# University of Alberta

Cortisol Decreases Prefrontal Glutamine Concentrations

by

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# Dedication

This thesis is a product of immense support and encouragement. I would like to thank all who aided me in this research. In particular, I would like to thank Dr. Nick Coupland for guiding me with this challenging project, and for ensuring that I would have a tremendous appreciation for the intricacies and nuances of psychiatric research. Dr. Coupland has given me a true appreciation for the hard work and dedication it takes to be successful, and I am privileged to have been his student.

I would like to fully dedicate this thesis to my wife, Amanda Neelam Bhardwaj, and my two children, Justin Neeraj Bhardwaj and Jalen Paul Bhardwaj. The support they have provided has been immeasurable. Thank you, Amanda, for putting up with the late nights, being supportive when I have been stressed, and single-handedly keeping our household running while I was engrossed in my research. Thank you, Justin and Jalen, for your unconditional love and for always succeeding in putting a smile on my face. Without the three of you, I would not have had the opportunity to experience psychiatric research and realize my true calling in life.

# Abstract

In rodents, stress and corticosteroids rapidly increase excitatory neurotransmission. During excitatory neurotransmission, glutamate concentrations are maintained by conversion of glutamine to glutamate. The hypothesis was that cortisol would alter human prefrontal glutamine or glutamate concentrations.

Glutamine and glutamate were measured in prefrontal cortex (n = 12) using 3.0 Tesla proton magnetic resonance spectroscopy ($^{1}$H-MRS) before and after intravenous cortisol (hydrocortisone 35mg), in a randomized, double blind, placebo-controlled, within-subjects design.

Glutamine decreased following cortisol compared with placebo (session by time, $F_{(2,22)} = 5.51$; $p = 0.012$), whereas glutamate did not change ($F_{(4,44)} = 0.71$; $p = 0.59$).

Glutamine may be utilized to maintain glutamate concentrations during increased excitatory neurotransmission following cortisol. A limitation is that $^{1}$H-MRS does not measure metabolic flux rates directly. The effects of cortisol on glutamine could be a useful measure of altered central glucocorticoid responses in psychiatric disorders.

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# Chapter 1

# Introduction

It is widely understood that neurotransmitter dysfunction often plays a critical role in mental illness and mood disorders. Thus, it is important to determine which neurotransmitters play key roles in disorders and how their function might be regulated with medications. However, identifying which neurotransmitters are linked with illnesses is a challenge. Over the past several decades, there have been numerous pharmacotherapies developed to modify neurotransmission in psychiatric disorders, particularly monoamine neurotransmission involving dopamine, noradrenaline and serotonin. However, it is very difficult to determine whether there are primary abnormalities with these neurotransmitter systems within living patients. Recently, there has been increasing interest in the role of glutamate, the most abundant excitatory neurotransmitter in the human brain, in psychiatric disorders, but methods to measure central glutamate or its metabolism *in vivo* have not existed.

Recently, 3.0 T proton magnetic resonance spectroscopy ($^{1}$H-MRS) methods have developed to the point that brain glutamate and its precursor, glutamine, can be measured quite selectively (Choi et al., 2007). This novel technology is the basic tool that has permitted the research presented in this thesis.

Given the critical roles of glutamate in excitatory neurotransmission and synaptic and structural neuroplasticity, the roles of stress and alterations in glucocorticoid regulation in several psychiatric disorders and the impact of stress

and glucocorticoids on glutamate neurotransmission, it is conceivable that methods to determine how stress and/or glucocorticoids affect glutamate neurotransmission may be relevant to understanding the pathophysiology of some psychiatric disorders. In the present study, using *in vivo* $^{1}$H-MRS, we aimed to determine how acute elevation of cortisol affects levels of glutamate and its precursor, glutamine, in the anterior cingulate/medial prefrontal cortex of the living human brain. It was necessary to investigate this relationship within a healthy population prior to designing studies in a clinical population. Thus, the research presented is data from healthy participants.

# Chapter 2

# An Overview of Cortisol

Corticosteroids, produced in the adrenal cortex, are a specific class of steroids that have similar skeletal structures. There are two basic types of corticosteroid: mineralocorticoids and glucocorticoids. Mineralocorticoids (e.g. aldosterone) are critical for balancing electrolyte concentrations via effects on the kidney. Glucocorticoids play key roles in regulating inflammation, stress responses, energy and lipid and protein metabolism.

Cortisol (corticosterone in rats) is one of a class of steroids known as glucocorticoids, which all share a common sterol skeleton. Cortisol is produced naturally in the human body, but can also be synthesized for medicinal purposes, with the synthetic version of the hormone termed ‘cortisone’. The first known synthesis of cortisone occurred in 1946 by Louis Sarett, an industrial chemist, while the first known therapeutic application occurred in 1948 by rheumatologist Dr. Philip Hench (Hillier, 2007). Up until present day, cortisone is a widely prescribed therapeutic medicine.

In the human body, cortisol is synthesized from cholesterol and secreted from the cortex of the adrenal glands, with a clear diurnal variation in serum cortisol, as the highest levels are present early after waking and the lowest levels are present approximately three hours after sleep onset. Severe alterations in cortisol availability occur in Addison’s Disease (decreased) and Cushing’s

Disease (increased), with more subtle dysregulation in major depressive disorder (MDD) and Post-Traumatic Stress Disorder (PTSD) (Checkley, 1996).

## 2.1 Synthesis

Cortisol is synthesized in the human body through a complex series of metabolic reactions initialized by cholesterol. Although cortisol is the primary export of the adrenal cortex, other regions of the gland secrete different hormones. For example, the medulla of the adrenal gland, lying beneath the cortex, secretes catecholamines. The synthesis of glucocorticoids involves a number of intermediate products and by-products, summarized in Figure 1.

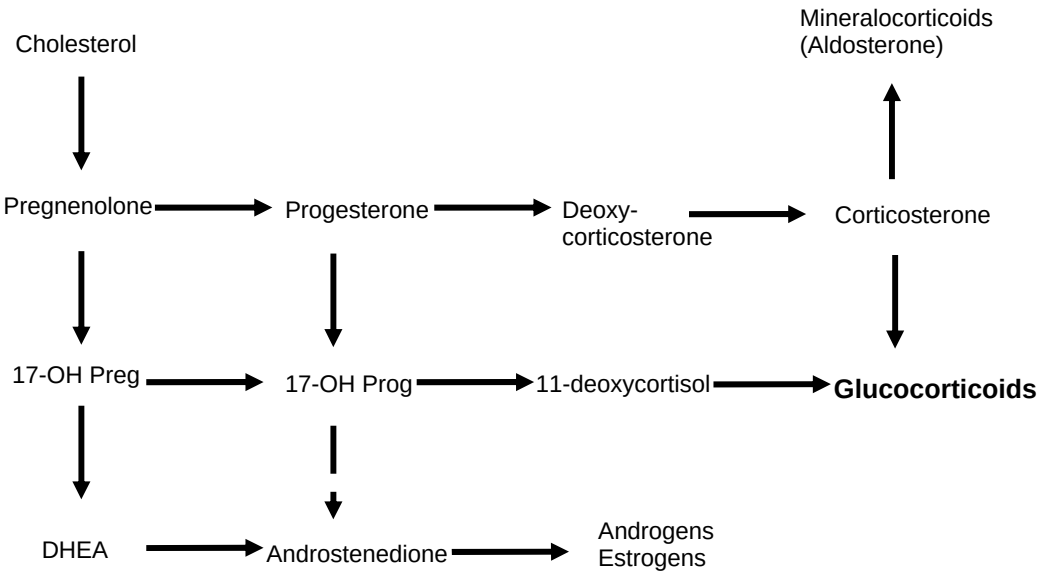


*Figure 1*: Glucocorticoid synthesis from cholesterol.

The reduction of cortisone from its inert state to its active state of cortisol involves the enzyme 11-beta hydroxysteroid dehydrogenase type I which activates the co-factor NADPH. Conversely, cortisol is converted back to cortisone by way of 11-beta hydroxysteroid dehydrogenase type II which activates the co-factor $NAD^+$ (Wan et al., 2009).

## 2.2 The Hypothalamic-Pituitary-Adrenal (HPA) Axis

The HPA Axis is a very complex self-regulatory mechanism in the human body. The intricacies and complexities of this system will not be discussed in detail. Instead, a brief overview of the mechanisms involved in the axis is provided.

An initial exogenous stressor leads to activation of the hypothalamus, which releases corticotropin releasing hormone (CRH). CRH is transported to the pituitary gland, where it stimulates release of adrenocorticotropic hormone (ACTH) into general circulation. In turn, ACTH acts on the adrenal, both to stimulate the release of cortisol acutely and to promote the growth of the adrenal cortex more chronically. In healthy humans, the increased cortisol would then have a negative feedback effect on this cascade of reactions, decreasing CRH and ACTH release and thereby regulating HPA activity.

In addition to actions at the levels of the hypothalamus and pituitary, actions of cortisol on glucocorticoid receptors in the amygdala, hippocampus and prefrontal cortex also play a role in regulating the HPA axis.

Feedback inhibition influences circulating cortisol concentrations and different mechanisms of feedback operate over different time intervals (Herman et al., 1989). For example, fast feedback operates over a period of several minutes and has been shown to involve a non-genomic effect of glucocorticoids on cannabinoid transmission in the hypothalamus (Di, Malcher-Lopes, Halmos & Tasker, 2003). However, chronic elevations of cortisol may act over hours. This 'delayed feedback' phenomenon has been assessed clinically using dexamethasone suppression, in which next day cortisol levels are measured after a night-time dose of dexamethasone (Miller et al., 1992). The dexamethasone test will be discussed further in Section 2.5.

## 2.3 Cortisol and Addison's Disease

Addison's disease, first described by Dr. Thomas Addison, involves chronic insufficient production of glucocorticoids from the adrenal glands, with symptoms that include headaches, diarrhea, mood and personality changes, vomiting, weight loss, and fatigue. Pathophysiological factors can include altered glucocorticoid synthesis, known as impaired steroidogenesis, adrenal dysgenesis, a congenital impairment of adrenal development, or adrenal destruction due to injury or infection. Another potential avenue for Addison's disease occurrence can be from sudden discontinuation of long-term corticosteroid replacement therapy, due to the lack of stimulation of adrenal growth and function in response to excessive negative feedback from exogenously produced glucocorticoid levels.

If it remains untreated, Addison's disease can potentially be fatal, but treatment is fairly straightforward via oral or injected glucocorticoid replacement.

## 2.4 Cortisol, Cushing's Syndrome and Cushing's Disease

Chronically elevated cortisol (hypercortisolism) is evident in Cushing's disease and Cushing's syndrome. However, there is a fundamental difference between the cause and severity of the two conditions. In Cushing's syndrome, elevated cortisol has a negative feedback effect on the pituitary, thereby decreasing the amount of ACTH released. This excessive cortisol release is often due to a cortisol-secreting adenoma in the adrenal gland. If an adenoma is implicated, it can be treated with surgery, or with medications that inhibit cortisol synthesis. In the event of surgery to remove the adenoma or the adrenal glands, corticosteroid replacement therapy must be undertaken for the duration of the individual's life.

Conversely, in Cushing's disease, the excess release of cortisol is propagated by increased ACTH release, most often due to a pituitary adenoma that leads directly to excessive ACTH production. In this case, the normal negative feedback loop fails to operate on the tumour tissue and adrenal production of cortisol is driven by high ACTH levels. Treatment for Cushing's disease is more complicated than Cushing's syndrome as it involves suppression or removal of the pituitary tumour, or, if necessary, the pituitary gland. Another course of treatment involves removal of the adrenal glands. In either case,

corticosteroid replacement therapy will, again, be necessary for the duration of the individual's life.

## 2.5 Cortisol and Depression

Major depressive disorder (MDD) is characterized by symptoms which include dysphoric mood, altered appetite, insomnia or hypersomnia, reduced motor activity or agitation, feelings of worthlessness, excessive guilt, reduced ability to think clearly or increased forgetfulness and hopelessness, or suicidal thoughts or ideation (Zimbardo, 1992). Although many people may experience some of these symptoms at some time, MDD is characterized by experiencing multiple symptoms persistently such that they lead to impaired daily functioning. The prevalence rates for MDD are high, with lifetime prevalence in the US estimated to be 17% (Kessler et al., 2003).

The diathesis-stress model of depression proposes that a genetic susceptibility (diathesis) is inherited and interacts with subsequent stressors that increase the risk of Major Depressive Episodes (MDEs) and trigger their onset. Early life stress may alter the perception of and physiological reactivity to stress, resulting in a hypersensitive response to stressors throughout life. Holsboer (2001) provided evidence for this model by demonstrating an abnormal functioning of the HPA axis in depressed people when coping with stresses. Many MDD patients synthesize more CRH while releasing more ACTH from the anterior pituitary, and ultimately release more glucocorticoids from the adrenal cortex (Pinel, 2003).

The link between MDD and alterations in cortisol secretion has been known for many years. Early research on this subject indicated that depressed patients have elevated levels of plasma cortisol (Gibbons, 1964). This has been corroborated by the dexamethasone suppression test (DST). Dexamethasone is a synthetic glucocorticoid that mimics the actions of cortisol in the HPA axis. Non-suppression of cortisol secretion following the administration of dexamethasone in the DST is an indicator of reduced negative feedback control that contributes to increased cortisol in MDD. The standard DST has subsequently been refined in the combined dexamethasone-CRH test (DEX-CRH test), in which reduced negative feedback is demonstrated as increased ACTH secretion when CRH is administered following dexamethasone. The DEX-CRH test increases sensitivity to reduced negative feedback to over 80% of MDD subjects compared with approximately 50% for the standard DST (Holsboer, 2001).

The link between elevated cortisol and depression is further evidenced by the increased rates of depression in patients with Cushing's syndrome (Checkley, 1996). In addition to evidence for reduced negative feedback in MDD, post-mortem and cerebrospinal fluid studies also provide evidence for CRH hypersecretion (Checkley, 1996). Checkley summarized three common elements in the HPA axis of MDD as being abnormal negative feedback, an increase in the central drive of the HPA axis, of which a rate-limiting step could be the expression of CRH, and adrenal gland hypertrophy.

## 2.6 Cortisol and PTSD

According to the Diagnostic and Statistical Manual of Mental Disorders-IV-TR (American Psychiatric Association, 2000), post-traumatic stress disorder (PTSD) is defined as a disorder resulting from very severe stressors that represent a threat of death or serious injury which can lead to a pattern of intrusive memories, intense emotional and physiological reactions to reminders of the stressful events. The ties between these reactions and memories of the incident can produce disruptive, stressful responses to stimuli that would previously have been innocuous. Simple acts or specific sensory experiences, either explicit or subconscious, can trigger these strong reactions.

In the clinical literature, there are varied reports of cortisol levels in PTSD patients. A prevailing theory has been that PTSD patients have lower levels of cortisol than normal, due to an increase in negative feedback. Although low basal cortisol levels in blood or saliva have not been consistently found, increased negative feedback has been found more consistently, using a modified low dose DST, in which the dexamethasone dose is lowered to 0.25-0.5 mg, compared with the standard 1 mg dose (Yehuda et al., 2004). Despite this increased negative feedback, PTSD subjects show evidence for increased central drive of the HPA axis, including increased CSF CRH levels (Baker et al., 1999) and increased release of ACTH when negative feedback is suppressed by preventing the synthesis of cortisol, using the cortisol synthesis inhibitor metyrapone (Yehuda et al., 1996). The increased negative feedback has been conceived as a compensatory response to counter increased central drive. However, it has also

been argued that initially low cortisol levels could predispose an individual to developing PTSD more readily than one with normal cortisol levels. For example, low cortisol levels in PTSD patients have been linked to increased catecholamine release, which may facilitate the storage of traumatic memories and contribute to hyperarousal (McGaugh, Gold, Van Buskirk & Haycock, 1975; Cahill & Alkire, 2003).

# Chapter 3

# An Overview of Glutamate and Glutamine

## 3.1 An Overview of Neurotransmitters

As dysregulation in neurotransmission plays a key role in a variety of mental illnesses, it is important to understand the basic mechanism by which synaptic transmission occurs. Signals are sent through the brain by way of electrical impulses that travel through neurons. At rest, the membrane potential of a neuron is approximately -70mV, with the actual value dependant on the relative permeability of the neuron to $Na^+$, $K^+$, $Cl^-$, and $Ca^{2+}$. The *equilibrium potential* of an ion occurs when there is no net flux of ions across the membrane, and thus little activity. However, as the permeability of the membrane to a particular ion increases, the resting potential of the membrane has a tendency to head towards the equilibrium potential. Described below are two ways in which a neuron is capable of sending a signal (Hille & Catterall, 2006).

*Voltage-dependent* ion channels are activated when the membrane is depolarized, thereby producing an action potential which initiates and propagates the electrical impulse. This action potential spike occurs when the resting potential reaches threshold and results in depolarization due to the opening of voltage-dependent $Na^+$ channels. The operation of the action potential is on the all-or-none principle, in that once threshold is reached, an action potential must

occur with no way of reversing the effect until the spike peaks. Once the spike reaches its peak, $K^+$ channels open to mediate spike depolarization. The $K^+$ channel activation is directly linked to inactivation of $Na^+$ channels. $K^+$ channels remain open for a length of time so that hyperpolarization may occur, which is when the voltage decreases to a level below the resting potential.

*Ligand-gated* channels do not rely solely on membrane permeability to ions, but, rather, work on chemical activation. In this instance, activation will occur when a ligand-binding site binds a neurotransmitter. Ligand-gated events allow for the influence of chemical neurotransmission on electrical impulses. Once the spike arrives at the neuronal terminal, there is an influx of ions (can be $Na^+$ or $Ca^{2+}$ depending on the receptor) at the pre-synaptic site where vesicles are stored. Once this influx occurs, vesicles will release neurotransmitters across the synaptic cleft, which will then bind to receptor sites on the post-synaptic neuron. Once bound, the electrical impulse is initiated through an influx and efflux of ions in the post-synaptic cell (Hille & Catterall, 2006).

This path of communication is quite rapid. However, there are also neuromodulatory mechanisms that result in slower neural communication due to a modulation of ion channels through phosphorylation. These slower routes of communication are through G-protein and second messenger coupling. In G-protein coupling, the act of a neurotransmitter binding will release a G-protein, which will then change the membrane permeability of the neuron to ions by way of the βγ G-protein subunit. Second messenger coupling is similar to G-protein coupling, but in this case the G-protein does not directly affect membrane

permeability. In this case, G-proteins will change the concentration of second messengers by way of enzymes. These messengers will then change the concentration of cytoplasmic protein kinases which in turn will alter the membrane permeability to ions (Hille & Catterall, 2006).

Ultimately, chemical transmission plays a very important role in the communication of neurons. However, there are some basic components that differentiate neurotransmitters from neuromodulators. The actions of neurotransmitters can be summarized as follows: they are synthesized in pre-synaptic neurons from which they are released, they interact with receptor sites on the post-synaptic neurons, and there is a means of terminating their action (often by way of a reuptake mechanism). Some messenger molecules capable of acting as neurotransmitters include serotonin, GABA, glycine, glutamate, acetylcholine and catecholamines. Conversely, most neuromodulators are synthesized in the cell body (soma) of the neuron and not the axon terminals. Further, as previously mentioned, neuromodulators are usually much slower acting than neurotransmitters. Some molecules that can function as neuromodulators include growth factors, cytokines, and neuropeptides. It is important to note that many neurotransmitters (e.g. serotonin) can also act as neuromodulators (Cooper, Bloom & Roth, 2003).

The G-protein receptor family is of particular interest in the medical fields as it is not only the largest of the receptor families, but also many psychiatric medications act on G-protein coupled monoamine receptors. Drugs that modify ligand-gated inhibitory neurotransmission by acting on GABA-A receptors are

also some of most widely used in psychiatry, including the benzodiazepines and previously the barbiturates.

### 3.2 An Overview of Glutamatic Acid (Glutamate)

Although some neurotransmitters are synthesized from amino acids, other amino acids themselves act as neurotransmitters. This class of neurotransmitters is plentiful in the central nervous system, with the most abundant being L-glutamic acid or glutamate (Vander, Sherman & Luciano, 2001).

In addition to acting as a neurotransmitter, glutamate is a very important amino acid involved in a number of other physiological functions, including acting as a precursor to GABA, synthesizing protein and peptides, and clearing and detoxifying ammonia from the brain (Dejong, Deutz & Soeters, 1996). Although glutamate is abundant throughout the human body, brain glutamate is synthesized within the CNS, since it is unable to cross the blood-brain barrier. Some brain regions with particularly high glutamate levels include the cortex, the hippocampus, the cerebellum and the dorsal horn of the spinal cord.

Glutamate is stored in vesicles at neuronal synapses until it is released by nerve impulses from the pre-synaptic neuron into the synapse. Once released, glutamate acts on post-synaptic receptors, including the AMPA, kainate and N-methyl d-aspartate (NMDA) types of ligand-gated ion channels. This initiates an influx of ions, resulting in cell depolarization, also known as an excitatory post-synaptic potential (EPSP). The primary differentiating factor between an EPSP and an action potential is that action potentials fire on an all-or-nothing principle

and have a maximum peak, but EPSPs are graded and can summate to the point of triggering an action potential.

### 3.3 Glutamate Biosynthesis and Degradation

Glutamate synthesis occurs in two ways: from glucose via the Kreb's Cycle, or from glutamine transported to glutamate neurons from glia. When glutamate acts as a neurotransmitter, replenishment of glutamate comes largely from glutamine supplied by glia, which is then converted to glutamate by the mitochondrial enzyme glutaminase (see Figure 2). Thus, a major role of glia is to take up glutamate from the synaptic cleft for conversion to glutamine by the enzyme glutamate synthetase. Glutamine is released from glia and taken up by neurons for conversion to glutamate. This metabolic cycle is critical for glutamate neurotransmission (Stahl, 2000).

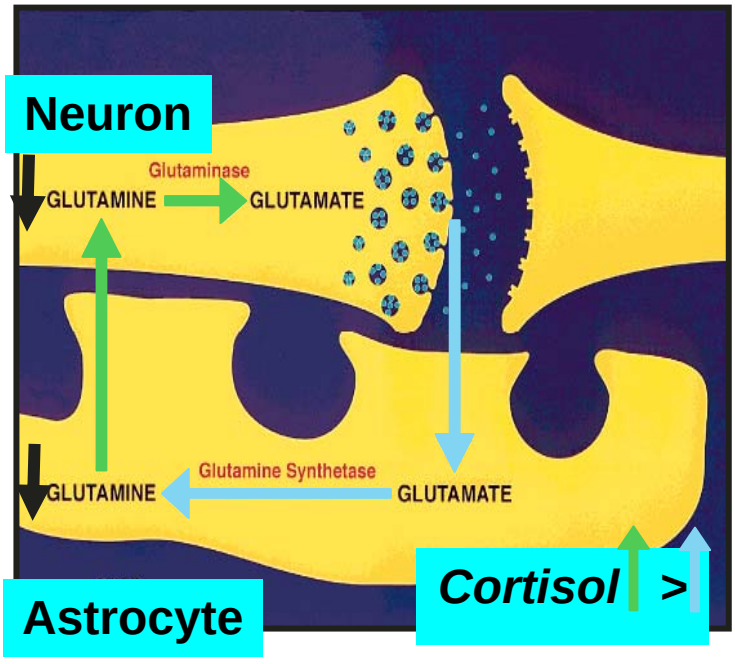


*Figure 2*: Glutamate metabolic cycle

Two $Na^+$-dependent transport pumps aid in the termination of glutamate action. One of the transport pumps is located on glial cells and acts by removing glutamate from the synapse. Another method of termination is removal of synaptic glutamate by reuptake into the neuron (Stahl, 2000).

### 3.4 Glutamate Receptors

Glutamate can bind both to ionotropic and metabotropic receptors. The ionotropic glutamate receptors are of the NMDA, AMPA and kainate types, whereas there are multiple subtypes of metabotropic glutamate receptors, $mGluR_1$ through $mGluR_8$.

The NMDA receptor is the most complex of the glutamate receptors, having six different binding sites and a high permeability to $Ca^{2+}$ ions. The receptor sites can be summarized as follows:

- A glutamate binding site will bind glutamate and other agonists to allow ions to enter the cell;
- A glycine (co-agonist of glutamate) binding site;
- A phencyclidine and non-competitive antagonist MK-801 binding site;
- A $Mg^{2+}$ binding site will block open channels;
- A $Zn^{2+}$ binding site will act as a NMDA channel blocker; and
- A polyamine regulatory site also exists that, when activated, will facilitate and regulate NMDA transmission.

The AMPA and kainate receptors have low permeability to $Ca^{2+}$ ions, but will be active when there is an influx of $Na^{+}$ ions. The binding profiles of these receptors are opposite to one another, as AMPA receptors will preferentially bind AMPA before all else, then glutamate, and finally kainate; whereas kainate receptors will preferentially bind kainate, then glutamate, and then AMPA.

$mGluR_2$, $mGluR_3$ $mGluR_4$, $mGluR_6$, $mGluR_7$and $mGluR_8$ receptors act to reduce the activity of cyclic adenosine monophosphate (cAMP), whereas $mGluR_1$ and $mGluR_5$ receptors activate the second messenger inositol triphosphate ($IP_3$) (Kandel & Siegelbaum, 2000).

# Chapter 4

# Glucocorticoids, Stress and Excitatory Neurotransmission

Stress is a term used to describe responses, with behavioural and biological components, to psychological or physical events or threats that place demands on an individual's resources. Although stress is provoked by demands that arise from the environment, the perception and appraisal of demands and of resources available to meet them are important components of stress reactions (Lazarus, 1966; McEwen, 2008). Biological components of the acute stress response include activation of the HPA axis and sympathoadrenal response. Termination of the acute response after brief stressors may restore homeostasis. Conversely, severe, repeated, or chronic stressors may alter adaptation to stress, leading to lasting changes in stress responsivity called allostasis (McEwen, 2008). These changes place altered demands on the organism that may lead to stress-related physical and psychiatric illnesses.

## 4.1 Glucocorticoids and the Brain

Glucocorticoids released by the HPA axis exert their effects through a variety of mechanisms. These include classical genomic steroid actions at intracellular mineralocorticoid receptors (MR) and glucocorticoid receptors (GR). Glucocorticoids enter the cell and bind to cytosolic receptors, which then interact

with chaperone proteins and are transported to the cell nucleus, where they regulate DNA transcription and thereby protein synthesis. This series of events leads to a relatively slow onset of effects. MR have a tenfold higher affinity to glucocorticoids than GR, such that when glucocorticoids are present at relatively low levels, MR are occupied. GR become occupied at higher levels of cortisol, during the peak of the daily rhythm or when the HPA axis is activated by stress (de Kloet, Vreugdenhil, Oitzl & Joels, 1998; Joels, 2008). Thus, during basal glucocorticoid release, MR are dynamically active, whereas during stress, MR are saturated and GR dynamically active. These patterns of receptor occupancy can contribute to U- or inverted U-shaped patterns of response to increasing glucocorticoid concentrations, where MR and GR have opposing actions. In addition to their classical actions, glucocorticoids can also exert rapid effects that do not depend on DNA transcription or protein synthesis, involving other actions that have recently come under investigation, as described below.

### 4.2 Glucocorticoids and Glutamatergic Neurotransmission

One line of evidence that has demonstrated relationships between stress, glucocorticoids and glutamate has been the use of *in vivo* microdialysis, a technique in which changes in extracellular glutamate can be measured from its passage across a dialysis membrane into artificial CSF flowing through a microscopic probe.

To examine extracellular glutamate levels during stress, Lowy, Gault & Yamamoto (1993) used *in vivo* microdialysis in the extracellular fluid in the CA3

hippocampal region of intact and adrenalectomized rats. The study protocol used physical restraint to induce stress, with microdialysis samples collected every 10 minutes. Samples were collected at baseline, then while the rat was in the restraining device (1 hour) and for an hour after the rat was removed. In adrenal-intact rats, the acute stressor rapidly elevated concentrations of extracellular glutamate in CA3 hippocampus. The maximum elevation was 76% over baseline, but with a short duration. In the adrenalectomized rats, there was a 30% decrease in basal extracellular glutamate, an attenuated stress response. These data suggested that corticosterone is a possible mediating factor for the effect of acute stress on glutamate.

Subsequently, Moghaddam, Bolinao, Stein-Behrens & Sapolsky (1994) showed that acute stress increased extracellular glutamate in the medial prefrontal cortex (PFC), striatum and nucleus accumbens, as well as hippocampus. In this study design, a plexi-glass restraining chamber was used and three groups of rats were examined: adrenalectomized rats implanted with a placebo pellet, adrenalectomized rats implanted with a corticosterone pellet, and an intact control group. There was a greatly attenuated glutamate response to stress in all brain regions measured in the adrenalectomized rats implanted with a placebo pellet, but no significant difference in the control rats or in the adrenalectomized rats implanted with a corticosterone pellet. This provided further evidence that the availability of corticosterone is required for the effect of stress on extracellular glutamate.

Bagley & Moghaddam (1997) investigated the effects of glutamate in the medial PFC and hippocampus further using a tail-pinch stressor. Specifically, they examined the temporal dynamics of the hippocampal and PFC glutamate response to repeated exposure to the same stressor, and the effect of diazepam treatment prior to stress administration, as benzodiazepines are effective anxiolytics (Finlay, Zigmond & Abercrombie, 1995). With probes implanted in the dorsal hippocampus and medial PFC, one group was given no treatment, the second group was given a saline treatment, and the third group was given diazepam. Measures of glutamate were taken during multiple tail-pinch procedures. In the control group, there was a rapid increase in extracellular glutamate concentration that was maintained throughout the duration of the stressor. However, with subsequent tail-pinches, the increase in glutamate was not as high as with the initial tail-pinch, thereby suggesting that the PFC may play an adaptive role in habituating to the stressor. However, it is important to be mindful that each subsequent tail-pinch occurred later in the diurnal rhythm, when the animal's basal cortisol levels were progressively lower. In animals given a saline injection, the initial tail-pinch did not produce as large an increase in extracellular glutamate as present in the control group, which may indicate that the injection procedure already produced some habituation to stress, eliciting a decreased reaction to the tail-pinch stressors. In the animals given a saline injection, the reaction to the first tail-pinch response was inhibited to a much higher degree than in the saline group, thereby suggesting that inhibitory post-synaptic GABA-A receptors modulate the glutamate response to stress (Tallman & Gallagher, 1985). However, the second

tail-pinch elicited a response similar to the first tail-pinch in the control group. The time frame of the second tail-pinch was such that diazepam may have been metabolized, suggesting that diazepam suppressed the initial response, but did not suppress the response to the second tail-pinch. The third tail-pinch exhibited a decreased glutamate concentration in comparison to the second, indicating that habituation may have occurred. In the dorsal hippocampus, there was a smaller increase in extracellular glutamate concentrations compared with the PFC, but also adaptation response was not apparent with repeated tail-pinches. The time lapsed between the diazepam injection and the first tail pinch was 30 minutes, with the second and third tail pinches occurring at 90 minute intervals. Although this is not a chronic stress paradigm, it may be indicative that the PFC may play a stronger role in stress adaptation than hippocampus.

In the above experiments on basal and stress-induced glutamate concentrations, glucocorticoid replacement in adrenalectomized rats was achieved by implanting corticosterone pellets at least 24 hours prior to testing. Subsequent microdialysis studies have investigated rapid effects of corticosterone on basal hippocampal glutamate.

Abraham, Juhasz, Kekesi & Kovacs (1996) infused dexamethasone directly into the hippocampus, with infusion into the striatum as a control measure. In addition to measuring glutamate by microdialysis, electrophysiological techniques were used to measure action potentials. Glutamate levels increased transiently to 280% of baseline at 30 minutes after injection. The glutamate response was selective to the hippocampus, while the

striatum showed no change. Further, infusion of cholesterol, a steroid precursor, into the hippocampus did not alter extracellular glutamate. Thus, the research suggests a direct link between glucocorticoids and excitatory neurotransmission.

Venero & Borrell (1999) also examined the effects of glucocorticoids on hippocampal extracellular glutamate. They established that corticosterone infused either peripherally or into the hippocampus increased concentrations of glutamate, but not GABA. This effect was apparent within 15 minutes in both sham operated and adrenalectomized rats, but significantly lower basal concentrations of glutamate were observed in the adrenalectomized rats. In addition to the rapidity of response, two other findings suggested a non-genomic action of corticosterone. The first was that pre-treatment with antagonists of MR and GR did not block the glutamate response. The second was that pre-treatment with anisomycin, a protein synthesis inhibitor, did not prevent the rapid effects of glucocorticoids on extracellular glutamate. These data suggest that the glutamate response involves a novel mechanism.

As previously mentioned, once corticosterone enters the brain it is able to bind to high affinity MR and lower affinity GR. In recent work, Karst et al. (2005) suggested that rapid effects may be dependent on a non-genomic pathway mediated by the actions of a novel MR. This was demonstrated through observation of miniature excitatory post-synaptic currents (mEPSCs) and paired-pulse stimulation in CA1 neurons in mouse hippocampal slices. In this model, addition of corticosterone increased glutamatergic synaptic activity within 10 minutes of administration. This effect was not prevented by the translational

inhibitor cycloheximide. The effect could be reproduced after pre-treatment with a GR antagonist and in hippocampal slices from GR knockout mice, but not by a highly selective GR agonist. In contrast, the effect was abolished following pre-treatment with a MR antagonist and in MR knockout mice. Although these data suggest the involvement of MR, affinity was tenfold lower than for classical MR and the effect was not diminished when corticosterone was conjugated with bovine-serum-albumin, which reduces entry into the cytosol. These data suggest the presence of a membrane MR that may mediate rapid non-genomic effects of glucocorticoids on excitatory neurotransmission at concentrations that occur during stress.

Expanding on this body of research, Olijslagers et al. (2008) examined pre- and post-synaptic MR in the CA1 region of the hippocampus. In addition to replication of the corticosterone effect, this study demonstrated the involvement of presynaptic extracellular signal-regulated kinase 1/2 (ERK1/2), since blockade of ERK1/2 phosphorylation and activation decreased the frequency of hippocampal mEPSCs.

In summary, microdialysis and hippocampal slice studies suggest that stress-induced glucocorticoid release can exert rapid effects on brain glutamate in regions that include hippocampus, amygdala and mPFC, although it is unclear whether the mechanisms are consistent between brain regions and species.

### 4.3 Glucocorticoids and Memory

Given that glucocorticoid receptors are plentiful in the hippocampus and prefrontal cortex in humans, regions that are critical for memory, it is important to note that acute administration of cortisol can exert rapid effects on human memory. The most thorough body of research on this topic is a meta-analysis of 16 studies on this subject conducted by Het, Ramlow & Wolf (2005). In line with pre-clinical evidence that glucocorticoids impair memory when administered prior to retrieval, but enhance consolidation of new memories (Roozendaal, 2002). Het et al. analyzed two categories of study. In the first category involving research in which glucocorticoids were given prior to memory retrieval tasks, impaired retrieval compared with placebo administration was demonstrated. In the second category, there was no overall effect when glucocorticoids were given prior to learning, although this would affect encoding as well as consolidation. Findings in individual studies ranged from no effect (de Quervain, Roozendaal, Nitsch, McGaugh & Hock, 2000), to mild enhancement (Abercrombie, Kalin, Thurow, Rosenkranz & Davidson, 2003), to impairment (Monk & Nelson, 2002).

It is important to note that more research is required with respect to specific types of memory (e.g. working memory, procedural memory or declarative memory), timing within the daily cortisol rhythm and timing with respect to memory phase. Effects on memory could be observed in some studies within 45 minutes and even as few as 15 minutes of glucocorticoid administration, suggesting that rapid non-genomic effects may occur in humans as well as rodents.

### 4.4 Non-Genomic Steroid Effects

The previous research discussed has demonstrated the role of rapid, non-genomic effects of glucocorticoids on glutamate transmission. Although relatively novel in our understanding of glucocorticoid effects, these findings are consistent with other evidence for rapid effects of steroid hormones, which will be discussed in this section.

A comprehensive review of non-genomic effects of steroids was reported by Haller, Mikics & Makara (2008). However, prior to a detailed discussion of non-genomic effects, it is important to give a reminder of the mechanism of how genomic effects occur. Upon release, glucocorticoids readily cross the cell membrane and bind to receptors and chaperone proteins within the cytoplasm. This ligand-receptor complex is then transported to the nucleus where it binds to transcriptional regulation sites on DNA. After binding, cell transcription is either inhibited or enhanced, depending on the type of steroid, type of receptor (e.g. mineralocorticoid or glucocorticoid) and the specificity of the genomic site. This type of action can take hours and last for days (Joels & de Kloet, 1992).

However, there are observed actions that appear and disappear quite rapidly, within minutes. For example Haller et al. (2008) noted that corticosterone rapidly elevated aggression towards intruders placed in a rat's home cage, or increased risk assessment behaviours occurred in rats in the elevated plus maze. Hence, one of the key features differentiating genomic from non-genomic is the function of time. Relocation of the ligand-receptor complex from the cytoplasm to the nucleus can take up to 30 minutes (Robertson, Schulman, Karnik, Alhemri &

Litwack, 1993), and subsequent transcription can take up to two hours (Kovaks & Sawchenko, 1996). Non-genomic effects have been observed to occur at a much more rapid rate. Another feature unique to non-genomic action is that rapid effects are not necessarily mediated by classical receptors. For example, hippocampal glutamate transmission in the mouse appeared to involve a low affinity MR (Karst et al., 2005). Non-genomic effects also do not rely on DNA translation or protein synthesis, since they can occur in the presence of inhibitors. Non-genomic effects have also been demonstrated in genome-free preparations, although since mitochondrial gene expression is also regulated by glucocorticoids, the preparation must not contain mitochondria.

Another proposed line of evidence is that conjugating glucocorticoids with bovine-serum albumin (BSA) reduces their ability to enter the cell cytoplasm (Daufeldt, Lanz & Allera, 2003). However, this might not be a strong relationship, as the BSA-conjugation may not completely abolish genomic mechanisms. Another mechanism for non-genomic effects may involve cytosolic protein-protein interactions during the formation of glucocorticoid ligand-receptor complexes.

From their comprehensive review of existing studies, Haller et al. (2008) summarized five potential mechanisms:

- Glucocorticoids might affect membrane fluidity by binding directly to membrane lipids (Carlson, Gruber & Thompson 1983).

- Glucocorticoids might bind directly to membrane receptors (Sze & Iqbal, 1994).
- Glucocorticoids might interact with cytoplasmic proteins, such as mitogen-activated protein kinase (MAPK).
- Protein-protein complexes might be produced during translocation of ligand-receptor complexes from the cytoplasm to the nucleus, as an alternative non-genomic pathway for classical cytosolic receptors.
- The GR might influence both genomic and non-genomic functions (Daufeldt, Klein, Wildt & Allera, 2006).

Further research is required to determine which mechanisms occur and also which of these influence glutamate neurotransmission in different brain regions.

# Chapter 5

# An Overview of Proton Magnetic Resonance Spectroscopy ($^1$H-MRS)

In order to translate animal experiments on the interactions between glucocorticoids and glutamate neurotransmission to clinical research, methods are needed to measure glutamate transmission in clinical research, either directly or indirectly. *In vivo* proton Magnetic Resonance Spectroscopy ($^1$H-MRS) has become a useful tool for measuring the brain neurochemistry of living individuals. $^1$H-MRS has impacted the field of psychiatric research by providing a non-invasive method of examining various neurotransmitters and metabolites in the human brain.

## 5.1 $^1$H-MRS: A Brief Introduction

$^1$H-MRS takes advantage of the concept that nuclei with an odd number of protons are capable of spinning and thereby able to produce a magnetic field. When a radio frequency (RF) pulse is applied, the nucleus orients to an energy level where it is less stable. Due to magnetic polarity, the nuclei will align either along the applied field to a lower energy state, or opposite the applied field to a higher energy state (Henry, Frederick, Moore, Stoddard & Renshaw, 2001). An observed signal is visible across a spectrum (see Figure 3) when the RF pulse is terminated and the nuclei return to their original state – a process termed

"relaxation". Relaxation can occur along three axes, termed X, Y, or Z. The relaxation time is termed T2 (transverse) along the X and Y planes, or T1 (longitudinal) along the Z plane (Salibi & Brown, 1998). The reversion of these compounds to their original energy level can be observed on a spectrum once a mathematical formula, the Fourier transform, resolves the time-dependent relaxation into an observable frequency across the spectrum. The observed signal does not hold a 1:1 ratio to its concentration in the human brain, as relaxation times result in a delay between signal detection and observation (termed echo time, or TE). The strength of the signal decays during this delay. Furthermore, relaxation times vary based on the compound and its environment (e.g. between brain regions), thus it is not straightforward to take relaxation effects into account when determining the absolute concentration values of metabolites (Salibi & Brown, 1998).

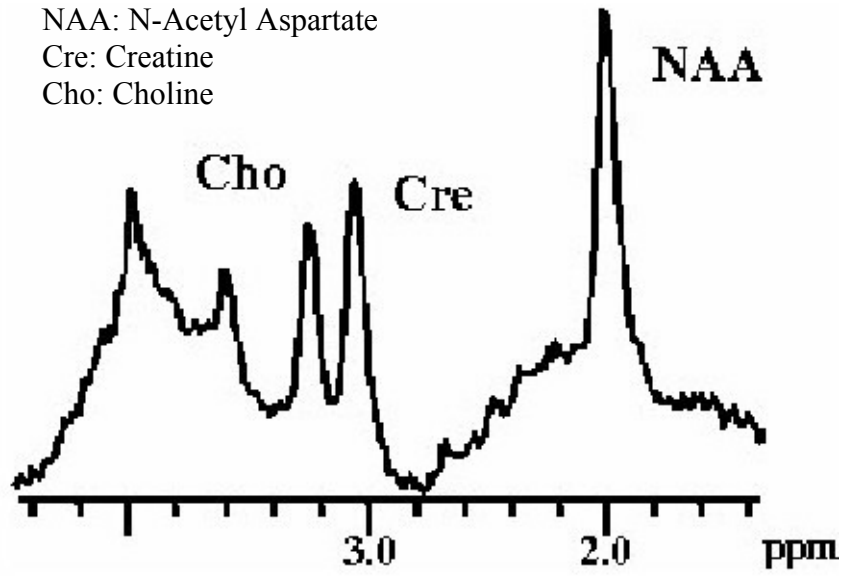


*Figure 3*: Chemical shift spectrum (STEAM sequence)

The magnetic field of a nucleus can be changed when a nearby nucleus imposes a polar disturbance, called coupling, on the nucleus of interest. This disturbance results in the signals of both nuclei splitting to form multiple separate peaks across the spectrum. The number of visible splits depends on the nuclei that surround and disturb the magnetic properties of the nucleus of interest. Furthermore, rather than simply changing in amplitude, the overall shape of signal derived from coupled spins can change markedly as echo time changes. It is important to be able to predict the lineshape at a given echo time in order to resolve signals from different nuclei that are close to or overlap each other in the frequency spectrum.

Another complexity in quantifying data is that signals of interest may also be overlapped by signals from macromolecules, although this is mitigated at longer echo times, because these signals decay much more rapidly than those of metabolites. Finally, since tissues contain such a high proportion of water, the water signal is massive compared with metabolite signals and has to be suppressed effectively.

## 5.2 Using $^{1}$H-MRS

Unlike Magnetic Resonance Imaging (MRI) or functional MRI (fMRI) it is not feasible to use MRS to examine the whole brain, and typically researchers adopt one of two methods, single voxel MRS, which examines a 3D volume of tissue, or chemical shift imaging, which examines multiple voxels within a 2D

slice of the brain (a voxel is a predetermined, computer-generated volume of interest).

Prior to commencing a scan, a standard set of tasks must be performed to ensure accurate data acquisition. First, the subject needs to be positioned comfortably, with sufficient head support provided to minimize movement during the scan. This is particularly important in prefrontal regions, which shift more than occipital regions because the head pivots on the occipital pole of the skull when subjects lie on their back. It is important to monitor head position at intervals across a scanning session to exclude displacement of the study voxel due to subject motion (see Figure 4).

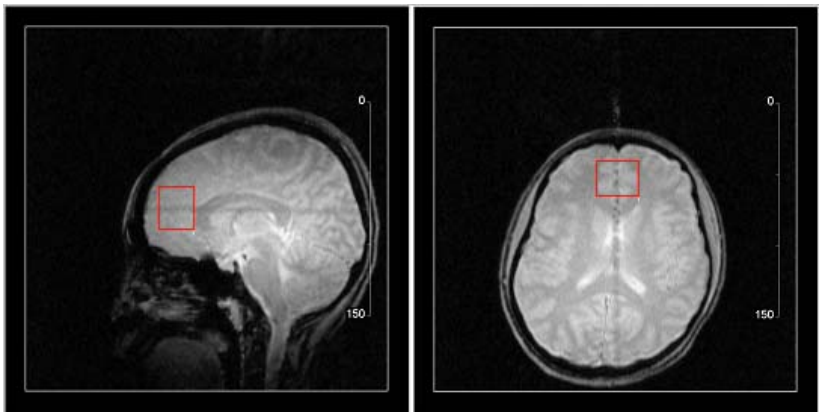


*Figure 4*: Prefrontal voxel placement

Because the static magnetic field in the scanner is not uniform throughout, one must ensure that magnetization in the area of interest is homogenous. This is accomplished through a process of shimming, which maps the field in the area of interest and introduces pulses to correct areas of inhomogeneity. When performed

correctly, this procedure provides better resolution with sharper, narrower and more easily discernable metabolic peaks. This process is commonly achieved with a pulse sequence protocol called FASTMAP (Gruetter, 1993), and at our centre additional fine tuning of the shims is performed using in-house software. Poor shimming leads to poor quality, noisy data. This is particularly important for studies of the prefrontal cortex because bone and air interfaces create magnetic field inhomogeneities and the prefrontal cortex is close to the air cavities of the sinuses. Hence, it is imperative to ensure that the shimming is performed accurately.

Another critical factor is the effective suppression of the water signal. Since water is so abundant and contains two hydrogen atoms, the water signal would be very large and broad, effectively masking all other compounds. Stanley, Drost, Williamson & Carr (1995) have previously reported that the intensity of the water signal is approximately 10,000 times greater than that of the proton-containing metabolites. There are many techniques for water suppression; for example, a popular method is the use of chemical shift saturation (CHESS) pulses (Elderkin-Thompson et al., 2004). CHESS involves the administration of a selective 90º pulse that will excite water, followed by a dephasing step. Repeatedly exciting and dephasing water molecules produces substantial suppression, as with each CHESS cycle the signal is decreased by a factor of 100-500.

Once shimming and water suppression have been performed to satisfaction, data is acquired by the utilization of pulse sequences. The two most

common single-voxel techniques used in $^{1}$H-MRS are Point Resolved Spectroscopy (PRESS) and Stimulated Echo Acquisition Mode (STEAM). PRESS and STEAM differ fundamentally in the pulse sequences used. PRESS utilizes an initial 90º RF pulse to excite the spins, followed by two 180º pulses to create the spin echo, whereas STEAM uses successive 90º pulses (Malhi, Valenzuela, Wen & Sachdev, 2002). The shorter pulses used in STEAM allow greater flexibility in compound measurements by allowing the researcher to view metabolites with shorter T2 relaxation times. The shorter STEAM sequence echo time also allows signal viewing with decreased signal decay (Malhi, et al., 2002). However, the longer echo time in PRESS sequences is also advantageous as the complete net magnetization is refocused to create the echo, thereby providing a much higher signal/noise ratio, resulting in a peak that is approximately double the size of observable peaks from STEAM sequences. The lower peaks and weaker signal/noise ratio in STEAM are due to a portion of the net magnetization producing the echo. Further, the longer echo time of PRESS sequences allows for discernment of major metabolic peaks, such as myo-inositol, that may appear hidden or clustered too closely with neighboring peaks in STEAM sequences. Additionally, the pulse used by STEAM is more sensitive to diffusion, potentially hindering the accuracy of measurements. Alternatively, STEAM is advantageous by allowing greater flexibility to determine voxel size, whereas PRESS results in a relatively smaller volume of interest. However, the size of the voxel should be determined by the area of the brain to be measured, which could then dictate the type of sequence used. Ultimately, both sequences are useful in measuring

different metabolites, and have been shown in the past to be effective when used in conjunction.

### 5.3 Clinical Research Applications of $^{1}$H-MRS

$^{1}$H-MRS has become a useful tool in psychiatric research. Measurements of major metabolites have provided a non-invasive avenue for obtaining insights into the chemical make-up of brain tissues in individuals suffering from psychiatric disorders. Of all major metabolites, N-acetyl aspartate (NAA) has been studied in the most detail and depth.

Having a large peak at 2.0 ppm (see Figure 3), NAA is an easily detected metabolite found exclusively in neurons, thereby functioning as a marker for neuronal density. NAA, along with glutamate, is a metabolite of N-acetylaspartyl-glutamate (NAAG). In early research, absolute values were not measured because T2 relaxation constants were not known. Ratio measures to creatine (Cr) levels were therefore used. Since creatine values are fairly constant (Passé, Charles, Rajagopalan & Krishnan, 1994) and Cr has a highly visible peak at 3.05 ppm (see Figure 3), NAA values were expressed as a ratio to Cr. In schizophrenia, a decreased amount of NAA has suggested a decrease in neuronal density in the hippocampus and prefrontal cortex as a key component of the illness (Steen, Hamer & Lieberman, 2005).

Choline (Cho) also produces a highly visible peak on the spectrum at 3.2 ppm (see Figure 3). Cho is a precursor and metabolic product in the metabolism of acetylcholine; however, the Cho peak measured by MRS also includes

phosphocholine and glycerophosphocholine signals (Auer et al., 1992). Cho has been investigated as a marker in cellular density and turnover, and high levels are present in certain types of tumor (Malhi et al., 2001). Cho levels have been studied in MDD, but the results are equivocal. Renshaw et al. (1997) showed a decrease in Cho in patients with MDD, while Hamakawa, Kato, Murashita & Kato (1998) demonstrated increased Cho.

Myo-inositol (m-Ino) is a glial cell marker that is actively transported into astrocytes and is usually measured from its resonance at 3.6 ppm in MRS studies. There is some evidence that m-Ino is decreased in the medial prefrontal cortex of patients with MDD (Coupland et al., 2005). Other studies have returned conflicting results, but are limited by using methods that are more susceptible to contamination by signals from water and macromolecules. Kar, Slowikowsky, Westaway & Mount (2004) published a review that provided evidence for an increase in m-Ino in patients with Alzheimer's disease, suggesting that m-Ino may be an indicator of gliosis in this disorder.

## 5.4 $^{1}$H-MRS and Neurotransmitters

Although measures of the easily visible peaks of NAA, Cr, Cho and m-Ino have been informative in psychiatric research, it is much more difficult to measure metabolites that are directly relevant to neurotransmission, such as glutamate, glutamine and GABA. This is because coupling splits their signals into smaller amplitude multiple peaks, due to either a very close overlap with other signals, or, in the case of GABA, a relatively low concentration. For example, the

GABA signal at 3.05 ppm is overlapped by the Cr signal, which is 1,000-fold larger. The glutamate and glutamine signals at 2.2-2.5 ppm overlap each other and cannot be distinguished using PRESS or STEAM at 1.5 T. There have been two main approaches to distinguishing glutamate and glutamine. One is to combine high magnetic fields (4.0-7.0 T) with short TE PRESS or STEAM (to minimize signal loss due to T2 relaxation) and then to use spectral fitting to estimate the contribution of each to the resulting signal lineshape. Limitations of this approach are that very high field scanners are not widely accessible and that strong macromolecule signals are present at short TE and have to be removed in the analysis. The other approach is to use specialized sequences, instead of PRESS or STEAM, that aim to preserve the signal from glutamate or glutamine, while suppressing the signal from the other metabolite. This is called spectral editing.

One recent development has been to use a spectral editing technique called spectrally selective refocusing to isolate the glutamate and glutamine signals from each other (Choi et al., 2006). Spectrally selective refocusing takes advantage of the fact that the C4 protons of glutamate and glutamine resonate at slightly different frequencies. Instead of using standard PRESS or STEAM to excite the whole spectrum, resonance selective sequences are used that produce excitation only within narrow frequency bands that do not overlap for glutamate and glutamine. As a result, spectra can be produced that include only the glutamate peak, or a glutamine peak that also includes small signals (<10%) from glutathione and NAA, which cannot be completely suppressed within PRESS. In practice, excitations are also added at additional frequencies that excite Cr, so that

the spectra include non-overlapping Cr peaks that can be used to obtain glutamate/Cr or glutamine/Cr ratios (Choi et al., 2006).

## 5.5 Limitations of $^{1}$H-MRS

Despite great advances in methodologies and measurement techniques, it is important to realize that this unique, non-invasive tool for measuring brain chemistry has limitations, which include selecting relevant, testable hypotheses and issues related to hardware, subjects and data analysis.

When presented with a relatively new, non-invasive apparatus that can measure in vivo brain metabolites, it is tempting to accumulate as much data as is humanly possible. In psychiatry, the reason for collecting data should, ultimately, be to test specific hypotheses or for clinical purposes, such as diagnosis or outcome prediction, and not because the technology to measure certain metabolites exists. Thus far, $^{1}$H-MRS measures of NAA and to a lesser extent m-Ino have proven to be relevant markers of neuronal and glial density, respectively, but are likely to be non-specific markers in relation to diagnosis and have not as yet been shown to have predictive value for disease outcome. In other cases, such as Cho or Cr measures, hypotheses can sometimes appear to be driven by the availability of the measurement, rather than any fundamental theory regarding the pathophysiology of the disorder.

There are also wide differences between research sites in terms of scanner hardware, software and sequences used, which means there is very little standardization across research sites. Although standardization is feasible,

particularly across similar scanning platforms, considerable effort is required to establish reliability across sites. This has obvious implications for trying to establish diagnostic standards.

The importance of shimming has been described above and it should also be noted that magnetic field homogeneities are not constant through the brain, but distortions are more severe in regions near sinuses, such as rostral and ventral prefrontal cortex and medial temporal lobe, and in regions with high iron content, such as the basal ganglia, compared with parietal and occipital grey matter or in white matter (Soreni et al., 2006). Time-consuming reproducibility data must be accumulated to ensure the precision of the measurements and it cannot be assumed that reliability obtained, for example, in occipital cortex will be matched by similar reliability in other regions (Passé et al., 1994).

Conducting scans on patients with psychiatric illnesses presents greater challenges than healthy volunteer studies. The consumption of caffeine or alcoholic beverages or smoking cigarettes may potentially alter metabolite concentrations and should be considered in the study design. For example, the prevalence of smoking in patients with schizophrenia is approximately 80% (Kuehn, 2006) – the highest of all psychiatric disorders. Care is therefore required to match for tobacco (and other substance) use and consideration be given to whether subjects should abstain prior to scanning, which may be a daunting task for patients. The scan procedure itself may produce stress that could influence neurochemical measures. Staying still within the scanner may be difficult for patients with inattention, agitation or movement disorders and it is advisable to

monitor movement in addition to providing comfortable head restraint. The length of data acquisition is a relevant factor here, since it is more difficult for subjects to remain still over long periods of time. As an example, our group previously used a sequence to obtain selective glutamate measurements that required a 17 minute acquisition. The present study would not have been possible without the development of the spectrally-selective refocusing method, which allows selective measurement of glutamate in 2.5 minutes and can therefore be repeated several times within an acceptable time frame.

In relation to data analysis, a challenge to the field is whether to measure absolute concentrations of metabolites or concentrations relative to Cr. Although Cr levels are relatively stable in healthy subjects, this might not necessarily be the case in psychiatric disorders. It is preferable to base concentrations on brain water (since osmolarity is under tight physiological control) and report measures either in 'institutional units' when predetermined T2 values are not available to adjust for relaxation effects, or as absolute concentrations when T2 values are available to adjust for this source of signal decay. Complicating matters, T2 values vary between brain regions and field strengths and can also possibly be influenced by disease. T2 measures specific to the research site and voxel location are therefore needed.

In measuring metabolite concentrations, whether relative to Cr, in institutional units or absolute concentrations, it is critical to take into account the fact that both metabolite concentrations and water content differ between CSF, grey matter and white matter. For most metabolites, the levels in CSF are tiny

relative to brain tissue, whereas differences between grey and white matter are metabolite-specific. The order of water content is CSF > grey matter > white matter. The volumes of CSF, grey matter and white matter in the voxel therefore need to be measured. The volume of the voxel that is CSF is excluded from absolute concentration measures because the signal all comes from brain tissue. It is important either to ensure that group comparisons are matched for grey and white matter content, or that the grey matter fraction (grey matter / brain tissue) is included as a covariate in the analysis. A variety of techniques can be used to measure the voxel makeup, including imaging or spectroscopic methods.

Having obtained the final measurements, it is important to question assumptions that cross-sectional measures of metabolite levels at a single time can be used to represent dynamic events, such as cell membrane permeability or neurochemical synthesis and degradation. Repeated measures over time or in response to physiological or pharmacological interventions might be more informative about dynamic processes. $^{13}$C-MRS can also be used to measure synthesis and degradation rates for certain neurochemicals, for example the rate at which $^{13}$C-labelled glucose is incorporated into glutamate and glutamine, using repeated measures during a period of $^{13}$C-glucose infusion. Interpretations of dynamic events based on single $^{1}$H-MRS measurements should be regarded cautiously.

# Chapter 6

# Hypothesis

The present study was based on the rationale that glucocorticoids can rapidly increase glutamate release in the prefrontal cortex and hippocampus (Mogghadam et al., 1993). Based on more detailed evidence from hippocampal studies glucocorticoids increase synaptic glutamate release via a pre-synaptic mechanism involving a low affinity membrane receptor (Karst et al., 1005; Olijslagers et al., 2008). This suggests that administration of a glucocorticoid may increase glutamate turnover and lead to a decrease in $^{1}$H-MRS-visible glutamate and/or glutamine. Following completion of the study, Jenstad et al., (2009) have provided evidence that although glutamate release and turnover increase during excitatory neurotransmission, glutamate concentrations are maintained by conversion of glutamine to glutamate. Since the effects of glucocorticoids on excitatory neurotransmission involve rapid non-genomic mechanisms, changes in glutamate and/or glutamine should be visible within thirty minutes of cortisol administration.

Thus, it was hypothesized that, compared with placebo, the acute administration of cortisol (hydrocortisone 35mg) intravenously would decrease prefrontal glutamate and/or glutamine concentrations. This hypothesis was tested in a randomized, double blind, placebo-controlled, within-subject design.

# Chapter 7

# Methods

The study was approved by the Biomedical Research Ethics Board of the University of Alberta. Subjects were informed of all aspects and risks involved in the study in which they were involved before providing written consent to participate.

Widely considered the gold standard of experimental research design, a randomized, double-blind, placebo controlled trial was conducted. The data were acquired from human participants in a within-subject design. Subjects attended for two separate test sessions, in which glutamate and glutamine were measured *in vivo* using Magnetic Resonance Spectroscopy ($^{1}$H-MRS) before and after the administration of hydrocortisone (35 mg) or placebo intravenously.

## 7.1 Research Participants

The inclusion criteria were for healthy male or female volunteers, aged 18-45 years. In order to reduce the likelihood of an endogenous cortisol response to novelty or stress from being in the scanner, all participants had previous experience of having a $^{1}$H-MRS scan during earlier studies. We therefore contacted subjects who had volunteered for previous research studies to see if they would be interested in participating in the study. Interested individuals were invited for assessment. The Anxiety Disorder Interview Schedule IV (Brown, DiNardo & Barlow, 2004) was used as a structured interview to exclude subjects

with a lifetime history of anxiety disorders, mood disorders, schizophrenia, substance use disorders, somatization disorder, hypochondriasis or anorexia nervosa. Potential participants with these illnesses were excluded to ensure that our sample would not include those who may have pre-existing alterations in brain neurotransmission or abnormal stress responses.

A medical history checklist was used to exclude significant medical conditions, particularly neurological or endocrine disorders that might alter subjects' baseline neurotransmitter levels or responses to cortisol. Height, weight, heart rate and blood pressure were recorded. Subjects with obesity or arterial hypertension were excluded.

Neurotransmission might be altered by use of alcohol or non-prescribed psychotropic drugs. Current use of alcohol and use of non-prescribed psychotropic drugs within the last year were recorded. Females who drank more than seven standard alcoholic drinks per week and males who drank more than 14 standard alcoholic drinks per week were excluded. Subjects were asked to abstain from alcohol for at least 48 hours before each scan. Subjects with repeated use of psychotropic drugs for a period more than one year in their lifetime, or any use within the last three months were excluded. Cigarette smoking of greater than one pack per day was also an exclusion criterion.

In a study of the effects of cortisol, it is important to ensure that exogenous corticosteroids have not been medically prescribed and are not being currently used by the participant. Hence, participants who had used corticosteroids of any variety over the past three months were also excluded.

Further, any usage of medications by the subject was recorded to allow the researchers to determine if these might have potentially confounding effects on cortisol or neurotransmission, in which case they were excluded.

Night shift-workers were excluded because shift-work can alter the diurnal rhythm of cortisol.

Subjects were further screened with a detailed checklist to exclude participants with contra-indications to magnetic resonance scans. During this process, participants were excluded if they wore a cardiac pacemaker, implanted neurostimulator, or implanted drug delivery system. The action of these devices could be severely altered by the strong magnetic field, resulting in a health risk to the participant. Further, any sort of surgery on the head, chest, vascular region, skeletal region, or joint replacement was also examined in detail. If there was a possibility of pin, screw or staple insertion without subsequent removal, the subject was excluded. Exclusion also occurred if there had been injury by a metallic foreign object that wasn't subsequently removed, or if the risk of such a condition existed, such as in the case of those who worked at some point as welders, lathe operators, sheet metal workers, or in similar trades.

For females, if there was the possibility of a pregnancy, or if an intra-uterine contraceptive device was worn, the participant was excluded. The safety of IUDs has not been tested at magnetic field strengths greater than 1.5T.

## 7.2 Procedures

Once included in the study, each participant was scheduled for two scans approximately one week apart, with each scan starting in the mid-afternoon at approximately 3:00pm. Scans were scheduled at this time due to the diurnal rhythm of cortisol, avoiding the morning hours when cortisol levels are at their highest.

The participant's first visit began at approximately 2:00pm, and included a brief health assessment and check of heart rate and blood pressure. The participant was accompanied to the University of Alberta's *In Vivo* Nuclear Magnetic Resonance Centre, where they removed any metallic objects from their person and changed into hospital scrubs in order to avoid introducing metals or dirt into the scanner area. An intravenous (IV) cannula was inserted, blood drawn for plasma cortisol and the cannula flushed with normal saline and locked. After cannulation the subject rested for 45 minutes before entering the scanner. Added to the time needed to set up the scans, this allowed 75 minutes for the offset of any cortisol response to venipuncture. After the rest period, the participant was fitted with earplugs and headphones to dampen the mechanical noise during the scans. The headphones also allow communication with the participant in the scanner. The participant was then given final instructions to remain as still as possible so that voxel positioning was not compromised during the scan.

Once the participant was comfortably in the scanner, the scan procedure (outlined in detail in section 7.3 and Figure 5) commenced. After voxel

placement, shimming and measurement of the CSF, grey matter and white matter content of the voxel, glutamate and glutamine acquisitions were obtained to determine their baseline levels. Mid-way through the scan (approximately 30 minutes) a blood sample was obtained and either saline placebo or hydrocortisone (35mg) was administered as a bolus through the IV cannula, which was then flushed and locked. In the first 10 minutes after the injection, the voxel was repositioned in case of any movement during the injection and shimmed. Further glutamate and glutamine acquisitions were obtained from 10 to 30 minutes post-injection. Upon scan completion a final blood sample was drawn. The blood samples were centrifuged at room temperature and the plasma was extracted and stored in a freezer at -80°C until cortisol analysis (see Figure 5).

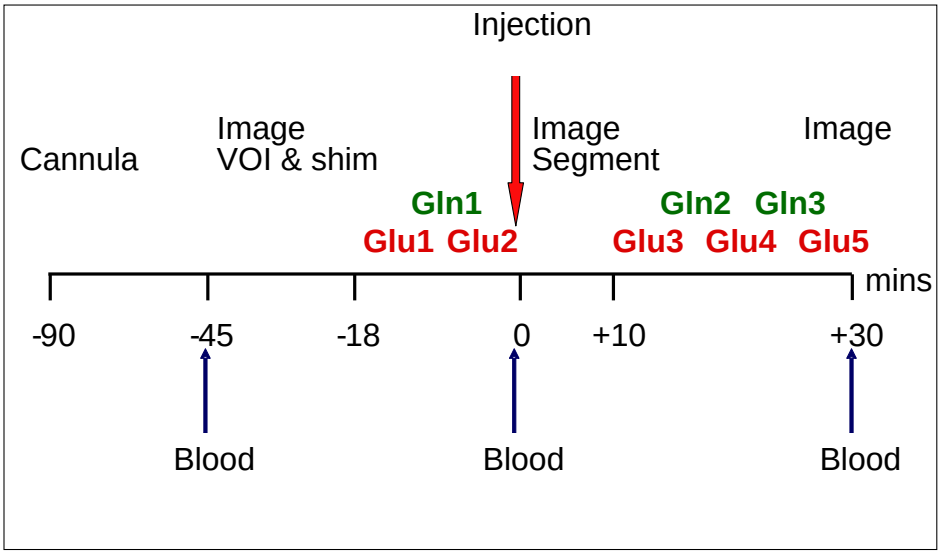


*Figure 5*: Research procedure (VOI = Volume of Interest)

The identical protocol was followed for the participant's second visit, with the only alteration being the study drug administered.

Some potential side effects of hydrocortisone administration include temporary puffiness and restless sleep, while in severe cases allergic reactions can include a rash, mouth or throat swelling, and difficulty breathing. A physician was available during the scans.

The voxel was located in the medial prefrontal grey matter across the mid-line above the callosal plane (between the genu and splenium of the corpus callosum) in order to avoid the region of high magnetic field inhomogeneity in ventral prefrontal cortex. The voxel was shimmed using FASTMAP and fine tuning of the shim using an in-house automated shimming routine.

### 7.3 $^{1}$H-MRS Protocol

Data were acquired using a 3.0 T magnet (Magnetic Scientific PCL) interfaced to a Surrey Medical Imaging Systems (SMIS) console. A 28cm diameter quadrature birdcage resonator coil was used for radiofrequency transmission and reception. A multi-slice gradient echo image (TR = 500ms, TE = 22ms, slice thickness = 5mm, 11 slices, resolution = 256 x 256), in the transverse, sagittal, and coronal planes, was used to visualize placement of the $^{1}$H-MRS voxel. The voxel (30 x 25 x 30 mm$^{3}$) was positioned across the midline in bilateral rostral anterior cingulate cortex and medial prefrontal cortex, above the callosal plane (between the lower borders of the genu and splenium of the corpus callosum, see Figure 6). This avoids the most ventral part of the prefrontal cortex, which has the highest magnetic field inhomogeneities.

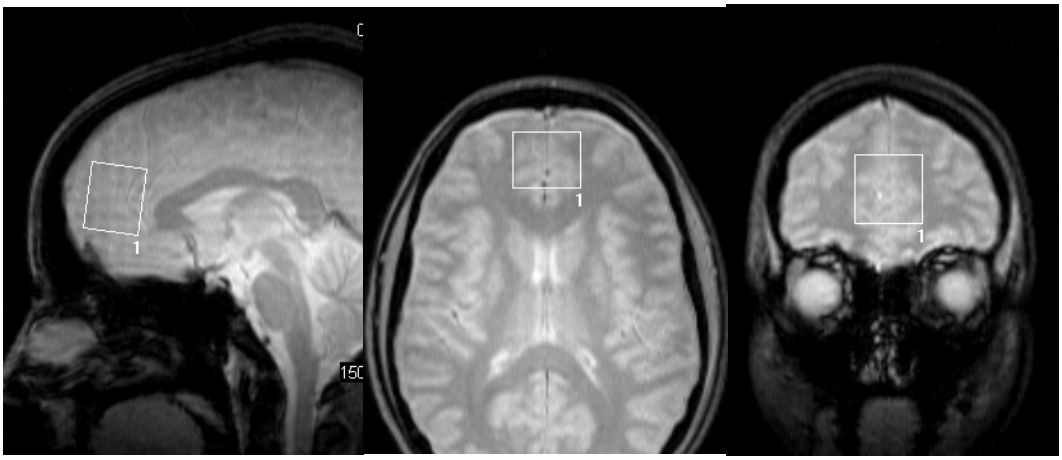

*Figure 6*: Voxel localization (30 x 25 x 30mm$^3$)

Shimming was carried out with FASTMAP (6-7 Hz) and fine tuning of the shim was performed using an automated in-house routine. Following shimming a double-inversion recovery PRESS sequence was used for segmentation to measure the grey matter, white matter, and CSF content of the voxel (Choi et al., 2006). The largest component of the voxel was grey matter, which has a higher glutamate concentration than white matter.

Five glutamate (-20, -8, +12, +22, +32 mins) and three glutamine (-15, +15, +25 mins) acquisitions were obtained using two spectrally selective refocusing sequences within a PRESS localization scheme. The glutamate sequence incorporated a triple-resonance selective 180º radiofrequency (RF) pulse (81.9-ms duration) with phase variations designed to edit the glutamate (2.35 ppm) and creatine (3.02 and 3.92 ppm) resonances. A separate sequence incorporated a 90.5-ms duration 180º RF pulse to edit the glutamine (2.39 ppm) and Cr resonances. The other spectral parameters were: carrier frequency = 3.0 ppm; TE = 128ms (glutamate) and 158 ms (glutamine); TR = 2.4 s; acquisition

time, at = 820 ms; dwell time, Δt = 400 μs; number of complex points = 2048; number of averages = 64 (glutamate) and 128 (glutamine); 64 and 128 step phase rotation. Variable-flip-angle water suppression was applied prior to the editing sequences. Spectral data were filtered with 2-Hz exponential and 4-Hz Gaussian functions and eddy current effects were removed by correcting the phase factor of individual data points, based on the phase factor of the water signal acquired with the 180º RF pulses tuned to the water resonance (Choi et al., 2006).

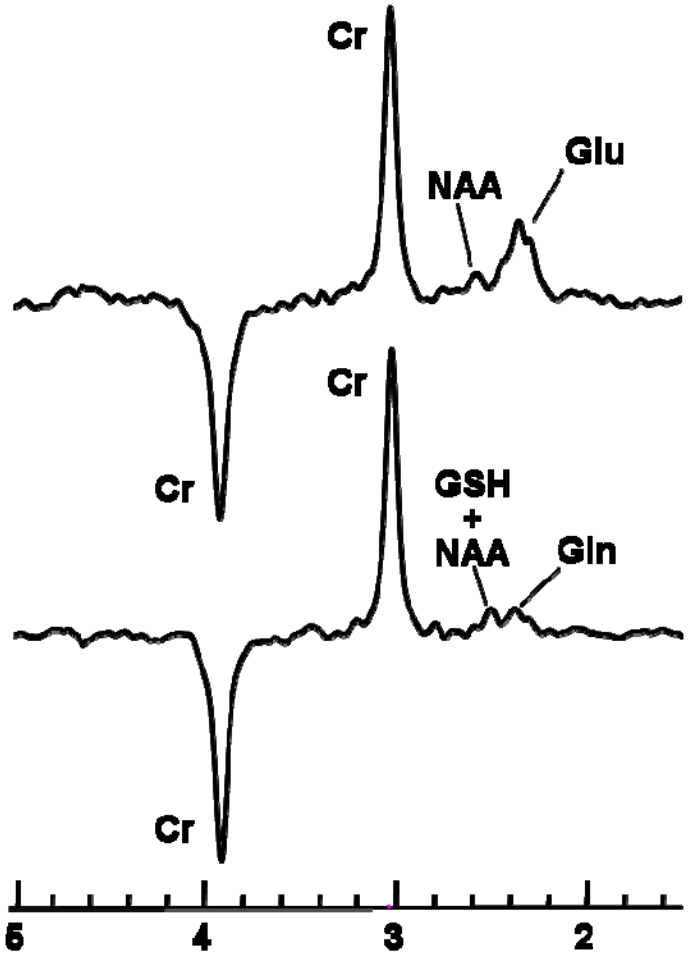


*Figure 7*: Chemical shift spectrum of glutamate and glutamine post-sequence

Glutamate and glutamine concentrations were calculated by integrating the area of their signal peak with respect to the brain tissue water fraction (i.e. excluding the CSF portion of the voxel). 97% of the peak within the interval 2.23-2.46 ppm of the chemical shift spectrum derives from glutamate. 90% of the peak within the interval 2.27-2.43 ppm is derived from glutamine. Data were adjusted for different water concentrations of grey matter (0.81 x 55.6 M) and white matter (0.71 x 55.6 M). In order to obtain absolute concentrations the data were also adjusted for relaxation effects using values for glutamate ($T_1$ = 1.2 sec, $T_2$ = 200 ms) obtained in a previous experiment with the same voxel size and position and (Choi et al., 2006b). $T_1$ and $T_2$ values for glutamine were assumed to be the same as for glutamate.

### 7.4 Plasma Cortisol

Plasma cortisol concentrations were assayed using an enzyme-linked immunosorbent assay (ELISA; Alpco Diagnostics, Salem, New Hampshire). Due to the marked elevation of cortisol levels in the plasma samples following hydrocortisone administration, serial dilutions were performed to bring the concentration of the samples within the linear range of the assay.

### 7.5 Statistical Analysis

Data were analyzed using repeated measures analysis of variance, including the within-subject factors of session (cortisol or placebo), time and their interaction. A significant session by time interaction indicates a differential

response to cortisol or placebo. The effects of time were also examined during the cortisol and placebo sessions separately, in order to confirm that there was a significant effect within the cortisol session and no effect during the placebo session. Due to the within-subject design, Mauchley's test for sphericity was performed in order to determine whether adjustments to the degrees of freedom were required due to within-subject correlation between session and/or time points. Post hoc comparisons were made between baseline and follow-up time points with each session and between the same time points in the cortisol and placebo sessions, using paired two-tailed *t* tests. Although the hypotheses were directional, two-tailed tests were used conservatively in the absence of empirical evidence for the direction of changes in glutamate or glutamine following cortisol administration. Plasma cortisol data were log transformed for analysis due to skew.

# Chapter 8

# Results

## 8.1 Plasma Cortisol

Plasma cortisol levels rose substantially during the hydrocortisone session as expected. The sphericity test was significant (Mauchley's W = 0.40; df = 2; p = .01) and a Greenhouse-Geisser adjustment to the degrees of freedom was used. The session by time effect was significant, $F_{1.25,13.7} = 88.82$; $p < .001$. Although plasma cortisol should increase following hydrocortisone, it was also notable that plasma cortisol declined during the placebo session (effect of time: $F_{2,22} = 4.07$; p = .03).

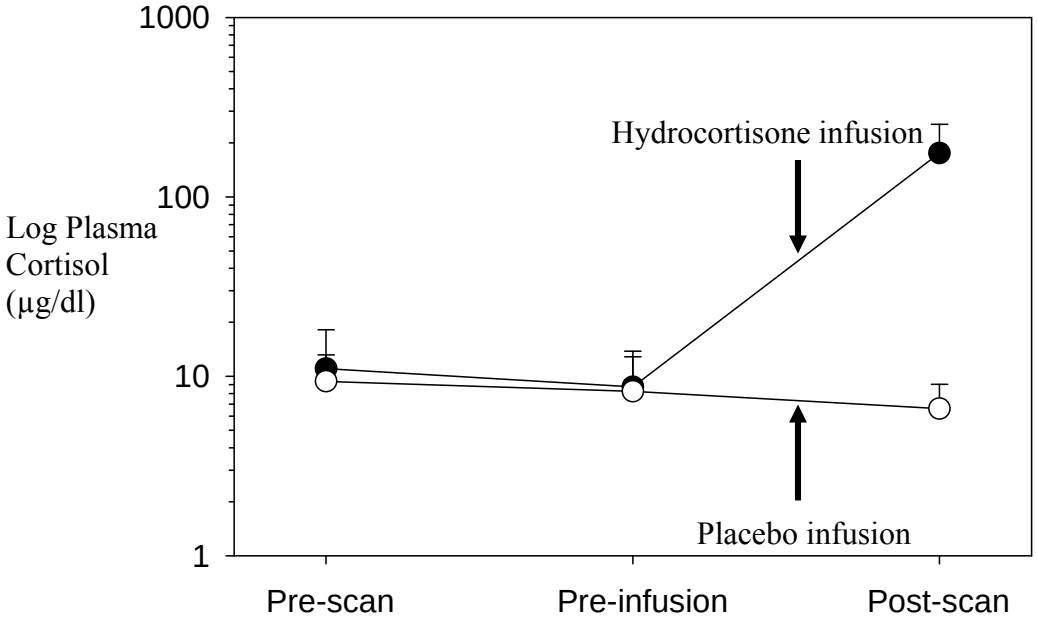


*Figure 8*: Plasma cortisol before and after infusion of hydrocortisone

## 8.2 Voxel Composition

A sample of an individual segmentation result is shown in figure 9. The composition of the voxel did not differ between the two test sessions: grey matter (session 1 = 66.3 ± 5.1 %; session 2 = 66.0 ± 3.8 %; t = .16; p = .88), white matter (session 1 = 21.6 ± 3.7 %; session 2 = 22.5 ± 5.1 % t = .49; p = .64) and CSF (session 1 = 12.4 ± 2.8 %; session 2 = 11.5 ± 3.1 %; t = 1.2; p = .27).

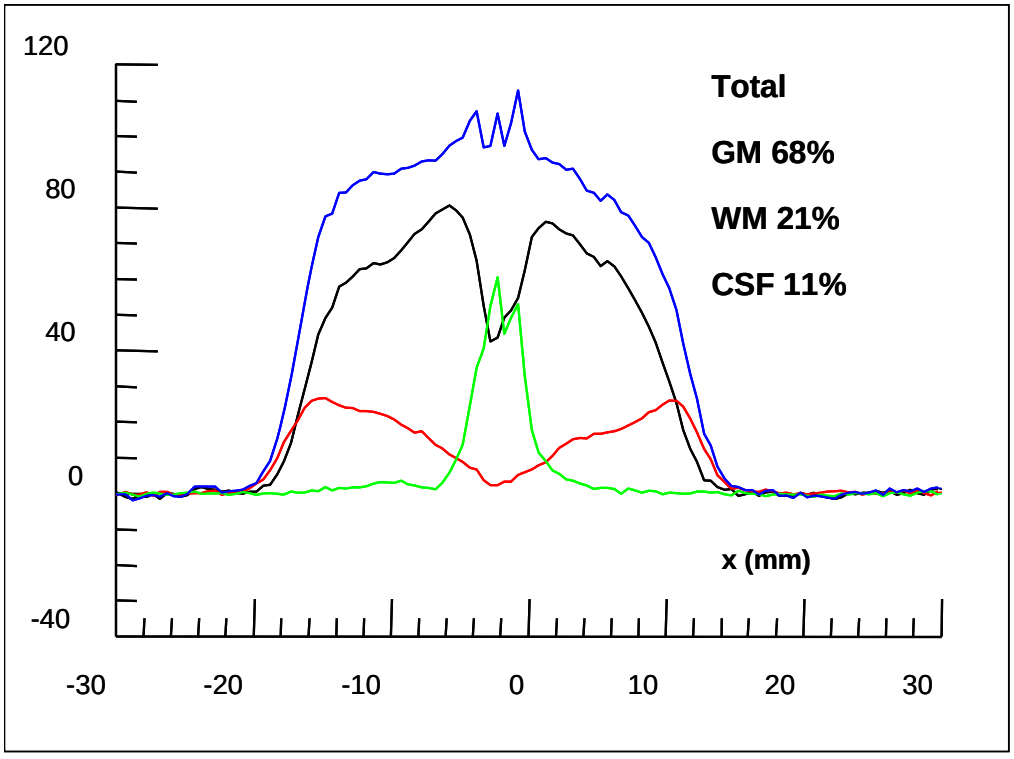


*Figure 9*: Voxel composition

## 8.3 Glutamate Concentration

There was no significant effect of hydrocortisone infusion on prefrontal glutamate. The sphericity test was not significant, W = 0.53; df = 9; p = .75 and the degrees of freedom were unadjusted. The session by time effect was not

significant, $F_{(4,44)} = 0.71$; $p = 0.59$. Furthermore, there was no effect of time during either the hydrocortisone session, $F_{(4,44)} = 0.26$; $p = 0.90$, or during the placebo session, $F_{(4,44)} = 0.97$; $p = 0.43$.

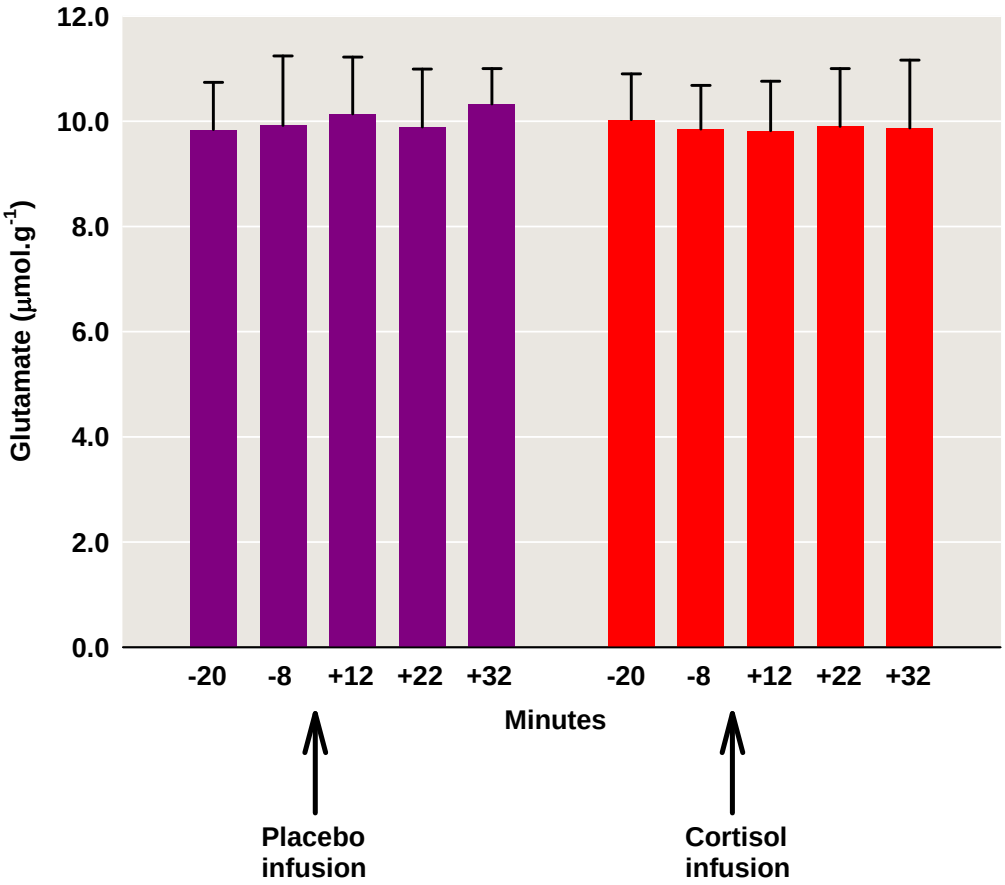


*Figure 10*: Glutamate concentrations (mean ± standard deviation) before and after the placebo and cortisol infusions.

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## 8.4 Glutamine concentrations

Glutamine concentrations decreased following hydrocortisone compared with placebo. The sphericity test was not significant, W = 0.77; df = 2; p = .26

and unadjusted degrees of freedom were used. The session by time effect was significant, $F_{(2,22)} = 5.51$; $p = 0.012$. The decrease in glutamine persisted from the first to the second time point: the time effect for the hydrocortisone session was significant, $F_{(2,22)} = 5.51$; $p = 0.012$ and post hoc tests showed that glutamine levels were decreased below baseline at both the +15 minute ($t = 2.94$; $df = 11$; $p = .013$) and + 25 minute ($t = 2.47$; $df = 11$; $p = 0.046$) time points. No significant time effect was observed for the placebo infusion, $F_{(2,22)} = 0.30$; $p = 0.74$. Compared with the same time points following the placebo infusion, glutamine concentrations did not differ at baseline ($t = 0.35$; $df = 11$; p .73) and were decreased at +15 minutes ($t = 2.34$; $df = 11$; $p = 0.039$), although the statistical significance of the decrease declined to trend level by +25 minutes ($t = 1.91$; $df = 11$; $p = 0.083$).

Glutamine concentrations decreased by 10.7 ± 13.0 % of baseline at + 15 minutes and by 6.6 ± 10.8 % of baseline at + 25 minutes following the hydrocortisone infusion.

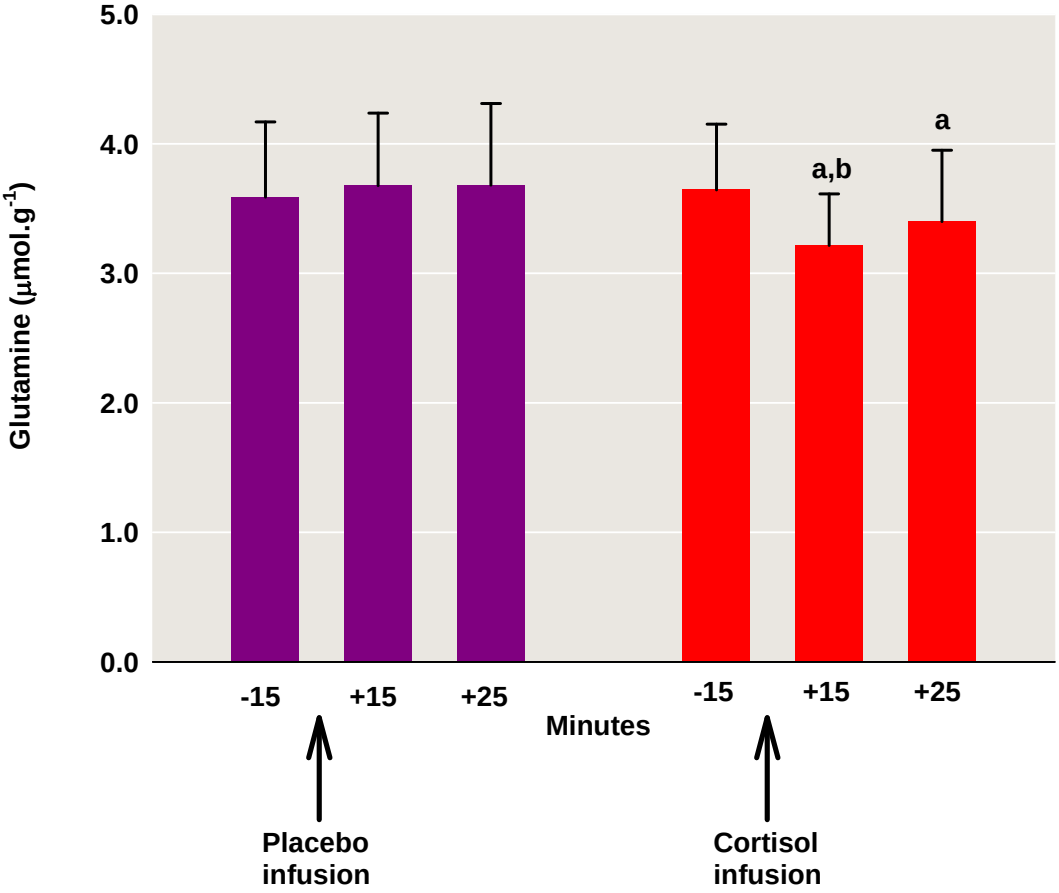


*Figure 11*: Glutamine concentrations (mean ± standard deviation) before and after the placebo and cortisol infusions, $p < .05$; [a]comparison with baseline; [b]comparison with placebo.

# Chapter 9

# Discussion

## 9.1 Major Findings

This study examined glutamate and glutamine concentrations before and after a single hydrocortisone injection of 35mg or placebo, in a randomized, double blind, within-subjects design. Metabolite concentrations were examined in a predominantly grey matter region of interest, the rostral anterior cingulate/medial prefrontal cortex.

The major result of the study indicates that exogenous hydrocortisone led to a rapid decrease in glutamine concentrations, in the absence of a change in glutamate concentrations. This is consistent with the hypothesis that cortisol would increase excitatory neurotransmission in this region, and consistent with increased turnover of glutamate and/or glutamine, leading to a decrease in their concentration.

The rapid time frame for the decrease in glutamine is consistent with a non-genomic effect, since the onset is not consistent with transcriptional events (Haller et al., 2008), but is consistent with evidence that stress rapidly increases release of glutamate into the extracellular space in prefrontal cortex and hippocampus (Mogghadam, 2003) and that glucocorticoids can rapidly increase synaptic glutamate in the hippocampus, independently of transcription (Venero & Borrell 1999; Karst et al., 2005).

Following the completion of the data collection for this study, independent research has provided evidence that an acute increase in excitatory neurotransmission is associated with an acute decrease in neuronal glutamine, rather than glutamate concentrations (Jenstad et al., 2009). One part of this study used quantitative immunogold labeling of intraneuronal glutamate and glutamine in conjunction with electrical stimulation of excitatory transmission in glutamatergic neurons of the rat hippocampus. Electrical stimulation resulted in a 20-30% decrease in intraneuronal glutamine in the dendrites and more markedly in the neuronal terminals, without significant changes in intraneuronal glutamate. The largest decrease in the glutamine/glutamate concentration ratio was in the neuronal mitochondria, the site of the conversion of glutamine to glutamate by phosphate-activated glutaminase. In another part of the study, using primary cultures of hippocampal pyramidal glutamatergic neurons, blockade of neuronal glutamine uptake transporters under depolarizing conditions led to a decrease in intraneuronal glutamate, consistent with glutamine uptake being required to replenish intraneuronal glutamate during excitatory neurotransmission. The Jenstad et al. (2009) research was limited with respect to interpreting the current findings by the fact that glutamate and glutamine were not also measured within astrocytes, since it is possible that increased glutamate release may lead to increased glutamine synthesis in astrocytes. If astrocytic glutamine synthesis occurred, the overall concentration of glutamine within the block of tissue contained within an MRS voxel might not change. Nevertheless, these data suggest that acute increases in excitatory neurotransmission are associated with

the maintenance of neuronal glutamate at the expense of consumption of glutamine; consistent with the interpretation that cortisol acutely increased excitatory neurotransmission in the present study. A possible heuristic explanation of these findings is that control of glutamate concentrations is critical for excitatory neurotransmission, whereas glutamine does not function as a neurotransmitter and its concentration may therefore not need to be under as tight control.

## 9.2 Strengths and Limitations

A major strength of the study is that it may provide an objective measure of cortisol effects on prefrontal neurochemistry. Previous studies of cortisol effects in healthy human subjects and in psychiatric disorders have focused on either peripheral effects (Checkley, 1996) or on cognitive measures (Het et al., 2005) that might be influenced by a number of other variables. The finding that cortisol has a measurable effect on neurochemistry might open the possibility of studying whether the effects of cortisol might differ between healthy subjects and subjects with psychiatric disorders, for example whether central responses to cortisol are decreased in MDD and increased in PTSD, as suggested by its peripheral effects.

A second strength of the study was the use of selective $^{1}$H-MRS techniques to measure glutamate and glutamine. Most $^{1}$H-MRS studies conducted at 1.5 T are not able to resolve glutamate and glutamine signals, or other contaminants in this region of the chemical shift spectrum, such as GABA or

glutathione. The inclusion of these signals in a compound of glutamate and glutamine (Glx) measure would probably have masked any changes in glutamine concentrations.

A third strength was that glutamate and glutamine concentrations were calculated as absolute values, rather than as ratios to Cr. The use of ratios to Cr might have reduced the power of the sample, by introducing variability of Cr levels into the calculations and have limited the interpretation of the results, in view of whether cortisol might alter Cr concentrations.

Another strength of the study is that by including healthy subjects who had prior experience of MR scans, we were able to show that plasma cortisol concentrations decreased during the placebo sessions. The absence of an increase in cortisol due to experimental stress may have helped to demonstrate an effect of exogenous cortisol.

Limitations of the study include the fact that baseline glutamine was measured only at one time point in each session. If baseline glutamine had been stable over at least two time points, this would have strengthened evidence that cortisol led to a significant change. However, the finding that there was no difference in baseline glutamine concentrations between the two sessions mitigates this as a source of error. Given that there was no change in glutamate concentrations, it would be practical to reduce the number of glutamate acquisitions in order to increase the number of baseline and post-infusion glutamine acquisitions in future studies.

Secondly, the glutamine peak includes small contributions from NAA and glutathione signals and is therefore not completely specific. However, simulation studies indicate that these sources of contamination do not exceed 10% of the signal (Choi et al., 2006). Since the decrease in glutamine exceeded 10%, it would be possible but not be likely that this resulted from a complete reduction in NAA and glutathione concentrations.

A third limitation was that the study examined the effects of only one dose of hydrocortisone and therefore does not provide evidence as to whether there is a dose-response relationship, or whether there is already a maximal effect at this dose. This would be important in planning future studies to assess whether changes in prefrontal glutamine in response to cortisol are different in psychiatric disorders. If the dose was too high, ceiling effects might prevent showing different responses in other subject groups.

The research presented did not examine sex differences or whether glutamine responses to cortisol might differ across the menstrual cycle. In moving forward to clinical studies, it might be important to exclude such effects, or to match patient and control groups carefully for sex and reproductive factors, such as menstrual phase or use of hormonal birth control.

Another limitation of the study was that it examined only rapid, non-genomic responses to cortisol. Although alterations in acute responses to stress and/or glucocorticoids may be relevant to understanding altered stress reactivity in psychiatric disorders, such disorders may involve repeated or chronic stress and it will also be important to study the effects of repeated or chronic differences in

cortisol levels, mediated by more classical effects on gene transcription (Joels et al., 2008). Indeed, such effects may be more relevant to chronic changes in HPA axis activity associated with psychiatric disorders.

### 9.3 Potential Contributions to Psychiatric Research

Psychiatric research using $^{1}$H-MRS has progressed rapidly from studies measuring the large singlet peaks of NAA and Cho, using ratios to Cr and without adjustment for grey and white matter voxel composition, to more recent studies using selective acquisitions and absolute quantification that takes account voxel tissue composition. The availability of methods for absolute quantification of glutamate and glutamine concentrations, together with the use of dynamic measures of metabolite concentrations in response to drug challenge advances the state of the art in the field. Furthermore, this new methodology provides new opportunities for clinical studies in patients with psychiatric disorders that were not previously available. In particular, although it has been shown that stress and glucocorticoids alter prefrontal glutamatergic neurotransmission, this has not previously been testable in living humans.

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