well as a number of 3α-reduced NASs.

I.4.3 The role of estrogens, progesterone and NASs in the development of PPD

Not only do the female hormones (estrogen and progesterone) rise during pregnancy with drastic decreases during the postpartum period but so do associated NASs, with a return to pre-pregnancy levels by 6-7 weeks postpartum (Pearson Murphy et al., 2001). The decline in female hormones and NASs during the postpartum period is thought to be a contributing factor to the development of postpartum mood disturbances. By exposing women to 8 weeks of treatment with estrogen and progesterone followed by an immediate withdrawal, Bloch et al. (2000) were able to mimic the effects of pregnancy and the postpartum, resulting in women who had a history of PPD developing symptoms of depression more frequently compared to healthy controls.

Very little is currently understood about the interactions between progesterone, estrogens, NASs and the glutamatergic system in relation to the development of MD during pregnancy and postpartum mood disturbances. Animal studies have shown some support for the interaction; however minimal human research exists in this area. Animal studies have shown that estrogen increases the number of NMDA receptors on dendritic spines by means of increased mRNA production, leading to an overall increase in NMDA-mediated Glu receptor activity (Woolley and McEwen, 1993; Smith, 1989). Additionally, progesterone has been shown to have inhibitory effects, reducing the number of dendritic spines and excitatory synapses (Woolley and McEwen, 1993).

I.5 The Medial Prefrontal Cortex

I.5.1 Structure and Function

The medial prefrontal cortex (MPFC) is an area of interest in the development of MD. The MPFC is involved in mood and emotions and has numerous connections with other structures in the brain, specifically the limbic system (Rigucci et al., 2010). The limbic-cortical-striatal-pallidal-thalamic circuits (LCSPT) are formed by connections between the orbital and MPFC, amygdala, hippocampal subiculum, ventromedial striatum, mediodorsal and midline thalamic nuclei and ventral pallidum (Ongur et al., 2003). Dysfunction of the LCSPT circuit alters neurotransmission, contributing to the emotional symptoms observed in MD (Drevets et al., 2004). The MPFC is also associated with two extended cortical circuits including the orbital prefrontal network and the medial prefrontal network (Drevets et al., 2008). The orbital prefrontal network includes sensory association areas involved in sensory integration (i.e. visual associated areas in the inferior temporal cortex) (Ongur and Price, 2000) as well as coding for affective characteristics of stimuli such as reward and aversion (Drevets et al., 2008). The medial prefrontal network does not have extensive sensory connections but is involved in introspective functions such as mood and emotions as well as visceral reactions to emotional stimuli, through prominent connections with limbic structures and visceral control structures (i.e. the hypothalamus) (Drevets et al., 2008, Ongur and Price, 2000). Alterations associated with these circuits and networks involving the MPFC are thought to play a major role in the development of MD and its symptoms.

1.5.2 MPFC and Mood

Various studies have shown the involvement of the MPFC in MD and mood. Positron emission tomography (PET) studies have shown increased blood flow to the MPFC in healthy female participants exposed to images meant to induce sadness (George et al., 1995) as well as changes in metabolism in the prefrontal cortex of MD patients (Kennedy et al., 2001). Functional magnetic resonance imaging (fMRI) studies have shown similar results to those of PET studies, with increased activity in the MPFC of healthy participants watching a video clip meant to induce sadness (Beauregard et al., 1998) and decreases in corticolimbic functional connectivity in MD patients (Anand et al., 2009). All of these studies implicate the MPFC as an area of interest in the pathophysiology of mood disorders.

1.5.3 The impact of hormones on the MPFC

Although the glutamatergic system has been shown to be of importance in the MPFC of MD patients, female hormone fluctuations have also been shown to have an impact on the MPFC. A PET study showed alterations in the activation of the MPFC of women when estrogen and progesterone were supressed and then added back one at a time, demonstrating that these female hormones play a role in neural activity associated with the MPFC (Berman et al., 1997). Alterations in the activation of the MPFC were also seen across the normal female menstrual cycle when estrogen and progesterone are fluctuating (Reiman et al., 1996). An ^{1}H-

MRS study showed that MPFC Glu levels fluctuate in relation to the hormonal fluctuations of estrogen and progesterone observed across the menstrual cycle as well, with a decrease in MPFC Glu levels observed during the luteal phase of the menstrual cycle (Batra et al., 2008). As pregnancy and the postpartum period are associated with alterations in female hormones similar to those of the menstrual cycle, only to a much greater magnitude, one might speculate that alterations of Glu in the MPFC are probable and potentially a contributing factor to the development of mood disorders during this time.

I.6 Magnetic Resonance Spectroscopy

I.6.1 Introduction to the phenomenon of magnetic resonance spectroscopy

I.6.1.1 Brief history

The phenomenon of nuclear magnetic resonance (NMR) was discovered in 1946 (Bloch et al., 1946; Purcell et el., 1946). However, it was not until the 1970's that NMR was applied to a biological system (Moon and Richards, 1973). Proton magnetic resonance spectroscopy (^{1}H-MRS) when applied to living systems allows for the assessment of *in vitro* and *in vivo* metabolism at the molecular level (Bottomley, 1989). In 1983, ^{1}H-MRS was first applied to study phosphorus in the human brain (Cady et al., 1983). With the introduction of ^{1}H-MRS and through rapid expansion of technical refinements it has become a tool in neuropsychiatric research for investigating cerebral physiology, biochemistry and pathology (Kauppinen et al., 1993).

I.6.1.2 Properties of nuclei that are NMR active

Atoms are made up of nuclear particles, which include protons and neutrons. Hydrogen is the most common nucleus used in MRS, exhibiting a single proton; as such this gives rise to the name ^{1}H-MRS (Plewes and Kucharczyk, 2012). The proton can be tipped out of alignment with the external magnetic field (B_0) causing it to precess. The rate of precession of the nuclei in the B_0 field is referred to as the Larmor frequency and is proportional to the gyromagnetic ratio as well as the strength of the B_0 (Plewes and Kucharczyk, 2012), as the strength of the B_0 increases so does the rate of precession.

I.6.1.3 The NMR phenomenon

Hydrogen has a spin quantum number of 1/2. When the B_0 is applied these nuclei are found in two states, either parallel with the B_0 or opposing the B_0, with more nuclei found to be parallel. This results in an imbalance which can be sampled in an NMR experiment (Plewes and Kucharczyk, 2012). Increasing the B_0 increases the number of parallel nuclei compared to opposed nuclei within a given sample, thus creating a greater number of nuclei with the same magnetization and as such a stronger signal.

I.6.2 The simple magnetic resonance spectroscopy experiment

I.6.2.1 How to perturb the nuclear equilibrium with radiofrequency pulses

In order to perturb the equilibrium state created by the B_0, radiofrequency (RF) pulses can be applied at the Larmor frequency of the nuclei. RF pulses are most efficiently absorbed by nuclei precessing at the same frequency. This causes the precessing nuclei to rotate into an excited state away from alignment with the B_0. The rotation is proportional to the applied RF energy. Following the RF pulse non-Bo aligned precessing nuclei induce an electromotive force (emf) which can be measured by an RF coil. The emf in the RF coil is amplified in receiver circuitry, digitized and stored as a time decaying signal or free induction decay (FID).

I.6.2.2 Conversion of time decaying signal into frequency spectrum using fast fourier transform

The Fourier transform is a mathematical equation that converts the time decaying signal arising from the nuclei into an array of its component frequencies or peaks, referred to as an NMR spectrum. The spectrum reflects the nuclear and electronic environment that exists in the target nucleus.

I.6.2.3 The concept of chemical shift

In a chemical bond the electronic environment is shared between nuclei. The nucleus which is more electronegative tends to steal negative charge from the less electronegative nucleus, resulting in a process known as deshielding. This

results in the deshielded nucleus experiencing a greater magnetic effect causing it to come into resonance at a higher frequency than its unbound state (Gadian, 1995). This concept can be used to target compounds with specific chemical bonds, using RF pulses, as the Larmor frequency changes based on shielding properties. Chemical shift is the term used to describe the effects of shielding reported in parts per million (ppm). In mathematical terms, it is the fractional difference between the Larmor frequency of a nucleus in a particular chemical bond and the frequency of a reference compound containing the same nucleus. Chemical shifts are independent of field strength in the sense that inherent linewidths are constant; however, an increase in field strength improves spectral resolution (Gadian, 1995).

I.6.2.4 The concept of spin-spin or J-coupling

In addition to the properties of shielding, the frequency of the NMR signal can also be altered by the state of neighboring nuclear spins (Gadian, 1995). This may result in the formation of multiplets, which are the splitting of spectral lines, in a process known as spin-spin or J-coupling. The magnetic field experienced by a nucleus can be changed based on the magnetic field produced from possible alignments experienced in the B_0 by neighboring spins, which are transmitted through the chemical bonds of a given molecule. Line separations within a multiplet depend on the molecular structure of a compound and are independent of field strength. The total area represented under the multiplet is the same as if there were no J-coupling. Spin decoupling is possible, by targeting the frequency of a specific chemical shift value with a continuous RF irradiation pulse.

I.6.2.5 T1 and T2 relaxation

The longitudinal relaxation time (T_1), commonly referred to as the "spin-lattice relaxation time", describes the recovery of magnetization back to parallel with the B_0. The transversal relaxation time (T_2), commonly referred to as the "spin-spin relaxation time", governs the decay of the signal (Boesch, 1999). Signal attenuation depends on repetition time (TR) and echo time (TE) of a given sequence. RF pulses can be applied numerous times during an MRS session; TR represents the time between RF pulses and defines if complete recovery to the original B_0 alignment occurs between pulses. This is dependent on the longitudinal relaxation time T_1 of a specific tissue. At short TR times, short T_1 values yield a larger signal than long T_1 values (Boesch, 1999). TE is the amount of time from an RF pulse to data acquisition. In a spin echo pulse sequence, the signal is progressively attenuated with longer TE and is based on the irreversible decay associated with T_2.

I.6.3 The in vivo MRS experiment

I.6.3.1 Introduction to the difficulties of in vivo MRS

Numerous difficulties arise when attempting to measure NMR signals in *in vivo* systems. Some of these difficulties include, localizing the signal to a given region of interest (ROI), supressing the water signal found within a given NMR spectrum, creating a homogenous field to collect data from, optimizing the pulse sequence for a target resonance recovery and analyzing a given spectrum.

I.6.3.2 Localization Methods

Before ^{1}H-MRS data can be collected, the region of interest (ROI) must be determined to detect the signal from a target volume of tissue. MRI can be used to produce structural images in the coronal, sagittal and axial planes in order to register a voxel to the desired location. Pulse sequences have been developed which can be used to obtain a well-defined spatial volume in order to observe changes in metabolite concentrations in the ROI. Point-resolved spatially localized spectroscopy (PRESS) (Bottomley, 1989) or stimulated-echo acquisition mode (STEAM) (Frahm et al., 1989) are two of the pulse sequences generally used. PRESS involves the use of a 90 degree pulse followed by two 180 degree pulses and produces a spin echo whereas STEAM uses three 90 degree pulses and produces a stimulated echo. The spins in the intersection of all three planes experience all three pulses and give rise to the signal in the ROI (De Graaf and Rothman, 2001). When comparing STEAM to PRESS, STEAM has a better localization accuracy and is less sensitive to T_2 relaxation, with the ability to collect a greater number of averages in a shorter period of time due to shorter acquisition time. PRESS has the advantage over STEAM in that it is more sensitive, making it a valid choice when measuring signals with long T_2 values.

Tissue composition of a given ROI also needs to be taken into consideration when assessing metabolite concentrations since the metabolites are not evenly distributed within different brain tissue types. GM is mainly composed of neuronal cell bodies, dendrites and unmyelinated axons whereas white matter (WM) is mainly composed of myelinated axon tracts. Cerebral spinal fluid (CSF)

is composed of a clear liquid that occupies the subarachnoid space and ventricular system within the brain.

I.6.3.3 Water suppression

NMR spectra are dominated by the signal from water (Kreis, 1997), which can lead to the distortion of other metabolite resonances. In addition, the large water signal does not allow for clear definition of a baseline, making resonance fitting ambiguous (De Graaf and Rothman, 2001). Varying methods for water suppression have been established and are required for stable *in vivo* ^{1}H-MRS. One of the more common methods is known as chemical shift selective saturation (CHESS) and uses a high frequency 90 degree pulse tuned to target only spins in water molecules, followed by a high intensity gradient to de-phase the spins. This leads to de-phased water molecules with no B_0 magnetization upon commencement of the MRS sequence (Haase et al., 1985). A double inversion recovery can also be used to supress the water signal. The water resonance can be inverted by frequency-selective inversion pulses, resulting in less sensitivity to pulse imperfections and relaxation time variations (Shen and Saunders, 1993). Water suppression allows for a more precise determination of metabolite concentrations.

I.6.3.4 Shimming

A process known as shimming ensures that like protons are resonating at the same frequency within a given sample, through applying small currents through shim coils. This ensures homogeneity of the B_0 and aids in producing

spectra of optimum quality. Fast Automatic Shimming Technique by Mapping Along Projections (FASTMAP) is a commonly used auto-shimming procedure (Gruetter, 1993).

I.6.3.5 Pulse sequence optimaization for target resonance recovery

The pulse sequence can be optimized to target specific resonances. In the case of STEAM, the mixing time and TE can be altered to target a specific metabolite. This becomes particularly important for targeting peaks such as Glu which has overlapping resonances with GABA, NAA and Gln.

I.6.3.6 Analysis of the spectrum

Analyzing the spectrum is complicated by the complexity of overlapping resonance peaks as well as unpredictable line shape and baselines. The LCModel method of analyzing spectra is used to analyze *in vivo* spectra as a linear combination of model *in vitro* spectra from individual metabolite solutions (Provencher, 2001). The LCModel uses a complete spectrum rather than individual peaks, allowing for resolution of overlapping peaks by incorporating maximum prior information into the analysis. Two metabolites with similar peaks in one region of the spectrum can be resolved if they have different peaks in another region of the spectrum (Provencher, 2001). In addition, multiplets due to J-coupling are accounted for in the LCModel. The LCModel finds the smoothest line shape and baseline consistent with the data by adapting to the data, with no fixed parameterization required. This permits flexibility while attempting to limit

concentration errors resulting from over or under-parameterization (Provencher, 2001).

I.6.4 Metabolites measured using ^{1}H-MRS

Several brain metabolites can be measured using ^{1}H-MRS. Which include GABA, NAA, creatine (Cr), Cho, Gln, Glu and myo-inositol. For the purpose of the research described in this thesis the main focus was on NAA, Cr, Cho and Glu.

NAA is a free amino acid found in both the peripheral and central nervous system. Although its function is poorly understood, it is thought to play a role in many neuropsychiatric disorders (Govindaraju et al., 2000). In the brain NAA is thought to be a marker of neuronal integrity and density and as such is mainly found in GM as opposed to WM. During the natural process of aging NAA levels are found to decrease parallel to the reduction in neuronal metabolism (Schuster et al., 2011). The acetyl peak from NAA is found at a chemical shift of 2.01ppm, while the aspartate peak is found at a chemical shift of 2.49ppm. The aspartate peak of NAA overlaps with the Glu peak making it difficult to target the Glu peak directly (Govindaraju et al., 2000).

Cr and phosphocreatine are measured together in ^{1}H-MRS and are found at a chemical shift of 3.03ppm (Govindaraju et al., 2000). The Cr peak has been shown to be very stable in the sense that it does not change in regards to natural aging or with many diseases that affect other metabolites. As such Cr in the past has been used as a concentration reference, although caution is warranted as Cr

has recently been shown to be decreased in tumors and stroke and increased in subjects experiencing myotonic dystrophy (Chang et al., 1998). In addition Cr and phosphocreatine work in balance with one another, when Cr rises phosphocreatine decreases and vice versa, as such the intracellular environment may be fluctuating based on the rise and fall of creatine and phosphocreatine, suggesting this may not be the best metabolite to use as a concentration reference.

The Cho peak is made up of glycerophosphorylcholine and phosphorylcholine as well as Cho and is referred to as total choline, with a chemical shift of 3.21ppm. Cho is an essential nutrient mainly obtained from the diet, specifically phospholipids. It is necessary for synthesis of acetylcholine, a neurotransmitter found in the brain, and also for phosphatidylcholine which is a marker of membrane integrity. Cho has been shown to be altered in numerous disorders, with reported increases in cancer, ischemia, head trauma, Alzheimer's disease and multiple sclerosis and decreases reported in liver disease and stroke (Rudkin et al., 1999).

One of the peaks of Glu is found at a chemical shift of 2.35ppm, which has been targeted for ^{1}H-MRS. It is the most abundant amino acid found in the brain but mainly localized to neurons and thus GM. Separation of the Glu peak from the glutamine (Gln) peak becomes difficult at low field strengths (i.e. less than 3T) and as such is often reported as glutamix (Glx) which is the combined signal of Glu and Gln. At low field strengths there is also overlap with the NAA-aspartate peak and the GABA peaks. Increasing the field strength greatly improves the ability to separate Glu from Gln, NAA-aspartate and GABA (Tkac et al., 2001).

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II. Prospective Assessment of Glutamate Levels in the Medial Prefrontal Cortex of Women at Risk for Postpartum Depression during Pregnancy and the Postpartum Period

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II.1 Introduction

The time following childbirth is a time of increased risk for depression, with 10-20% of women experiencing an episode of postpartum depression (PPD). This risk increases up to 65% in women with a history of PPD (Gaynes et al., 2005). According to the DSM-IV-TR, PPD is defined as an episode of major depression (MD) occurring within the first 4 weeks following childbirth (American Psychiatric Association, 2000). However, expert consensus based on recent epidemiological data suggests that this time frame should be expanded to 3 months postpartum (Leahy-Warren et al., 2011). PPD has been divided into early vs. late onset, with early onset PPD occurring within the first 6-8 weeks following childbirth and late onset PPD occurring after 8 weeks postpartum. Risk factors for early onset PPD differ from those of late onset PPD (Bloch et al., 2006). A history of premenstrual dysphoric disorder (PMDD), major depression (MD) or prior PPD increase the risk of developing early onset PPD (Gaynes et al., 2005; Tronick and Reck, 2009). Late onset risk factors include low level of education and low socioeconomic status (Grote et al., 2010). A substantial percentage of women diagnosed with PPD also have an onset of symptoms during pregnancy (Stowe et al., 2005**;** Josefsson et al., 2001).

Glutamate (Glu) is the major excitatory neurotransmitter found in the central nervous system (Pittenger et al., 2007). Dysregulation of the glutamatergic system has been implicated in the pathophysiology of MD (Mitchell & Baker, 2010). *In vivo* proton magnetic resonance spectroscopy (^{1}H-MRS) is a non-invasive technique currently available for measuring brain metabolite

concentrations (Stanley, 2002; Soares et al. 1996). ^{1}H-MRS investigations have shown medial prefrontal cortex (MPFC) levels of glutamix (Glx), a combined signal of Glu + glutamine (Gln), to be decreased in individuals diagnosed with MD (Hasler et al., 2007; Auer et al., 2000; Pfleiderer et al., 2003). A more recent study by Merkl et al. (2011) used a ^{1}H-MRS technique that allowed Glu to be measured independently from Gln, and found decreased Glu levels in the MPFC of MD patients. In addition, novel agents such as the N-methyl-D-aspartate (NMDA) Glu receptor antagonist, ketamine, have been shown to have a rapid-onset antidepressant effect (Berman et al., 2000; Zarate et al., 2006). These data all suggest a key role of the glutamatergic system in the pathophysiology of MD.

A role for hormonal changes in the development of MD during pregnancy and PPD are suspected. Levels of estrogen and progesterone, as well as of associated neuroactive steroids (NASs), gradually rise during pregnancy, reaching an apex during the third trimester with a drastic decline following parturition (Tulchinsky et al., 1975; Lin et al., 1972; Ahmed et al., 1972; Bloch et al., 2003; Buckwalter et al., 1999). The menstrual cycle shows similar fluctuations in female hormones to that of pregnancy and the postpartum, although of a lesser magnitude. Published research from our group has demonstrated alterations in MPFC Glu levels across the menstrual cycle in relation to hormonal fluctuations (Batra et al., 2008), suggesting that female hormone fluctuations impact MPFC Glu levels. However, to our knowledge, brain Glu levels have never been measured in humans during pregnancy and the early postpartum period.

The objective of the current study was to evaluate Glu levels in the MPFC of women at a high risk for developing early onset PPD, high risk women (HRW), compared to healthy controls (HCs), during pregnancy and the early postpartum (up to 7 weeks), using 3-Tesla (3T) ^{1}H-MRS. We hypothesized that MPFC Glu levels will decrease during the early PP in HRW, with a gradual return of Glu levels to HC levels during the late postpartum period.

II.2 Methods

II.2.1 Participants:

Forty-one healthy women without any current or past psychiatric history (HC) and twenty two healthy high risk women with a past history of PMDD, MD or PPD (HRW) were recruited from advertisements and through collaborations with health institutions in Edmonton, Canada. Each woman was recruited according to the guidelines of the Health Research Ethics Board of the University of Alberta. After a complete description of the study to the subjects, written informed consent was obtained.

All participants were administered the Structured Clinical Interview for DSM-IV-TR of Axis I disorders (First et al., 2002) to screen for any current or lifetime Axis I psychiatric disorders. Participants meeting criteria for current psychiatric disorders were excluded from participation in the study. Participants had not used any street or recreational drugs in the previous 6 months or during the study, nor had they used any form of hormonal treatment. Participating

women were not taking any medications, psychotropic drugs or herbal products with psychotropic activity 3 months prior to entering the study or at any time during the study. Other exclusion factors included potential confounding factors such as brain injury or classical contraindications to MRS and any medical conditions that could interfere with the study including endocrine or neurological disorders (e.g. seizure disorders). The Edinburgh Postpartum Depression Scale (EPDS) was administered to all participants at all visits (Cox et al., 1987). If a woman developed PPD during the course of the study, she was excluded from participation and all data points were removed from analysis as it is possible that Glu dysregulation precedes overt PPD symptomatology. Accordingly, follow-up continued for at least 13 weeks postpartum in order to ensure that none of the participants went on to develop late onset PPD.

II.2.2 ^{1}H-MRS

The ^{1}H-MRS sessions were scheduled 2-3 weeks prior to delivery, 10 days postpartum, 3 weeks postpartum, 5 weeks postpartum and 7 weeks postpartum.

^{1}H-MRS was performed in the Peter S. Allen MR Research Centre, University of Alberta Hospital, Edmonton, Canada, using a 3T magnet (Magnex Scientific, Concord, California) equipped with a spectrometer (Surrey Medical Imaging System, Surrey, United Kingdom) and a quadrature birdcage resonator. A 2x3x3 cm^3 voxel (for segmentation and spectroscopy) was positioned perpendicular to and centered on the midline. Shimming to ~0.05 p.p.m. was accomplished by using both FASTMAP (Gruetter, 1993) and an in-house

autoshim routine. The optimal in vivo Glu and Gln contrast to background (McEwen, 2012), determined using numerical simulation (Thompson, 2001), used an echo time (TE) equal to 240 ms, mixing time (TM) equal to 27 ms, and repetition time (TR) equal to 3 s. Spectra were the sum of 512 averages, acquired in 16 blocks of 32 averages. The in vivo data were analyzed using the LCModel (version 6.0-1) analysis program (Provencher, 1993). The metabolite basis spectra used in the LCModel analysis were derived by numerical simulation and included N-acetylaspartate (NAA), creatine plus phosphocreatine (t-Cr), myo-inositol, N-acetylaspartylglutamate, taurine, lactate, aspartate, glycine, alanine, gamma-aminobutyric acid, glycerophosphorylcholine plus phosphorylcholine (t-Cho), and Glu. We only report metabolite measures for Glu, NAA, t-Cr, and t-Cho in the MPFC which were within the reliable threshold for the % standard devition of the fit (< 15%). The segmentation data were used to scale the water data, used for quantification, for established differences in the water content of gray matter (GM) and white matter (WM). In addition, these data allowed us to eliminate the cerebrospinal fluid (CSF) water volume that contributes to the total water signal, so that the quantified metabolite concentrations relate to the tissue space of the GM and WM. The water peak area was utilized as the denominator in concentration calculations after removing the non-brain signal contribution from CSF.

A full description of the methods used to obtain ^{1}H-MRS data used in this manuscript can be found in a recent publication by our group McEwen et al. (2012).

II.2.3 Statistical Analysis

Statistical analyses were performed using SPSS version 19.0. A p-value of less than 0.05 was considered for statistical significance for main level factors and a p-value of less than 0.10 for interaction terms. All statistical testing was performed with two-tailed tests.

Linear mixed (LM) modeling was used to examine the pattern of recovery for metabolites and tissue composition scores over the five time points (pregnancy, 10 days postpartum, 21 days postpartum (3 weeks), 35 days postpartum (5 weeks) and 49 days postpartum (7weeks)) because non-linear equations provided the best fit for predicting metabolites and tissue composition scores over the 7 weeks. Another feature of the LM modeling allowed us to use available data at each time period (as not all participants had complete data sets) unlike repeated measures ANOVA analysis that requires complete datasets over all time periods. The LM models include parameters that estimate either metabolites or tissue compositions at pregnancy and the rate of change during the pregnancy. The square of time is also included as an estimate of change in the rate for some models because of a quadratic relationship over time for Glu and GM scores. The model had two levels which consisted of one level for the within-individual change over time and the other for between-individual differences in change over time.

In the multivariate models, variables were selected using both forward selection and backward elimination procedures. Forward selection starts at a

simple model, then considers all of the reasonable one-step-more-complicated models and chooses the one with the smallest p-value for the new parameter. This continues until no addition parameters have a significant p-value. Backward selection starts at a complicated model and removes the term with the largest p-value, as long as that p-value is larger than 0.05. All the variables in the base model with $p<0.2$ were included in the forward selection and backward elimination models and only those variables with $p<0.05$ were kept in the final model. Interaction between time and group was included based on $p<0.1$.

II.3 Results

The number of data points included at each sample time point were for HCs, pregnancy (21), 10 days (19), 3 weeks (31), 5 weeks (27), and 7 weeks (16); for HRW, pregnancy (13), 10 days (8), 3 weeks (16), 5 weeks (12), and 7 weeks (13). Missing data points are explained by women not willing to undergo the initial pregnancy MRS visit, women unable to attend visits for personal reasons and poor data quality which resulted from movement occurring during the course of the MRS scan. Mean values and standard deviations for metabolites, tissue compositions and EPDS scores are presented in Table II-1.

There was no statistically significant age difference between HCs and HRW (29.34±4.73 and 30.92±4.31; $p=0.19$, $t=1.33$, $df= 63$). There was a significant interaction between group and time for MPFC Glu levels (Table II-2). The significant interaction indicates that the pattern of change over time is

different between women in the HC group and women in the HRW group with MPFC Glu levels being lower during pregnancy and the very early postpartum in the HRW group, returning to HC levels as the postpartum progresses (Figure II-1). Subanalysis revealed no significant changes in Glu over time for the HC group whereas there was a significant increase in Glu levels over time for the HRW group (Table II-2). This interaction remained statistically significant after correcting for tissue composition changes in %GM (ie. treating %GM as a covariate) (Table II-2) as %GM was shown to significantly increase over time for both groups (Table II-2) (Figure II-2).

Of interest, t-Cho significantly changed over time (Table II-2) with an increase observed for both groups from the pregnancy time point up to 7 weeks postpartum, with no significant differences observed between groups (Figure II-3). There were no significant differences observed in other water quantified brain metabolites (NAA, t-Cr) (Table II-2) or %WM and %CSF (Table II-2).

There were no statistically significant differences in EPDS scores between HC and HRW groups (Table II-2). The Pearson correlation coefficient was used to assess the association between depressive symptoms (based on scores from the EPDS) and Glu in HCs and HRW at each time point. There were no statistically significant correlations between Glu and scores on the EPDS in either group at any of the time points.

II.4 Discussion

The results of this ^{1}H-MRS investigation suggest that MPFC Glu levels fluctuate during pregnancy and the early postpartum period in women at a high risk for PPD, but not in HCs. To the best of our knowledge this study is the only investigation to report on brain Glu levels during pregnancy and the postpartum period. We therefore cannot make direct comparisons to other studies.

Our research group has recently shown that MPFC Glu levels are increased in un-medicated women suffering from PPD (McEwen et al., 2012). These findings suggest an association between Glu dysregulation and the pathophysiology of PPD. Our findings of lower Glu levels in HRW initially appear difficult to reconcile with our findings in PPD women. However, as none of the HRW went on to develop PPD, the decrease in Glu observed in the HRW group may reflect a compensatory mechanism which contributes to the prevention of developing PPD in at risk women. Interestingly, the period of greatest risk for developing PPD is the very early postpartum (Stowe et al., 2005) when the HRW showed the lowest MPFC Glu levels compared to HCs. MPFC Glu levels in HRW return to normal as the postpartum progresses and the risk for PPD decreases at approximately 5 weeks postpartum (figure II-1), coinciding with the return to normal female hormone levels and associated NASs (Buckwalter et al., 1999; Pearson Murphy et al., 2001). This observation further supports the notion of a compensatory mechanism to the fluctuation of female hormones and associated NASs in HRW as the alteration of MPFC Glu levels appears to be a unique feature of pregnancy and the early postpartum period in this group.

Our results of altered MPFC Glu levels in HRW can also be discussed in relation to studies of MD. Although our previous results suggest potential differences in MPFC Glu levels observed in PPD and MD, comparison of our HRW can be made to patients at risk of developing a future episode of MD as both populations can be considered at risk of developing depressive symptomatology. A MRS investigation by Taylor et al. (2009) showed that MPFC Glu levels in un-medicated, remitted MD patients return to HC levels between depressive episodes. This observation is further supported by Price et al. (2009) who also found a return to normal Glx levels in remitted MD patients. This suggests women at risk for MD do not display long lasting trait related dysregulation in Glu or Glx levels. These results are consistent with our results as HRW show a return to HC levels as the postpartum progresses, with only an initial difference in MPFC Glu levels during the high risk period of the early postpartum. Combined, these results suggest that there are no chronic trait related MPFC Glu level differences in either women at risk for MD or PPD.

Although there were no time or group effects for EPDS scores, the trend towards a difference between groups in EPDS scores (p=0.06) indicates that the HRW group may show greater subsyndromal depressive symptoms compared to the HC group. However, it is unlikely to have an impact on our results, as the difference in EPDS scores between groups is not clinically significant, as neither group reached pathological ranges. In addition, the EPDS data did not match the Glu results, showing no time by group interaction. Additional analysis revealed no correlations between Glu levels or EPDS scores at any of the time points.

Significant changes in %WM and %CSF were not observed during this study. However, we did observe a significant increase in %GM in the MPFC from pregnancy up to 7 weeks postpartum. As there were no differences between the HC group and the HRW group, this suggests that the change in %GM is associated with pregnancy and the early postpartum, which normalizes as the postpartum period progresses.

Observed decreases in %GM during pregnancy and the early postpartum with a progressive increase in %GM later during the postpartum, are somewhat consistent with previous investigations. An MRI study by Oatridge et al. (2002) compared healthy pregnant women to those with preeclampsia and found a decrease in overall brain size in healthy pregnant women, with a significant increase in brain size, compared to pregnancy, by 6 weeks postpartum and a return to pre-pregnant levels by 6 months postpartum. These results obtained for the whole brain are consistent with our results of a decrease in % GM in the MPFC during pregnancy with an observed significant increase across the early postpartum. In addition, a study by Kim et al. (2010) used voxel based morphometry to assess %GM in the prefrontal cortex of mothers at two different time points, 2-4 weeks postpartum and 3-4 months postpartum, and found a significant increase in %GM from the first measurement to the second. However, as no additional time points in between were measured it is difficult to determine when the increase in %GM occurred, and the %GM may have already been increasing from pregnancy to the 2-4 week postpartum time point as would be suggested by our findings. The decline in %GM during pregnancy and the early

postpartum may be linked to common cognitive complaints experienced by many women during this time, often referred to as “baby brain” (Sharp et al., 1993). The decrease in %GM observed during pregnancy and the postpartum may have provided an evolutionary advantage to women by focusing on neural connections necessary for providing for the developing fetus and newborn baby. From a MRS methodological point of view, it is important to note that the change in %GM seen during pregnancy and the early postpartum should be taken into consideration for subsequent MRS investigations of brain metabolites during pregnancy and the postpartum and lead to statistical correction of metabolite results for tissue composition.

MPFC t-Cho levels significantly increased over time from pregnancy up to 7 weeks postpartum in both the HC and HRW groups. This has been reported in a previous MRS study comparing pregnant women, with and without preeclampsia, to non-pregnant women and found an overall decrease in t-Cho levels in pregnant women compared to non-pregnant controls (Rutherford et al., 2003). The demands of the developing fetus have previously been indicated as a cause for the decreased brain t-Cho levels observed in healthy pregnant women (Caudill, 2010). As such our data describing the most significant decrease in t-Cho levels during pregnancy and a gradual return to pre-pregnant levels across the postpartum period, equally in both groups of subjects, would be expected.

The hormonal changes occurring during pregnancy and following parturition may be contributing to the alterations observed in MPFC Glu levels. By the end of the third trimester of pregnancy, estrogens (estradiol, estriol and

estrone) levels rise to 50 times the highest levels observed during the menstrual cycle, while progesterone levels rise to 10 times that of the menstrual cycle (Bloch et al., 2003). Childbirth brings on a drastic drop in both estrogens and progesterone with a return to early follicular phase menstrual cycle levels by 3-7 days postpartum (Bloch et al., 2003). Levels of associated NASs have also been shown to rise during pregnancy and remain elevated up to 7 weeks postpartum (Pearson Murphy et al., 2001). Interestingly, MPFC Glu levels in HRW women return to the levels of HCs by 5 weeks postpartum, concurrent with the return to normal levels of female hormones and associated NASs (Bloch et al., 2003, Pearson Murphy et al., 2001).

Estrogen and progesterone have been shown to have an effect on the MPFC. Alterations in activation of the MPFC have been observed in PET studies where estrogen and progesterone were suppressed and then added back one at a time (Berman et al., 1997). Additionally, changes in activation of the MPFC have been observed across the menstrual cycle in relation to fluctuations of both estrogen and progesterone (Reiman et al., 1996). Our group has also shown that alterations of MPFC Glu levels during the menstrual cycle are influenced by female hormone fluctuations (Batra et al., 2008).

Animal studies have shown estrogen to be responsible for increasing the number of NMDA receptors in the brain, as well as increasing NMDA receptor activity (Woolley and McEwen, 1993; Smith, 1989). Furthermore, estrogen has been shown to prevent Glu-related excitoxicity through improving astrocyte function and Glu reuptake from the extracellular space (Pawlak et al., 2005, Mong

& Blutstein, 2006). In contrast progesterone has an opposite effect reducing the number of excitatory synapses within the brain (Woolley and McEwen, 1993). Estrogen and progesterone appear to regulate glutamatergic activity in animal models. Further research is needed to correlate our observations of changes in Glu levels with estrogen and progesterone levels.

The relatively small sample size is one limitation to our study. Ideally, all participants would have completed all scans, but the missing data points are unfortunate but inherent to these types of complex MRS investigations. One weakness of this investigation is monitoring of MPFC Glu levels only continued up to 7 weeks postpartum, continuation of measurements up to 6 months postpartum would have been ideal, however difficult to maintain participation for this amount of time and costly. A longer follow up period combined with pre-pregnancy measurements would have allowed us to determine when or if our various measurements eventually returned to pre-pregnant levels. One of the strengths of this investigation was using water as the reference metabolite, resulting in water quantified target metabolites. In a number of MRS studies, metabolite concentrations are referenced to another metabolite such as t-Cho (Jansen et al., 2006); however, the use of a reference metabolite, as seen with the fluctuations of t-Cho in this investigation, can be problematic, producing apparent fluctuations in target metabolites as levels of the reference molecule itself may be fluctuating.

From an ethical point of view, accumulating evidence exists for the safety of performing MRI and ^{1}H-MRS during pregnancy, especially during late

pregnancy when the fetus is fully developed (Clements et al., 2000; Heerchap et al., 2003; Kok et al., 2004; Kreis et al., 2002; Girard et al., 2006). Babies born in women who underwent ^{1}H-MRS during pregnancy in our investigation did not present any postnatal problems.

Our study is the first extensive prospective study to measure changes in brain metabolites from the third trimester of pregnancy across the postpartum period. The observed difference in water-quantified MPFC Glu levels between HCs and HRW during the early postpartum with a return to normal control levels as the postpartum progresses, contributes to the understanding of the biological factors playing a role in the increased risk associated with developing early onset PPD in high risk women. Future work is needed to determine the relationship between the hormonal changes and Glu alterations occurring during this time in the role of developing MD during pregnancy and PPD and to assess how metabolite levels and tissue composition vary compared to pre-pregnant levels.

Table II-1: *Water-quantified metabolite concentrations and tissue compositions in the MPFC of HC and HRW groups*

HC					
	Pregnancy	**10 Days PP**	**3 Weeks PP**	**5 Weeks PP**	**7 Weeks PP**
Metabolites					
Glu	6.74±1.39	6.93±1.74	7.03±1.62	6.51±1.20	6.60±2.00
NAA	8.99±1.44	9.66±1.53	9.00±1.62	8.60±1.25	9.12±1.25
t-Cr	8.65±3.42	9.54±2.91	10.41±3.18	9.60±2.54	9.47±2.99
t-Cho	1.37±0.24	1.79±0.36	1.77±0.36	1.72±0.35	1.89±0.35
Tissue Composition					
%GM	46.53±10.66	51.59±7.61	51.21±8.59	52.30±9.27	49.34±11.35
%WM	30.94±10.14	26.48±6.99	30.73±6.59	28.89±7.16	31.41±8.17
%CSF	22.54±10.24	21.91±6.88	17.42±6.39	18.19±8.02	18.08±8.81
EPDS Score	2.76±2.59	5.47±3.79	3.84±3.41	3.52±4.39	3.81±3.23
HRW					
	Pregnancy	**10 Days PP**	**3 Weeks PP**	**5 Weeks PP**	**7 Weeks PP**
Metabolites					
Glu	5.56±1.86	6.54±1.79	5.96±0.91	6.88±1.89	6.78±1.48
NAA	9.18±0.99	9.05±1.28	8.69±1.08	8.59±1.46	8.96±1.98
t-Cr	9.27±2.50	10.19±2.99	9.00±1.84	9.31±2.86	9.97±3.31
t-Cho	1.43±0.37	1.46±0.36	1.71±0.28	1.78±0.33	1.82±0.77
Tissue Composition					
%GM	47.32±7.81	48.30±7.19	54.19±7.97	55.03±13.22	54.89±9.06
%WM	29.88±9.07	26.24±8.30	27.28±6.05	26.23±11.53	28.43±5.29
%CSF	20.47±10.73	25.45±8.72	18.53±9.17	18.76±6.97	16.68±8.10
EPDS Score	4.62±4.84	6.00±3.02	6.50±4.23	5.42±4.60	4.00±3.13

Abbreviations: HC = healthy controls, HRW = high risk women, PP = postpartum, Glu = glutamate, NAA = N-acetyl-aspartate, t-Cr = creatine plus phosphocreatine, t-Cho = glycerophosphorylcholine plus phosphorylcholine, %GM = % grey matter, %WM = % white matter, %CSF = % cerebrospinal fluid, EPDS = Edinburgh postnatal depression scale.

All values are reported as means ± standard deviation.

Table II-2: *Description of trajectories from linear mixed models fitted for water-quantified metabolites: Glu, NAA, t-Cr, t-Cho and tissue compositions: %GM, %WM and %CSF outcomes*

Factor	Coefficient	95% CI	p-value
Outcome: Glu			
Intercept	6.833	6.325, 7.340	<0.001
Group (HRW vs. HC)	-1.178	-2.031, -0.326	0.007
Time (in Days)	-0.006	-0.022, 0.010	0.469
Interaction: Time by Group	0.031	0.005, 0.058	0.020
Sub-Analysis[1] within HRW			
Intercept	5.679	4.994, 6.365	<0.001
Time (in Days)	0.025	0.003, 0.047	0.025
Sub-Analysis[1] within HC			
Intercept	6.816	6.305, 7.327	<0.001
Time (in Days)	-0.006	-0.022, 0.010	0.468
Outcome: Glu adjusting for %GM (%GM as a covariate)			
Intercept	6.583	5.226, 7.940	<0.001
Group (HRW vs. HC)	-1.173	-2.026, -0.321	0.007
Time (in Days)	-0.006	-0.022, 0.010	0.459
Interaction: Time by Group	0.031	0.004, 0.057	0.023
Outcome: NAA			
Intercept	9.170	8.731, 9.608	<0.001
Group (HRW vs. HC)	-0.221	-0.830, 0.388	0.471

Time (in Days)	-0.006	-0.018, 0.005	0.263
Outcome: t-Cr			
Intercept	9.645	8.764, 10.525	<0.001
Group (HRW vs. HC)	-0.280	-1.526, 0.966	0.655
Time (in Days)	0.008	-0.013, 0.030	0.449
Outcome: t-Cho			
Intercept	1.546	1.428, 1.663	<0.001
Group (HRW vs. HC)	-0.061	-0.220, 0.098	0.446
Time (in Days)	0.007	-0.004, 0.010	<0.001
Outcome: %GM			
Intercept	47.594	44.419, 50.768	<0.001
Group (HRW vs. HC)	2.038	-1.632, 5.708	0.271
Time (in Days)	0.338	0.091, 0.586	0.008
Time-Square	-0.005	-0.010, -0.001	0.028
Outcome: %WM			
Intercept	29.629	27.404, 31.854	<0.001
Group (HRW vs. HC)	-1.854	-4.553, 0.845	0.174
Time (in Days)	0.0003	-0.069, 0.070	0.993
Outcome: %CSF			
Intercept	19.163	16.518, 21.809	<0.001

Group (HRW vs. HC)	-0.451	-4.504, 3.602	0.825
Time (in Days)	-0.030	-0.081, 0.021	0.251
Outcome: EPDS Score			
Intercept	4.135	2.970, 10.752	<0.001
Group (HRW vs. HC)	1.580	-0.078, 3.238	0.061
Time (in Days)	-0.015	-0.044, 0.013	0.285

Abbreviations: CI = Confidence Interval, HRW = high risk women, HC = healthy controls, Glu = glutamate, NAA = N-acetyl-aspartate, t-Cr = creatine plus phosphocreatine, t-Cho = glycerophosphorylcholine plus phosphorylcholine, %GM = % grey matter, %WM = % white matter, %CSF = % cerebrospinal fluid, EPDS = Edinburgh postnatal depression scale.

[1] Sub-analysis was conducted to examine the Glu pattern over time within each group (HRW and HC) because we found the interaction between time and group to be significant. The interaction for time and group for the other outcomes were found to be non-significant.

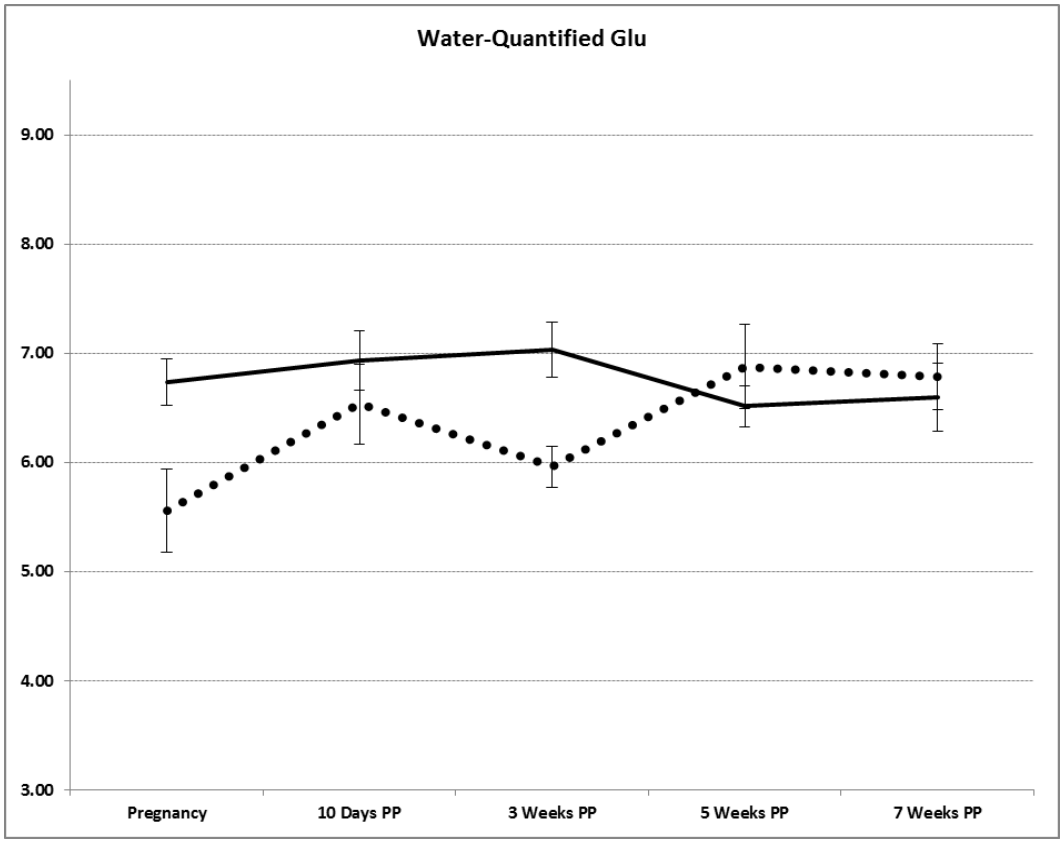

Figure II-1: Change in Glu from the pregnancy time point up to 7 weeks postpartum

HCs

HRW

Abbreviations: HCs = healthy controls, HRW = high risk women, Glu = glutamate, PP = postpartum

Values are reported as the mean ± standard error

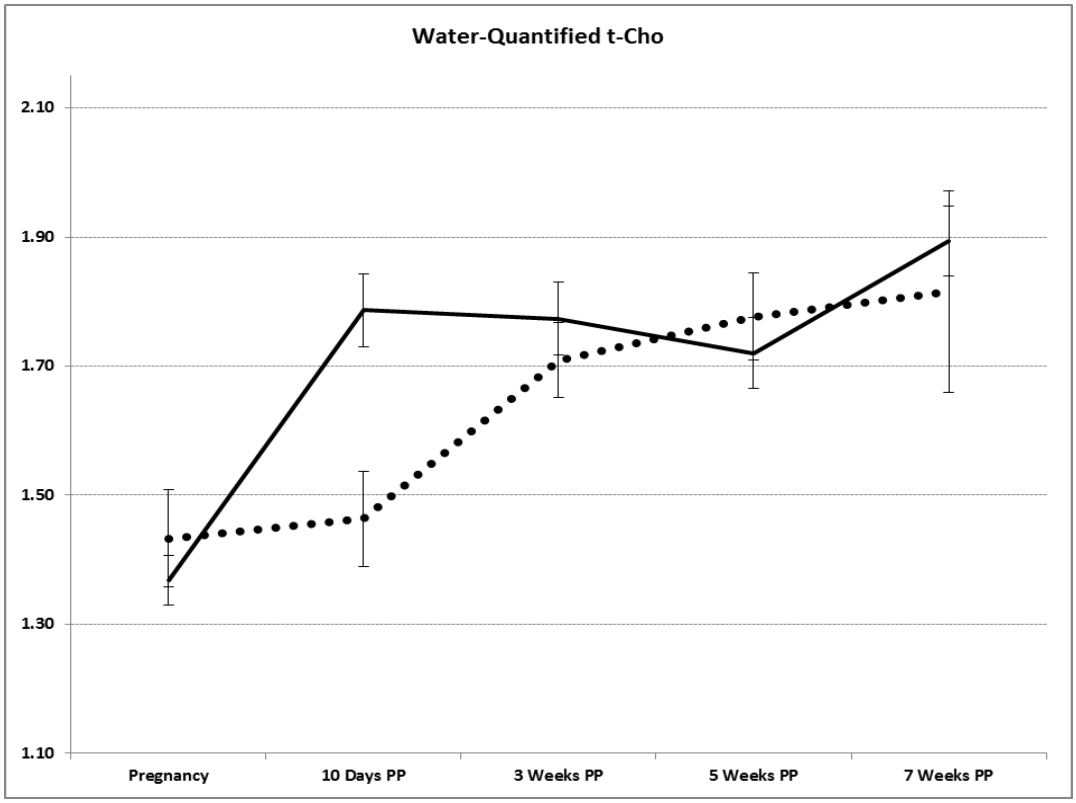

Figure II-2: Change in t-Cho from the pregnancy time point up to 7 weeks postpartum

▬ HCs

▪▪▪ HRW

Abbreviations: HCs = healthy controls, HRW = high risk women, t-Cho = glycerophosphorylcholine plus phosphorylcholine, PP = postpartum

Values are reported as the mean ± standard error

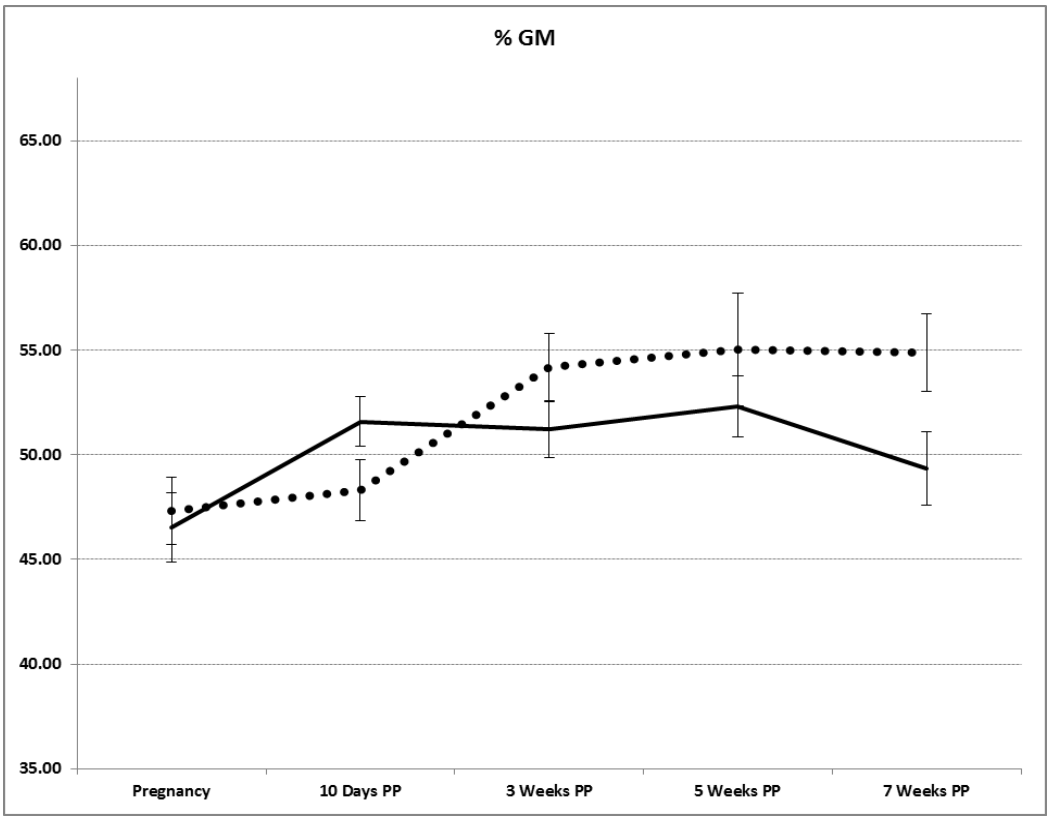

Figure II-3: Change in %GM from the pregnancy time point up to 7 weeks postpartum

HCs

HRW

Abbreviations: HCs = healthy controls, HRW = high risk women, %GM = % Grey Matter, PP = postpartum

Values are reported as the mean ± standard error

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III. Glutamate Levels in the Medial Prefrontal Cortex of Healthy Pregnant Women Compared to Non-Pregnant Controls

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III.1 Introduction

Pregnancy is associated with physiological alterations that are essential in supporting the developing fetus and preparing a woman for birth (Carlin et al., 2008). Although peripheral alterations occurring during pregnancy have been relatively well studied, very little is known about the cerebral changes associated with pregnancy. Without information on the normal brain alterations associated with pregnancy, it is difficult to investigate the brain mechanisms responsible for the impact of pregnancy on mood, either directly during pregnancy or indirectly during the postpartum period.

A great number of women present with symptoms of major depression (MD) during pregnancy (Evans et al., 2001; Bennet et al., 2004). MD during pregnancy has been associated with an increased risk of low birth weight and preterm birth (Grote et al, 2010), which are the leading causes of neonatal, infant and child mortality and morbidity. Furthermore, depressive symptoms occurring during pregnancy have been identified as a risk factor for developing postpartum depression (PPD), with 17-51% of women who develop PPD having an onset of depressive symptoms during pregnancy (Stowe et al., 2005**;** Josefsson et al., 2001). PPD is defined as MD with onset within the first four weeks postpartum (American Psychiatric Association, 2000). However, epidemiological studies have shown MD with an onset within the first three months postpartum to be a more acceptable time frame (Elliot, 2003; Wisner et al., 2006). PPD is often characterized by a depressed mood, loss of interest in usual activities, guilt, worthlessness, excessive worry about the baby as well as a loss of interest in the

baby. PPD not only has effects on the mother but also may affect the mother-infant relationship, which has short term and long term impacts on child development including social, behavioral, cognitive, emotional and physical (Rahman et al., 2004; Downey and Coyne, 1990; Goodman et al., 1993; Murray & Cooper, 2003; Ramchandani et al., 2005). Research further shows that children of PPD mothers are more likely to develop depression themselves by their mid-teen years (Murray et al., 2011). As there are no *in vivo* human normative data of brain neurochemicals during pregnancy, it is very difficult to speculate on the possible brain mechanisms associated with these clinical observations.

Glutamate (Glu) is the major excitatory neurotransmitter, found widespread throughout the brain and accounting for at least 60% of the synapses in the central nervous system (Pittenger et al., 2007; Javitt, 2004). We were specifically interested in measuring Glu concentrations in the brain of pregnant women, as an increasing body of evidence supports the involvement of the glutamatergic system in the pathophysiology of MD (Mitchell & Baker, 2010), a hypothesis supported by *in vivo* proton magnetic resonance spectroscopy (^{1}H-MRS) investigations of brain metabolites and more specifically medial prefrontal cortex (MPFC) glutamate concentrations (Merkl et al., 2010). It is therefore clinically relevant to investigate the influence of a normal pregnancy on brain Glu levels.

The MPFC is also an area of interest in our investigation as it has been shown to be sensitive to hormonal fluctuations. Pregnancy and the postpartum period show a similar pattern of hormonal fluctuations to that of the menstrual

cycle, with a gradual rise in reproductive hormones followed by an abrupt decrease, but to a much greater extent (Bloch et al., 2003, Hendrick et al., 1998, Zonana and Gorman, 2005, Buckwalter et al., 1999). Estradiol levels increase to 50 times the greatest menstrual cycle levels by the third trimester of pregnancy, while plasma progesterone levels increase to 10 times the greatest menstrual cycle levels by the third trimester, both dropping drastically following childbirth. Our research group has previously shown, using i*n vivo* ^{1}H-MRS, that hormonal fluctuations associated with the menstrual cycle impact brain MPFC Glu levels (Batra et al., 2008). Furthermore, positron emission tomography studies have shown alterations in the activation of the MPFC as a result of both pharmacological manipulation of female hormones (Berman et al., 1997) and natural fluctuation of female hormones during the menstrual cycle (Reiman et al., 1996).

Based on principles of MRI and using similar machinery, i*n vivo* ^{1}H-MRS is the only noninvasive technique that can directly assess levels of certain neurochemicals, particularly Glu in localized brain regions (Stanley, 2002; Soares et al. 1996). Concerns surrounding MRI and ^{1}H-MRS during pregnancy have greatly subsided. Accumulating evidence exists for the safety of performing MRI and ^{1}H-MRS during pregnancy, especially during late pregnancy when the fetus is fully developed (Clements et al., 2000; Heerchap et al., 2003; Kok et al., 2004; Kreis et al., 2002; Girard et al., 2006).

The objective of this study is to evaluate Glu levels in the MPFC using 3 T ^{1}H-MRS in healthy pregnant women. We hypothesized that Glu levels are

reduced in the MPFC during late pregnancy in comparison to non-pregnant healthy controls.

III.2 Methods

III.2.1 Participants:

Twenty one healthy pregnant near term women without any current or past psychiatric disorder (pregnant HC: pHC) and fourteen non-pregnant healthy controls in the follicular phase of their menstrual cycle (Follicular Phase HC: FPHC) were recruited from advertisements and through collaborations with health institutions in Edmonton, Canada. Each woman was recruited according to the guidelines of the Health Research Ethics Board of the University of Alberta. After a complete description of the study to the subjects, written informed consent was obtained.

All participants were administered the Structured Clinical Interview for DSM-IV-TR of Axis I disorders to screen for any current or lifetime Axis I psychiatric disorders. Participants meeting criteria for current psychiatric disorders were excluded from participation in the study. Participating women were not taking any medications, psychotropic drugs or herbal products with psychotropic activity 3 months prior to entering the study or at any time during the study. Participants had not used any street or recreational drugs in the previous 6 months or during the study, nor had they used any form of hormonal treatment (within the previous three months for FPHCs) that could interfere with the

interpretation of our results. Other exclusion factors included potential confounding factors such as brain injury or classical contraindications to MRS and any medical conditions that could interfere with the study including endocrine or neurological disorders (e.g. seizure disorders). Exclusion criteria specific to FPHCs included any factors which could alter hormonal levels at the time of scan such as lactation, giving birth in the previous 6 months, having had an abortion in the previous three months and irregular menses.

III.2.2 ^{1}H-MRS

The ^{1}H-MRS sessions were scheduled 2-3 weeks prior to delivery for pHCs and during the follicular phase of the menstrual cycle for FPHCs.

^{1}H-MRS was performed in the Peter S. Allen MR Research Centre, University of Alberta Hospital, Edmonton, Canada, using a 3T magnet (Magnex Scientific, Concord, California) equipped with a spectrometer (Surrey Medical Imaging System, Surrey, United Kingdom) and a quadrature birdcage resonator. A 2x3x3 cm^3 voxel (for segmentation and spectroscopy) was positioned perpendicular to and centered on the midline (Figure III-1). Shimming to ~0.05 p.p.m. was accomplished by using both FASTMAP (Gruetter, 1993) and an in-house autoshim routine. The optimal in vivo Glu and Gln contrast to background (McEwen, 2012), determined using numerical simulation (Thompson, 2001), used an echo time (TE) equal to 240 ms, mixing time (TM) equal to 27 ms, and repetition time (TR) equal to 3 s (Figure III-2). Spectra were the sum of 512 averages, acquired in 16 blocks of 32 averages. The in vivo data were analyzed

using the LCModel (version 6.0-1) analysis program (Provencher, 1993). The metabolite basis spectra used in the LCModel analysis were derived by numerical simulation and included N-acetylaspartate (NAA), creatine plus phosphocreatine (t-Cr), myo-inositol, N-acetylaspartylglutamate, taurine, lactate, aspartate, glycine, alanine, gamma-aminobutyric acid, glycerophosphorylcholine plus phosphorylcholine (t-Cho), and Glu. We only report metabolite measures for Glu, NAA, t-Cr, and t-Cho in the MPFC (Figure III-3) which were within the reliable threshold for the % standard devition of the fit (< 15%). The segmentation data were used to scale the water data, used for quantification, for established differences in the water content of gray matter (GM) and white matter (WM). In addition, these data allowed us to eliminate the cerebrospinal fluid (CSF) water volume that contributes to the total water signal, so that the quantified metabolite concentrations relate to the tissue space of the GM and WM. The water peak area was utilized as the denominator in concentration calculations after removing the non-brain signal contribution from CSF.

A full description of the methods used to obtain ^{1}H-MRS data used in this manuscript can be found in a recent publication by our group McEwen et al. (2012).

III.2.3 Statistical Analysis

Statistical analysis was performed using SPSS statistics. A two-tailed t-test was used for independent sample analysis of the differences between groups. ANCOVA analysis was used to adjust for tissue composition changes in relation

to water-quantified metabolite changes. Age, %GM and %CSF were treated as covariates in this analysis. Statistical significance was defined to be $p \leq 0.05$. All results are reported as means and standard deviation.

III.3 Results

Two-tailed, unpaired t-test results showed a significant difference between pHCs and FPHCs in water quantified Glu and t-Cho levels and in %GM as well as %CSF. pHCs showed a significant decrease in both water quantified Glu and t-Cho compared to FPHCs (Table III-1) as well as a significant decrease in %GM and a significant increase in %CSF (Table III-1). A univariate ANOVA was performed to determine if significant changes in water quantified Glu were a result of significant changes in tissue composition and the results showed that the change in Glu levles was no longer statistically significant between groups when adjusting for %GM and %CSF (Table III-2). As t-Cho is not localized to a specific tissue composition univariate ANOVA analysis was not performed on this metabolite.

The average age of participants was 28.83±5.20. Although relatively similar between groups, age was found to be significantly different between pHCs and FPHCs (30.24±4.35, 26.71±5.80, p=0.048). Upon univariate ANOVA analysis age was shown not to be a contributing factor to changes observed in water quantified Glu (Table III-2).

III.4 Discussion

The present ^{1}H-MRS study reveals a decrease in MPFC Glu levels and t-Cho levels during pregnancy. This investigation also shows that pregnancy is associated with dramatic changes in tissue composition in the MPFC during pregnancy. After correction for tissue composition changes MPFC Glu levels were no longer significantly decreased in women during pregnancy.

We found a significant decrease in MPFC %GM and a significant increase in MPFC %CSF in pregnant women compared to non-pregnant women. There have been no previous investigations of changes in tissue composition in the brain of pregnant women. However, a MRI study by Oatridge et al. (2002) comparing healthy pregnant women to those with preeclampsia suggested a decrease in overall brain size and an increase in ventricular size in healthy pregnant women during pregnancy, with a return to pre-pregnant levels by 6 months postpartum. These results obtained for the whole brain are consistent with our results of alterations of tissue composition in the MPFC.

The underlying cause of the increase in % CSF may be a result of increases in body circulatory changes and extracellular fluid volume associated with placental and hormonal activity (Royek et al., 1999, Oatridge et al., 2002). Pregnancy has been described as a state of altered water homeostasis facilitated by the movement of water at the blood-brain interface and between the brain and CSF (Amiry-Moghaddam et al., 2003). Aquaporins (AQP) are a family of channel-forming transmembrane proteins which facilitate the movement of water

and other solutes across the plasma membrane of cells. The gene expression of AQP4, the most predominant AQP in the brain is has been shown to be significantly increased in the rat brain during pregnancy (Quick & Cipolla, 2005). Whether such a phenomenon occurs in the brain of pregnant women remains to be determined.

Our investigation is the first investigation of % GM in the brain of pregnant women. There is however, one prospective investigation (Kim et al, 2010) that has measured brain tissue composition prospectively at two time points: 2-4 weeks postpartum and 3-4 months postpartum. This investigation showed an increase in %GM from 2-4 weeks postpartum to 3-4 months postpartum in the prefrontal cortex, parietal lobes, and midbrain areas. Based on our results, we may speculate that the decrease in %GM in the MPFC already takes place during pregnancy and normalizes progressively during the postpartum (Oatridge et al.'s morphologic data suggest a normalization at 6 months). Although remaining to be demonstrated, Kim et al.'s results combined with our results suggest that various brain areas including the MPFC undergo a decrease in % GM during pregnancy. The evolutionary advantage of these changes in tissue composition remains to be determined.

In addition, %GM has been shown to be decreased in the MPFC of patients suffering from MD. A study by Frodl et al. showed a significant decrease in GM in the right dorsomedial prefrontal cortex in patients suffering from MD compared to healthy controls, over a 3 year period. This was even more pronounced in patients who had not remitted compared to those who had (Frodl et

al., 2008). Furthermore, a study by Amico et al. (2011) showed decreased GM in the dorsolateral prefrontal cortex in patients with a family history of MD, compared to those without a family history of MD, suggesting decreased GM as a risk factor for developing depressive symptoms. The decreased GM observed in healthy pregnant women in our investigation, may thus also reflect vulnerability towards depression during pregnancy (and by extension during the postpartum).

As Glu is mainly found in GM, it was necessary to control for %GM in the MPFC voxel. The results of the univariate ANOVA analysis showed the statistically significant difference in Glu was lost when tissue composition was taken into consideration. The logical interpretation is that MPFC Glu levels are fluctuating during pregnancy due to changes in %GM and Glu levels are potentially unchanged in healthy pregnant women.

Hormone fluctuations are thought to play a key role in the development of depressive symptoms during pregnancy and the postpartum period. It is possible that hormone fluctuations are contributing to the differences observed in tissue composition between pHCs and FPHCs. Menopause is also a time in the female reproductive cycle when hormone fluctuations are occurring and the risk of developing depressive symptoms are high. Although direct comparison between menopausal women and women during pregnancy cannot be made, research has shown that estrogen hormone replacement therapy during menopause can alter GM tissue composition (Robertson et al., 2009).

A significant decrease in t-Cho was also observed in this study. The decreases seen in water quantified t-Cho in pregnant women compared to non-pregnant controls may be a result of the bodies attempt to support fetus development during pregnancy. An MRS study comparing pregnant women with and without preeclampsia to non-pregnant women also found a general decrease in t-Cho levels in pregnant women compared to non-pregnant controls (Rutherford et al., 2003). During early pregnancy choline is required for the growth of the placenta and choline demands continue to rise across pregnancy with the greatest choline demands occurring during the third trimester, necessary for fetal organ growth and more specifically membrane biosynthesis (Caudill, 2010). The increased choline consumption by the fetus, essential to its development, may potentially be a reason for the observed decrease in t-Cho levels observed in the MPFC of pregnant women.

To the best of our knowledge there is only one additional ^{1}H-MRS study investigating brain neurochemical changes during pregnancy; however, in relation to pre-eclampsia. NAA, t-Cho, t-Cr and lactate were measured on a 1.5T magnet, finding a significant decrease in t-Cho levels in pregnant women compared to non-pregnant controls (Rutherford et al., 2003). Glu was not measured during this study making it difficult to make comparisons to our current Glu findings, in addition changes in tissue composition where not taken into consideration.

In the past, it has been difficult to collect data on pregnant women as there has been a lot of controversy surrounding MRI and ^{1}H-MRS during pregnancy and the safety associated with this method of data collection. Recently, these

concerns have subsided and studies have shown MRI to be a safe method of data collection for pregnant women, especially during the third trimester when the fetus is fully developed (Clements et al., 2000; Heerchap et al., 2003; Kok et al., 2004; Kreis et al., 2002; Girard et al., 2006). Accordingly, babies born in women who underwent ^{1}H-MRS during pregnancy in our investigation did not present any postnatal problems.

A number of MRS investigations use metabolite concentrations referenced to another metabolite such as t-Cho (Jansen et al., 2006); however, the use of a reference metabolite, as seen with the fluctuations of t-Cho in this investigation, may be problematic, resulting in apparent fluctuations in target metabolites as levels of the reference molecule itself may be increasing and decreasing. One of the strengths of this investigation was using water as the reference metabolite, resulting in water quantified target metabolites.

Our study is the first systematic investigation of changes in brain metabolites and tissue composition during pregnancy. Our findings of decreased %GM, during pregnancy, may contribute to a better understanding of the impact of pregnancy on depression and the risk associated with developing PPD. Additional work is needed to better understand the potential role of female hormones on Glu during pregnancy. Our findings of alterations in GM and CSF content suggest that refined MRI investigations are necessary to fully assess the change in brain structure occurring during pregnancy.

Table III-1. *Water-referenced metabolite concentrations and tissue composition in pHCs and FPHCs.*

	pHC (n=21)		FPHC (n=14)		Group	
	Mean	SD	Mean	SD	*P*	t (df=33)
Metabolite						
Glu	6.74	1.39	8.53	1.55	0.001*	3.58
NAA	8.99	1.44	10.00	1.48	0.052	2.02
t-Cr	8.65	3.42	10.09	2.88	0.203	1.30
t-Cho	1.37	0.24	1.83	0.42	0.0002*	4.11
%GM	46.53	10.66	60.43	6.98	0.0001*	4.29
%WM	30.94	10.14	26.60	4.57	0.144	1.50
%CSF	22.54	10.24	12.96	6.10	0.004*	3.14

Abbreviations: pHC = pregnant healthy control, FPHC = follicular phase healthy control, Glu = glutamate, NAA= N-Acetylaspartate, t-Cr = creatine plus phosphocreatine, t-Cho = glycerophosphorylcholine plus phosphorylcholine, GM= grey matter, WM = white matter, CSF = cerebrospinal fluid.

Brain metabolites are measured in institutional units

*** A significant difference indicated between groups**

Table III-2. *Comparisons of water-referenced MPFC Glu concentrations in pHCs and FPHCs*

	Mean ± SD of Glu		***p-value* ***
	pHC	FPHC	
	6.74 ± 1.39	8.53 ± 1.55	<0.001
Covariates			
Age	6.70 ± 1.51	8.59 ± 1.53	<0.001
GM Only	7.06 ± 1.51	8.04 ± 1.58	0.106
GM and CSF	7.09 ± 1.52	8.01 ± 1.59	0.131

Abbreviations: pHC = pregnant healthy control, FPHC = follicular phase healthy control, Glu = glutamate, GM= grey matter, CSF = cerebrospinal fluid.

*** p-values are based on univariate ANOVA**

All values are reported as means ± standard deviation.

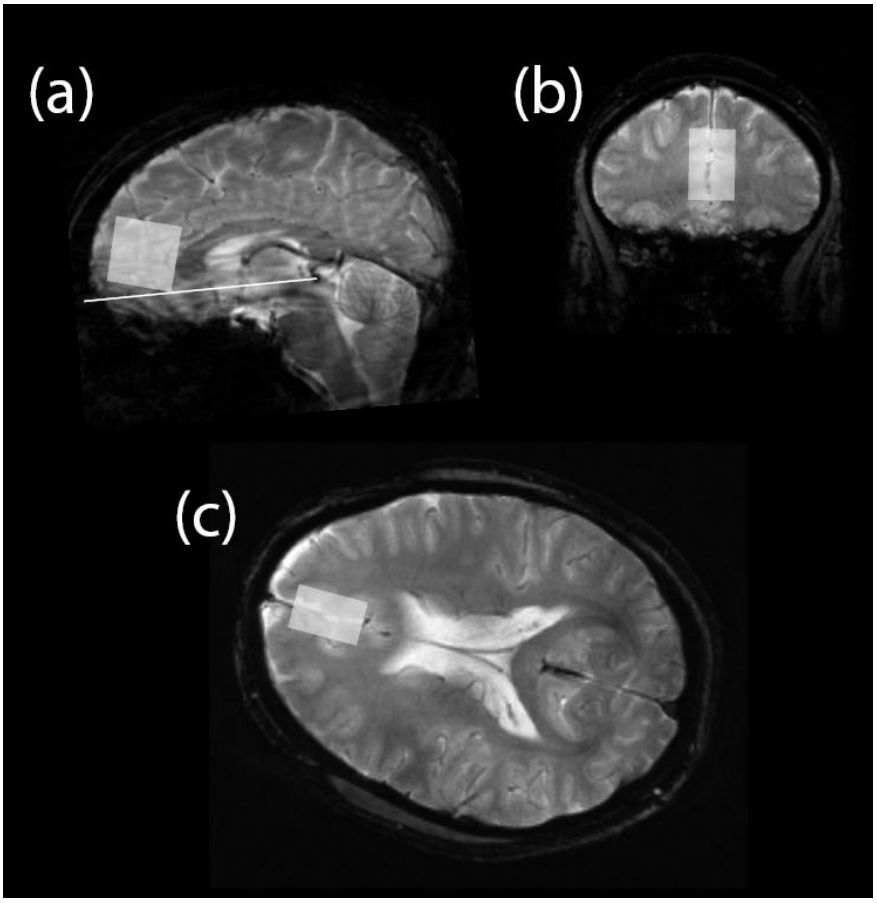

Figure III-1: Medial prefrontal cortex voxel shown in A) mid-sagittal section with the posterior inferior corner contacting the anterior commissure-posterior commissure line, B) coronal, and C) transverse views.

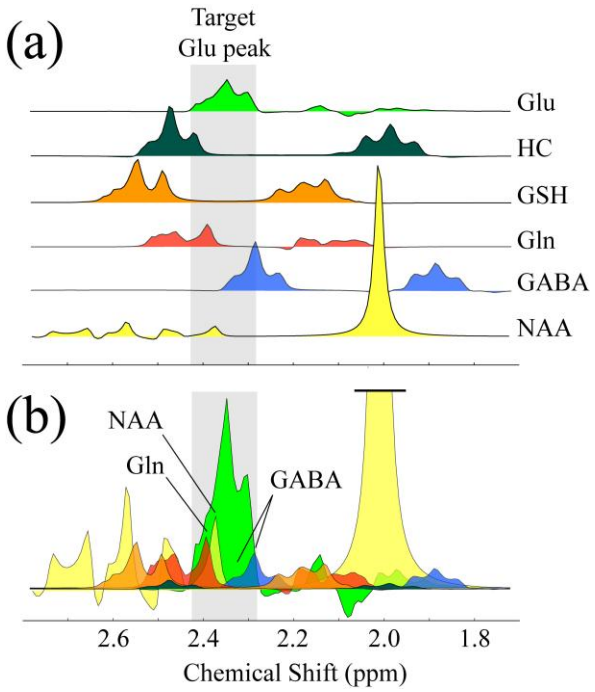

Figure III-2: (a) Simulated metabolite MR spectra for STEAM {TE, TM} = {240, 27ms} centered on the spectral region surrounding the Glu multiplet at ~2.35 ppm. Metabolites at equimolar concentrations include: Glutamate (Glu), glutamine (Gln), glutathione (GSH), homocarnosine (HC), γ-aminobutyric acid (GABA), N-acetylaspartate (NAA).

(b) Scaled metabolite spectra based on typical literature concentrations relative to Glu at 100%, NAA (120%), GABA (15%), HC (3%), GSH (20%), Gln (40%). No significant overlap with the target Glu signal arises from GSH or HC. Since the NAA-aspartate signal amplitude is well characterized by its singlet, its overlap as a contaminating signal can be readily accounted for during LCModel analysis. Within the Glu target band only Gln (Gln peak / Glu peak ~ 8%) and GABA (~7%) contamination will have a minor impact on quantification under these optimized STEAM acquisition timings.

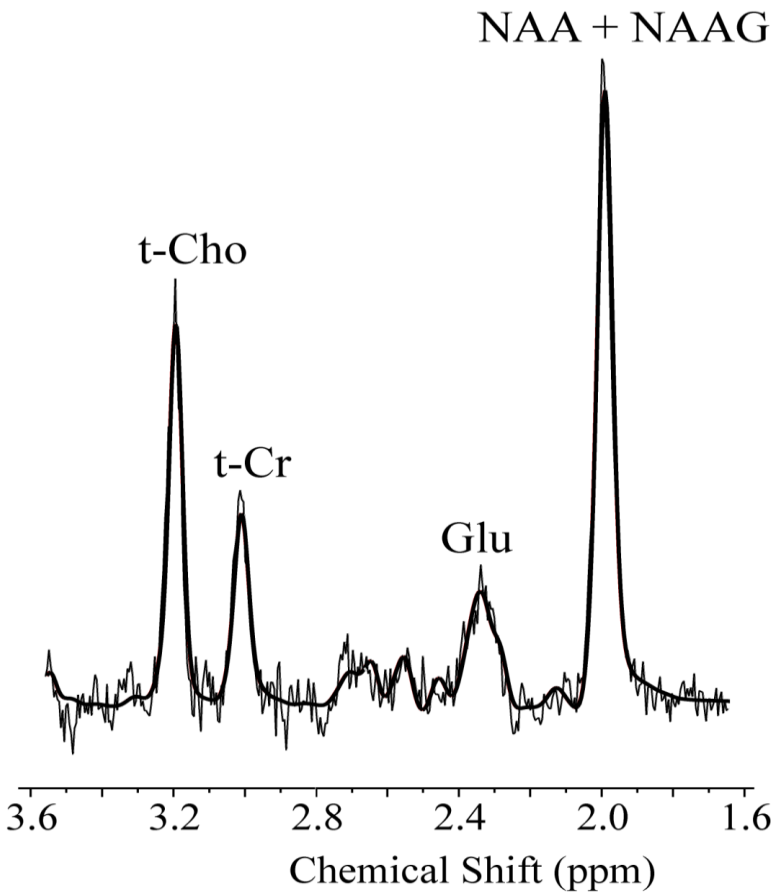

Figure III-3: Sample STEAM localized MRS data acquired from the medial prefrontal cortex and with sequence timings optimized for recovering signal from glutamate (STEAM TE, TM = 240, 27 ms). The spectra illustrates the unfiltered data superimposed with the LCModel fit.

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IV. Conclusion

Minimal information is available about the cerebral changes that occur during pregnancy and the early postpartum period when women are at an increased risk for developing depressive symptoms. Gaining more insight into these cerebral changes in healthy women is essential to understanding the pathophysiology of PPD.

We have shown that MPFC Glu levels are increased in PPD women (McEwen et al., 2012), a result which contradicts previous findings in MD patients. Whether the role of MPFC Glu in the pathophysiology of PPD and MD truly differ or not remains to be determined. It is conceivable that the dysregulation of MPFC Glu levels in depression differ based on whether they are measured early after the onset of depressive symptoms (as in our PPD patients) or after a more chronic course (as in most MRS investigations of MD patients).

We have now demonstrated that MPFC Glu levels are decreased in women at risk for developing PPD, including women with a history of mood sensitivity to fluctuations of female hormones, compared to healthy controls during late pregnancy and the early postpartum. Considering that MPFC Glu levels in high risk women return to the levels of healthy controls during the early postpartum, when the risk of developing PPD is the highest, and none of the high risk women ultimately went on to develop PPD, it is logical to consider that these MPFC Glu alterations may represent a homeostatic compensatory mechanism aimed at avoiding the development of PPD symptomatology. This would be explained as a

response to the dramatic female hormone fluctuations occurring during pregnancy and the postpartum. Interestingly, the chronological return to healthy control levels of MPFC Glu coincides with the return to normal concentrations of female hormones and associated NASs during the postpartum period (Bloch et al., 2003). This major influence of female hormones on MPFC Glu levels is further supported by our previous findings of fluctuations of MPFC Glu levels in association with the fluctuations of female hormones and NASs during the menstrual cycle (Batra et al., 2008). However, further research is required to determine the exact effect of female hormones and NASs on the glutamatergic system and the role both play in developing depressive symptoms during pregnancy and the postpartum.

Whatever the interpretation of our results, the combinations of these studies seem to indicate a major role of MPFC Glu levels in the development of PPD.

Pregnancy has long been a contra-indication for MRI investigations. However, there has been an accumulation of evidence indicating that ^{1}H-MRS and MRI are safe during pregnancy, especially during the third trimester when the fetus is fully developed (Clements et al., 2000; Girard et al., 2006). As a result, our investigation is the first investigation of brain Glu levels in pregnant women. Interestingly, although MPFC Glu levels appear to be decreased in healthy control pregnant women compared to healthy controls in the follicular phase of the menstrual cycle, this difference disappeared after control for tissue segmentation,

more specifically for %GM composition. This suggests that there are no real changes in MPFC Glu levels in healthy non-at-risk pregnant women.

This decrease in %GM in pregnant women compared to healthy controls is consistent with the results of our prospective study which indicates that %GM is decreased during pregnancy and the early postpartum and progressively increases as the postpartum progresses (both in healthy controls and in women at risk of developing PPD). Other brain volumetric studies are consistent with our results and suggest a progressive return to pre-pregnancy levels of %GM after a few months postpartum (Oatridge et al., 2002, Kim et al., 2010). This suggests that changes in %GM are brought on by pregnancy which normalizes as the postpartum progresses. Common cognitive complaints fondly referred to as "baby brain" may be a result of the changes in %GM observed during pregnancy and the postpartum period.

%CSF was also shown to be significantly increased in pregnant women compared to non-pregnant controls; however, no significant differences were observed over time across the postpartum period between high risk women and healthy controls. This implies that the alteration of %CSF is pregnancy-related, potentially due to increased body circulatory changes, and is supported by a study that found increased ventricular size in pregnant women (Oatridge et al., 2002). From a methodological point of view, the combination of our results for %GM and %CSF suggest that future MRS investigations of brain metabolites should control for tissue composition.

Our research stresses the importance of closely looking at tissue composition in any investigations of the brain during pregnancy and the postpartum period. Our research also provides strong evidence for the role of the glutamatergic system in the development of mood disorders during pregnancy and the postpartum. As future investigations are undertaken and the pathophysiology of PPD is better understood, the development of specific prophylactic or therapeutic approaches will become increasingly feasible, benefiting women who may be at an increased risk of developing postpartum depression based on mood sensitivities to fluctuations in female hormones.

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V. Appendix

INCREASED GLUTAMATE LEVELS IN THE MEDIAL PREFRONTAL CORTEX IN PATIENTS WITH POSTPARTUM DEPRESSION

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ABSTRACT:

The medial prefrontal cortex (MPFC) is a key brain area in depressive symptomatology; specifically Glutamate (Glu) has been reported to play a significant role in major depression (MD) in this area. MPFC Glu levels may be sensitive to ovarian hormone fluctuations, with pregnancy and the postpartum period being associated with the most substantial physiological alteration of female hormones. It is therefore logical to measure MPFC Glu levels in women with postpartum depression (PPD). Using in vivo magnetic resonance spectroscopy (MRS) at a field strength of 3 Tesla, we acquired single-voxel spectra from the MPFC of 12 women with PPD and 12 healthy controls (HCs) matched for postpartum scan timing. Water-referenced MPFC Glu levels were measured using a specific MRS technique that allowed us to be specific for Glu with very little glutamine contamination. The concentrations of other water-quantified brain metabolites such as glycerophosphorylcholine plus phosphorylcholine (t-Cho), N-acetylaspartate (NAA) and creatine plus phosphocreatine (t-Cr) were measured in the same MR spectra. MPFC Glu levels were higher in women with PPD [7.21 ±1.20] compared to matched HCs [6.04±1.21] ($p<0.05$). There were no differences between groups for other brain metabolites measured. These findings suggest an association between Glu dysregulation in the MPFC and PPD. Whether the pathophysiology of PPD differs from the pathophysiology of MD remains to be determined. Further investigations are needed to determine the chronological associations between the occurrence of symptoms of PPD and the onset of changes in MPFC Glu levels.

Keywords: Glutamate; postpartum; depression; magnetic resonance spectroscopy; medial prefrontal cortex; women

Introduction

Postpartum depression (PPD) is a common mood disorder that occurs in up to 20% of women after childbirth (Gaynes et al., 2005). According to the DSM-IV-TR (American Psychiatric Association, 2000), PPD is limited to a diagnosis of major depression (MD) with an onset within 4 weeks of birth. However, based on epidemiological data, it has been recommended by many researchers in the field that 3 months postpartum is a more suitable timeframe for defining postpartum onset of depression (Elliot, 2003; Wisner et al., 2006). Women who suffer from PPD experience feelings of inadequacy and hopelessness, which can often persist from months to years after childbirth. The damaging role that PPD plays in the mother-infant relationship may result in suboptimal cognitive and emotional development in the child which can subsequently increase the risk of depression in the child into adolescence (Ramchandani et al, 2005; Murray and Cooper, 2003; Goodman et al., 1993; Downey and Coyne, 1990; Rahman et al., 2004; Sinclair and Murray, 1998; Moehler et al., 2007, Murray et al., 2011). Although psychosocial aspects of PPD are well researched, there is a dearth of biological investigations into the pathophysiology of this disorder. As a result it is not clear whether the pathophysiology of PPD differs from the pathophysiology of MD.

In vivo magnetic resonance spectroscopy (MRS) is the only noninvasive technique that can directly assess levels of neurochemicals, such as the excitatory neurotransmitter glutamate (Glu), in localized brain regions (Stanley, 2002; Soares et al., 1996). The ability to selectively measure Glu by MRS is confounded

by overlapping resonances with glutamine (Gln) due to a similarity in the chemical structures of the two.

MRS investigations have suggested dysregulation of glutamix (a combination of Glu and Gln) in patients with MD (Yuksel and Ongur, 2010). Although the glutamatergic hypothesis of depressive symptomatology has been prominent for a period of time, the recent clinical findings of the rapid onset of antidepressant activity of the NMDA antagonist ketamine (Berman et al., 2000, Zarate et al., 2006) have established the glutamatergic hypothesis of depression as one of the most promising avenues of research.

Various neuroimaging investigations have suggested that the MPFC is a key brain area for depressive symptomatology (Price and Drevets, 2010). Recent animal studies (Li et al., 2010; Li et al., 2011, Autry et al., 2011) suggest that the rapid antidepressant response to ketamine is related to its synaptogenesis activity in the MPFC. Furthermore, data indicate that MPFC Glu levels are influenced by ovarian hormone fluctuations (Batra et al., 2008). Of note, pregnancy and the postpartum period are associated with the most substantial physiological alteration of female hormones (Buckwalter et al., 1999, Bloch et al., 2003). As PPD is defined as an episode of MD occurring during the postpartum phase, it stands to reason that glutamatergic dysregulation in the MPFC may play a key role in depressive symptomatology observed in PPD.

Our objective in this report was to examine whether Glu levels in the MPFC are decreased or increased in women with PPD compared to healthy women.

Methods

Subjects:

Twelve women suffering from PPD (only 1 suffering from PPD as defined by the DSM-IV-TR, presenting with symptoms within the first 4 weeks postpartum and the other 11 suffering from PPD with onset of symptoms in the more commonly accepted time frame of within the first 3 months postpartum) were matched with twelve healthy control women (HCs) based on postpartum scan time. Women were brought in for the MRS scan as soon as possible after first contact was made. One PPD woman and her matched HC were scanned at 3 weeks postpartum, two PPD women and their matched controls were scanned at 5 weeks postpartum, one PPD woman and her matched HC were scanned at 7 weeks postpartum, two PPD women and their matched HCs were scanned at 9 weeks postpartum and six PPD women and their matched HCs were scanned at 3 months postpartum. All participants were recruited from advertisements and through collaborations with health institutions in Edmonton, Canada. All subjects were compensated for their time. Each woman was recruited according to the guidelines of the Health Research Ethics Board of the University of Alberta.

After a complete description of the study was provided to the subjects, written informed consent was obtained.

Eligible women were not taking any psychotropic drugs or herbal products with psychotropic activity 3 months prior to entering the study or at any time during the study. Participants were excluded if they had used any street or recreational drugs in the previous 6 months or during the study; or if they used any form of hormonal contraception. A pregnancy test ensured that women were not pregnant. Other factors that excluded participants included potential confounding factors such as brain injury or classical contraindications to magnetic resonance imaging.

All participants were administered the Structured Clinical Interview for DSM-IV-TR of Axis I disorders to screen for any current or lifetime Axis I psychiatric disorders. Specific exclusion criteria for HCs included any current or past Axis I psychiatric illness. PPD women had to meet diagnostic criteria for MD with an onset of symptoms within the first 3 months postpartum. The Edinburgh Postpartum Depression Scale (EPDS) and the Beck Depression Inventory (BDI) were administered to all participants in order to screen for PPD. MRS sessions for both PPD patients and HCs were scheduled during the follicular phase of the menstrual cycle (with the exception of women who were scanned early in the postpartum at a time when menstruation has not yet resumed).

MRS:

MRS was performed in the Peter S Allen MR Research Centre, University of Alberta, Edmonton, Canada, using a stimulated echo acquisition mode (STEAM) sequence (Frahm et al., 1989) and a 3 T magnet (Magnex Scientific, Concord, CA) equipped with a spectrometer (Surrey Medical Imaging System, Surrey, UK) and a quadrature birdcage resonator. A $2 \times 3 \times 3\ cm^3$ voxel (for segmentation and spectroscopy) was positioned such that the 2cm dimension was perpendicular to, and centered on, the midline. The center sagittal slice was subsequently used to first register the voxel such that the posterior edge touched the rostrum of the corpus callosum in the mid-sagittal plane and inferior edge lay along the anterior commissure– posterior commissure (AC–PC) line. The voxel was then rotated until the corners of the anterior edge were equidistant from the brain surface, while maintaining one corner contacting the AC–PC line, and an edge contacting the corpus callosum (Figure 1).

Shimming to 0.05 p.p.m. was accomplished by using both FASTMAP (Gruetter, 1993) and an in-house autoshim routine. The optimal in vivo Glu and Gln contrast to background, determined using numerical simulation, used a TE equal to 240 ms, mixing time (TM) equal to 27 ms, and repetition time (TR) equal to 3 s (Thompson and Allen, 2001). The long TE time resulted in minimal macromolecule contamination due to their short T_2 relaxation time (Behar et al, 1994). Spectra were the sum of 512 averages, acquired in 16 blocks of 32 averages. This warranted each of the 16 subspectra to be analyzed for spectral artifacts because of subject movement or hardware fluctuations before their

final summing (Zhu et al, 1992). Where necessary, re- registering of each of the 16 subspectra to the same frequency reference before summing was allowed. The in vivo data were analyzed using the LCModel (version 6.0-1) analysis program (Provencher, 1993). The metabolite basis spectra used in the LCModel analysis were derived by numerical simulation and included N-acetylaspartate (NAA), creatine plus phosphocreatine (t-Cr), myo-inositol, N-acetylaspartylglutamate, taurine, lactate, aspartate, glycine, alanine, γ-aminobutyric acid, glycerophosphorylcholine plus phosphorylcholine (t-Cho), and Glu. This analysis gave reliable measures of Glu, NAA, t-Cho, and t-Cr in the MPFC, with Cramer–Rao Lower Bound of the fit for Glu $<13\pm4\%$, for NAA $<3\pm1\%$, for t-Cr $<9\pm3\%$, and for t-Cho $<5\pm1\%$ in PPD patients. We only report for Glu, NAA, t-Cr, and t-Cho. Glu measures from LCModel analysis typically had a standard deviation of the fit o20% and were therefore deemed reliable. Selection of the target Glu signal at 2.35 p.p.m. was optimized using numerical simulation by minimizing contamination from overlapping signals (Figure 2), by assessing TE and TM space of the simulated response of Glu, Gln, NAA, GABA, glutathione (GSH), and homocarnosine spin systems to find the optimal Glu signal-to-background(TE,TM = 240,27ms). The reduced SNR at this longer TE required us to select a relatively large $2x3x3cm^3$ volume, and signal average for ~26 min (512 averages, TR = 3 s). Moreover, placement across the midline to maximize the Glu-rich GM was critical to yield a good Glu signal. Under these optimal timing conditions, contamination from other metabolites was Gln 8%, NAA 11%, and GABA 7%. Signals with minimal

contamination include GSH and homocarnosine. A representative spectrum used for LCModel analysis is shown in Figure 3.

Segmentation of the frontal brain region was performed using a double-inversion recovery 1-D projection method (Hanstock and Allen, 2000). The segmentation data were used to scale the water data, used for quantification, for well-characterized values of water content in gray matter (GM) 80%, and white matter (WM) 65%. In addition, these data allowed us to eliminate the CSF water volume that contributes to the total water signal, so that the quantified metabolite concentrations relate to the tissue space of the GM and WM. All computations necessary for calculating experimental timings before acquisition and for data analysis were performed using the MATLAB program environment.

The water data for quantification were acquired at several TE values (TE = 20, 40, 60, 80, 100, 150, 200, 250, 300, 350, 400, 450, 500, 700, 900, 110, 1300, 1500 ms; TR=12000 ms; 2 averages per TE value). Using customized processing routines in MATLAB, the water data were imported, and filtered, Fourier-transformed, and phase and baseline corrected. The water peak area from each spectrum in this TE series was determined and these area data were fitted to a multi-exponential using a non-negative least-squares algorithm. This fitting yielded both the T_2 relaxation time coefficients contributing to the decay and their relative proportions. In addition, it permitted an estimation of the water peak area at a theoretical TE of 0ms.

Metabolite quantification

Three series of data were used for quantification which were acquired from the same selected voxel: Metabolite peak area estimates are extracted from the LCModel output; segmentation information for GM, WM, and cerebrospinal fluid (CSF) compartment sizes are used to estimate water concentration in the selected brain voxel; and internal water data acquired at different TE values are used as the reference MR signal standard.

Metabolite and water MR signals (S_{metab} and S_{water} respectively) and concentrations (C_{metab} and C_{water} respectively) are related by the simple expression:

$$S_{water} / C_{water} = S_{metab} / C_{metab}$$

After rearranging the simple expression, the metabolite concentration can be readily calculated.

$$C_{metab} = (C_{water} \times S_{metab}) / S_{water}$$

The term C_{water} is calculated by accounting for the GM:WM compartments:

$$C_{water} = \text{Pure Water Conc} \times [(GM_{segment} \times GM_{water}) + (WM_{segment} \times WM_{water})]$$

where:

$$GM_{water} = 0.8 \times \text{Pure Water Conc}$$

WM_{water}= 0.65 x Pure Water Conc

Pure Water Conc = 1000 / MW_{water} = 55.56M

The term S_{water} is calculated from NNLS fitting of the water data acquired at multiple TE values and extrapolation of the brain water compartment to a theoretical TE = 0 ms.

The term S_{metab} can be used directly as measured ($S_{metabTE240}$) or can be scaled to account for the effects of metabolite T_2 and the different number of averages used for water suppressed and non-suppressed acquisitions.

$S_{metab} = S_{metabTE240} \times SF_{T2metab} \times SF_{av}$

Where for data acquired at TE = 240 ms, and using 512 or 2 averages (water suppressed or non-suppressed):

Scaling Factor number of averages = SF_{av} = sqrt 2 / sqrt 512,

Scaling Factor for metabolite T_2 = $SF_{T2metab}$ = exp (-240/T_{2metab})

Note that T_2 values for metabolites were assigned based on averaged literature values for NAA (350 ms), Cr (150 ms), and Cho (310 ms), and estimated for Glu (380ms) based on expected normal brain concentration values for the GM:WM mix sampled in our studies. Also note that the scaling factor accounting for metabolite T_2 is only to provide numerical values in the mM range, and that the same values are applied to all data. This allows comparison to reported data. One could equally well report the data in institutional units by using the measured metabolite signal $S_{metabTE240}$.

Analysis

A two-tailed t-test was used for independent sample analysis of variables between HCs and PPD women. Additional covariate analysis was also performed treating %GM as a covariate. Statistical significance was defined to be $p \leq 0.05$. An analysis of the relationship between the EPDS and BDI scores and neurochemical concentrations was conducted using the Pearson correlation coefficient.

Results

The mean age of the women with PPD was 28.67 ± 7.45 years and the mean age of the HCs was 29.08± 4.89 years (t=0.43, df=11, p=0.68). None of the PPD women had current co-morbid psychiatric disorders, but 5 out of the 12 PPD women had a history of MD. MPFC Glu levels were significantly increased in PPD women compared to matched HCs (see Table 1). In all cases MPFC Glu levels were higher in the PPD woman compared to her matched HC. There were no statistically significant differences between PPD women and matched HCs for the other MPFC metabolite levels (NAA, t-Cr and t-Cho) or tissue composition (GM, WM and CSF) (Table 1). Although GM was shown not to be significant in the MPFC voxel, a lower p value (p=0.10) lead us to perform a covariate analysis treating %GM as a covariate. When the covariate analysis was performed Glu remained marginally significant between the two groups (p=0.067) with elevated

Glu levels observed in PPD women. No significant differences were observed upon covariate analysis for the other metabolites, including NAA which is also mainly present in GM.

The Pearson correlation coefficient was used to assess the association between depressive symptoms (based on scores from the BDI or EPDS) and water-referenced neurochemicals, including Glu, in PPD women. There was no statistically significant correlation between water-referenced neurochemicals, including Glu, and scores on either EPDS or BDI in PPD women. BDI and EPDS scores were not available for two PPD women. Results of the analysis between EPDS and BDI scores with Glu were r= -0.281 (p=0.43) and r=-0.165 (p= 0.65), respectively; t-Cho results were r=-0.078 (p=0.83) and r=0.0.099 (p=0.79), respectively; NAA results were r=-0.106 (p=0.77) and r=-0.037 (p=0.92), respectively; and t-Cr results were r=-0.002 (p=0.99) and r=-0.074(p=0.84), respectively.

Discussion

This study suggests that Glu levels in the MPFC are increased in PPD women compared to HCs. To the best of our knowledge, no other study has investigated brain Glu levels in PPD. However, MRS investigations have been performed in patients with MD. Most MRS investigations of the MPFC in MD have measured Glx (Glu + Gln) and found decreased levels of Glx in the MPFC of patients suffering from MD. A MRS investigation performed by Hasler et al.

found that twenty unmedicated MD patients demonstrated a decrease in Glx concentrations in the MPFC when compared to controls (Hasler et al., 2007). Two other studies in the anterior cingulate cortex (ACC) support findings of decreased Glx in MD patients compared to healthy controls (Auer et al., 2000, Pfleiderer et al., 2003). However, without Glu and Gln levels being individually measured it is difficult to discuss these findings in relation to our results. Clear resolution of the Glu signal from Gln is not considered feasible at lower field strengths such as 1.5 Tesla. By increasing the field strength this ability is improved (Tkac et al., 2001); however, although it has been shown that shorter echo times (TE) can produce a reliable distinction of the Glu peak from Gln (Mullins et al., 2008), the best timing conditions need to have the PRESS sequence echo timings set to be asymmetric (Jutras et al., 2009). In addition, the use of an optimized STEAM sequence with a longer TE in our study enabled us to measure the Glu signal with minimal contamination from the Gln signal or macromolecules.

A study by Merkl et al. used a field strength of 3T (TE=80ms) to measure water-quantified Glu concentrations, with little contamination from the Gln signal, in the prefrontal cortex of MD patients. Merkl et al. found Glu to be reduced in the ACC in MD patients compared to healthy controls (Merkl et al., 2010). This study suggests that Glu levels in the MPFC are decreased in MD, which is opposite to our results of increased MPFC Glu levels in PPD women. In contrast to Merkl et al's results, in a post-mortem study Hashimoto et al. found elevated Glu levels in the frontal cortex of MD patient brains compared to healthy control brains (Hashimoto et al., 2007).

Both male and female participants were used in the previously mentioned studies, and although closely matched for age (Auer et al., 2000) and age and sex (Hasler et al., 2007, Pfleiderer et al., 2003, Merkl et al., 2010), phase of the menstrual cycle was not taken into consideration for female participants who were not menopausal, and may have altered the results. Indeed, our research team has shown that Glu levels are decreased during the luteal phase compared to the follicular phase of the menstrual cycle (Batra et al., 2008).

Pregnancy and the postpartum period are associated with fluctuations in female hormones (i.e. estrogen and progesterone) and associated neuroactive steroids (NASs) that have been hypothesized to be a contributing factor to the pathophysiology of PPD. Levels of estrogen, progesterone and NASs (such as pregnenolone and allopregnanolone) rise during pregnancy and return to pre-pregnancy levels in the postpartum (Bloch et al., 2003, Gilbert et al., 2005). Bloch et al. induced symptoms of depression in euthymic women with a history of PPD, but not in HCs, by exposing women to 8 weeks of hormonal treatment with both estrogen and progesterone followed by an immediate withdrawal period meant to mimic pregnancy and the postpartum (Bloch et al., 2000).

The link between Glu and female hormones in relation to depression in humans is poorly understood at this time. Our research group has demonstrated that MPFC Glu levels fluctuate in relation to the rise and fall of female hormones and associated NASs during the menstrual cycle (Batra et al., 2008). NASs as well as estrogen and progesterone are able to cross the blood-brain barrier, acting as neuromodulators by binding to neurotransmitter receptors and altering neuronal

excitability (Finocchi and Ferrari, 2011; Rupprecht, 2003). In animal studies, estrogen has been shown to increase the number of NMDA receptors on dendritic spines by means of increased mRNA production, as well as to increase NMDA-mediated glutamate receptor activity (Woolley and McEwen, 1993; Smith, 1989). Progesterone on the other hand, has been shown to have inhibitory effects, reducing the number of dendritic spines and excitatory synapses (Woolley and McEwen, 1993). Animal studies have also shown both that NASs can modulate glutamatergic neurons (Zamudio-Bulcock and Valenzuela, 2010) and that Glu impacts the production of NASs (Remage-Healey *et al.*, 2008). Considering the known interactions between female hormones, NASs and glutmatergic activity, it is therefore possible that the fluctuations of female hormones and NASs in the postpartum contribute to the increased MPFC Glu fluctuations observed in PPD women. It is important to note that our current findings apply to the follicular phase of the menstrual cycle, not the luteal phase (for those women who have resumed menses).

Only one MRS study has previously been performed in PPD women. That pilot study, which consisted of 9 PPD women, found that GABA levels in the occipital cortex did not differ between PPD women and HCs scanned within six months postpartum and who had not yet resumed menstruation (Epperson et al., 2006). Since Glu is the major excitatory neurotransmitter in the cortex and its action is counterbalanced by the inhibitory action of GABA, this study is remotely relevant to our investigation. However, considering the region-specific alterations of brain metabolites in MD (Hasler et al., 2007), an absence of GABA level

dysregulation in the MPFC of PPD women cannot be inferred from the data obtained from the occipital cortex.

We acknowledge the contingency that the increased MPFC Glu levels in PPD women may be correlated to past psychiatric disorders of these women and more particularly to MD rather than to the current PPD. However, this contingency is not supported by previous MRS studies which have suggested that alterations in Glx levels associated with MD resolve to levels that are similar to that of controls following clinical treatment (Yildiz-Yesiloglu and Ankerst, 2006). Furthermore, Taylor et al. (2009) compared levels of Glx in the ACC of unmedicated individuals with a history of MD to those of controls and found no significant differences between groups. Consistent with these findings, there were no differences in Glu levels in the 5 PPD women with a history of MD (7.17±1.47) relative to the 7 PPD women without a history of MD (7.25±1.01) ($t=-0.11$, $df=10$, $p=0.92$).

We did not find a correlation between the severity of depressive symptoms and MPFC Glu levels, and this is consistent with the results of other MRS investigations performed in MD patients (Hasler et al., 2007; Sanacora et al., 1999). Indeed, in those investigations, which demonstrated dysregulations of Glu levels in various brain regions of MD patients, no correlations were observed between the severity scores of various depression scales and the Glu levels (Sanacora et al., 2004). However, a greater sample size would have allowed for a more subtle analysis of the relationship between Glu levels and the severity of PPD symptoms, as has been done in the current study.

As priority was given to matching for postpartum scan time, women were not matched for breastfeeding. Coincidentally when breastfeeding was taken into consideration, there were only 3 out of 12 pairs that were unmatched for breastfeeding. Breast feeding status is therefore an unlikely confounding factor.

The relatively small sample size is a limitation to our study, replication with a larger sample size would allow for further analysis into the underlying pathophysiology of PPD. Although Glu concentration was our main variable, the MRS methodology chosen allowed for concomitant measurements of other metabolites and we reported on these. As a result of these multiple comparisons, a type I error cannot be excluded; however we do not have the sample size to allow for Bonferroni corrections. Furthermore, our sample is a mix of PPD women with early or late onset PPD, which has been defined as being either before or after 6 to 8 weeks from delivery, respectively (Dennis, 2004). Indeed, it has been suggested that the pathophysiology of early onset and late onset PPD may differ (Dennis, 2004). The heterogeneity of our PPD population is a commonly occurring issue in published PPD research (Stowe et al., 2005). In most published PPD studies, women with either a pregnancy onset, with an early onset or with a late onset are included and analyzed as a whole.

Referencing Glu to water has a significant advantage (compared to referencing to other metabolites) for the interpretation of MRS data. Indeed, we can ensure that the Glu level fluctuations that we observed are not related to the fluctuation of another brain metabolite used as an internal reference. While an advantage of MRS is the measurement of total tissue Glu, it does not differentiate

intracellular and extracellular glutamatergic activity (Valentine and Sanacora, 2009). Therefore, the precise involvement of the mechanism accountable for Glu dysregulation remains unclear. ^{13}C-MRS technology has the potential to be used for the measurement of the precise involvement of the glutamatergic system by combining intravenous infusion of ^{13}C-labeled glucose, or other precursors, with MRS (Valentine and Sanacora, 2009); this type of investigation would enable us to improve our overall understanding of the observed changes in Glu levels.

In conclusion, this is the first report investigating brain Glu levels in PPD women. Our findings of increased MPFC Glu levels have to be tempered by the fact that the difference between MPFC Glu levels in PPD women and HCs was only marginally statistically significant in the covariate analysis for GM content. Our results certainly contrast with findings of lower MPFC Glu levels in MD patients. Replication of our findings is necessary and future MRS investigations with sample sizes sufficient to control for PPD onset (early vs late) and including women suffering from MD as an additional control group, will help us refine our understanding of the role of Glu in the pathophysiology of PPD.

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Table 1. Water-referenced metabolite concentrations and tissue compositions in women with PPD and matched healthy subjects.

	PPD Patients (n=12)		Control subjects (n=12)		Group	
	Mean	SD	Mean	SD	*p*	t(df=11)
Metabolite						
Glu	7.21	1.20	6.04	1.21	0.02*	2.77
NAA	9.89	1.07	9.10	1.28	0.19	1.41
t-Cr	11.87	3.23	10.22	2.57	0.11	1.72
t-Cho	1.97	0.28	1.84	0.30	0.25	1.20
%GM	57.88	8.22	50.03	13.47	0.10	1.83
%WM	27.29	6.58	29.73	10.66	0.62	0.52
%CSF	14.83	11.67	20.25	9.48	0.27	1.17

PPD, Postpartum depression; HCs, healthy controls; GM, grey matter; WM, white matter; CSF, cerebrospinal fluid; Glu, glutamate; NAA, N-acetylaspartate; t-Cr, creatine plus phosphocreatine; t-Cho, glycerophosphorylcholine plus phosphorylcholine.

Brain metabolites are measured in institutional units.

*A significant difference indicated between groups

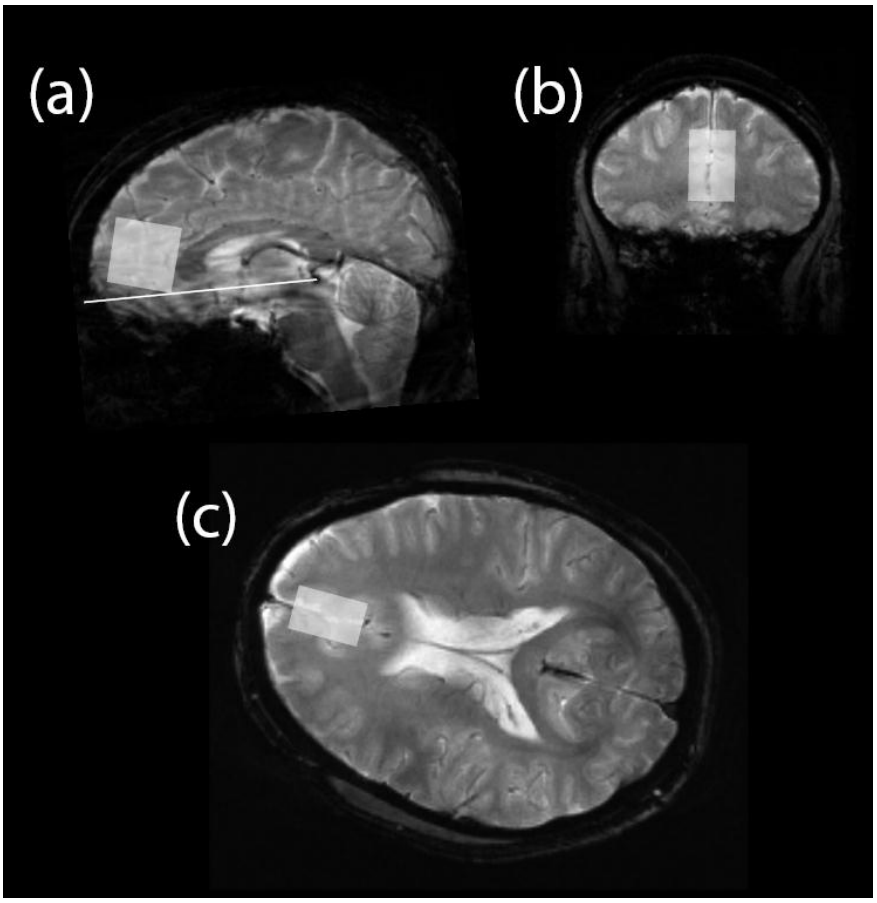

Figure 1: Medial prefrontal cortex voxel shown in A) mid-sagittal section with the posterior inferior corner contacting the anterior commissure-posterior commissure line, B) coronal, and C) transverse views.

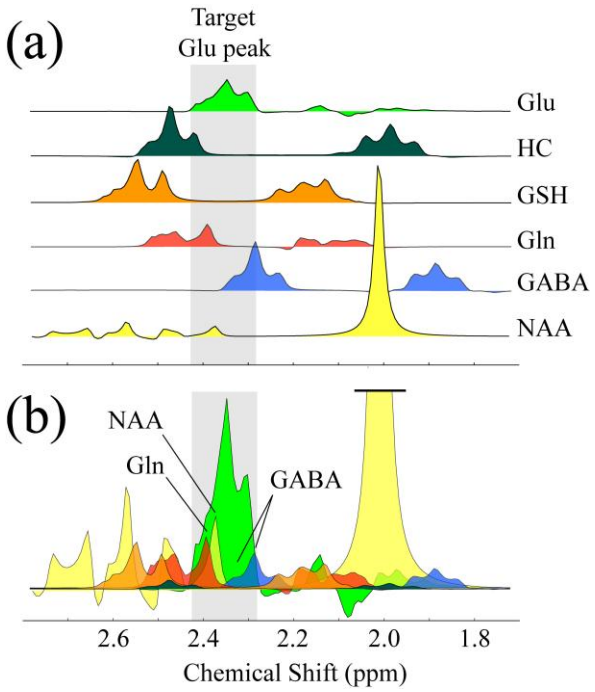

Figure 2: (a) Simulated metabolite MR spectra for STEAM {TE, TM} = {240, 27ms} centered on the spectral region surrounding the Glu multiplet at ~2.35 ppm. Metabolites at equimolar concentrations include: Glutamate (Glu), glutamine (Gln), glutathione (GSH), homocarnosine (HC), γ-aminobutyric acid (GABA), N-acetylaspartate (NAA).

(b) Scaled metabolite spectra based on typical literature concentrations relative to Glu at 100%, NAA (120%), GABA (15%), HC (3%), GSH (20%), Gln (40%). No significant overlap with the target Glu signal arises from GSH or HC. Since the NAA-aspartate signal amplitude is well characterized by its singlet, its overlap as a contaminating signal can be readily accounted for during LCModel analysis. Within the Glu target band only Gln (Gln peak / Glu peak ~ 8%) and GABA (~7%) contamination will have a minor impact on quantification under these optimized STEAM acquisition timings.

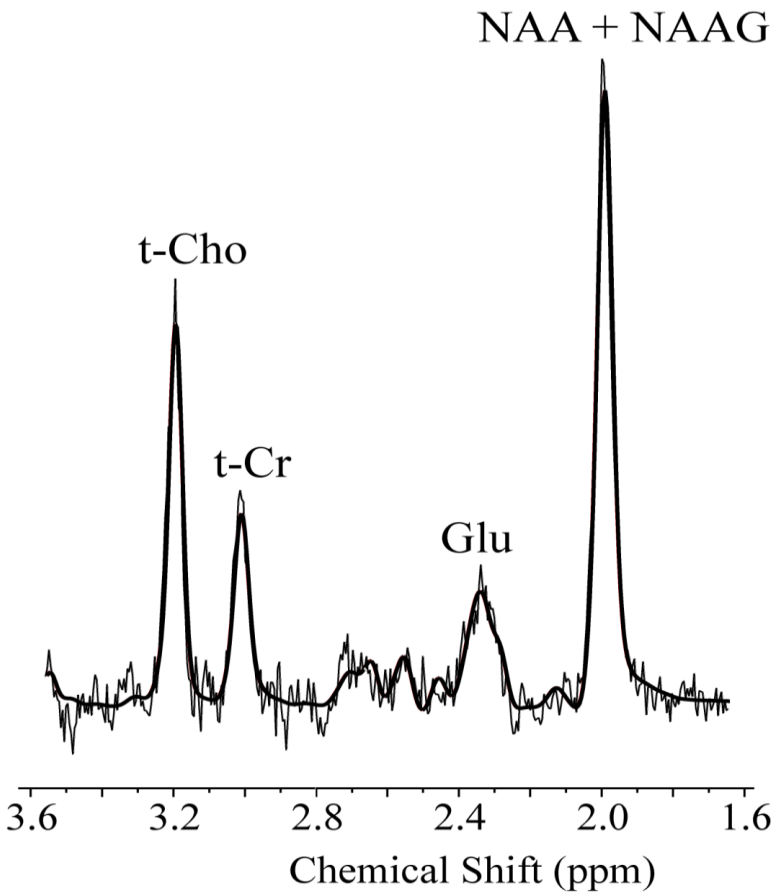

Figure 3: Sample STEAM localized MRS data acquired from the medial prefrontal cortex and with sequence timings optimized for recovering signal from glutamate (STEAM TE, TM = 240, 27 ms). The spectra illustrates the unfiltered data superimposed with the LCModel fit.