AN INTEGRATED APPROACH FOR DETECTING METABOLIC SHIFTS IN RESPONSE TO DRUG ADMINISTRATION AND DISEASE BY MAGNETIC RESONANCE SPECTROSCOPY

By

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To Chelsea and Barrett
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<tr>
<td>ADP</td>
<td>Adenine diphosphate</td>
</tr>
<tr>
<td>ALA</td>
<td>Alanine</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenine monophosphate</td>
</tr>
<tr>
<td>ASP</td>
<td>Aspartate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine triphosphate</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BCAA</td>
<td>Branched chain amino acids</td>
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<tr>
<td>BOLD</td>
<td>Blood oxygen level dependence</td>
</tr>
<tr>
<td>BW</td>
<td>Band width</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CHO</td>
<td>Choline</td>
</tr>
<tr>
<td>CPMG</td>
<td>Carr-Purcell-Meiboom-Gill</td>
</tr>
<tr>
<td>Cr</td>
<td>Creatine</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>dDNP</td>
<td>Dissolution Dynamic Nuclear Polarization</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNP</td>
<td>Dynamic Nuclear Polarization</td>
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<td>DWI</td>
<td>Diffusion weighted imaging</td>
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<td>FID</td>
<td>Free induction decay</td>
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<td>fMRI</td>
<td>Functional Magnetic Resonance Imaging</td>
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<tr>
<td>FWHM</td>
<td>Full width at half maximum</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-amino butyric acid</td>
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<td>GDH</td>
<td>Glutamate dehydrogenase</td>
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<td>GLC</td>
<td>Glucose</td>
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<td>GLN</td>
<td>Glutamine</td>
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<td>GLT</td>
<td>Glutamate transporter</td>
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<td>GLU</td>
<td>Glutamate</td>
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<tr>
<td>GLUT</td>
<td>Glucose transported</td>
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<td>GS</td>
<td>Glutamine synthetase</td>
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<tr>
<td>HIPP</td>
<td>Hippocampus</td>
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<td>HP</td>
<td>Hyperpolarization</td>
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<td>HRMAS</td>
<td>High resolution magic angle spinning</td>
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<td>IACUC</td>
<td>Institute for Animal Care and Use Committee</td>
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<td>ISIS</td>
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<tr>
<td>JRES</td>
<td>J-resolved spectroscopy</td>
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<tr>
<td>KBr</td>
<td>Potassium bromide</td>
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<tr>
<td>KIC</td>
<td>2-ketoisocaproate</td>
</tr>
<tr>
<td>LAC</td>
<td>Lactate</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LNAA</td>
<td>Large neutral amino acids</td>
</tr>
<tr>
<td>LPM</td>
<td>Liters per minute</td>
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<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MDPV</td>
<td>3,4-Methylenedioxyxpyrovalerone</td>
</tr>
<tr>
<td>MI</td>
<td>Myo-inositol</td>
</tr>
<tr>
<td>MM</td>
<td>Macromolecules</td>
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<tr>
<td>MR</td>
<td>Magnetic resonance</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<tr>
<td>MRS</td>
<td>Magnetic Resonance Spectroscopy</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MSME</td>
<td>Multi slice multi echo</td>
</tr>
<tr>
<td>MW</td>
<td>Microwave</td>
</tr>
<tr>
<td>NAA</td>
<td>N-Acetyl aspartic acid</td>
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<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
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<tr>
<td>NAD+</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>NOESY</td>
<td>Nuclear Overhauser effect spectroscopy</td>
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<tr>
<td>PA</td>
<td>Phenylalanine</td>
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<tr>
<td>PAH</td>
<td>Phenylalanine hydroxylase</td>
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<tr>
<td>PC</td>
<td>Phosphocholine</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatine</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PKU</td>
<td>Phenylketonuria</td>
</tr>
<tr>
<td>PPM</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PQN</td>
<td>Probabilistic quotient normalization</td>
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<tr>
<td>PRESS</td>
<td>Point resolved spectroscopy</td>
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<td>PROJECT</td>
<td>Periodic refocusing of J-evolution by coherence transfer</td>
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<td>Pyruvate hydrate</td>
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<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>RARE</td>
<td>Rapid imaging with refocused echoes</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-Noise ratio</td>
</tr>
<tr>
<td>STEAM</td>
<td>Stimulated echo acquisition mode</td>
</tr>
<tr>
<td>TAU</td>
<td>Taurine</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TEMPO</td>
<td>2,2,6,6-Tetramethyl-1-piperidinyloxy</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>TSP</td>
<td>Trimethylsilylpropanoic Acid</td>
</tr>
<tr>
<td>UDG</td>
<td>Uridine diphosphate glucose</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
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Psychostimulant drug abuse is associated with profound psychological, behavioral, and neurochemical effects. Cocaine and bath salt constituents both act on the dopamine reabsorption system, blocking removal of neurotransmitters from the synapse. Furthermore, these drugs have been shown to have a neurometabolic affect, dysregulating basal metabolic processes such as glucose transport and oxygen utilization. Neurochemical profiling of regions of reward in the brain reveals a distinct metabolic response to drug administration and disease. *In vivo* spectroscopic analysis of small molecule metabolites in the brain provide key biomarkers that elucidate dysregulation of specific metabolic pathways.

The purpose of this study is to investigate the metabolic pool shifts associated with acute psychostimulant administration through a multimodal magnetic resonance approach, both *in vivo* and *ex vivo*. Our findings indicate a decoupling of normal neuron astrocyte cooperative exchange and a significant overall increase in excitatory neurotransmitter biosynthesis on administration of cocaine and MDPV. In addition, we observe a shift in lactate production when using hyperpolarized pyruvate as a tracer of
cerebral metabolic function. *In vivo* and tissue analytical techniques were also applied to characterize metabolic shifts with late stage neurological decline due to Phenylketonuria. These studies demonstrate how novel metabolic flux measurements, combined with high field magnetic resonance measurements of metabolic pools, can provide key metabolic insights into the effect of acute drug action and inherited metabolic disease on brain metabolism. The finding presented here identify potential biomarkers for neurological decline and present novel approaches for understanding the indirect metabolic effects of acute psychostimulant administration.
CHAPTER 1
PRINCIPLES OF BRAIN METABOLISM

A Brief History of Cerebral Metabolism and Magnetic Resonance Applications

The study of cerebral blood flow and metabolism has long been the subject of intensive study in field of biomedical research. The earliest record connecting cerebral blood flow to brain activity dates to the Greeks where they observed that compression of the carotid arteries had a profound effect on the state of consciousness to the extent that arterial compression was used as an early form of anesthesia\(^1\). The first laboratory based study was performed in the late 1800’s where investigators noted that a change in cerebral blood pressure directly caused an effect on cerebral vessel diameter\(^2,3\). Later, the field of cerebral metabolism was advanced by Kety and Schmit when the first nitrous oxide study was conducted to experimentally determined the value of cerebral blood flow\(^4,5\). This work was revolutionary because, for the first time, cerebral blood flow was observed in an un-anaesthetized human. While the nitrous oxide method uncovered that it was possible to monitor gas exchange and diffusion in the brain, it did not answer the questions of regional functional activation or energy source utilization. The next major scientific landmark came with the application of autoradiography of nonvolatile radioactive carbon tracers\(^6\). The first experiment connecting blood flow to metabolic function was carried out in 1975 by Kennedy and Sokoloff by administering \([^{14}\text{C}]\)deoxyglucose to animals\(^7\). Here, Kennedy demonstrated that it was possible to monitor glucose consumption and utilization by region, the first step towards metabolic mapping of the brain. In 1986 Fox and Raichle contributed a seminal work in the field of cerebral metabolism when they observed a coupling between cerebral blood flow and oxygen consumption\(^8\). Here, they demonstrated, through the use of \(^{15}\text{O}\)-labeled
radiotracers, that cerebral blood flow was dramatically increased with brain stimulation, while metabolic rates were only moderately increased (29% and 5% increase respectively), indicating that blood flow could be uncoupled from metabolic rates in vivo. This discovery spurred further investigation of local metabolic activity independent of blood flow. The end of the 20th century saw an explosion of modern technology development for neuroscience including the mapping of functional activation, blood flow, and quantification of key metabolites. These advancements relied on molecular imaging via positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS).

The introduction of non-invasive MRSI techniques into cerebral metabolic research has been instrumental in understanding the structure, function, and chemical makeup of the brain. Nuclear Magnetic Resonance (NMR) is the foundation of all magnetic resonance techniques and has played a fundamental role in understanding cerebral metabolic function because it can provide chemical, anatomical, and flow information without the patient being exposed to ionizing radiation sources. While the phenomenon of NMR was discovered in 1940 by Felix Bloch and Edward Purcell, spatially localized MR, through the application of magnetic field gradients, was not discovered until 1973, independently by Peter Mansfield and Paul Lauterbur 9-11. Their work laid the foundation for the advancement of MR based techniques including functional MRI (fMRI) via blood oxygen level dependence (BOLD), diffusion weighted imaging (DWI), and magnetic resonance spectroscopy (MRS). Although much emphasis is placed on the anatomical imaging capability of MR, rich biochemical
information is possible through the implementation of cerebral MR spectroscopy. MRS uses strong magnetic fields and radiofrequency (RF) waves to determine levels of chemicals present in the tissue of interest\textsuperscript{12}. Specifically, MRS provides a platform for analyzing individual chemical compounds with high spatial (millimeter) and temporal (sub-second) resolution\textsuperscript{13}. Multinuclear approaches are currently being conducted to answer fundamental cerebral metabolic questions by tuning RF coils to specific frequencies. For example, neurotransmitter synthesis and activity can be monitored through both $^{13}$C and $^1$H approaches while bioenergetics and pH measurements can be obtained through $^{31}$P MRS. Neurochemical profiling through MRS has been shown to be an invaluable diagnostic tool for ADHD, depression, cancer, bipolar disorders, Parkinson’s disease, substance abuse, mitochondrial diseases, and many others\textsuperscript{14-19}.

Molecular profiling of drug action has been of particular interest in recent years due to the recent substance abuse epidemic\textsuperscript{20}. In substance use disorders, which affect the US and worldwide populations, altered cerebral metabolism is hallmark. Studies using PET have shown dysregulation of glucose metabolism during periods of acute drug action and drug craving\textsuperscript{21-23}. NMR studies have shown shifts in energy and membrane associated metabolites and MRI have shown changes in CBF\textsuperscript{24-27}. Details on the application of NMR to the study of cerebral metabolism in drug use disorders are further detailed in Chapters 3 and 4.

**Substrate/Product Reactions**

This chapter details basics metabolic pathways for the purpose of describing the biochemical mechanisms that are foundational to this work. For the sake of brevity, enzymatic reactions that are not pertinent to this work have been omitted. Metabolism, generally described, is the complete series of biochemical reactions (pathways) which
enables the following: providing high energy substrates to cells, maintaining basic functions, and enabling cell-type specific biosynthesis. Eukaryotic metabolism is uniquely partitioned into organelles, each with their own metabolic pathways. Mitochondria are the site of ATP generation, through respiration, as well as glutamate synthesis, fatty acid oxidation, and gluconeogenesis. Mitochondria are structurally composed of an outer membrane and an inner, highly folded, membrane (crista). The crista surrounds the mitochondrial matrix and is the site of ATP synthesis. Although mitochondria are central players in many metabolic processes, neurometabolic reactions occur throughout multiple cellular compartments as well as in the extracellular spaces between cells.

Metabolic pathways are a series of coupled chemical reactions in which molecules (substrates) are converted into different molecules (products) through the input of an activation energy (ΔG). In the absence of catalysts, the activation energies (ΔGuncat) for each reaction are very large and the reactions proceeds very slowly. The interaction of a substrate with an enzyme catalyst dramatically reduces the activation energy (ΔGcat) required to generate a product (Figure 1-1). In a single-substrate model, one substrate (S) is converted into a product (P) through an enzyme-substrate (ES) and enzyme-product transition (EP) complex as described by Equation 1-1.

\[
E + S \leftrightarrow ES \leftrightarrow EP \leftrightarrow E + P
\]  

(1-1)

The interaction of the enzyme with the substrate significantly decreases the activation energy required to form the product. The sum of the activation energies required to form the ES and EP is substantially less than it would be to form the transition state without the enzyme presents thus the enzyme, often dramatically, increases the rate of the
reaction. For a two-substrate reaction, a tertiary complex (EAB) is formed with the first substrate (A) and the second substrate (B) via three distinct mechanisms: ordered sequential, random sequential, or ping-pong\textsuperscript{30}. For the purposes of this work, only ordered sequential and random sequential mechanisms will be discussed (Figure 1-2). The ordered sequential two-substrate model is a reaction where the enzyme forms a complex with each substrate individually to form the EAB transition complex. An example of an ordered sequential mechanism is the pyruvate to lactate conversion by the lactate dehydrogenase enzyme (LDH) and is of particular interest of this work. In the forward reaction, NADH always binds first to LDH then pyruvate binds as the second substrate into the transition state. NADH is then oxidized into NAD\textsuperior, followed by the release of the lactate product. Pyruvate to lactate conversion is a key reaction that is characterized in later chapters as an indicator of the glycolytic state of the cell. LDH kinetics can be exploited as a direct maker of energy metabolism as discussed later in this chapter. By contrast, random sequential mechanisms are bound and released in no particular order. Random sequential reactions are common among ATP kinases, including the creatine to phosphocreatine reactions and are fundamental to bioenergetic reactions in the brain. Together, these two mechanisms outline important enzymatic processes carried out in basal neuro-metabolic reactions and are key to important metabolic reactions characterized in this work.

**Enzyme Kinetics**

In addition to the free energy component of enzymatic reactions, rate velocities should also be considered. Substrate-to-product conversion as a function of time is an indicator of the metabolic state of the cell or organ system\textsuperscript{31}. Many endogenous and
Exogenous factors can shift this rate including negative feedback mechanisms, enzymatic function, and substrate availability.

\[ v = \frac{d[A]}{dt} = k[A] \]  

(1-2)

Enzyme kinetics is the analysis of the dynamic rate velocity of metabolic reactions where the velocity of the reaction is equal to the change in initial compound concentration \((S)\) over change in time \((t)\) and \(k\) is the rate of the reaction as expressed in Equation 1-2. This rate constant can then be applied in the enzyme catalyzed formula where \(k_1\) is the rate of ES transition complex formation and \(k_2\) is the rate of formation of the product (Equation 1-3).

\[ E + S \xleftrightarrow{k_1} ES \xleftrightarrow{k_2} E + P \]  

(1-3)

In a biological system, substrate concentrations are often much greater than the concentration of enzyme. In this environment, \(k_2\) rates are the slower and the rate determining reaction step. The maximum rate of an enzyme mediated reaction is reached when the enzyme is completely saturated with substrate and varies between biological enzymes. This value \((V_{max})\) is calculated by fitting an exponential curve to a substrate titration and is the theoretical maximum velocity of the reaction. Since kinetic rate does not increase infinitely, experimentally, this maximum value is plotted as a function of substrate concentration with the \(V_{max}\) equal to the highest velocity value for a specific enzyme.

Leonor Michaelis and Maud Menten first introduced their acclaimed Michaelis-Menten equation in 1913 while studying the interaction of the Invertase enzyme with varied sucrose concentration. They noted that the addition of more sucrose to their reaction vessel increased the rate at which the sucrose was metabolized. The
Michaelis-Menten constant ($K_M$) is equal to the substrate concentration at which the velocity of the reaction is half of the $V_{max}$ (Figure 1-3). When $S >> K_M$, the reaction velocity ($v_0$) approaches $V_{max}$ rapidly in a first order reaction. Alternatively, if $S << K_M$ the reaction corresponds to a near zero order reaction (Equation 1-4).

$$v_0 = V_{max} \frac{[S]}{[S]+K_M} \quad (1-4)$$

### Regulation of Metabolic Activity

*In situ* regulation of enzymatic activity is crucial for basic biological function. By specifically controlling the rate of individual reactions, a biological system can react to exogenous or endogenous stimuli. There are two general categories for enzymatic regulation based on time scale: regulation of enzymatic quantity (slow) and regulation of enzymatic activity (rapid). Slow regulation of enzyme quantity is accomplished by increasing or decreasing the amount of enzyme present in the biological system. This type is achieved through regulation of gene expression, mRNA stability, enzymatic post-translational turnover, or production of stores of inactive zymogens. Rapid regulation of enzymatic activity *in vivo* is key to expeditious reactivity to metabolic needs on a time scale that cannot be accomplished by changes in enzyme quantity. As discussed previously, concentration of available substrate dictates the rate of enzymatic activity as observed in basic Michalis-Menten kinetics. Additionally, other mechanisms are present to further regulate enzyme activity on a sub-second to minute timescale. The first class of rapid enzyme regulation is the inhibitors. Competitive inhibition is accomplished when an inhibitor reversibly binds to the active site of an enzyme's active site, preventing the formation of the enzyme substrate complex. By contrast, noncompetitive inhibition is accomplished when the inhibitor binds to the non-active site on the enzyme, changing
the protein conformation and altering the affinity for the substrate at the active site. Finally, uncompetitive inhibition is when the enzyme-substrate complex is bound by an inhibitor, prohibiting release of the product from the enzyme-substrate complex.

Furthermore, allosteric regulation and covalent modification are two mechanisms that do not directly affect the enzyme active site while regulating the activity at the active site. Allosteric regulation is an oligomeric regulatory process in which the enzyme itself is cooperatively regulated (either positively or negatively) through a site other than the active site. In allosteric regulation, ligands bind to a non-active site on one or more oligomeric subunits causing a conformation change and a change in the velocity of the reaction at the active site. By contrast, covalent modification may involve reversible or irreversible reactions in which an inactive enzyme is activated by addition of a covalently bonded group. By far the most common form of this type of regulation are phosphorylation events such as observed in glucose catabolic regulation. Regulation of enzymatic processes, either directly or indirectly, alter the affinity for substrate metabolism shifting the biochemical equilibrium and affecting the rate of a reaction.

**Anaplerosis and Cataplerosis**

Most metabolic pathways are a complex network of simple biochemical reactions. Taken as a whole, these pathways are regulated at specific steps to control the pools of available intermediates needed to carry out basic or specialized metabolic functions. Metabolism is in constant fluctuation as pools are depleted and replenished by upstream carbon sources. The term “anaplerotic sequences” was coined by Hans Kreb’s mentee Hans Kornberg describing the influx of “ingredients”, such as carbohydrates, acetate and glyoxylate, to replenish bacterial TCA cycle intermediates. By contrast, cataplerosis is a system in which the intermediate pools are being depleted
for the purpose of biosynthesis\textsuperscript{34}. The balance between anaplerotic and cataplerotic events is crucial for basic cell function as well as the cell’s ability to response to external stimuli. Furthermore, exchange of all nitrogen and carbon intermediates are accomplished through either a cataplerotic or anaplerotic reaction. The distribution of metabolic pools involved in major metabolic pathways indicates the biosynthetic needs of the cell and are specifically discussed later in this chapter.

**Cerebral Metabolism**

The brain has an extraordinarily high basal metabolic rate with resting energy demands, on the order of 20\% of the bodies’ total metabolic consumption, despite only being \(\sim 2\%\) of the total body weight\textsuperscript{35-37}. Additionally, 10\% of the blood flow from resting cardiac output is used to oxygenate the brain\textsuperscript{38}. This high resting energy demand is required to sustain a variety of functions including maintaining resting state biochemical reactions, biosynthesis of neurotransmitters, and maintenance of electrochemical gradients\textsuperscript{36}. Because of very low glycogen storage capabilities, the brain requires constant replenishing of glucose to maintain basic functioning even to the point of shunting from other organ systems during periods of starvation or hypoglycemia\textsuperscript{39}. Circulating glucose is the requisite energy source for the brain. Glucose is transported into the cell through specific glucose transporters (GLUT) after which it undergoes glycolysis to pyruvate and enters the TCA cycle through Acetyl-CoA. Through the TCA cycle, glucose is oxidized to produce \(\text{CO}_2\), water, and ATP (through the electron transport chain). The brain’s high energy requirements are primarily due to replenishing electrochemical gradients after neurotransmission. Following neuronal synaptic transmission, the membrane-bound enzyme, \(\text{Na}^+\), \(\text{K}^+\)-ATPase, restores resting membrane potential necessary for continued transmission\textsuperscript{40}. Additionally, \(\text{Na}^+\), \(\text{K}^+\)-
ATPase is responsible for excitatory neurotransmission, Ca^{2+} regulation, and maintenance of cell volume. Basal neuronal ATPase activity is the central reason the brain has such high resting ATP requirements. In addition to its high energy requirements, the brain is a highly heterogeneous organ with complex network systems. These networks have their own unique modular organization, characterized by extensive axonal projections and synaptic connectivity. While neuronal electrochemical exchanges at the synapse have been well characterized, the metabolic profiles necessary for cerebral function as it responds to stimuli has not been fully characterized. Cerebral metabolic processes are fundamental to every action performed by the organ system including maintaining basic cellular function by producing ATP, reestablishing electrochemical gradients, and producing or recycling neurotransmitters.

**Substrate Delivery and Blood Brain Barrier**

The brain is separated from the peripheral vasculature by the blood brain barrier (BBB). As a result of the protection conferred by the BBB, the brain maintains its own cerebral blood flow and metabolic exchange in an autoregulatory capacity, which is independent of the peripheral blood flow to an extent. The blood brain barrier is made up of tight junctions between the endothelial cells lining the cerebrovascular interface. The so-called “gliovascular unit” contains a microvascular network surrounded by multiple astrocytic perivascular endfeet. The physical interface barrier between the BBB and the brain prevents infection and the selective bidirectional transport or exchange of nutrients and metabolic intermediates. The BBB allows free diffusion of gases (O\textsubscript{2} and CO\textsubscript{2}) and many small lipophilic molecules while inhibiting transport of large molecules and hydrophilic metabolites. Hydrophilic metabolites are actively transported from the microvasculature into the brain by transporters proteins. These are especially important
when considering the influx of metabolic constituents such as glucose, amino acids, and metabolic intermediates as discussed in this work. Glucose, the major energy source for brain metabolism, is actively transported through the BBB by GLUT1. GLUT1 facilitates transendothelial transport of glucose via its presence on endothelial cells on both the microvasculature-facing surface and the interstitial-side facing astrocytic processes\textsuperscript{44}. Furthermore, some small monocarboxylate metabolites, including pyruvate, lactate, and ketone bodies, have been shown to be transported across the BBB by Monocarboxylate transporter 1 (MCT1)\textsuperscript{45,46}. The MCT and GLUT transporters are highly expressed on both side of the BBB, with $V_{\text{max}}$ rates of 91 and 1,420 nmol/min/g, respectively\textsuperscript{46}. Although normally impermeable to large and hydrophilic molecules, disruption of the BBB has been reported in traumatic brain injury and stroke, leading to neuroinflammation, ionic imbalance, and edema\textsuperscript{47-49}. Permeability of the BBB has also been shown to be affected by ultrasound disruption and certain anesthetics\textsuperscript{50,51}. It has been reported that the general anesthetic, isoflurane, has measureable effects on BBB permeability to pyruvate\textsuperscript{52}. Therefore, it is important to carefully regulate the dose of general anesthetic administered during metabolic experiment, especially in the case of hyperpolarized tracer experiments as discussed in later chapters. Furthermore, when considering metabolic transport and glucose delivery it is important address intercellular and extracellular volume fractions. Quantification of extracellular volumes is an indicator of cellular effusion, metabolic clearance, and cellular transport\textsuperscript{53-55}. Previous sodium MRI studies have observed a 1:2 ratio of intercellular to extracellular sodium concentration in the brain at steady state\textsuperscript{56}. By comparison, diffusion MRI work has indicated that glucose is nearly equally distributed between intracellular and
extracellular spaces while vascular glucose was 2-3 times higher than it is in the brain. This suggests that the rate limiting glucose transport in the brain is through the BBB\(^5\).

**Astrocyte and Neuronal Cell Metabolism**

The brain is composed of four major cell types, neurons, astrocytes, oligodendrocytes, and microglia\(^4\). Although neurons are the primary cell type involved in CNS neurotransmission and communication, the other cell types comprise roughly half of the brain's cells have distinct and specialized functions. Microglial cells compromise 10-15% of the total number of cells and are specialized to carry out immune responses in the central nervous system\(^5\). Lipid rich oligodendrocytes myelinate the axon of adjacent neurons increasing the speed of neuronal action potentials\(^5\). The last two major cell types in the brain, astrocytes and neurons, are of particular interest to this work because they have distinct metabolic profiles that are complementary and coupled through metabolic transport mechanisms\(^2\).

As previously discussed, brain's high energy demands are due primarily to the ATP required to restore and maintain synaptic and actions potentials as well as generate and recycle neurotransmitters. Of these, synaptic potential has been shown to require the most energy\(^6\). Neuronal synaptic transmission is a temporary depolarization of the presynaptic membrane potential resulting in either release or inhibition of ion channels which release neurotransmitters into the synaptic cleft. Excitatory synaptic potential, through glutamate neurotransmission, has been shown to be responsible for a majority of energy expenditure (~80%)\(^6\). Because of this high energy demand, the neuron is constant need for a replenished ATP supply especially under periods of high neurotransmission. Metabolically, the neuron is highly active with multiple mitochondria that migrate and respond to neuronal activity. Neuronal
mitochondria are dynamic and modal within the neuron, dependent on the metabolic needs of the cell\textsuperscript{63}. Neurons rely heavily on oxidative metabolism to generate the ATP required for basal cellular function as well as to reestablish electrical action potentials. Paradoxically, neurons consume a relatively small amount of glucose directly, they primarily rely on carbon input through lactate supplementation from astrocytes to maintain their significant ATP demands\textsuperscript{64}. Astrocyte-derived lactate is delivered through the MCT1 isoform from the astrocyte end feet\textsuperscript{35}.

By contrast, astrocytes have highly glycolytic metabolisms and are the main consumers of glucose uptake in the brain via the astrocytic GLUT1. Glucose transported into the astrocyte is preferentially converted to lactate and supplied to neurons through the lactate shuttle, playing an important supportive role through the metabolic coupling with neurons.

**Metabolic Intercellular Coupling**

Prior work has shown the exchange metabolic substrates between astrocytes and neurons can vary with energy demands\textsuperscript{65}. This regulation of intercellular exchange of energy substrates directly correlates with neuron activity and provides a protective component for neurons under hypoxic or glucose depleted episodes\textsuperscript{66-68}. Glucose undergoes glycolytic breakdown to two molecules of pyruvate. Pyruvate then either enters the TCA cycle as Acetyl-CoA or is converted to lactate via the Lactate Dehydrogenase 5 (LDH5) enzyme\textsuperscript{69}. Immunohistochemical analysis of LDH isoforms have shown that LDH5 is highly expressed in astrocytes while neurons express only low levels of the LDH5 isotype\textsuperscript{70}. Furthermore, LDH5 demonstrates a high affinity for pyruvate, promoting a more glycolytic equilibrium and producing high levels of lactate for delivery to the neuron\textsuperscript{70}. Glucose-derived lactate, from astrocytes cells, is supplied to
the neuronal cells via MCT1,4 transporters located on neuronal membranes\textsuperscript{35}. The lactate shuttle between the neuron and astrocyte facilitates an additional level of metabolic regulation for neuronal function. Lactate, supplied from astrocytic glycolysis, has been shown to have a neuroprotective effect on neurons during periods of high synaptic activity\textsuperscript{71}. Lactate’s role in cooperative metabolism should not be understated. It has been shown that lactate alone can supply the basal metabolic needs of the TCA cycle without any contribution from glucose directly\textsuperscript{72}.

Neurotransmitters also have a cerebral metabolic effect. The two most abundant neurotransmitters in the mammalian CNS are glutamate and gamma-aminobutyric acid (GABA). Glutamate is traditionally described as an excitatory neurotransmitter while GABA is an inhibitory neurotransmitter. Structurally, the two molecules only differ by an extra carboxylic acid moiety on glutamate. Both are released from the presynaptic neuron at the axon terminus via synaptic vesicle transport (Figure 1-4). Neurons with a high density of glutamate receptors on the post-synaptic membrane are classified as excitatory neurons. By contrast, neurons expressing high GABA receptors are inhibitory GABAergic neurons. In addition to the neurotransmitter effects on post-synaptic receptors, both molecules are recycled by astrocytes into glutamine for the purpose of recycling nitrogen and carbon pools as well as preventing damage to the neuron during periods of high neurotransmission. Glutamate is especially present in the cortical and limbic structures of the brain\textsuperscript{73}. Glutamate is a cataplerotic product of the TCA cycle, produced from alpha-ketoglutarate by Glutamate Dehydrogenase (GDH). Extracellular glutamate in the synaptic cleft can be transported into astrocytes by the excitatory amino acid transporter 1 (EAAT1 or GLT1) and converted into glutamine. This is
facilitated by astrocytes to prevent glutamate accumulation and subsequent neurotoxicity\textsuperscript{74,75}. Conversion of the glutamate neurotransmitter to the non-excitatory glutamine by astrocytes accomplishes three major goals: neutralization of excitotoxic molecules, recycling of glutamate into glutamine energy stores, and cooperative feedback by stimulating increased glucose uptake and lactate output in astrocytes\textsuperscript{76-78}. GLT1 is highly expressed on the cell surface of astrocytes near the neuronal synaptic cleft and sparsely expressed on the neuronal presynaptic membrane\textsuperscript{79}. Within the cytoplasm of the astrocyte, glutamate is converted into glutamine by glutamine synthetase (GS). Glutamine is supplied back to the neuron where glutamine is readily available for production of additional glutamate\textsuperscript{35}.

There is considerably less known about regulation of exchange for other metabolites between astrocytes and neurons, such as aspartate. Aspartate is, like glutamate, a cataplerotic product of the TCA cycle. Aspartate is primarily produced from oxaloacetate and can by synthesized by both neurons and astrocytes\textsuperscript{80}. Aspartate transport is bidirectional between neurons and astrocytes through the glutamate/aspartate (GLAST) transporter\textsuperscript{81}.

Branched chain amino acids (BCAAs; valine, leucine, and isoleucine) also play an important role in neuron-glial intercellular exchange. BCAA’s are introduced into the exchange from the vascular system through astrocytic transport\textsuperscript{80}. They are commonly presented as nitrogen donors for neuronal glutamate synthesis through α-ketoisocaproate exchange\textsuperscript{82-84}.

**Lactate Metabolism**

Otto Warburg proposed his Nobel prize winning hypothesis in 1924 on the importance of cellular glycolysis as an indicator of cellular cancer\textsuperscript{85}. In this work, he
noted the rate of glycolysis increased in cancer cells shifting the pyruvate/lactate equilibrium towards higher production of lactate. There are several explanations to the reason behind the “Warburg effect” in cancer cells including mutation of tumor suppressors, activation of oncogenes, or survival in hypoxic conditions\textsuperscript{86,87}. This observation has proven very popular in modern day metabolic approaches towards cancer diagnosis especially in the field of magnetic resonance spectroscopy and hyperpolarization. Polarization of pyruvate provides an excellent point of view for quantifying this glycolytic reaction and is an effective means to diagnose cancer nodes especially in regions where tumor masses are difficult to image\textsuperscript{88-90}. In these works, LDH flux towards lactate is used as a diagnostic marker for highly glycolytic tumor cells providing accurate nodes for interventional treatment. LDH is an important enzyme for not only cancer diagnosis, but sheds light on the metabolic state of a cell. Lactate also provides an important energy bridge between neuron and astrocytic metabolism. As previously described, the glial glycolytic product lactate is an important substrate for TCA oxidative metabolism in neurons. Although LDH activity is an accurate predictor of brain cancer\textsuperscript{91}, we show that it is also useful in gaining insight into the metabolic balance between astrocytes and neurons.

Lactate dehydrogenase is a tetrameric cytosolic enzyme with 5 isotypes encoded by two genes, LDH-A and LDH-B, to produce LDH-M and LDH-H monomers respectively. Each LDH tetramer is a combination of the LDH-M/H monomeric units leading to M\textsubscript{4}, MH\textsubscript{3}, M\textsubscript{2}H\textsubscript{2}, M\textsubscript{3}H or H\textsubscript{4}. The LDH-1 isoenzyme is composed of four H monomeric units while LDH-5 is a homotetramer of the LDH-M monomers. LDH-M monomers have a higher affinity for pyruvate while LDH-H monomers have a higher
affinity for lactate. A LDHM:LDHH ratio is often used to quantify the glycolytic vs. oxidative state of the cell with a high level of LDH-M present in more glycolytic cells. As previously described, the brain expresses higher levels of isoforms 1 and 5 in the neuron and astrocyte, respectively. This difference indicates the astrocyte is more glycolytic while the neurons has more oxidative capacity. Regulation of LDH can occur either through long term regulation of protein expression or more rapid post-transcriptional modification of LDH via substrate regulation. Compensatory LDH protein expression has been reported by Hypoxia Induced Factor 1 (HIF1), estrogen, cyclic AMP, and other pertubations. Regulation of the LDH protein has been previously reported at the transcriptional level under to hypoxic conditions. LDH activity on the timescale relevant to this work is primarily accomplished through substrate regulation or enzymatic availability. LDH is a non-allosteric enzyme and so is only directly regulated through substrate availability or concentrations of pyruvate, lactate, NAD+, and NADH. Indirect control of LDH activity through substrate availability can occur via regulation of phosphofructokinase and pyruvate dehydrogenase activity. Additionally, LDH availability has been shown to decrease under cytotoxic conditions due to release from cytoplasm.

In regards the Warburg effect, the brain displays a unique feature due to the glycolytic state of the astrocyte and the redox state of the neuronal cell. The brain undergoes a reverse Warburg effect in which it increases oxidative phosphorylation from the lactate substrate. This occurs in the neuron when lactate is preferentially converted to pyruvate to enter the TCA cycle. The reverse Warburg effect is complemented by the redox switch hypothesis which states that when lactate is
delivered to the neuron by astrocytes, NAD+/NADH ratios are shifted towards a more reduced state which decreases glycolysis through GAPDH to increase neuronal biosynthesis and reduce harmful reactive oxygen species\textsuperscript{99}.

Together, the coupling of astrocyte and neuronal lactate metabolism through LDH ensures overall neuronal competence and health. This complementary system is complex, reacting to the energy needs and electrochemical expenditure of the neuron. Exchange of metabolic intermediates are both dynamic and adaptable, able to respond to stimuli by supportive cooperation. The work highlights disruption of these metabolite pools as biomarkers for psychostimulant drug administration. Due to the complexity of this metabolic system, a multmodel MR based approach was used to quantify metabolic pools as well as pyruvate to lactate kinetics to characterize the metabolic state of the brain in response to acute drug administration.
Figure 1-1. Free energy diagram. $\Delta G^{\text{uncat}}$ and $\Delta G^{\text{cat}}$ are the changes in free energy of the uncatalyzed and catalyzed reactions respectively.
Figure 1-2. Two-substrate enzyme models. A) Ordered sequential mechanism. B) Random sequential mechanism.
Figure 1-3. Michaelis-Menten saturation curve. $V_{\text{max}}$ is equal to the rate velocity at maximum value and $K_m$ is the enzyme concentration at half of the $V_{\text{max}}$. 
Figure 1-4. Neuron/Astrocyte metabolic exchange diagram. Cerebral metabolism is primarily supplied through circulating glucose, through either direct metabolism into neuronal cells or through glycolytic astrocytic metabolism. Lactate is shuttled between the cells to supply neuronal metabolic needs and for biosynthesis of neurotransmitters GABA and glutamate. Abbreviations: Lac – lactate, Glc – glucose, N donor – nitrogen donor (BCAA, etc), Pyr – pyruvate, Glu – glutamate, Gln – glutamine.
CHAPTER 2
METABOLOMICS METHODS

Introduction to Metabolomics

Metabolomics is the study of low molecular weight compounds (<1.5 kDa) in biological tissue and biofluids for the purpose of identifying changes in response to disease, external stimuli, or degeneration\(^{100-103}\). The terms metabonomics and metabolomics were coined by Nicholson \textit{et al} and Fiehn \textit{et al} respectively in landmark articles on the multiparametric characterization of biological metabolites\(^{104,105}\).

Metabolomics is the quantitative measurements of phenotypic markers, ideally a simultaneous acquisition of all observable metabolic pathways. In concert with other areas of “Omic” biology (e.g., genomics, proteomics, transcriptomics), the field of metabolomics is a search for biomarkers of health and disease in a complex and rich dataset. Metabolomics has two major branches, targeted and untargeted. Targeted metabolomics is specific to well-defined and known metabolic pathways, including techniques such as \textit{in vivo} spectroscopy, isotopomer NMR, and mass spectrometry\(^{106}\). Targeted metabolomics, more recently, includes dissolution dynamic nuclear polarization (dDNP). dDNP is a technique by which transient polarized tracers are administered and enzymatic processes are observed in real time. By contrast, untargeted metabolomics is the global metabolic analysis of a sample that contains unknown compounds or unidentified shifts in pathways\(^{107}\). Untargeted metabolomics includes \textit{in vivo} and \textit{ex vivo} approaches to metabolic profiling of systems in response to stimuli or disease. Together, these two approaches have been utilized to elucidate neurometabolic shifts associated with neurologic and neuropsychiatric diseases\(^{108}\).
Magnetic Resonance Spectroscopy

Magnetic resonance spectroscopy (MRS) is a non-invasive and non-radioactive analytical technique that can be used to quantify biochemical compounds within biological tissue. While MRI primarily characterizes protons in water to spatially localize contrasted behavior of water in different biological contexts, MRS resolves chemical composition by obtaining spectral information that can be spatially localized or unlocalized. Localized spectroscopy is dependent on proton MR images to provide the structural information for MRS voxel localization. MRS is a suitable approach for monitoring in vivo neurochemical profiles, metabolic reactions, and energetics for species at millimolar and higher concentrations. Although many non-zero spin nuclei can be detected, the focus of this work is on the $^{13}$C, $^1$H, and $^{31}$P nuclei. Improvements in magnetic field homogeneity and stability, stronger magnetic field gradients, focused pulse sequence design, and radiofrequency (RF) coil design have substantially improved the attainable spectral resolution, spatial localization, and sensitivity of MRS in recent years. The advent of ultra-high (>7 T) magnetic field strengths for MRS in humans provides exciting possibilities for analysis of metabolic pools at low biological concentration and with improved spatial sensitivity (reduced voxel size).

Principles of MRS

When magnetically active nuclei (for this discussion spin = $\frac{1}{2}$) are placed in a static magnetic field ($B_0$) the nuclear spins align with the applied field. The spins in the bulk magnetization have a magnetic dipole moment vector ($\vec{\mu}$) which is proportional to the nuclear gyromagnetic ratio constant ($\gamma$) and the applied magnetic field ($B$) $^9$. The
Bloch equation describes the magnitude of the magnetic moment in reference to the magnetic field strength (Equation 2-1).

\[
\frac{d\mu}{dt} = \gamma \vec{\mu} \vec{B}
\]  

(2-1)

When spins are aligned with the static magnetic field they do not generate detectible signals, but when acted upon by an external force, such as a transient transverse magnetic field, the spins generate signals at a frequency known as the Larmor frequency. The Larmor frequency \( (\omega) \) is dependent on the inherent nuclear gyromagnetic ratio \( (\gamma) \) and the external magnetic field \( (B_0) \)

\[
\omega = -\gamma B_0
\]  

(2-2)

The behavior of NMR active spins, such as protons, may be influenced by applying an external field in the form of a radiofrequency (RF) pulse (i.e. a transient transverse magnetic field in the form of a magnetic “pulse”). An oscillating electrical current flowing through a loop of wire generates an associated oscillating magnetic field \( (B_1) \) orthogonal to the electrical loop, as described by Faraday’s law. The frequency of the oscillating current should be equal to the Larmor frequency of the nucleus in observation, which is within the radiofrequency range for all nuclei discussed in this work, and hence, these are typically described as RF pulses. The maximum signal is obtained when the nuclear magnetic moment is “tipped” into the transverse plane by a full 90° pulse (Figure 2-1). This rotational angle \( (\theta) \) is achieved by applying the pulse for an optimized power \( (B_1) \) and duration \( (\tau) \) at the nuclear Larmor frequency to achieve excitation into the transverse (XY) plane.

\[
\theta = \gamma B_1 \tau
\]  

(2-3)
Once a tip angle is applied, rotational precession of spin ensembles about the transverse plane exerts a reciprocal magnetic field, and, in turn, electromotive force, which is observed by the detection RF coil. The magnitude of the magnetization vector in the transverse plane is directly proportional to the intensity of the induced current in the RF coil. However, this magnetization is transient and its coherent lifetime is dependent on several factors including intra and inter-molecular dipole interactions and $B_0$ inhomogeneities. Two relaxation mechanism ($T_1,T_2$) are used to describe the processes by which the magnetic moment changes over time. $T_1$, or spin-lattice relaxation, describes the spin’s return to equilibrium in terms of its magnetization in the $z$ coordinate ($M_z$). The faster this relaxation the shorter the value of $T_1$. Experimentally, $T_1$’s are calculated by conducting inversion recovery or saturation recovery experiments in which delay times between pulses are varied and an exponential curve is fitted through the relaxation term (Figure 2-2). $T_2$, spin-spin relaxation, times are defined as a bulk phase coherence in the transverse plane. By comparison, $T_2$ times are either shorter or equal to $T_1$ times.

**Chemical Shift and J-Coupling**

In NMR spectroscopy, variations in local fields at the molecular level cause nuclear spins of the same species to become magnetically non-equivalent. This non-equivalence effect, which is caused by electronic shielding which depends on the chemistry of neighboring functional groups, is called chemical shift. Chemical shift is expressed in parts-per-million (ppm), due to its magnetic field dependence, and is a referenced value with well-defined shifts. In addition to well characterized chemical shift values, indirect nuclear spin-spin couplings (j-coupling) values are often used to identify bonded interactions and are expressed in hertz. J-coupling is a phenomenon by which
two nuclear spins interact through hyperfine electron interactions. J-coupling is field independent and, under conditions of good signal resolution, can provide information to assign metabolic constituents.

**Magnetic Field Gradients and Slice Selection**

In addition to the bulk field ($B_0$), all modern MRI magnets contain an array of electronic coils capable of generating transient magnetic field gradients using direct currents. These gradient coils are oriented in the three orthogonal directions (XYZ) and can be rapidly switched on and off to encode spatial information out of nuclear spin phase/frequency shifts. For voxel localized spectroscopy, field gradients are necessary for creating a magnetic, and therefore frequency, gradient through space (Figure 2-3). This spatial dependent frequency is used to calculate the excitation of pulse needed. The position of slice ($\Delta r$) is selected by selecting the frequency of the RF pulse, while the thickness of the slice is determined by the bandwidth and shape of the RF pulse according to Equation 2-4.

$$\Delta r = \frac{\Delta \omega_{slice}}{\gamma G_{slice}}$$  \hspace{1cm} (2-4)

Slice selective excitation is accomplish when the gradients are on during the RF pulse. When this approach is applied in three dimensions, a singular voxel can be localized at the junction of three slices. Use of spoiler gradients to dephase outer voxel signals can improve spatial precision and reduce outer voxel contamination. At high frequency with large excitation bandwidths, it should be noted that there is a large risk of chemical shift displacement, this issue is discussed further in subsequent sections.
**Benefits of Single Voxel Spectroscopy**

In the field of neuro-metabolism, single voxel spectroscopy (SVS) has the benefit of collecting chemical information, a chemical shift (spectrum) that is spatially constrained to a small voxel (cubic millimeters). This is especially important in an organ system as chemically heterogeneous as the brain\(^{109}\). Additionally, SVS can be acquired rapidly (<20mins) with relatively low signal averaging and so has the benefit of temporal resolution which is especially important when investigating rapid metabolic changes in response to drug administration. Because of many technical advances, including stronger field gradients and higher magnetic fields, single voxel brain spectroscopy has become an important modality for biochemical analysis of cerebral structures.

**Bioenergetics by \(^{31}\text{P} \text{NMR}\)**

Another important application of MRS in the field of neuroscience is the excitation of phosphorus nuclei to collect information on bioenergetic and phospholipid compounds through \(^{31}\text{P} \text{MRS}\). In vivo \(^{31}\text{P} \text{MRS}\) began in the late 1970s to study cellular metabolism\(^{110}\). Like \(^{1}\text{H} \), \(^{31}\text{P} \) is also spin \(\frac{1}{2}\) and nearly 100% naturally abundant, which provides an excellent platform for near real-time acquisition of high energy phosphates present in the brain. Unlike the relatively narrow chemical shift bandwidth (~10ppm) of \(^{1}\text{H} \text{MRS}\), \(^{31}\text{P} \) metabolites span a wider chemical shift range which can be both advantageous and problematic. The advantages of \(^{31}\text{P} \) include much better peak dispersion (due to the chemical shift range), no need for solvent suppression, and high \(^{31}\text{P} \) concentration in the brain. Despite the advantages of performing \(^{31}\text{P} \text{NMR}\), there are several distinct technical challenges. Notably, the chemical shift artifact is especially concerning at high fields. Chemical shift artifact is a phenomenon where spectral resonances that are spatially shifted appear frequency shifted. This problem arises due
to the RF bandwidth exciting outer voxel resonances during gradient application. As would be expected, this challenge is worse at higher fields due to the higher bandwidth. This artifact can be calculated and corrected for by adjusting the acquisition of the voxel or by increasing the bandwidth.

$T_1$ values at 11.1 T have been experimentally determined both for *in vivo* brain spectra and for *in vitro* conditions (Figure 2-5). $T_1$ times were calculated by performing inversion recovery experiments over randomized delay times ($\tau$) and fitting an exponential equation through the data points (Figure 2-5B). Additionally, $^{31}$P spins in metabolites generally have shorter $T_2$ values than $^1$H spins which make signal acquisition with traditional STEAM or PRESS sequences difficult. To adjust for this, the ISIS pulse sequence is typically employed because it retains magnetization in the z-plane except for a short readout period during the FID acquisition$^{111}$.

Through $^{31}$P MRS, simultaneous biochemical events are detectable, including high energy phosphate (ATP) metabolism, pH, phosphocreatine levels, free magnesium levels, and coenzymes such as NAD$^{112}$. Acquisition of *in vivo* $^{31}$P MRS over a high bandwidth contains all three phosphates present in ATP and that ratio can be an indicator of intercellular energetics (Figure 2-6). Cerebral ATP is made primarily as a product of oxidative phosphorylation and the majority of ATP is consumed to power electrochemical pumps and maintaining neurotransmitter production$^{113}$. $^{31}$P MRS of ATP has been shown to reflect cerebral function and health as well as diagnostic tool for neurodegenerative diseases$^{113}$. Steady state ATP pools, therefore, are important indicators of neuronal health and reflective of momentary energetic expenditure. In addition to steady state $^{31}$P MRS, saturation and magnetic transfer NMR experiments
can provide in vivo phosphate exchange kinetics although these experiments are outside of the scope of this work. In addition to ATP spectroscopy, $^{31}$P MRS provides information on redox molecules (NAD+/NADH) that are important indicators of the energetic state and can be indicators of diseases such as diabetes, aging, cancer, and others. As a product of the TCA cycle and a coenzyme for the LDH reaction, cellular redox molecules provide useful information on not only the energetics of the brain, but, indirectly, the exchange capacity between neurons and astrocytes. NAD+ and NADH are structurally nearly identical, so their chemical shifts are very similar making linewidth constraints, chemical shift referencing, and spectral fitting absolutely necessary. High resolution spectroscopy (often on phantoms) is helpful in correctly assigning redox resonances. For these reasons, redox spectroscopy can be very challenging in vivo.

**Dynamic Nuclear Polarization**

Nuclear magnetic resonance (NMR) is an invaluable targeted tool for determining chemical structure and carbon metabolite flux in vitro, in vivo, and ex vivo. The power of NMR spectroscopy in biomedical research cannot be understated, providing the foundation for multiple applications including protein structure and dynamics, metabolomics, free water diffusion and imaging, and many others. In recent years, applications have been pursued that utilize molecular imaging of in vivo carbon metabolic pathways. Although $^{13}$C NMR has the possibility to provide key insights, it is inherently insensitive due to the low gyromagnetic ratio ($\gamma$) of carbon in addition to the 1.1% natural abundance of the magnetically active $^{13}$C isotope. In addition, in vivo spectroscopy is notorious for poor signal to noise ratios (SNR) and difficulty resolving
individual signals due to the predominance of noise while scanning and the poor homogeneity in living systems. The development of dissolution dynamic nuclear polarization (DNP) enables carbon signal enhancement of 10,000 fold or more \textit{in vivo} \textsuperscript{118}. This technique enables the measurement of metabolic flux in real time due to drastically increasing the signal to noise ratio (SNR). This technique is superior in many ways to other metabolic imaging techniques in that it does not rely on radioactive labeling of carbons and multiple doses of tracers can be applied in a short period of time without the necessity to wait for the body to clear the radioactive metabolites.

\textbf{Hyperpolarization Spin Dynamics}

When a spin system is placed in a magnetic field the magnetic moments of all nuclei and electrons are aligned with the magnetic field. For a spin \( \frac{1}{2} \) nuclei this alignment is separated into either parallel (spin up) or antiparallel (spin down) to the applied magnetic field (Figure 2-7). The proportion of spin up to spin down determines the NMR signal. This ratio is dependent on the temperature of the system and the applied magnetic field, as described in the Boltzmann equation (where \( \frac{N_\beta}{N_\alpha} \) is the ratio of spin populations, \( E \) is energy difference between the spin states, \( k \) is Boltzmann’s constant and \( T \) is the temperature (Equation 2-5).

\[
\frac{N_\beta}{N_\alpha} = e^{\frac{-E}{kT}} \tag{2-5}
\]

\textit{In vivo} \textsuperscript{13}C is inherently insensitive due to several factors including the low gyromagnetic ratio for \textsuperscript{13}C, and therefore low energy differences, high physiological temperature (~300K), and low \textsuperscript{13}C natural abundance. Albert Ovehauser was the first to propose a theory of polarization transfer between high \( \gamma \) electrons and low \( \gamma \) \textsuperscript{13}C nuclei (\( \gamma_{	ext{electron}}/\gamma_{\text{carbon}} = 2,616 \)) \textsuperscript{119}. At low temperature (~1K) and high magnetic field, electrons
are almost 100% polarized even at modest magnetic fields (Figure 2-8). By irradiating a free electron near the electron resonance frequency this electron polarization can be transferred to low $\gamma$ nuclei and exploited$^{89,120}$. Polarization transfer at high fields and low temperatures enables up to five orders of magnitude gain in $^{13}$C polarization relative to steady state polarization at physiologic temperatures.

**Solid State Polarization Theory**

Dissolution DNP has two procedural stages, polarization transfer in the cold solid state and sample transfer (solution state) to a living system in an imaging magnet. Initial polarization is obtained by brute force methods of applying high field strength (5 T) and low temperature (<1.2 K) to the sample. Under these conditions, the electrons near 100% polarization. Microwave induced polarization transfer from a high $\gamma$ spin (electron) to a low $\gamma$ spin ($^{13}$C) requires coupling via a combination of thermal mixing and/or the solid effect. Thermal mixing is the dominant mechanism in my proposed DNP experiment because of the width of the electron spin resonance (ESR) line is much larger than the NMR frequency$^{117}$. Thermal mixing is a three spin, microwave induced polarization transfer from two coupled electrons to single nuclei$^{121}$. Under these conditions there are strong dipole-dipole couplings between electron spin baths which can be described as a single spin temperature. Irradiation of the spin bath near the electronic Larmour frequency causes coupled electrons to flip in the spectral diffusion process$^{122}$. Through conservation of energy, when the difference in frequency between these two electron spins equals the nuclear frequency, a coupled nuclear spin is also flipped into one of the nuclear Zeeman energy states$^{117}$. Because the $T_{1e}$ time of the electron (~900 milliseconds at 3.35 T and 1.2 K) is relatively short compared to the $T_{1n}$
of the nuclei (hours at 3.35 T and 1.2 K), the “flipped” electrons are quickly recycled back to their ground state and can polarize additional nuclei. Thermal mixing allows large gain in signal in the solid state and therefore increases the signal to noise ratio of a labeled $^{13}$C metabolite.

**Experimental Solid State Polarization**

Optimal radical and $^{13}$C substrate concentrations have been experimentally determined and optimized for maximum polarization in the solid state. Solid state polarization has been optimized in two stages, brute force polarization of the electrons and microwave induced polarization transfer to the nuclear spins. The brute force cooling step has been optimized by using a liquid helium bath and applying a vacuum of 0.7 (+/- 0.1) mbar to induce evaporative cooling of the sample to ~1.2 K. Once the sample has been cooled, polarization transfer from electron to the $^{13}$C occurs by microwave irradiation of the sample. Preliminary experiments have been carried out to optimize the microwave frequency for polarization transfer (Figure 2-9). In addition, a microwave power array was carried out to ensure sufficient microwave power was applied to the sample (data not shown). We have taken alternative measures for sample preparation to ensure that the electron-to-nucleus transfer is optimized and that dissolution parameters are at their maximum performance. These steps include sample preparation, optimization of substrate and stable radical concentration, and selection of stable radical.

**Sample Preparation**

Numerous substrates have been the target of polarization because of their potential as biological tracers for *in vivo* applications and to study their inherent NMR properties. By far the most popular substrate for polarization is the [1-$^{13}$C] pyruvic acid
molecule. Among the properties that make pyruvic acid (PA) so popular is its role in
important metabolic pathways, relatively long carbon $T_1$ relaxation time (~60 s), and its
self-glassing properties. Glassing is the process of vitrification of an aqueous solution
without formation of crystals. It is an important step in DNP sample preparation to
homogeneously distribute the radical and the polarization substrate. As an acid, PA self-
glasses without adding potentially hazardous glassing agents such as ethanol, glycerol,
DMSO, etc. This is beneficial when polarizing pyruvic acid with a pH insensitive radical
such as OXO-63 because the sample can be made to be very concentrated. Excluding
OXO-63, most commonly used radicals are pH sensitive so the pH neutral sodium
pyruvate salt should be used. Sodium pyruvate dissolved in water not self-glassing and
requires an addition of a glassing agent to produce a homogeneous sample for
polarization. We have found that through rapid freezing of the sample by spraying
sodium pyruvate, acetate, or butyrate aqueous solution into nitrogen cooled isopentane
we can achieve an expeditious freeze without the need for a glassing agent. In addition
to removal of the glassing agent, this approach has improved polarization potential as
well as faster build-up times as compared to samples with glassing agents (Figure 2-10). From this work, we demonstrated an improved sample preparation technique for
aqueous samples by implementation of an isopentane bath. This approach reduces the
need for potentially toxic glassing agents, decreases solid-state buildup time and retains
or improves $^{13}$C polarization. This technique has broad application especially in
dissolution DNP for in vivo studies.

**Solution State Polarization**

The second stage of dissolution DNP is rapid melting and transfer of the
polarized $^{13}$C substrate in the solution phase to a live animal. In our preparation, solid-
state polarization is reached in tens of minutes and then the sample is dissolving and
transferred to a custom designed pump, via room temperature helium gas as a motive
force, and then injected, at a fixed rate, into an animal stabilized in a 4.7 T or 11.1 T MR
scanner. Helium gas is used during the dissolution to prevent air contamination in the
sample space of the cryostat. As the sample is warmed to physiological temperature
and removed from the strong magnetic field, the $^{13}$C polarization begins to relax back to
thermal equilibrium due to the inherent $T_1$ time of the nuclear spins. The $T_1$ relaxation
rate is magnetic field dependent so it is partially preserved by ensuring that the sample
remains in the magnetic fringe field of nearby superconducting magnets$^{125}$. If the
sample crosses through zero-field the spin states lose their hyperpolarized (HP) state.

**Spin Lattice Relaxation Effect and RF Observation**

After dissolution, the sample begins exponential relaxation back to the thermal
Boltzmann energy distribution of states, so rapid $^{13}$C observation is the primary goal.
The nuclear magnetization exponentially decays proportionally to the inverse of the $T_1$
of the sample. This relaxation effect can be described by Equation 2-6

$$M_z(t) = M_0 e^{-t/T_1}$$

(2-6)

where $M_z$ and $M_0$ are the remaining and initial magnetization respectively. It should be
noted here that $T_1$ is affected by magnetic field and by chemical environment, so
selection of substrates with optimal $T_1$ times is paramount to the success of dissolution
DNP. Furthermore, to observe the HP spins it is necessary to tip the spins into the $x,y$-
plan using RF pulses. These pulses effectively remove the spins from the observable
pool proportional to $\sin \theta$. Although 90° pulses return the greatest observation signal,
the signal is effectively “used up” in one transient. Since biochemical kinetics are
typically one of the goals of DNP experiments, a series of small tip angle (<30°) is generally applied in rapid succession (1-2s) to sample the magnetization without exhausting it too quickly126. In vivo effective T₁’s of the substrates are considerably smaller than reported T₁ values for their pure form due to interactions with oxygen and other biological paramagnetic ions. Nonetheless, we can observe ¹³C polarized pyruvate and its conversion to lactate, alanine, and bicarbonate over the course of 1-3 minutes in vivo for kinetic modeling of substrate-product reactions in a time dependent model.

**Kinetic Modeling**

Kinetic flux modeling of enzymatic processes can be performed with DNP in vivo and in vitro. These models are made possible because of the chemical specificity and high SNR achieved through dDNP. Although multiple products are observed in a typical HP pyruvate experiment, lactate kinetics are often the primary focus of the modeling because of the significance of the enzymatic conversion step involved and due to the high SNR of lactate as compared to the other products. As discussed in Chapter 1, pyruvate-to-lactate conversion provides a relevant semiquantitative indicator of intracellular glycolytic production. In the brain, the pyruvate-to-lactate conversion rate is an indicator of the neuron-to-astrocytic energy exchange rate and provides insight into the neuronal oxidative function of the brain under certain conditions127. Due to the transient nature of HP signals, especially in vivo, rapid acquisition is necessary for observation of signal and for accurate kinetic modeling. HP metabolic flux is based on the Michaelis-Menten foundational concepts discussed in Chapter 1. In its simplest form, the biochemical rate constant $k$ is a measure of enzymatic rate flux from substrate-to-product. This value $k$ is dependent on substrate concentration, which we
assume is constant over the time course of HP observation (Equation 2-7). Additionally, since the substrate concentration is greater than both physiological substrate concentration and product concentration we assume that the rate is unidirectional and first order on the observable time scale (Equations 2-8/9).

\[ S \xrightarrow{k} P \tag{2-7} \]

\[ \frac{d(S)}{dt} = -k(S) \tag{2-8} \]

\[ \frac{d(P)}{dt} = k(S) \tag{2-9} \]

These differential equations describe the flux between substrate (S) and product (P) in a simple two chemical compartment/unidirectional model. Terms must be added to the equations to account for relaxation due to T₁ and RF “read” pulses (θ) in the Bloch formalism where \( M_S \) and \( M_P \) are the z-magnetization of the substrate and product respectively.

\[ \frac{dM_S}{dt} = -kM_S - M_S(R + (1 - \frac{\cos \theta}{TR}) \tag{2-10} \]

\[ \frac{dM_P}{dt} = kM_S - M_P(R + (1 - \frac{\cos \theta}{TR}) \tag{2-11} \]

Fitted data from HP experiments provide input values for substrate and product magnetization and kinetic values can therefore be solved.

Although this basic model is appropriate for the experiments performed in this work, more complex models have been described\textsuperscript{128,129}. These alternative kinetic models account for multiple physical compartments, intercellular/extracellular transport, and bidirectional kinetic exchange. The two-pool bidirectional/unidirectional models can be an accurate predictor of \textit{in vivo} metabolic function but our data fit well to the single compartmental model\textsuperscript{128}. 

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High Resolution Magic Angle Spinning

In vivo spectroscopy of biological tissue suffers from inherently poor line shapes due to anisotropic NMR interactions and susceptibility differences. These interactions contribute to spectral resolution that is poorer than solution state NMR approaches, but has the added advantage that the tissue has not been chemically extracted or fractionated as is the case with solution state samples. High Resolution Magic Angle Spinning (HRMAS) is a technique by which tissue is directly loaded into an NMR magnet in the solid state without any extraction protocols. As an added benefit, ease of setup for HRMAS analysis is advantageous and produces a potential for high throughput workflows. Additionally, simple sample preparation is beneficial for retaining biological stability by reducing the time needed to load samples into the NMR spectrometer. HRMAS has wide ranging applications for multiple tissue types as well as broad applications in multinuclear observations and complex pulse sequence design.

HRMAS Theory

In solution, dipolar interactions between nuclei and chemical shift anisotropies averaged out in solution by Brownian motion. By contrast, molecular motions are more restricted in solid-state samples leading to broadened line shapes. Magic Angle Spinning (MAS) is an approach to average out these anisotropic interactions by orienting the sample at a fixed angle. Dipolar interactions are averaged to zero when oriented at the magic angle (54.7 ° in respect to the magnetic field) significantly narrowing spectral line shape\textsuperscript{100}.

\[ 3 \cos^2 \theta - 1 = 0 \quad (2-12) \]

Additionally, by spinning the sample, these interactions are averaged and with a strong narrowed central line and weaker side band in the interaction manifold separated by the
frequency of spinning. In practice, the frequency of spinning for HRMAS is maintained at a frequency high enough to remove the sidebands from the observation bandwidth\(^{100}\). The application of this theory to solid-state samples was first described by Andrew et al and Lowe in 1959\(^{130,131}\). HRMAS has wide biological applications in metabolomics, including, but not limited to, isolated cell suspensions, hepatic tissue, brain tissue structures, intestinal tissue, lymphatic tissue, and many others\(^ {100}\).

**HRMAS Probe Design**

HRMAS probes are unique in their individual design but retain several basic components. The first and most crucial component of the HRMAS probe is the stator/rotor assembly. In the loaded position, this assembly is fixed near the magic angle of 54.7° with pins for fine angle adjustment. Typically, the stator has an air driven turbine mechanism which floats the rotor away from the stator walls and drives the rotation of the rotor itself. The stator and rotor diameter vary depending on the sample being analyzed. The rotor is a small cylinder (~30-50 μL) with a turbine drive cap fitted on the end. This drive cap has fins which spin as the drive air passes across them. The frequency of rotor rotation is typically measured by a fiberoptic spin rate monitor. For biological stability, it is also important that the HRMAS probe has the means to control sample temperature. This is accomplished through a variable temperature unit that cools or warms the air flowing over the rotor. The final component germane to all HRMAS probes is the RF coil centered over rotor. The nuclei observable by this RF coil is dependent upon the probe’s application. Some probes are designed to include additional hardware components, such as high powered gradient coils, sample ejector system, etc.
Sample preparation

As previously mentioned, the sample preparation for HRMAS is minimal. For the experiments performed in this work we used a 4mm Zirconium rotor with a Kel-F drive cap. The rotor volume was 80 µL and allowed for Kel-F sample inserts to be used. The inserts had a volume of ~40 µL and included a gas and screw cap. The sample inserts were helpful for the workflow of this project because multiple samples could be prepared at one time and stored at -20 °C until needed. Tissue samples were previously flash frozen in liquid nitrogen and were stored at -80 °C. 10 µL of D$_2$O was loaded into sample insert and ~20-30 µL of brain tissue was quickly loaded into the rotor on ice. To remove any air trapped by the tissue insertion, the inserts were briefly spun on a benchtop centrifuge and placed into a labeled Eppendorf tube. It is important to keep the tissue cold because this slows the metabolic reaction rate and maintains brain tissue structure needed to load the samples into the inserts. A chemical shift reference can be added to the D$_2$O, but was not for these samples because of the well conserved chemical shift fingerprint. Additionally, exact tissue weights can be measured prior to loading the inserts for normalization, but we found this to be an inconsistent method of normalization and relied on statistical normalization in post processing$^{132}$. A step-by-step protocol for HRMAS of brain tissue can be found in Appendix B.

Technical Challenges

Although sample preparation is relatively straight forward and can be mastered after some practice, there are significant technical challenges associated with HRMAS of brain tissue specific to this project that will be address herein. The brain contains 66-85% water, depending on the region of the brain. Furthermore, water is at a concentration of ~55 molar while important biological metabolites in the brain are on the
order of millimolar concentration. As is common in all biological $^1$H NMR experiments, proton signal from water dominates the chemical shift spectrum. Suppression of water signal is a fundamental element of biological $^1$H NMR and can be accomplished through several approaches. The most common method, and the method used in this work is water presaturation. Presaturation is accomplished by applying a long, low power pulse at a defined frequency offset. Correctly setting this offset on unsuppressed water, is an important step ensuring that water is properly suppressed. In addition to setting the suppression frequency, it is also pertinent to set the suppression power high enough to achieve adequate suppression without affecting nearby resonances. Additional water suppression approaches can also be applied including gradient assisted suppression.

Another issue common to HRMAS of brain tissue is the abundance of lipids in brain tissue. Lipids resonate between 0-2ppm and overlap with many metabolites of interest including lactate, alanine and the branch chain amino acids (Figure 2-11a). Selective observation of the metabolites without the lipid background is accomplished by exploiting difference in $T_2$ times by utilizing the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. CPMG is a $T_2$ weighted spin echo experiment comprised of a train of $90^\circ$ - $\left[ - 180^\circ - \tau \right]$$n$ acquisition pulses$^{133,134}$. Overlapped fat signals in brain tissue can be dramatically suppressed by optimization of the delay times between pulses by effectively weighting the echo acquisition away from molecules with short $T_2$ times (lipids). Spin echo NMR experiments often suffer from homonuclear spin-spin interactions, or J-modulation, that can be attenuated through implementation of rapid refocusing$^{135}$. Insertion of an additional 90° pulse at the midpoint of the CPMG experiment reduced the J-modulation of the spin echo experiment. Implementation of
this Periodic Refocusing of J Evolution by Coherence Transfer (PROJECT) sequence resulting in “in-phase” spectra with improved filtering of unwanted macromolecule signal as compared to CMPG (Figure 2-11B). In addition to relaxation edited approaches, lipids and macromolecules NMR signals can be suppressed by differences in molecular diffusion coefficients. By applying pulsed field gradients to a NMR experiment, macromolecule NMR signals can be attenuated from a complex soluble metabolic sample.

Normalization

Qualitative normalization is another issue common to HRMAS applications in metabolomics. Common approaches include normalization to tissue weight, unsuppressed water normalization, and post-processing statistical normalization. We have found that statistical normalization is the most robust approach with the highest reproducibility. Normalization in post-processing can be accomplished by peak area normalization to a known and unchanging metabolite (i.e. creatine, NAA, etc), total signal area, or quotient normalization. Specific normalization approaches used in this work are discussed in further detail in subsequent chapters.

Solution State NMR of Tissue Extracts

Solution state NMR spectroscopy and mass spectrometry are the two major analytical platforms traditionally utilized for collecting metabolomics data. While NMR spectroscopy is inherently less sensitive than mass spectrometry, it has important benefits including high reproducibility, it is non-destructive to the sample, and it can identify unknown compounds based on structure. NMR based metabolomics has applications ranging from biomedicine to plant biology and soil science. In addition to steady-state metabolomics, isotopomer NMR techniques can be used to investigate flux...
and pathway activity. These experiments rely on selective stable radiolabeling of metabolites and observation of downstream products. Natural abundance NMR spectroscopy is a snapshot of the metabolic state of the system in question. Furthermore, NMR spectroscopy has the benefit of analyzing a broad range of biological specimens including serum, tissue, and urine. The advent of high field magnets with favorable magnetic field homogeneity has boosted the sensitivity of NMR spectroscopy and improved the spectral resolution attainable to analyze hundreds of compounds in complex biological samples. For example, metabolomics of neurodegenerative disease has become increasingly popular in recent years toward discovery of biomarkers as a diagnostic tool.

**Dissection and Metabolic Quenching**

Brain tissue contains a complex mixture of hundreds of metabolites. Polarity of the metabolites of interest is an important first selector of extraction protocol. Metabolic degradation is another important consideration when preparing the tissue. Like HRMAS, it is important to first quench any metabolic reactions by rapidly freezing the sample in liquid nitrogen immediately after dissection of the desired region. This step stabilizes the metabolic compounds and stops additional enzymatic reactions from taking place. Contamination artifacts should be considered when dissecting brain tissue. This includes cleanliness of the dissection area and removal of extraneous blood and adjacent tissue. As in other dissection methods, brain heterogeneity should be considered. Careful and rapid dissection of brain tissue is of high importance in cerebral metabolic analysis. Once the tissue has been frozen in liquid nitrogen it should be stored at -80 °C until extraction can be performed. Homogenization of tissue can be
accomplished with a variety of methods including mortar and pestle or handheld motorized homogenization at \( < 0 \, ^\circ \text{C} \).

**Metabolic Extraction**

There are multiple extraction techniques for non-targeted NMR metabolomics. Regarding the present work, we focus on extraction of polar metabolites from non-polar lipids. Polar extraction by the method described in this work retains organic acids, polar amino acids, redox molecules, organophosphate compounds and other important cerebral biomarker compounds (creatine, glucose, TCA cycle intermediates, etc). Additionally, the protocols (see Appendix C) for this analysis were optimized to reduce the impact of oxidation on the sample\(^{141}\). The two most common extraction protocols for non-targeted NMR metabolomics are protein precipitation by perchloric acid or two-phase polar extraction with methanol/chloroform\(^{140}\). Reproducibility, throughput, tissue composition, and product yield should be considered when selecting extraction protocols. The two-phase extraction technique relies on the principle that a compound mixture can be separated by hydrophobicity with fatty acids occupying the hydrophobic phase while hydrophilic compounds remain in the aqueous phase. This method was selected for this work due to methanol/chloroform’s superior performance for extraction of tissue with high lipid content. Methanol/chloroform extraction was first described by Folch *et al* in 1951 as an approach to extract lipid compounds from brain tissue\(^{142}\). Since its inception, methanol chloroform extraction has been optimized with the addition of water to select for varying degrees of polarity\(^{114,143-145}\). After the sample has been extracted the next step is removal of extraction solvent by lyophilization. This step ensures that the sample is concentrated enough for NMR analysis and that minimal solvent suppression is needed during the NMR experiment. \( \text{D}_2\text{O} \) with or without
chemical shift reference is then added to the dried sample for a concentrated sample that is prepared for NMR analysis. Trimethylsilylpropanoic Acid (TSP) is a common chemical shift reference for metabolic profiling experiments. TSP is a partially deuterated compound with nine chemically equivalent protons bonded to a trimethylsilyl group. This molecule has a single $^1$H resonance references to 0 ppm and has been shown not to interact with other compounds in the extracted NMR sample, although some TSP-peptide interactions have been reported$^{146,147}$.

**NMR Analysis**

NMR analysis of biofluids including serum, urine, and tissue extracts can be performed through 1D metabolic profiling. As detailed in the HRMAS spectroscopy section, $T_2$ weighted CMPG sequences are often preferred when lipids are present in the sample. For polar extracted samples, lipid signals have been almost completely eliminated. Therefore, metabolic profiling can be performed with a 1D Nuclear Overhauser Spectroscopy (NOESY) experiment (Figure 2-13). Although $H_2O$ has been removed during the extraction procedure, solvent suppression is still necessary to attenuate residual $H_2O$ signals. NOESY experiments are sufficient for basic metabolic profiling, especially when chemical shift resonances are well assigned, coupling constants are known, and peaks are well resolved$^{148}$. Creatine/Phosphocreatine (Cr, PCr) peaks have been assigned together although there are three distinct peaks; restricted creatine, mobile creatine, and phosphocreatine.

A 2D J-Resolved spectroscopy (JRES) sequences can be used when resonances are overlapping which resolves the peaks in the second dimension$^{149}$. 2D JRES homonuclear sequence separates the J-coupling and chemical shift into two independent dimensions$^{150}$. Processing 2D JRES spectra involves tilting the spectrum
to reveal chemical shift information in the 1st dimension and J-coupling information in the second dimension (Figure 2-14). JRES is useful for identifying metabolic peaks where the shifts are not known *a priori* or when the peaks are too overlapped to assign.
Figure 2-1. Magnetic excitation in the rotating frame. Blue arrows indicated magnetic moment as it is excited by the B1 field.
Figure 2-2. Gamma-ATP T<sub>1</sub> time measured by inversion recovery at 11.1 T by inversion recovery experiments. Delay times (D1) sampled randomly up to 25 seconds. Peak integral values used to calculate intensity. Calculated T<sub>1</sub> = 1.6 s.
Figure 2-3. Diagrammatic representation of pulsed field gradient. Nuclei in the sample precess at the Larmour frequency ($\omega$) in the presence of the bulk field ($B_0$). A single Z gradient coil is represented by red bars with a magnitude (G) over distance ($\Delta r$).
Figure 2-4. Energy level diagram of spin state populations as a function of magnetic field strength ($B_0$). Two spin states ($\alpha/\beta$) are separated by an energy of the nuclear magnetic moment ($\Delta E$) as described in the Larmour equation.
Figure 2-5. T₁ times collected at 11.1 T. A) Stacked plot of inversion recovery experiment for in vivo ³¹P time of the brain with assignments. B) Representative T₁ data and fits for α-ATP resonances in vivo and in vitro experiments. C) Experimentally determined T₁ values at 11.1 T with coefficient of determination (R²) values.
Figure 2-6. Representative $^{31}$P spectrum with fitted and assigned peaks. Assignment abbreviations$^{151}$: Pi – inorganic phosphate, PME – phosphomonoester, PDE - phosphodiester, PCr – phosphocreatine, UDG – uridine diphosphate glucose.
Figure 2-7. Simulated percent of total spin polarization of electron (purple), $^1$H (green), $^{13}$C (red) at 5 T. Polarization transfer is indicated by blue arrow from high $\gamma$ electrons and low $\gamma$ nuclei.
Figure 2-8. Dynamic nuclear polarization in the solid state. A) $^{13}$C nuclear spin population represented at thermal equilibrium and after electron polarization transfer. B) relative signal of [1-$^{13}$C] acetate before microwaves (MW) turned on (red 20x) and after polarization transfer (blue 1x).
Figure 2-9. Microwave frequency optimization sweep. Solid state polarization of [1-\(^{13}\)C] sodium pyruvate with 4-oxo-TEMPO (4-oxo-2,2,6,6-tetramethyl-1-piperidinyloxy) radical. Maximum polarization present at ~140.7 GHz.
Figure 2-10. The effect of glassing agents on solid state buildup time. Polarization buildup curves at 5 T and ~1.1 K for 3.0 m sodium acetate, 4-oxo-TEMPO solutions. Traditional glassing agent for DNP experiments is deuterated ethanol (EtOH-D₆). This graph illustrates the abbreviated build up time associated with isopentane bath sample preparation (D₂O/isopentane).
Figure 2-11. Hyperpolarized signal exponential decay due to $T_1$ effects and RF pulses. Simulation calculated from a $T_1$ of 20 sec, RF pulse angle of $20^\circ$, and a repetition time of 1 sec.
Figure 2-12. HRMAS pulse sequence comparison. A) Macromolecule (MM) suppression using NOESY (Red) vs. CPMG (Blue). B) J-modulation resolution with “perfect echo” PROJECT (Green) vs. CPMG (Blue). Assignment abbreviations: Tau – taurine, MI – myoinositol, GPC – glycerophosphorylcholine, PC – phosphocholine, Cho – choline.
Figure 2-14. Representative 1H JRES spectrum of brain extract for metabolic assignments of overlapping resonances. First dimension (F1) is the J-coupling of metabolites in the spectral dimension (F2). Selected metabolic coupling constants presented. Abbreviations: t – triplet, d – doublet, Val – valine.
CHAPTER 3
COCAINE INDUCED CHANGES IN METABOLIC FLUX AND POOL SIZE

Cerebral Shift in Metabolic Pools and Flux in Response to Acute Cocaine Administration

The high-energy requirements of the brain are sustained by a unique metabolic relationship between astrocytes and neurons. The tight coupling of neuroenergetics between astrocytes and neurons regulates and responds to neural activity as well as synaptic remodeling. Here, we show how cocaine administration shifts neurometabolism at a fundamental level. Using a novel approach combining dynamic nuclear polarization-enabled metabolic flux measurements with steady state magnetic resonance measures of metabolite pools, we reveal acute cocaine administration disrupts the balance of oxidative and non-oxidative metabolic pathways between neurons and astrocytes, respectively. These results demonstrate significant metabolic shifts in response to cocaine administration, providing insight into the observed short term effects of cocaine use. These findings also illustrate how in vivo metabolic flux measurements can probe the health of and synergy between neurons and astrocytes. The approach we demonstrate can be combined with more traditional, correlative MRI approaches to understanding brain development, function, cognition, and neurodegeneration to enable the identification of early metabolic markers for specific neurodegenerative diseases as well as provide a more fundamental understanding of brain function and health at the molecular and cellular levels.

Introduction

Cocaine continues to rank among the major illicit drugs of abuse causing severe substance use disorders. Its acute reinforcing effects are in part due to its direct binding to dopamine transporters (DAT) and blockade of presynaptic dopamine (DA) reuptake
in mesocortical and mesolimbic brain regions\textsuperscript{152-155}. A consistently reported finding has been that acute and chronic cocaine administration causes significant alterations in cerebral glucose utilization, impairs mitochondrial metabolism, and these effects are associated with cytotoxicity and autophagic cell death\textsuperscript{156-163}. Impairments in cellular bioenergetics and suboptimal cellular ‘health’ can in turn adversely impact other neurophysiological processes such as synaptic plasticity and neuronal excitability, efficacy of intracellular signaling cascades, DAergic and glutamatergic neurotransmission, all of which are reportedly altered by chronic cocaine exposure\textsuperscript{164-166}. While general neurometabolic changes related to glucose utilization following cocaine administration is strongly supported, there is much less understood about the in vivo intracellular metabolic pathways that are adversely affected by cocaine or that can contribute to impaired cerebral metabolic activity. Impaired energy utilization in cocaine subjects may arise through glycolytic enzymatic pathway dysfunction and/or impaired intercellular exchange, which, up until now, has been difficult to measure in real-time and in situ.

Glucose is the primary source of energy for the brain, generated first through glycolytic oxidation to pyruvate and then astrocyte-neuronal intercellular mitochondrial respiration\textsuperscript{167}. Astrocytes are the primary site of circulating glucose uptake transported through the blood brain barrier by the GLUT1 transporter\textsuperscript{80}. Astrocytes have a high glycolytic rate as compared to neurons and preferentially convert glucose to lactate stores via high expression of lactate dehydrogenase 5 (LDH5)\textsuperscript{69}. Lactate is shuttled between the neuro-astrocyte intercellular space and converted back into pyruvate in the neuron by Lactate Dehydrogenase 1 (LDH1) isoform where it can be utilized for
oxidative functions such as REDOX production, biosynthesis, and ATP generation. The exchange facilitates astrocytic control of neuron function and can be a marker of neuronal stress and dysfunction. Analysis of downstream metabolic pool sizes and pyruvate flux can be an important marker of cellular respiration and provide insight into energetic and biosynthetic state of cerebral function. Previous studies have illustrated acute cocaine’s effect on the cataplerotic biosynthetic pathways of glutamate and aspartate. In addition to metabolic pool shifts, cocaine has been shown to have profound regional effects on glucose transport. In recent years it has been shown that the coalition of hyperpolarization techniques, namely dDNP, with traditional NMR and MRI modalities has the potential to overcome previous signal-to-noise (SNR) constraints. Hyperpolarization of metabolites with a known chemical shift can be used to observe product conversion, providing both a snapshot of product pool sizes and real-time enzyme kinetic flux values. The flux between these metabolites are indicative of the metabolic state of an organ system and have been exploited to diagnose metabolic diseases such as the cancer Warburg effect, heart disease, and pulmonary inflammation.

Methods and Methods

Animals

Experiments and procedures received prior approval by the Institutional Animal Care and Use Committee of the University of Florida and followed all applicable NIH guidelines. Adult male Long Evans rats (250-350 grams) obtained from Charles River Laboratories (Wilmington, MA, USA) were housed in pairs in a temperature and humidity-controlled vivarium (12 h light-dark cycle with lights off at 1900 h) with food and water available ad libitum. Subjects were administered a single intraperitoneal (i.p.)
injection of cocaine hydrochloride (Sigma, St. Louis, MO) dissolved in sterile isotonic saline solution at a dose of 10 mg kg\(^{-1}\) while under anesthesia 12-13 minutes prior to either metabolic pool analysis or \textit{in vivo} flux measurements by hyperpolarized [1-\textsuperscript{13}C] pyruvate. Control subjects followed the same time course of anesthesia and analysis, but did not receive an i.p. injection. Experiments were performed at randomized times over the course of the day.

**Metabolic Flux Measurements Using Hyperpolarized [1-\textsuperscript{13}C] Pyruvate**

Subjects were anesthetized with Piralmal isoflurane (Bethlehem, PA) delivered in medical grade oxygen (2.5-3.25 % at a flow rate of 2 LPM oxygen) ~30 minutes prior to imaging. A catheter was placed in the left leg femoral vein for efficient delivery of hyperpolarized (HP) substrate. Respiration was regulated at 40-50 breaths per minute and body temperature was maintained at 35-37 °C by a recirculating water bath. After cannulization, the catheter was connected to an automated injector, and subjects were positioned in an MRI cradle with an Agilent 60 mm I.D. linear \(^1\text{H}\) (200 MHz) volume coil and a homebuilt 15 mm surface \textsuperscript{13}C (50 MHz) coil\textsuperscript{180}. The cradle was then positioned in a 4.7 Tesla (T) / 33 cm horizontal-bore MRI system (Oxford/Agilent) for landmarking, shimming, and RF calibration.

**Dynamic Nuclear Polarization and Dissolution**

Dissolution DNP has two procedural stages, hyperpolarization of metabolic substrates in a frozen solid state followed by sample dissolution and transfer for injection into the subject in an imaging magnet. For the polarization step, 3M \textsuperscript{13}C-enriched sodium pyruvate, pH 7.4 (Cambridge Isotopes, Boston, MA) and 50 mM 4-oxo TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy) (Sigma, St. Louis, MO) were prepared in a 400 μL volume of 2:1 D\textsubscript{2}O:ethanol-D\textsubscript{6} (V/V). Glassed sample beads were formed by
dripping this solution into a liquid nitrogen bath and were then transferred into a Teflon sample cup. The sample was polarized for approximately 1 hour in 5 T magnet (Bruker, Billerica, MA) at 1.1 (± 0.03) K in a custom built cryostat by irradiating at 140 GHz / ~50 mW (Virginia Diodes Inc., Charlottesville, VA) to elicit polarization transfer from the TEMPO radical to the pyruvate $^{13}$C spins. Next, heated (120 °C) phosphate buffered saline was injected into the polarizer sample space at 12 bar to melt the sample and transfer it to a custom built automated injector for injection into the subject in the 4.7 T MRI system (Agilent VNMRS). Each subject received ~1.5 mL of polarized pyruvate at a final blood concentration of ~14 mM. The acute cocaine study group received an i.p. injection of 10mg/kg/ml cocaine 12-13 min prior to injection of hyperpolarized substrate. The time for dissolution and injection was ~8 s. Typical polarization levels were ~15% at the point of injection. The trigger for dissolution of the hyperpolarized $^{13}$C-enriched pyruvate also triggered the 4.7 T MRI system to begin $^{13}$C spectral acquisition. Spectra of the injected pyruvate and its products were acquired every 1 s for a total of 200 transients using a small angle pulse (~18°, 7 μsec) and 144 ms acquisition time (2048 pts). n=7 subjects the for the control group, n=7 subjects for the cocaine group.

**In Vivo $^1$H MRS**

After being anesthetized, subjects were positioned in an MRI cradle with a homebuilt $^1$H (470 MHz) transceive volume coil and a Bruker 2x2 receive only phased array surface coil and placed in an 11.1 T MRI system (Bruker AV3HD running Paravision 6.0.1). TurboRARE image slices were collected and used to localize a 6.8mm x 5.0mm x 2.6mm voxel in the cortex. After global field mapping and shimming, iterative voxel shimming was performed to optimize resolution in the region of interest. Single voxel $^1$H spectra were collected using a STEAM pulse sequence with VAPOR
water suppression and outer volume suppression with the following parameters: ns = 128, 1 K points, 2 s recycle time, 8 KHz bandwidth and 5.5 ms echo time. The acute cocaine study group received an i.p. injection of 10mg/kg/ml cocaine after positioning and shimming, and $^1$H spectra were collected ~12 min after injection. Spectra were manually phased in Topspin, and no line broadening was applied. Baseline correction and peak fitting was performed using Mnova v11.0 software. n=10 subjects the for the control group, n=5 subjects for the cocaine group.

**In Vivo $^{31}$P MRS**

Animals were fixed in an MRI cradle with a homebuilt $^1$H (470 MHz) transceive volume coil and a homebuilt 2 cm $^{31}$P (190 MHz) surface coil and placed in an 11.1 T MRI system (Bruker AV3HD). The volume $^1$H coil was utilized for shimming the whole brain region and TurboRARE image slices were used to localize a 14mm x 10mm x 27mm voxel for data acquisition with adiabatic refocusing pulses. $^{31}$P spectra were acquired using an ISIS pulse sequence with 32 scans, 50 ppm sweep width, 2 s recycle time and 1 K points. Data were acquired at the same time intervals as for $^1$H MRS. The spectra were processed with 5Hz exponential line broadening and phase corrected in Topspin. Baseline correction and peak fitting were performed with MNova. n=10 subjects the for the control group, n=5 subjects for the cocaine group.

**Ex Vivo Metabolic Pool Analysis**

Acute and control subjects were briefly anesthetized and received an i.p. injection of cocaine 12-13 mins prior to decapitation. The left cerebral hemispheres were immediately removed and flash frozen in liquid nitrogen and kept at -80 °C for use in mass spectrometry and solution state NMR experiments. The right cerebral cortex tissue, corresponding to the sensitive region detected by the $^{13}$C coil in the DNP
experiment and the $^1$H coil in the MRS experiments, were flash-frozen and stored as previously described for high-resolution magic angle spinning (HR-MAS) experiments. The frozen left hemisphere tissue samples were lyophilized to dryness overnight and pulverized to a fine powder using a Precellys (Bertin Instruments, France) homogenization system.

**Targeted Organic Acid LC-MS**

$\sim$5mg of lyophilized powder was homogenized in 500 µL of 50:50 acetonitrile/water with 0.6 % formic acid using the Precellys bead-based homogenizing system. A 50 µL aliquot of homogenate for organic acids and a 100µL homogenate for amino were aliquoted and immediately stored at -80 °C. Another 5mg of lyophilized powder was homogenized in 1000 µL of 5% TCA and 10µL of heavy isotope-labeled internal standards for Acetyl and Malonyl CoA. Liquid chromatography / mass spectrometry measurements were performed using a Thermo Scientific UltiMate 3000 UHPLC/TSQ Quantiva triple quadrupole mass spectrometer.

**Polar Metabolites Analysis by Solution State NMR**

$\sim$75 mg aliquots of brain lyophilized tissue were extracted overnight at -20 °C with 1 ml of 1:1:1 chloroform:methanol:water. Samples were centrifuged at 10,000 xg, 4 °C, for 40 m, and the polar phase was collected and lyophilized to dryness. The polar metabolites were reconstituted in 500 µL D$_2$O an external chemical shift reference of 1 mM 3-(trimethylsilyl)propionic-2,2,3,3,-d$_4$ acid (TSP) reference in a stem coaxial insert. $^1$H NMR spectra were collected using a 600 MHz Bruker AVII system with a 5 mm CryoProbe. 1D spectra were collected at 27 °C using 1D-$^1$H-Nuclear Overhauser Effect SpectroscopY (1D-NOESY) with water presaturation pulses. For all spectra, 32 scans were collected with a 5 s relaxation delay, 32 K data points, and a 7.2 KHz spectral
width. For each spectrum -0.1 Hz exponential and 0.02 Gaussian adipozation was applied. Whittaker smoother baseline correction was applied to each spectrum. Peak assignments were confirmed by previously published work and by 2D J-resolved spectroscopy (2D-JRES). n=8 subjects the for the control group, n=7 subjects for the cocaine group.

**High Resolution Magic Angle Spinning (HR-MAS) Analysis**

Approximately 20 mg of frozen intact brain tissue + 10 μL D₂O was packed directly into a Bruker Kel-F rotor insert for a 4 mm Zirconia rotor. ¹H NMR spectra were collected at 4 °C, 5 KHz MAS, using a 600 MHz Bruker AV3 HD system with a 4 mm HRMAS probe. 1D spectra were collected using a T₂ weighted, Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence with water suppression. For all spectra, 128 scans were collected with a 5 s relaxation delay, 16 K points, and a 6 KHz sweep width. HRMAS spectra were individually phased and 2 Hz exponential line broadening was applied in Topspin. Baseline correction via Whittaker smoothing and peak fitting were done in MNova. n=8 subjects the for the control group, n=7 subjects for the cocaine group.

**Post Processing of Metabolic Flux Data**

Hyperpolarized metabolic flux data analysis was conducted using in house MATLAB (Mathworks, Natick, MA) scripts. The spectra were automatically phased using a minimum entropy approach and baseline corrected. Each spectral set had Gaussian apodization applied to it, using a matched filter approach. For each time series of spectra, the Gaussian filter width was matched to the line width of the highest intensity spectrum, which provides the highest possible SNR. The individual metabolic peaks were assigned using published ¹³C chemical shifts, and integrated as a function of
time. The metabolite values were taken as a sum of the integrated values over the period in which the given metabolite was distinguishable from the noise.

**Modeling of metabolic flux kinetics**

Kinetic models were fit to the time evolution curves of the hyperpolarized pyruvate and lactate resonances. The time dependent pyruvate magnetization was fitted with an Exponentially Modified Gaussian (EMG) function (Equation 3-1) and lactate magnetization \( M_{\text{Pyr}} \) served as input for the differential equation in the Bloch-McConnell formalism (Equations 3-2)\(^{182}\). This modification accounts for the gradual, rather than instantaneous, arrival of the pyruvate bolus in the brain.

\[
M_{\text{Pyr}} = \frac{R_{\text{Pyr}}}{2} e^{\frac{R_{\text{Pyr}}}{2} (2\mu + R_{\text{Pyr}}\sigma^2 - 2t)} erf\left(\frac{\mu + R_{\text{Pyr}}\sigma^2 - t}{\sqrt{2}\sigma}\right)
\]  
\[
\frac{dM_{\text{Lac}}}{dt} = k_{LDH} M_{\text{Pyr}} - M_{\text{Lac}} (R_{\text{Lac}} + (1 - \frac{\cos\theta}{TR}))
\]  

\(M_{\text{Pyr}}\) and \(M_{\text{Lac}}\) are the pyruvate and lactate \([1^{-13}\text{C}]\) magnetization over time, respectively; \(R\) is the effective relaxation term that accounts for loss of signal due to \(T_1\) relaxation as well as flow out of the measured region; the RF excitation tip angle \(18^\circ\) and scan repetition times \(TR\) (1 s) are defined by the acquisition parameters; \(\mu\) and \(\sigma\) refer to the peak time and width of the Gaussian component of the pyruvate curve. Due to the high concentration influx of pyruvate, compared to physiological levels, the system is assumed to be significantly far from equilibrium so that it can be considered unidirectional and first order\(^{183}\). The differential equation was solved using the Euler method, and fitted to the measured product curves to obtain values for \(k\), the conversion rate constant.
NMR spectral deconvolution and statistical analysis

All NMR peak areas were integrated by fitting mixed Lorentzian-Gaussian line shapes to the spectra in MNova to enable deconvolution of overlapping multiplets. All $^1$H and $^{31}$P spectra were referenced to lactate (1.32 ppm) and $\alpha$-ATP (-10 ppm) respectively. A simulated annealing approach was taken to reduce residual error. Peak areas were normalized with a probabilistic quotient normalization approach$^{132}$. These data were first filtered for outliers with the Dixon’s Q-test at a 95% confidence interval. The null hypothesis was then tested and statistical significance for change in individual metabolites was determined with a non-parametric Wilcoxon-Mann-Whitney test in MATLAB (significant difference $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

Results

Acute Cocaine’s Effect on Pyruvate Flux by DNP

Figure 3-1a shows average $^{13}$C spectra over all subjects in the two groups after infusion of hyperpolarized [1-$^{13}$C] pyruvate. Pyruvate and observable products for both the acute cocaine group and control group are shown. For both groups a total of five resonances could be observed: pyruvate, lactate, alanine, pyruvate hydrate, and bicarbonate. Since cocaine is a known vasoconstrictive agent, data was normalized to total carbon signal$^{184}$. Quantification of each of the resonances (Figure 3-3B) indicates cocaine administration induces a significant shift away from glycolytic conversion of pyruvate as demonstrated by a significant drop in the lactate to total carbon ratio, from 0.31 to 0.23 ($p = 0.005$). The spectro-temporal evolutions for the pyruvate and lactate resonances averaged over the two subject groups are shown in Figures 3-1C. Kinetic analysis of the pyruvate and lactate curves show the enzymatic flux from pyruvate to lactate has decreased in animals administered cocaine (Figure 3-3D). From these data,
we observe real-time \textit{in vivo} pyruvate conversion to lactate is decreased in acute cocaine animals suggesting that, as a whole, lactate production is suppressed. Thus, acute administration of a single dose of (10mg/ml/kg) cocaine significantly suppresses the conversion of pyruvate to lactate as observed in the HP signal areas summed over time.

\textbf{In Vivo MRS Quantitation of Major Metabolites}

\textsuperscript{1}H and \textsuperscript{31}P single voxel spectra collected during the psychoactive period of cocaine activity (12-13min) after administration are shown in Figure 3-2. \textsuperscript{1}H spectra were localized to the cortical region of the brain. Fitting of spectral line-shapes indicate a suppression of \textit{in vivo} levels of lactate and GABA after acute cocaine administration. (Figure 3-2A). Levels of glutamate, aspartate, and creatine are significantly increased. \textsuperscript{31}P spectra collected using the whole brain region were used to quantify bioenergetics shifts on cocaine administration (Figure 3-2B). Fitting of major energetic components indicate a significant increase in \(\beta\)-ATP and NADH and a significant decrease in the \(\gamma\)-ATP resonance after cocaine administration.

\textbf{Ex Vivo analysis of Metabolic Shift on Cocaine Administration}

High Resolution Magic Angle Spinning (HRMAS) experiments were conducted on cerebral cortex tissue during the same period as previously described. \textsuperscript{1}H T\textsubscript{2} weighted CMPG spectra were acquired of the tissue showing a significant increase of cataplerotic metabolites aspartate and glutamate while a significant decrease of cerebral lactate was observed (Figure 3-3). To further confirm these results on more global scale, we performed NMR and MS of lyophilized left hemisphere tissue (Figure 3-3/Table 3-1). Solution state NMR was performed on polar extracts of acute cocaine and control tissue with improved spectral resolution as compared to \textit{in vivo} and HRMAS.
approaches. Significant metabolites demonstrated consistent findings across all
correlative techniques.

**Discussion**

Our data indicate that a single injection of cocaine causes a significant 49% 
reduction in pyruvate to lactate conversion. Furthermore, acute cocaine administration 
significantly decreases glycolytic metabolic pools while significantly increasing oxidative 
catatropic products, as observed using multiple metabolomics characterization 
modalities.

Importantly, we extend the findings of past research using the novel dDNP 
technique to demonstrate that cerebral metabolic activity is shifted by cocaine 
administration toward a more oxidative state. This shift is also observed through in vivo 
and ex vivo characterization of metabolite pools. This multi-modal approach provides 
key insights into cocaine’s effect on brain metabolism. While in vivo MR approaches 
provide high physiological accuracy, they often lack the precision measurements of 
multiple metabolites which can be obtained through ex vivo approaches. Even with the 
advent of fast FIELDMAP/MAPSHIM algorithms and implementation of high order 
shimming, in vivo spectroscopy suffers from inherently poor line shapes which 
convolutes overlapping metabolites\textsuperscript{185,186}. While ex vivo analysis has the benefit of 
 improved spectral resolution, effects of tissue manipulations, chemical environment, and 
metabolic stability must be considered. Spectral overlay of in to ex vivo approaches 
display remarkable agreement in the frequency domain while providing a platform for 
metabolic assignment and deconvolution. \textsuperscript{13}C, \textsuperscript{31}P, and mass spectrometry techniques 
further support the \textsuperscript{1}H findings and provide a more complete picture of the complex 
metabolic effects of cocaine administration on brain function.
These are the first studies to demonstrate that the effects of cocaine administration on *in vivo* pyruvate flux can be measured and coupled to metabolic pool analysis on the same timescale. Our results are consistent with past research using *in vitro* 2DG assays in rats, and $^{18}$F-2-fluoro-2-deoxy-D-glucose (FDG) PET in cocaine dependent human subjects$^{156,157,159,187}$.

dDNP is rapidly becoming a cost effective and clinically applicable alternative to PET modalities because of three major benefits; the ability to observe downstream metabolic products, a much faster tracer clearance time, and no exposure to radioactive isotopes of CT irradiation$^{88,90,188}$. Carbon hyperpolarization is necessary for *in vivo* metabolic flux measurements via NMR spectroscopy, due to the large amount of signal averaging that would otherwise be required to overcome the inherent insensitivity of the $^{13}$C isotope. This insensitivity arises due to a combination of poor Boltzmann nuclear polarization, low natural abundance of $^{13}$C, and inherently broader *in vivo* line widths$^{89,189}$. The development of dDNP enables carbon signal enhancement of greater than four orders of magnitude *in vivo*$^{190-193}$. This enhancement is achieved by transferring polarization from high gamma spins (unpaired electrons) to low gamma nuclei ($^{13}$carbon) at low temperature (~1 K) via microwave irradiation. This polarization is retained for several minutes, while it is dissolved in heated buffer and injected into the animal subject. The polarized metabolite can then be observed *in vivo* as it is metabolized into well-characterized products. Through dDNP, we demonstrate that normal cerebral metabolism of the glycolytic intermediate pyruvate is significantly shifted. Brain mitochondrial metabolism of $[1,^{13}$C] pyruvate yields four products and the distribution of only the labeled product pool of lactate and enzymatic fluxes are shifted
as a result of cocaine administration. The reaction from pyruvate to lactate is catalyzed by the enzyme LDH and pyruvate to lactate flux values have been calculated for each test group. *In vivo* enzymatic flux calculations are inherently complex due to the convolution of multiple enzymatic steps with changes in blood perfusion. The monocarboxylate family of transporters (MCT) is responsible first for transport of pyruvate and lactate into the brain then as the lactate shuttle between neuron and astrocyte cells\(^36\). Expression of these transporters have been shown to be disrupted in the presence of cocaine, although not significantly by the 12-13 minute timescale used in the acute cocaine experiment presented in this work\(^{194}\). High dose cocaine administration (80mg/kg ip) has shown to have cytotoxic effects and increases circulating LDH levels, although this dose far exceeds the cocaine challenge presented in this work\(^{98}\). In addition, cocaine has been shown to have a local neural vasodilation, heart rate, and blood pressure affects which might play a substantial role in delivery of the hyperpolarized substrate to the brain\(^{52,195}\). Although we did not observe a significant difference in \(^{13}\)C SNR between all of the test subjects, further experimentation is needed to fully separate perfusion/transport effects from enzymatic rates.

To further validate the importance of dDNP and metabolic flux calculations we investigated steady-state metabolic pools by using traditional *ex vivo* techniques such as mass spectrometry and NMR. High-Resolution Magic Angle Spinning (HRMAS) of cortex tissue sampled in the dDNP experiments demonstrated findings consistent with increased overall cellular oxidative metabolism including significant increases in excitatory neurotransmitter pools and significantly decreased glycolytic production of lactate. Solution state NMR data agreed with HRMAS data while also showing a
significant decrease in glucose and selected amino acids. LC/MS data also showed significantly decreased amino acid profiles observed through NMR approaches (Table 3-1). Together these results indicate amino acids are being depleted as a nitrogen source for neurotransmitter synthesis as has been previously reported\(^{80}\). Additionally, TCA cycle intermediates are not significantly changed, which suggests either a balance between anaplerotic and cataplerotic pools or that glutamate synthesis is primarily synthesized by amino acid pools. Lactate levels are significantly decreased across NMR based approaches when cocaine is administered indicating a dysfunction in astrocytic delivery to neurons or a carbon source for neurotransmitter synthesis. Furthermore, our solution state NMR and MS data suggests that astrocytic production of glutamine (which is difficult to deconvolute with other approaches) is also suppressed. Decreased astrocytic activity would also present as an overall decrease in pyruvate to lactate flux, a finding that is consistent with our DNP results. In addition to the metabolic shifts described, we also observed a significant shift in \textit{in vivo} bioenergetics as a result of acute cocaine administration. \textit{In vivo} \(^{31}\)P spectroscopy of whole brain energetics demonstrate a reduced \(\gamma\)-ATP pool and a significantly increased \(\beta\)-ATP. Additionally, we observed a significant shift in the NAD+/NADH ratio toward a more reduced state. This reduced state has been previously been proposed by Cerda’n \textit{et al} under periods of high glutamate transmission\(^{99}\). \textit{In vivo} \(^{31}\)P NAD+/NADH deconvolution is inherently difficult due to the proximity of the resonances and the relative metabolic abundance, so further experimentation is needed to confirm these results\(^{196,197}\).

This study demonstrates, for the first time, \textit{in vivo} observation of a hyperpolarized glycolytic intermediate, pyruvate, as a marker of cocaine’s effect on brain metabolism.
This technique has been shown to be a robust means of calculating metabolic product pool distribution and pyruvate kinetic conversion values by overcoming previous signal to noise restraints. The technique used in this study provides a more complete understanding of whole brain energetics especially when combined with metabolic pools analysis. Although these data outline a preliminary method by which cerebral metabolism can be observed, additional hyperpolarized isotopomer analysis are needed to fully understand the cerebral metabolic shifts associated with drug challenge.

Our results support previously published data that cocaine has a significant acute metabolic effects. It has recently been shown that the role of lactate in the brain is more complex than previously thought, and can be used as a supporting source of energy generation \textit{in vivo}^{198}. Quantitative kinetic analyses of pool size dynamics can provide important flux values that are used as markers of healthy brain function and normal substrate handling. Our present results indicate that acute cocaine administration initially shifts the cerebral flux equilibrium away from lactate production. Near steady state metabolic profiles have shown decreased lactate levels within the areas of the nucleus accumbens \textit{in vivo} when cocaine is administered acutely\textsuperscript{161}. Our metabolic pool results also show a significant decrease in lactate pool sizes in addition to the flux data supporting the hypothesis that cocaine actively shunts the cytosolic production of lactate during the psychoactive period of cocaine action.
Figure 3-1. Acute cocaine’s effect on pyruvate metabolism. A) Average brain spectra from all rodent groups administered with acute cocaine or control. B) Metabolite/total carbon ratios for pyruvate and its metabolically active products. Significant changes were observed only in lactate metabolic product. C) In vivo time evolution curves for observation of hyperpolarized [1-\textsuperscript{13}C] pyruvate and [1-\textsuperscript{13}C] lactate. An EMG algorithm was used to fit the influx pulse and subsequent relaxation of each peak. D) k values for conversion of pyruvate to lactate calculated from time evolution curves.
Figure 3-2. $^{31}$P and $^1$H in vivo MRS. A) Representative $^1$H spectra with metabolic assignments, fit of the control spectrum, and fit residual displayed as shaded peak. B) Representative $^{31}$P spectra with fitting of the control spectrum, assignments and fit residual shown. Shaded in peaks indicate significant changes. Abbreviations: MM – macromolecules, Lac – lactate, Gln – glutamine, Glu – glutamate, Asp – aspartate, Tau – taurine, Ins – inositol, UDG – uridine diphosphate glucose.
Figure 3-3. Comparison of representative $^1$H spectra from intact tissue (HRMAS) and solution NMR of polar metabolite extracts. Polar extract (top) and HRMAS (bottom) spectra show significantly shifted metabolic pools denoted with asterisks. Insets from PQN normalized spectra show significantly increased or decreased metabolites common to both techniques (center). Annotation: dd—doublet of doublets, m—multiplet, t—triplet, d—doublet.
Figure 3-4. Model of cocaine induced metabolic shifts in neuron/astrocyte exchange hypothesis. Significant increase aspartate and neurotransmitter glutamate while significant glutamine levels were observed. Additionally, redox shifts in ADP and NADH were also observed.
Table 3-1. Panel of significant metabolic changes due to acute cocaine administration. Table is organized by technique beginning with MR approaches; solution state NMR of polar brain metabolites, HRMAS of cortical tissue, single voxel $^1$H spectroscopy of cortex, and single voxel $^{31}$P spectroscopy of whole brain. Bottom panel are significant changes in amino acids and malonyl CoA as measured by targeted mass spectrometry. $^1$H values were scaled to lactate. $^{31}$P were scaled to β-ATP. * near edge of detection limit.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>p-value (wilcoxon)</th>
<th>Control (± sterror)</th>
<th>Acute (± sterror)</th>
<th>Percent Change (± sterror)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polar Extract ($^1$H)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine monophosphate (AMP)</td>
<td>0.0093</td>
<td>2.1 (0.15)</td>
<td>2.7 (0.080)</td>
<td>24% (9.4)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.0022</td>
<td>3.8 (0.29)</td>
<td>5.0 (0.14)</td>
<td>32% (11)</td>
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<tr>
<td>Glucose</td>
<td>0.0070</td>
<td>0.38 (0.13)</td>
<td>0.12 (0.0069)</td>
<td>-69% (10)</td>
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<tr>
<td>Glutamate</td>
<td>0.0093</td>
<td>33 (1.4)</td>
<td>39 (0.58)</td>
<td>18% (5.4)</td>
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<tr>
<td>Glutamine</td>
<td>0.0080</td>
<td>21 (1.0)</td>
<td>17 (0.35)</td>
<td>-19% (4.0)</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.040</td>
<td>0.15 (0.013)</td>
<td>0.10 (0.012)</td>
<td>-34% (10)</td>
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<tr>
<td>Lactate</td>
<td>0.040</td>
<td>100 (5.3)</td>
<td>88 (2.2)</td>
<td>-12% (5.0)</td>
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<td>Valine</td>
<td>0.014</td>
<td>1.2 (0.14)</td>
<td>0.74 (0.07)</td>
<td>-39% (9.3)</td>
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<td><strong>HRMAS ($^1$H)</strong></td>
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<tr>
<td>Aspartate</td>
<td>0.014</td>
<td>3.1 (0.56)</td>
<td>5.19 (0.37)</td>
<td>65% (32)</td>
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<td>Glutamate</td>
<td>0.0012</td>
<td>26 (1.5)</td>
<td>33 (0.93)</td>
<td>28% (8.4)</td>
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<td>Lactate</td>
<td>0.0060</td>
<td>100 (5.0)</td>
<td>77 (4.14)</td>
<td>-23% (5.6)</td>
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<td><strong>In Vivo ($^1$H)</strong></td>
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<tr>
<td>Aspartate</td>
<td>0.013 *</td>
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<td>39 (6.2)</td>
<td>177% (65)</td>
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<td>Creatine</td>
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<td>630 (17)</td>
<td>706 (8.7)</td>
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<td>GABA</td>
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<td>190 (5.8)</td>
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<td>Lactate</td>
<td>0.0010</td>
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<td>35 (4.6)</td>
<td>-64% (5.3)</td>
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<td><strong>In Vivo ($^{31}$P)</strong></td>
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<tr>
<td>β-ATP</td>
<td>0.040</td>
<td>1 (0.028)</td>
<td>1.1 (0.037)</td>
<td>12% (4.8)</td>
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<td>γ-ATP</td>
<td>0.019</td>
<td>0.90 (0.019)</td>
<td>0.82 (0.021)</td>
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<td>NAD/NADH</td>
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<td>12 (0.90)</td>
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<td>NS</td>
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<td>0.16 (0.00064)</td>
<td>NS</td>
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<tr>
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<td>0.17 (0.012)</td>
<td>0.16 (0.00064)</td>
<td>NS</td>
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<tr>
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<td>0.08 (0.0050)</td>
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<td>90% (23)</td>
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Table 3-1. Continued

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<th>Acute (± sterror)</th>
<th>Percent Change (± sterror)</th>
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<td>nmol/mg dry powder wt.</td>
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<td>Glutamate</td>
<td>0.032</td>
<td>30 (1.2)</td>
<td>38 (1.3)</td>
<td>25% (6.8)</td>
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<tr>
<td>Glutamine</td>
<td>0.032</td>
<td>47 (4.5)</td>
<td>30 (2.3)</td>
<td>-35% (8.0)</td>
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<tr>
<td>Histidine</td>
<td>0.016</td>
<td>0.62 (0.025)</td>
<td>0.32 (0.027)</td>
<td>-49% (4.9)</td>
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<tr>
<td>Leucine</td>
<td>0.016</td>
<td>0.58 (0.054)</td>
<td>0.37 (0.021)</td>
<td>-36% (6.9)</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.032</td>
<td>1.7 (0.21)</td>
<td>1.1 (0.096)</td>
<td>-37% (9.4)</td>
</tr>
<tr>
<td>Malonyl-CoA</td>
<td>0.016</td>
<td>0.80 (0.14)</td>
<td>0.40 (0.0050)</td>
<td>-50% (8.5)</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.016</td>
<td>0.29 (0.021)</td>
<td>0.19 (0.0045)</td>
<td>-34% (4.9)</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.048</td>
<td>0.29 (0.11)</td>
<td>0.25 (0.0055)</td>
<td>-12% (3.7)</td>
</tr>
<tr>
<td>Valine</td>
<td>0.016</td>
<td>0.65 (0.047)</td>
<td>0.31 (0.016)</td>
<td>-52% (4.3)</td>
</tr>
<tr>
<td>3-Hydroxybutyrate</td>
<td>NS</td>
<td>0.13 (0.0046)</td>
<td>0.10 (0.0048)</td>
<td>-19% (4.7)</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>NS</td>
<td>0.014 (0.000089)</td>
<td>0.016 (0.00099)</td>
<td>20% (11)</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>NS</td>
<td>26 (0.66)</td>
<td>25 (0.88)</td>
<td>-4.4% (4.2)</td>
</tr>
<tr>
<td>Alanine</td>
<td>NS</td>
<td>2.7 (0.039)</td>
<td>3.1 (0.35)</td>
<td>-13% (13)</td>
</tr>
<tr>
<td>Arginine</td>
<td>NS</td>
<td>1.6 (0.63)</td>
<td>0.64 (0.06)</td>
<td>-59% (17)</td>
</tr>
<tr>
<td>Asparagine</td>
<td>NS</td>
<td>0.46 (0.046)</td>
<td>0.41 (0.018)</td>
<td>-11% (9.9)</td>
</tr>
<tr>
<td>Citrate</td>
<td>NS</td>
<td>0.50 (0.026)</td>
<td>0.48 (0.029)</td>
<td>-2.8% (7.9)</td>
</tr>
<tr>
<td>Citrulline</td>
<td>NS</td>
<td>0.12 (0.0099)</td>
<td>0.10 (0.0086)</td>
<td>-13% (10)</td>
</tr>
<tr>
<td>Fumarate</td>
<td>NS</td>
<td>0.15 (0.0058)</td>
<td>0.16 (0.0061)</td>
<td>8.0% (6.0)</td>
</tr>
<tr>
<td>Glycine</td>
<td>NS</td>
<td>3.9 (0.30)</td>
<td>3.1 (0.27)</td>
<td>-20% (9.4)</td>
</tr>
<tr>
<td>Lactate</td>
<td>NS</td>
<td>71 (5.3)</td>
<td>65 (2.2)</td>
<td>-8.5% (7.5)</td>
</tr>
<tr>
<td>Malate</td>
<td>NS</td>
<td>0.93 (0.052)</td>
<td>0.97 (0.053)</td>
<td>3.9% (8.1)</td>
</tr>
<tr>
<td>Proline</td>
<td>NS</td>
<td>0.54 (0.03)</td>
<td>0.45 (0.024)</td>
<td>-17% (6.6)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>NS</td>
<td>0.13 (0.026)</td>
<td>0.092 (0.0048)</td>
<td>-27% (16)</td>
</tr>
<tr>
<td>Serine</td>
<td>NS</td>
<td>3.7 (0.048)</td>
<td>3.5 (0.080)</td>
<td>-6.0% (2.5)</td>
</tr>
<tr>
<td>Succinate</td>
<td>NS</td>
<td>2.0 (0.078)</td>
<td>2.13 (0.0061)</td>
<td>6.5% (5.14)</td>
</tr>
<tr>
<td>Threonine</td>
<td>NS</td>
<td>3.3 (0.39)</td>
<td>2.54 (0.21)</td>
<td>-24% (11)</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>NS</td>
<td>0.093 (0.0092)</td>
<td>0.091 (0.00072)</td>
<td>-2.2% (9.8)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>NS</td>
<td>0.34 (0.026)</td>
<td>0.31 (0.035)</td>
<td>-9.4% (12)</td>
</tr>
</tbody>
</table>
CHAPTER 4
BATH SALTS INDUCED CHANGES IN CEREBRAL METABOLIC POOLS

Bath Salts Induced Changes in Cerebral Metabolic Pools

Chronic use of illicit and psychostimulant 3,4-Methylenedioxyxpyrovalerone (MDPV) can cause severe and adverse effects including extreme aggression, psychosis and violence\(^{199}\). Additionally, use of MDPV blocks of reuptake of dopamine neurotransmitters, cerebral connectivity, and cerebral metabolism. We detail here a significant increase in excitatory neurotransmitter levels in the reward regions of the brain during the psychoactive period of MDPV administration. Furthermore, we present herein a multi-parametric approach toward cerebral metabolomics as a detector of metabolic dysfunction due to administration of bath salts.

**Introduction**

MDPV is a synthetic cathinone derivative and one of the active ingredients of the psychoactive designer drugs known as "bath salts"\(^ {199} \). MDPV is a schedule I drug according to the USDA classification and is one of the most common compounds found in biological fluid collected from bath salts associated overdose and intoxication related emergency room visits\(^ {200} \). In addition to its acute psychoactive effects, MDPV has also been shown to elicit increased empathic and euphoric responses\(^ {199} \). After a prolonged acute “rush” period (3-4 hours), MDPV “crash” has been shown to promote extreme bouts of violence, depression, anxiety, tachycardia, combative behavior, and even death\(^ {201-203} \). Similar to cocaine, MDPV prevents dopamine reuptake but with the added blockade of norepinephrine transporters\(^ {200} \). MDPV is up to 10 times more efficacious and potent than cocaine on the dopamine levels *in vivo*\(^ {200} \). MDPV produces similar connectivity responses to that of cocaine, demonstrating significant reductions in
connectivity between the prelimbic area and the nucleus accumbus (NAc), with both eliciting functional loss of plasticity between these two reward regions\textsuperscript{199}. At high doses, acute MDPV creates functional loss of connectively between striatal, frontal cortex (PFC) and hypothalamic (HIPP) regions. Although there has been limited work performed on the cerebral metabolic effect of MDPV it has been shown to have a phasic effect on nucleus accumbens glucose levels\textsuperscript{195}. Here we show a significant change in energy metabolism and cerebral metabolic pool size of the reward circuitry in response to acute MDPV administration.

**Materials and Methods**

All experiments were prior approved by the University of Florida Institutional Animal Care and Use Committee of the University of Florida (IACUC). Adult male Long Evans rats (250-450 grams) obtained from Charles River Laboratories (Wilmington, MA, USA) were housed in pairs in a temperature and humidity-controlled vivarium (12 h light-dark cycle with lights off at 1900 h) with food and water available ad libitum. For all experiments, subjects were administered a single intraperitoneal (i.p.) injection of Methylendioxyppyrovalerone (MDPV) dissolved in sterile isotonic saline solution at a dose of 3 mg ml\textsuperscript{−1} kg\textsuperscript{−1} while under anesthesia 60 minutes prior. Controls followed the same time course of anesthesia and received sterile saline solution i.p. at a dose of 1 ml kg\textsuperscript{−1}.

**Metabolic Measurements Using Hyperpolarized [1-\textsuperscript{13}C] Pyruvate**

Subjects were anesthetized with Piralmal isoflurane (Bethlehem, PA) delivered in medical grade oxygen (2.5-3.25 % at a flow rate of 2 LPM oxygen) ~20 minutes prior to imaging. A catheter was placed in the left leg femoral vein for delivery of hyperpolarized (HP) substrate. After cannulization, the femoral catheter was connected to an
automated injector, and animals were positioned in an MRI cradle with an Agilent 60 mm I.D. quadrature $^1$H (470 MHz) volume coil and a homebuilt 25 mm surface $^{13}$C (118 MHz) coil. The cradle was then positioned in an 11.1 Tesla (T) / 33 cm horizontal-bore MRI system (Oxford/Agilent) for landmarking, shimming, and RF calibration.

**Dynamic Nuclear Polarization and Dissolution**

3M [1-$^{13}$C] sodium pyruvate (Cambridge Isotopes, Boston, MA) and 50 mM 4-oxo TEMPO (2,2,6,6-Tetramethyl-1-piperidinyloxy) (Sigma, St. Louis, MO) were prepared in a 400 μL volume of 2:1 D$_2$O:ethanol-D$_6$ (V/V). Glassed sample beads were formed by dripping this solution into a liquid nitrogen bath and then transferred into a Teflon sample cup. The sample was polarized for approximately 1 hour in 5 T magnet (Bruker, Billerica, MA) at 1.1 (± 0.03) K in a custom built cryostat by irradiating at 140 GHz / ~50 mW (Virginia Diodes Inc., Charlottesville, VA) to elicit polarization transfer from the TEMPO radical to the pyruvate $^{13}$C spins. Typical solid state polarization levels were ~15% of theoretical maximum at the point of injection. Next, heated (120 °C) phosphate buffered saline was injected into the polarizer sample space at 12 bar using helium gas as a motive force to transfer the melted sample into a custom built automated injector for injection into the subject in the 11.1 T MRI system (Bruker AV3HD). The time for dissolution and injection was ~9 s. Each subject received ~1.5 mL of polarized pyruvate at a final blood concentration of ~14 mM. Although this volume (~3 ml/kg) is well within the maximum recommended intravascular bolus volume of 5 ml/kg, the rate of injection (~45 ml/min) is well above the recommended maximum rate of injection (3 ml/min)$^{204}$. Rapid injection is associated with transient tachycardia and haemodialalation and could have dramatic effects on blood pressure and substrate delivery times$^{204}$. This effect is controlled for by matching injection volumes and rates of treated and control groups.
Spectra of the injected pyruvate and its metabolic products were acquired every 1 using a small angle pulse (~20°, 50 μs) for each transient. n=7 subjects for the control group, n=7 subjects for the MPDV group.

**Post Processing of Metabolic DNP Data**

Hyperpolarized metabolic flux data analysis was conducted using in house MATLAB (Mathworks, Natick, MA) scripts. The spectra were automatically phased using a minimum entropy approach and baseline corrected. Each spectral set had Gaussian apodization applied to it, using a matched filter approach\(^{181}\). For each time series of spectra, the Gaussian filter width was matched to the line width of the highest intensity spectrum, which provides the highest possible SNR. The individual metabolic peaks were assigned using published \(^{13}\)C chemical shifts\(^{89}\).

**In Vivo MRS**

After being anesthetized, animals were positioned in an MRI cradle with a homebuilt \(^1\)H (470 MHz) transceive volume coil and a Bruker 2x2 receive only phased array surface coil and placed in an 11.1 T MRI system (Bruker AV3HD running Paravision 6.0.1). TurboRARE image slices were collected and used to localize a 6.8 x 2.6 x 5 mm voxel in the prefrontal cortex. Global field mapping and shimming were performed followed by iterative voxel shimming to optimize resolution in the region of interest. Single voxel \(^1\)H spectra were collected using a STEAM pulse sequence with VAPOR water suppression and outer volume suppression with the following parameters: ns = 128, 1K points, 2s recycle time, 8K Hz bandwidth and 5.6 ms echo time. Spectra were manually phased in Topspin, and no line broadening was applied. Baseline correction and peak fitting was performed using Mnova v11.0 software. For \(^{31}\)P experiments, animals were fixed in an MRI cradle with a homebuilt \(^1\)H (470 MHz)
transceive volume coil and a homebuilt 2 cm $^{31}$P (190 MHz) surface coil and placed in an 11.1 T MRI system (Bruker AV3HD). The volume $^1$H coil was utilized for shimming the whole brain region and TurboRARE image slices were used to localize the voxel for data acquisition. $^{31}$P spectra were acquired using an ISIS pulse sequence with 32 scans, 50 ppm sweep width, 2 s recycle time and 1 K points. Data were acquired at the same time intervals as for $^1$H MRS. The spectra were processed with 5Hz exponential line broadening and phase corrected in Topspin. Baseline correction and peak fitting were performed with MNova. n=6 for control groups and n=6 for MDPV subjects.

High Resolution Magic Angle Spinning (HRMAS)

20 mg of frozen tissue + 10 μL D$_2$O was packed directly into a Bruker Kel-F rotor insert for a 4 mm Zirconia rotor. $^1$H NMR spectra were collected at 4 °C, 5 kHz MAS, using a 600 MHz (Bruker AV3 HD) with a 4 mm HRMAS probe. 1D spectra were collected using a PROJECT pulse sequence with water presaturation. The PROJECT sequence applies a T$_2$ filter in a similar manner to the CPMG sequence used previously (Chapter 3), but utilizes so called ‘perfect echoes to suppress J modulation in the resultant spectra, and thus improve quantitation of metabolites, while maintaining the filtering of unwanted macromolecular and lipid signals$^{137}$. For all spectra, 128 scans were collected with a 5 s relaxation delay, 16 K complex points, and a 6 KHz sweep width. HRMAS spectra were individually phased, 0.02 Gaussian line broadening was applied in Topspin. Polynomial baseline correction and peak fitting was applied in MNova. n=7 for control subjects and n=8 for the MDPV group.

NMR Spectral Deconvolution and Statistical Analysis

NMR peak areas were fitting with a mixed Lorentzian-Gaussian line shapes to the spectra in MNova. All $^1$H and $^{31}$P spectra were referenced to lactate (1.32ppm) and
α-ATP (-10 ppm) respectively. Multiplet lineshapes were constrained by matched widths at full-width half max. A simulated annealing approach was taken to reduce residual error. Peak areas were normalized with a probabilistic quotient normalization approach. These data were first filtered for outliers with the Dixon’s Q-test at a 95% confidence interval. The null hypothesis was then tested and statistical significance was determined with a non-parametric Wilcoxon-Mann-Whitney test in MATLAB (significant difference *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001).

Results

Acute MDPV’s Effect on HP Lactate Production by DNP

Figure 4-1 shows average $^{13}$C spectra over all subjects in the two groups after infusion of hyperpolarized [1-$^{13}$C] pyruvate. Pyruvate and observable products for both the MDPV cocaine group and control group are shown. For both groups a total of five resonances could be observed: pyruvate, lactate, alanine, pyruvate hydrate, and bicarbonate. Quantification of each of the resonances (Figure 4-1B) indicates MDPV administration induces a significant increase in lactate production (p-value = 0.01) From these data we observe real-time in vivo pyruvate conversion to lactate is increased in MDPV animals suggesting that, as a whole, lactate production is increased. Thus, acute administration of a single dose of MDPV (3mg/ml/kg) significantly increases the conversion of pyruvate to lactate as observed in the HP signal areas summed over time.

In Vivo MRS Quantitation of Major Metabolites

$^1$H single voxel spectra collected during the psychoactive period of MDPV activity (60min) after administration are shown in Figure 4-2. $^1$H spectra were localized to the PFC region of the brain. Fitting of spectral line-shapes indicate a significant increase in
glutamate (p-value = 0.02) and aspartate (p-value = 0.04) between control and MDPV groups which agree with findings presented ex vivo. $^{31}$P single voxel spectroscopy was also performed for MDPV and control group, but no significant differences were found between the two groups (Figure 4-2).

**Ex Vivo Analysis of Metabolic Shifts on MDPV Administration**

HRMAS spectra for cerebral cortex tissue sections are shown in Figure 4-3. A significant increase in the cataplerotic metabolites aspartate and glutamate is observed on MDPV administration for both the hippocampus and the prefrontal cortex regions. These data are consistent with findings in acute cocaine administration during the psychoactive period. Concurrently, an increase in cerebral lactate is observed although not significantly. To further confirm these results, we also performed solution NMR on the polar metabolites of the PFC and HIPP (Figure 4-4). Solution state NMR spectra for polar extracts of brain tissue improved spectral resolution compared to *in vivo* and HRMAS approaches enabling further quantitation of less abundant metabolic species. These data confirmed HRMAS finding for both regions and indicated a significant increase in lactate pools. Significant decreases in lactate pools and flux are contradictory to the finding presented in acute cocaine administration and could be the result of the difference in the administration periods between the two drugs. Furthermore, significant decreases in glucose and increases in adenosine monophosphate (AMP) levels indicating increased glucose and ATP consumption when MPDV was administered acutely.

**Discussion**

The most characterized reward regions in the brain are the dopamine, glutamate, and GABA circuitry from the ventral tegmental area (VTA) to the nucleus accumbens,
prefrontal cortex and hippocampus$^{205}$. MDPV disrupts the dopamine neurotransmitter system by inhibiting the reuptake of dopamine resulting in overstimulation of the post-synaptic membrane especially in dopamine rich regions$^{206}$. As outlined in paralleling work on cocaine, this neurotransmitter accumulation also has profound gluco-metabolic effects$^{195}$. We investigated the response to MDPV in two reward regions (prefrontal cortex and hippocampus) with untargeted and targeted metabolic approaches. HRMAS of prefrontal cortex regions had significantly increased levels of catapleurotic metabolites aspartate and glutamate at 1 hour after MDPV injection as compared to the control (Figure 4-3). Glutamate is a known excitatory neurotransmitter synthesized primarily in the neuron$^{207}$. Synthesis of glutamate is accomplished through catapleurisis out of the TCA cycle or through nitrogen contributions from branched chain amino acids from the astrocyte and delivered to the neuron$^{84,208}$. Dopaminergic projections from the VTA extend into the both the PFC and Hipp from which then both innervate the NAc through glutamatergic neuronal projections. We observe here a significant increase in glutamate levels for both PFC and Hipp regions. Interestingly, we observed a significant decrease in glutamine in the hippocampus and a decreased, but not statistically significant, glutamine levels in the PFC tissue. This suggests that there is a failure of astrocytes to fully clear glutamate production in the hippocampus. Glutamate is cleared from the synaptic cleft by astrocytes and converted to glutamine to prevent potentially cytotoxic accumulation of glutamate concentrations$^{209}$. Psychostimulant drugs such, as cocaine and substitutive amphetamines, have been shown to have an effect on glutamate activity and transport$^{210,211}$. We observed a significant increase in glutamate levels through HRMAS and in vivo MRS approaches on the PFC at 1 hour after MDPV
administration (Figure 4-3). MDPV has a prolonged psychostimulant effect, as compared to cocaine. It has been shown to retain its effects on reward and functional connectivity at 1 hour while after the vasoconstrictive and temperature phases\textsuperscript{199}.

These \textit{in vivo} data agree with data collected on tissue samples at the same time interval further indicating a shift toward excitatory neurotransmitter transmission in the PFC. Furthermore, the increase in aspartate levels indicate a shift toward TCA cycle catapleurosis. TCA cycle pyruvate is derived both directly through glucose transport and through cooperative lactate shuttling through astrocyte glycolytic metabolism\textsuperscript{212}. Because of the significant increases observed in lactate pools with solution state spectroscopy, we hypothesized a global shift in lactate production as a carbon source for neurotransmitter synthesis. To test this, we utilized HP pyruvate as a tracer of glycolytic function. HP pool analysis showed pyruvate derived lactate was significantly increased as compared to control (Figure 4-1). These data indicate a shift towards the glycolytic conversion of pyruvate as an energy source. Our hypothesis is that increased lactate production from pyruvate is an indicator of increased astrocytic function to supply neuronal cells through the lactate intermediate.

\textbf{Conclusion}

MDPV is a potent stimulant that has significant psychological, physiological, and metabolic effects through its blockade of dopamine, serotonin, and norepinephrine. We show here that these effects should also include increased glutamatergic action and lactate production, although whether this action is direct or indirect in currently unknown. Through investigation of global and local reward region metabolomics we can conclude that acute MDPV administration causes a shift in metabolic function during the psychoactive period. These data agree with previously published work on cocaine’s
effect on brain metabolism and provide insight into a drug which is relatively new and understudied. Experiments on acute cocaine administration demonstrated a similar significant increase in aspartate and glutamate levels in the cortex regions (Chapter 3). These comparable metabolic profiles, although on a different timescale, indicate to us that these drugs have analogous effects on excitatory neurotransmitters production in the cortex region of reward. Both drug administrations were observed in the psychoactive phase of drug action and caused a 28% and 17% increase in glutamate levels in cocaine and MPDV tissue respectively. Additionally, acute cocaine caused a significant rise in cortex aspartate level of 65% compared to MDPV’s 53%. This disparity could be due in part to the extended timeline of MPDV observation (13 minutes vs. 60 minutes). The half-life of cocaine is approximately half of MPDV, which indicates that disparities in glutamate pools is not directly coupled to drug stability but might instead be connected to other factors such as vasoconstriction.200,213 Interestingly, we also observed a reversal of lactate production in hyperpolarized pyruvate to lactate conversion between cocaine and MDPV. In our cocaine model, we proposed the decreased lactate production as an astrocytic failure to effectively produce deliver lactate to neurons. Increased lactate production in acute MDPV administration is an indicator of effective astrocyte shuttle function and increased glutamate and aspartate synthesis or decreased neuronal lactate utilization. In vitro experiments have shown that increased glutamate concentrations stimulate increased lactate pools to supplement neuronal oxidative phosphorylation.214 Our steady state and dDNP pool data indicate that there is an increase in lactate pools, although there is no significant increase in lactate to pyruvate conversion kinetics. These comparisons together suggest that at 60
minutes post MDPV injection astrocytes are still producing lactate but neurons have ceased to utilize astrocytic lactate. Decreased glucose concentrations, at this time point, suggested that neuronal oxidative supply is reliant on glycolysis over lactate. These data indicate a switch in neuronal energy utilization, although additional time points and TCA cycle metabolomics by targeted mass spectrometry are needed to fully understand the disparity between cocaine and MDPV. Finally, we show here that acute MDPV administration causes significant changes in both neurotransmitter synthesis and lactate production in the cerebral regions of reward.
Figure 4-1. Increased cerebral lactate production by dissolution DNP. A) Average representative $^{13}$C spectrum with HP metabolites assigned. B) Fitted areas for prefrontal cortex regions normalized as a percentage of total carbon signal. C) Representative in vivo time evolution curves for observation of hyperpolarized $[1^{13}$C] pyruvate and $[1^{13}$C] lactate. An EMG algorithm was used to fit the influx pulse and subsequent relaxation of each peak. D) $k$ values for conversion of pyruvate to lactate calculated from time evolution curves. n=7 for control, n=7 for MDPV.
Figure 4-2. In vivo MRS of prefrontal cortex and whole brain. A) Representative 1H STEAM spectrum with metabolic assignments of prefrontal cortex with fits and fit residual (red). B) fitted areas for prefrontal cortex regions. n=6 for Control, n=6 for MDPV. C) Representative 31P ISIS spectrum with metabolic assignments of whole brain fits and fit residual (red). D) Fitted areas of bioenergetics molecules with no significant change observed. n=5 for control groups and n=4 for MPDV subject groups.
Figure 4-3. Cerebral metabolic response to MDPV by solution NMR. A) Representative solution NMR 1H NOESY spectrum. B) Fitted data comparisons for prefrontal cortex regions. C) Fitted data comparisons for hippocampus.
Figure 4-4. Cerebral metabolic response to MDPV by HRMAS. A) Representative HRMAS 1H PROJECT spectrum. B) Data comparisons for prefrontal cortex regions. C) Data comparisons for hippocampus. Abbreviations: Lac – Lactate, Ala – Alanine, NAA – N-Acetyl Aspartic Acid, GABA - gamma-amino butyric acid, Glu – Glutamate, Gln – Glutamine, Asp – Aspartate, Cr – Creatine, Tau – Taurine, MI – Myo-inositol. n=7 for control, n=8 for MDPV.
CHAPTER 5
PHENYLALANINE DISRUPTS CEREBRAL GLUTAMINE LEVELS

Phenylketonuria (PKU) is a metabolic genetic disorder that is characterized by the loss of function of phenylalanine hydroxylase (PAH), the enzyme needed to metabolize the amino acid phenylalanine. Untreated, this disease leads to severe mental disabilities and low IQ. Treatment currently consists of strict phenylalanine depleted diets after diagnosis is performed in infancy. Although much is known about the neurological impact of PKU from a neurological perspective, little is known about the direct neurometabolic component of PKU. We present here in vivo and ex vivo approaches towards metabolic profiling of cerebral shifts associated with a murine model of human PKU.

Introduction

PKU is a non-sex linked, autosomal recessive disorder that affects ~1:12,000 babies across all ethnic groups\textsuperscript{215}. PKU was discovered in 1934 by Asbjørn Følling through the detection of high levels of phenylpyruvic acid in two severely mentally disabled children\textsuperscript{216}. Diagnosis is performed in the first weeks of life with a heel prick to screen for increased circulating phenylalanine levels\textsuperscript{217}. Although there is currently no cure for PKU, early detection of the disease should initiate a strict dietary regime lacking phenylalanine. Currently, dietary restriction is the foundational treatment to prevent neurological impairment and include reduction of high protein foods\textsuperscript{218}. Because of the protein rich dietary restrictions, supplementation of vitamins, minerals, and amino acids (especially tyrosine) is paramount\textsuperscript{219}. Some childhood patients are unable or unwilling to adhere to a strict phenylalanine depleted diets and often this diet is relaxed during adolescent years. If left untreated, PKU can cause severe neurological impairments and
developmental delays including microcephaly and epilepsy. Untreated cases of PKU can result in decreased cerebral myelination and reduced production of neurotransmitters dopamine, serotonin, and norepinephrine. Furthermore it has been shown that PKU causes depletion of large neutral amino acid (valine, methionine, isoleucine, leucine, tyrosine, histidine, and tryptophan) transport from the blood to the brain. In addition to reduction in LNAAs, dysregulation of circulating glutamine levels has also been reported. It is our central hypothesis that PKU causes severe metabolic dysregulation in the brain tissue of effected animals. We present here a case for glutamine depletion in the cortex and striatum regions of PKU affected animals both in vivo and ex vivo. This study is the first of our knowledge to observe this glutamine depletion in brain regions of living animals and suggests cause of neurological impairments observed in severely affected PKU individuals.

**Materials and Methods**

**Animal Model and Care**

All animal experiments were performed with the approval of the University of Florida Institute for Animal Care and Use Committee. Adult female PAHenu2/enu2 and PAHWT/enu2 (12-20g) animals on the BTBR genetic background as orthologues for human PKU and control respectively were used for the study as previously described. Animals were housed in sets of five in a temperature and humidity-controlled vivarium (12 h light-dark cycle with lights off at 1900 h) with food and water available ad libitum. Diets were not phenylalanine depleted.

**In Vivo Magnetic Resonance Spectroscopy**

For all studies, subjects were anesthetized with Piralmal isoflurane (Bethlehem, PA) delivered in medical grade oxygen (1-1.5 % at a flow rate of 2 LPM oxygen).
Respiration was regulated at 50-60 breaths per minute and body temperature was maintained by warming the probe to 37 °C through recirculating water. ¹H imaging and spectroscopy was performed on a AVIII HD 750 MHz spectrometer using a commercial 30 mm Bruker linear volume coil (Billerica, MA). The mouse was placed under anesthesia by ventilation with 3% isoflurane in an O₂ gas using an enclosed chamber. The anesthetized mouse was then transferred to an MRI/S-compatible animal coil and placed into the isocenter of the magnet. Multi Slice Multi Echo (MSME) MRI sequences were taken to localize voxel placement and to optimize shimming regiments. 2 x 1 x 3 mm and 2 x 2 x 3.5mm regions were selected in the cortex and striatum structures respectively and voxel localized shimming was performed. Point RESolved Spectroscopy (PRESS) spectra were collected with the following parameters: nt – 128, BW – 9.5ppm, points – 2048, TE – , TR – 3 s. VAPOR water suppression was applied for solvent suppression for each voxel. 2 Hz exponential line broadening and manual phasing was applied to each spectrum in Topspin (Bruker). n = 5 for control cortex region, n = 7 for control striatum, n = 4 for PKU cortex region, n = 5 for PKU striatum region.

High Resolution Magic Angle Spinning (HRMAS) Acquisition

Animals were briefly anesthetized by isoflurane followed by rapid decapitation. Cortex and striatum tissue was dissected and flash frozen in liquid nitrogen. Approximately 20 mg of frozen tissue + 10 μL D₂O was packed directly into a Bruker Kel-F rotor insert for a 4 mm Zirconia rotor. ¹H NMR spectra were collected at 4 °C, 5 kHz MAS, using a 600 MHz (Bruker AV3 HD) with a 4 mm HRMAS probe. 1D spectra were collected using a T₂ weighted, Car-Purcell-Meiboom-Gill (CPMG) pulse sequence with water suppression. For all spectra, 128 scans were collected with a 5 sec
relaxation delay, 16 K complex points, and a 10 ppm sweep width. 1 Hz exponential line broadening was applied and each spectrum was manually phased in Topspin (Bruker). n = 11 for control cortex region, n = 10 for control striatum, n = 9 for PKU cortex region, n = 9 for PKU striatum region.

**Post Processing and Statistical Analysis**

Bernstein Polynomial baseline correction was applied and mixed Lorentzian/Gaussian line shapes were fit in MNova v11.0 (Santiago de Compostela, Spain). All $^1$H were referenced to creatine (3.02 ppm). Multiplet lineshapes were constrained by matched widths at full-width half max. A simulated annealing approach was taken to reduce residual error in MNova. Peak areas were normalized with a probabilistic quotient normalization approach$^{132}$ (Dieterle, F, 2006). These data were first filtered for outliers with the Dixon’s Q-test at a 95% confidence interval in MATLAB (Mathworks, Natick, MA). A null hypothesis was then tested and statistical significance was determined with a non-parametric Wilcoxon-Mann-Whitney test in MATLAB (significant difference $^*p < 0.05$).

**Results and Discussion**

$^1$H Magnetic Resonance Spectroscopy

We observed the metabolic profile associated with the advanced PKU phenotype and compared to phenotypically silent control mice. These data display marked suppression of glutamine at 2.34 ppm in both cerebral structures. Constrained fitting of the glutamine resonance demonstrated a nearly 50% reduction in glutamate in the tissue of the murine cortex and striatum (Figure 5-1). In vivo cortex data also shows a significant increase in myo-inositol levels (Figure 5-1). Glutamine is an important precursor to neurotransmitters $\gamma$-amino butyric acid (GABA) and glutamate in the brain$^{228}$. 
Neurotransmission recycling exchange between glial cells and neurons is essential for healthy cerebral function. Glutamine production reduces glutamate neurotoxic events and prevent neuronal damage\textsuperscript{75}. Reductions in circulating glutamine levels has been previously reported for PKU patients\textsuperscript{225}. This is the first study, to our knowledge, where cerebral glutamine levels have been reported as significantly suppressed \textit{in vivo} in both the cortex and striatum regions. Previous studies on PKU subjects have focused on circulating and cerebral phenylalanine pools and kinetics\textsuperscript{229-233}. Cerebral phenylalanine levels are 2-4 times lower in the brain than it is in the plasma, micromolar and millimolar concentrations respectively. Fitting and statistical analysis of steady state phenylalanine levels from baseline levels with \textit{in vivo} approaches is unreliable which is why we applied our approach toward investigating other potential biomarkers of PKU neurological decline\textsuperscript{231}.

Untreated, PKU causes severe mental impairment which has been linked to demyelination of the cerebral white matter\textsuperscript{221}. Hypomyelination of PKU affected is thought to be the cause of many of the neuropsychological delays observed in PKU patients. Myo-inositol is one of the most common metabolites in the brain and is easily observable by magnetic resonance spectroscopy. Dysregulation of myo-inositol levels have been observed in neurological disorders such as hemiplegic migraines, multiple sclerosis, diabetes mellitus, brain cancer, and others\textsuperscript{234-237}. Myo-inositol is primarily responsible for maintaining the cell membrane and myelin structure. Damage to the myelin sheath often results in increased concentrations of free myo-inositol \textsuperscript{238}.

**High Resolution Magic Angle Spinning**

HRMAS is analytical tool for metabolic observation of intact tissue. HRMAS has the added benefit of simple tissue preparation loaded directly into a rotor. Additionally,
HRMAS provide well resolved spectral peaks by orienting the sample at the magic angle to average out the dipolar molecular interactions. The resolution improvement over in vivo spectroscopy is more than an order of magnitude providing the spectral information needed to resolve metabolic J-coupling. We present here a similar significant decrease in glutamine across both the cortex and striatum tissue (Figure 5-3). Overall, the HRMAS findings confirms observations made in the in vivo measurements and outline as significant reduction in glutamine levels. Spectral fitting of in vivo data, while constrained and peak picked, cannot fully deconvolve overlapping peaks. HRMAS provides a platform for more precise spectral assignments and peak fitting. While HRMAS is highly precise technique, confirmation of metabolic shifts in vivo provides a complementary accuracy and physiological relevance for tissue that has not been manually dissected. Furthermore, ex vivo HRMAS tissue has been exposed to hypoxic conditions and has undergone a level of enzymatic and metabolic decomposition regardless of the proficiency and speed of dissection. Complementary technical approaches, as utilized here, are an effective means towards improving both the accuracy and the precision of metabolic profiling for diseases such as PKU.

**Conclusion**

Here we demonstrate a significant decrease in glutamine concentrations across both in vivo and ex vivo approaches for cortex and striatum regions of the brain of a murine model of human PKU. We present this significant decrease in glutamine concentrations is a potential therapeutic target for treating neurocognitive decline in PKU patients. As previously discussed, deviations for strict dietary restrictions is a major contributor for psychological disorders associated with PKU. Low cerebral glutamine levels in the brain has been shown in a variety of neurological diseases and by
preventing healthy neurotransmitter recycling. We present glutamine here as an important cerebral biomarker for an advanced PKU phenotype and as a possible target for treatment. Furthermore, we believe that glutamine deficiency might also be present in other amino acid metabolic disorders. We also further show that the complementary MRS *in-vivo* / NMR *ex-vivo* analysis previously demonstrated on cocaine and MDPV drug models, also provides significant new information regarding metabolic dysfunction in the brain in preclinical disease models.
Figure 5-1. *In vivo* glutamine suppression in PKU mice cortex. A) Voxel localized region for cortex spectroscopy. B) Representative $^1$H spectrum from control (top) and PKU animals (bottom). C) Significant metabolic changes for cortex regions. Abbreviations: MM – Macromolecules, NAA – N-Acetyl Aspartic Acid, Glu – Glutamate, Gln – Glutamine, Asp – Aspartate, Cr – Creatine, Tau – Taurine, MI – Myo-inositol
Figure 5-2. Glutamine suppression in PKU mice striatum. A) Voxel localized region for striatum spectroscopy. B) Representative $^1$H spectrum from control (top) and PKU animals (bottom). In C, significant metabolite changes for striatum regions.
Figure 5-3. Glutamine suppression in PKU mice tissue. A) Representative $^1$H HRMAS spectrum at 600 MHz. B) Fitted data from cortex tissue. C) Fitted data from striatum.
CHAPTER 6
CONCLUSION AND FUTURE DIRECTION

In the body of work, I have presented multimodal approaches toward neurometabolic profiling of psychostimulant drug administration and PKU associated neurodegeneration. Through investigation of these conditions, I have developed protocols and workflows for *in vivo* and *ex vivo* neurometabolomics at high and ultra-high magnetic fields (4.7 – 17.6 T). In addition, I have implemented flux measurements and calculations for real-time enzymatic processes through dissolution DNP *in vivo*. My work has outlined novel biomarkers of neuronal/astrocyte interactions and highlighted metabolic dysregulations during cocaine and MPDV administration. Furthermore, complementary *in vivo* and *ex vivo* data collected from a human orthologue PKU model presents convincing evidence of glutamine’s role in PKU neuronal damage. These results are preliminary and much more work is needed to fully understand the findings presented herein.

**Future Directions**

The work conducted here are a first step in understanding the relationship between drug administration and cerebral metabolism. Much more work is needed to fully elucidate the direct and indirect effects that neurotransmitter blockade has on intercellular exchange. Furthermore, due to the high structural and metabolic heterogeneity of the brain, local profiling is paramount toward connecting biochemical processes with function and behavior. Dissolution DNP is a relatively new field so technical improvements are in continued progression. Hyperpolarized metabolic imaging required the $^{13}$C acquisition of HP substrate and products assigned by discrete resonance frequencies. Due to the exponential signal decay due to relaxation effects
and RF sampling, this chemical observation is only currently possible on the seconds to minutes time scale. This rapid signal decay presents both problems and opportunities. Rapid relaxation is beneficial because only single pass metabolism is observed, in contrast to isotopomer experiment with complex labeling schematics of multiple turns of a given cycle, should be considered. The major downside to current hyperpolarization is the truncated timeframe for which useable signal can be acquired. Because collection of spectral, spatial, and temporal information are all important for understanding biological processes, compromises are often made to which of these three to observe. For this work, the acquisitions were performed as an array of small tip angle pulses spatially constrained by the coil dimensions. By contrast, single time point chemical shift imaging can be utilized to collect a singular 2D data from HP injections at the maximum signal intensity\textsuperscript{189}. In recent years, pulse sequences have been optimized to acquire accelerated, spatially resolved spectra\textsuperscript{239}. These approaches include rapid spiral chemical shift imaging (CSI), sparse sampling, and $T_2$ based sequences (balanced steady state free precession)\textsuperscript{240-242}. Implementation of these sequences for neurometabolic imaging would be advantageous for studies on drug’s metabolic effect because of the spatial and temporal components. Furthermore, spatial localization of small regions of reward far away from the RF coil (NAc) could provide additional information about the metabolic state of the brain. Implementation of spectral-spatial pulses would be the logical next step for this neurometabolic project.

Hyperpolarized $[1^{-13}C]$ pyruvate is a model compound for dissolution DNP experiments because it both has a long $T_1$, is non-toxic and higher than physiological doses, and provides important data on the glycolytic state of the organ system in
question. Although a majority of hyperpolarization experiment have been performed with pyruvate, other metabolic tracer should be considered, especially for cerebral measurements. Because we observed a significant shift is glutamate biosynthesis in our $^1$H pool experiments, hyperpolarized glutamate is a logical target for consideration. [1-$^{13}$C] pyruvate is not observable through the TCA cycle because the labeled nuclei are converted to lactate. HP [2-$^{13}$C] glucose has been shown to produce observable HP glutamate, glutamine, and aspartate in the human brain with stochastic low-power decoupling at 3 T$^{243}$. Additionally, HP of 2-keto[1-$^{13}$C]isocaproate (KIC), a branched chain amino acid, provides an indirect measurement of glutamate/glutamine synthesis and could be used as a marker of neuronal/astrocyte intercellular exchange$^{244}$. KIC has a relatively long T$_1$ of 55 seconds and is able to cross the blood-brain barrier providing an excellent platform for studying drug action of brain metabolism$^{89}$.

In addition to the future direction of hyperpolarized experiments, I believe further investigation is needed applying the techniques described here towards pharmacokinetic analysis of the drugs administered. We selected the metabolic sampling timings based on previously published psychoactive period. Although it was outside the time constraints realistic to this project, sampling additional time points of drug action would provide a better understanding of changing neurometabolic shifts. It has been shown that both cocaine and MPDV have a dynamic effect on glucose brain metabolism$^{195}$. Probing these effects at varying time points across the psychoactive period and throughout withdrawal phases would provide a better understanding of the acute and long term effects of these drugs.
Conclusion

In conclusion, I feel privileged to have been a part of these projects and to have had a hand in contributing to the rich scientific knowledge surrounding neurometabolic research. Multimodal approaches towards understanding the effects of drug metabolism on neuron/astrocyte interaction have resulted in novel findings. Further, these data have indicated a glutamate/glutamine/lactate disruption not previously reported. Ultra-high field MRS of PKU coupled with complementary tissue profiling has presented convincing evidence of glutamine’s role in PKU associated neurodegeneration. The findings presented here have promising applications towards continued investigation.
Goal: The purpose of this protocol is to observe brain pyruvate metabolism through DNP. This protocol covers the in vivo application of hyperpolarized pyruvate injection and observation. This protocol does not include solid state polarization protocols which vary between instrument. The audience of this protocol is an individual familiar with basic animal handling with basic non-survival surgical procedures including femoral vein catheterization. Additionally, s/he should have familiarity with rapid hyperpolarized fluid handling and instruments included herein.

MATERIALS:

CHEMICALS AND REAGENTS:
1. Catheter lock – sterile heparinized saline (500IU/mL)
2. Tissue adhesive (VETBOND)
3. Alcohol swab
4. Isoflurane (USP)

EQUIPMENT:
1. Catheter - “Micro-renathane” catheters – MRE-025 (0.025 OD x 0.012 ID)
2. Catheter Needle - 27G needle with tip removed
3. 25G needle for catheter guide
4. 1mL syringe
5. Hair clippers
6. Alcohol swab
7. Cellulose Eye Spears (to control bleeding)
8. Sterile monofilament
9. Sterile surgical suture
10. Surgical tools (surgical clip, forceps, hemostat, etc)
11. Surgical microscope
12. Tape
13. Surgical pad
14. Isoflurane vaporizer
15. Nose cone for anesthesia
16. DNP injector kit
17. Syringe pump

PROTOCOL:

Preparation of DNP injector
1. 7.5mm ring size (~1mL of injection fluid)
2. grease hydraulic piston
3. place hydraulic piston in injector
4. Fill dead space above piston with water
5. Screw in cover plate over water to ensure no air bubbles
6. Fill outflow catheter port with saline to ensure no air bubbles are injected
7. Do not tilt

Preparation of syringe pump
1. Set up syringe pump (Note: pump is magnetic so should be set up outside magnetic field)
2. Plug in VGA to VGA connected to DNP
3. Plug into power and turn on
4. Pump regime should be set to pump at maximum rate for a volume of 5mL dispense volume. Consult manual to check or change these values
5. Ensure hydraulic (water) line to injector is free of bubbles

Preparation of RF and cantilever
1. Begin by setting up cradle/cantilever
   a. Turn on spectrometer
   b. Turn on recirculating water bath
   c. Prepare cradle with anesthesia/water tubing/respiratory monitor/RF coils
      (1H volume TX/RX : 13C surface coil)

Femoral vein catheterization
1. Surgical equipment and animal setup
   a. Place rat in induction box at 3LPM isoflurane and 1.5-2% O₂ rate
   b. Fill syringe with heparin lock solution ~1ml
   c. Connect syringe to 27 G needle and remove sharp tip. Connect blunt needle to ~7cm catheter section.
   d. Cut animal end of catheter to a sharp bevel
   e. Fill catheter with heparin lock ensuring no air bubbles
2. Place animal supine on surgical area and tape left leg straight.
3. Catheter placement
   a. Clip hair from surgical site and swab with alcohol
   b. Make ~2cm incision above femoral artery/vein
   c. Retract skin to expose femoral artery/vein
   d. Isolate femoral vein by removing connective tissue
   e. Insert sterile 25G needle, advancing towards the head to create hole for catheter insertion
   f. Before removing needle have catheter prepared from insertion **this transition needs to be done quickly to ensure hole does not clot and close
   g. Quickly remove needle and insert catheter line
   h. Advance catheter towards the head **this should be done with little or no resistance
   i. Ensure catheter is patent by withdrawing blood and injecting small (~50uL) amount of saline **if blood does not withdrawal or saline injection pools around catheter insertion site, readjust catheter placement until these parameters are met.
   j. Secure catheter by placing suture caudal to the insertion site
k. Suture the skin over the catheter and further secure the skin and catheter with VETBOND
l. Carefully place the catheterized animal in the cradle with the head facing the rear of the magnet continually checking catheter patency
m. Connect isoflurane line
n. Connect water recirculating bath lines
o. Connect respiratory monitor

4. Placement of RF coils and magnet placement
   a. Place the $^{13}$C coil over the head, centering coils over the brain
   b. Slide cradle back into volume $^1$H coil so as centered
c. Check tuning/matching of $^{13}$C coil with bore simulator
d. Secure animal fully in cantilever
e. Insert cantilever into magnet just outside of bore
f. Place DNP injector behind animal’s legs and secure
g. Connect hydraulic line to DNP injector
h. Test catheter patency (blood should still withdrawal)
i. Carefully connect catheter line to DNP injector ensuring no air bubbles
j. Secure all lines and center in magnet
k. Connect RF to preamplifiers

5. Imaging and shimming
   a. Check respiration rate (40-60 is normal)
b. Run frequency/power on $^1$H channel
c. Run global shimming
d. Acquire $^1$H image
e. Run voxel localized shimming on region of the $^{13}$C coil
f. Acquire test $^{13}$C spectrum (fat signal should be visible in 4-8 scans)
g. Animal is now ready for DNP injection
Goal: The purpose of the protocol is to carry out $^1$H high resolution magic angle spinning on brain tissue at 4°C for metabolic analysis with high reproducibility. The audience of this SOP is an individual familiar with basic NMR and Bruker software with an understanding of specific HRMAS probe design.

MATERIALS:
CHEMICALS AND REAGENTS:
1. D$_2$O
2. Frozen tissue sample (~20mg)

EQUIPMENT:
1. 4mm HRMAS Probe
2. 600 MHz NMR Spectrometer (AMRIS Facility)
3. 4mm rotor (80µL volume)
4. Rotor cap
5. Kel-f 4mm insert (Bruker BL4)
6. Kel-f insert cap
7. Micro-screwdriver (included with Bruker order)
8. Forceps
9. Benchtop centrifuge (optional)

PROTOCOL:
Setting the Magic Angle:
1. Open Topspin 3.2
2. Turn on the BCU II cooling unit to from the Temperature Control Suite panel on Topspin 3.2. VTU state should be “On” and target flow rate should be ~800-1200 lph.
3. Set target temperature to desired temperature.
4. BCU will take ~20min to cool the probe to 4°C
5. In the Temperature Control Suite panel change the target gas flow to 200 lph. Insert the KBr standard into the probe. Using MAS II unit, touch the insert button. Once the sample is properly inserted then change the target gas flow back to 800-1400 in Topspin. (Check gas flow Standby gas flow should be around 200-500 lph.
6. On the MAS II unit, manually increase the bearing pressure to ~1000 mB then increase the drive pressure slowly until reaching desired spinning speed (± 10%). On the MAS II unit, switch to automatic mode and set desired spinning rate and press “Go”. Spinning rate for KBr should be 4-5 KHz.
7. Wait for the temperature and spinning to become stable before moving on.
8. Create a new KBr experiment by typing edc into the command line of Topspin and make new experiment. Nucleus should be on the X-broadband channel.
9. Use edhead to define the probe and check that the correct slice is being used. If not already selected, define the probe as 4mm HR-MAS 1H/2H-13C Z-GRD.
10. Tune and match the probe by typing wobb (red/big silver screws).
11. Type **zg** and enter to start experiment, **efp** to process, phase **ph**.
12. You should obtain a spectrum with a large center resonance and smaller spinning sidebands separated by the frequency of the rotor frequency.
13. Measure the center peak Full Width of the Half Max (FWHM) by zooming into the region and typing **pps** followed by **hwc**. FWHM of the 5th spinning side band should be around should no larger than 8% of the center peak.
14. To optimize the magic angle - type **gs** opening a window showing the free induction decay (FID). The FID should display rotational echoes (spikes). Turn the gold knob on the probe until the peaks protruding from the FID are as prominent as possible.
15. Redo steps 10 and 11 until the magic angle is optimized.

**Sample Preparation:**

1. Weigh and record the weight of the HR-MAS rotor insert before tissue is added.
2. Add 10 uL D₂O to the insert.
3. Add ~20 mg of tissue. Note: attempt to make the tissue transfer as quickly as possible and if possible on ice or dry ice to reduce metabolic degradation.
4. Air bubbles should be removed by quickly spinning the insert on a benchtop centrifuge or by inserting a needle into the airspace and removing the air.
5. Insert the gas spacer (optional) and cap.
6. Weigh and record the weight of tissue + insert weight to calculate the weight of the tissue alone.
7. Sample insert can be kept in ependorph tube on ice during transit to NMR facility.
8. Sample inserts can be loaded directly into HRMAS insert compatible rotors (80uL volume).
9. Secure fin cap onto rotor and insure that spinning marker is visible (this can be darkened with a black marker).

**Loading sample:**

1. Load the rotor as previously described in Magic Angle section.
2. Set spinning speed to desired frequency (5 kHz)
3. Allow several minutes for the temperature and spinning to become stable before moving on.

**Acquiring spectra:**

1. **Lock** sample on D₂O.
2. Open pulse sequence and name accordingly.
3. After sample is as desired temperature, tune and match on proton the probe by typing **wobb** (yellow screws).
4. Calibrate the correct 90 time by following directions on Bruker manual (this only should be done once and periodically checked).
5. Acquire a ‘zgpr’ spectrum to check water suppression and shims by typing **zg** and enter to start experiment, **efp** to process, phase **apks** (apk).
6. To improve the shims, begin by reading in old shim set (rsh). In gs mode, decrease delay time for rapid acquisition (~0.5-1s) and touch up shims on BSMS window. Zooming in on lactate at 1.3ppm is a good indicator of shim and lineshape quality.
7. While in gs mode on a zgpr sequence, set O1 frequency for optimal water suppression.
8. Run rga to set receiver gain.
9. Set up sequence of experiments and queue them. Sample acquisition parameters for select sequences have been included below.
APPENDIX C
EXTRACTION OF POLAR METABOLITES FROM FROZEN BRAIN TISSUE BY TWO-PHASE METHOD

Goal: The purpose of this protocol is to extract polar metabolites from brain tissue for the purpose of NMR spectroscopy while retaining stability of redox molecules through the two-phase method. The audience of this SOP is an individual familiar with basic laboratory benchtop practices.

MATERIALS:

CHEMICALS AND REAGENTS:
1. Argon (or Helium) gas for degassing
2. Chloroform
3. Methanol
4. Deionized H₂O
5. 1x PBS
6. 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TSP)
7. Deuterium oxide (D₂O)

EQUIPMENT:
1. Scale
2. Ventilated hood
3. Vortex
4. 5-20 mL glass vials
5. Hand-held tissue homogenizer
6. Isopentane bath on dry ice (-20 °C)
7. Glass centrifuge tube
8. Ice bath
9. Lyophilizer

PROTOCOL:
1. Degas all solvents and glassware with Argon prior to extraction.
2. Weigh brain tissue (on ice). Tissue should be between 25-100 mg.
3. Add 1mL (per 50mg tissue) of ice cold methanol in chloroform resistant glass tube
4. Add 1mL (per 50mg tissue) of ice cold chloroform
5. Homogenize on ice with hand-held homogenizer
6. Add 1mL (per 50mg tissue) of ice cold water
7. Rapidly cool sample to -20 °C in isopentane bath on dry ice
8. Vortex thoroughly
9. Transfer methanol/chloroform/water/brain tissue homogenate into chloroform resistant centrifuge tube.
10. Centrifuge at 10,000 x g at 4 °C for 30 mins.
11. Two phases will form, separated by a layer of precipitated protein. The lower (heavier) phase consists of methanol, chloroform and dissolved lipids, whereas the upper (lighter) phase consists of water, methanol and dissolved water-soluble metabolites.

12. Transfer upper phase to an appropriate $\geq$15 ml tube (plastic resistant to methanol, or glass). Keep on ice.

13. Lyophilize to dryness.

14. Store sample at -80 °C until immediately before NMR experiment.

15. Prepare deuterated PBS (DPBS) with 50 $\mu$M TSP and degas with argon.

16. Add 500 $\mu$l DPBS to dried sample and transfer into 5 mm NMR tube (high quality tube).

17. Run NMR experiments.

**REFERENCE:**
Adapted from Lutz A, *et al.* "Metabolomic Analysis of Rat Brain by High Resolution Nuclear Magnetic Resonance Spectroscopy of Tissue Extracts"
Goal: The purpose of this experiment was to ensure full adiabatic inversion over a large bandwidth at 11.1 T by resetting transmitter offset.

Experimental Parameters: ATP + phosphate buffered saline phantom was placed in NMR tubes adjacent to surface 31P coil (190 MHz). Data collected on PV 6.0.1.
- Pulse sequence – spec1dIR (Inversion Recovery T1)
  - NS - 1
  - SW(ppm) - 63.073
  - Repetition time - 3 seconds
  - Excitation Flip Angle - 90° (hard pulse)
  - Refocusing Flip Angle - 180° (sech pulse)
  - Working Frequency 1 - 190.557989 MHz
  - Working Frequency 2 - 190.5599789 MHz

Figure D-1. Inversion (red) vs. recovery (blue) pulse at working frequency 1 with sum (green)
Figure D-2. Inversion (red) vs. recovery (blue) pulse at working frequency 2 with sum (green)
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BIOGRAPHICAL SKETCH

Daniel Downes was born and raised in Winter Park, Florida. He was homeschooled from elementary school through high school. Although homeschooled, Daniel was very active in sports and extracurricular adventures. He attended Palm Beach Atlantic University for his undergraduate work, majoring in biochemistry. Daniel graduated in four years with high honors. Following his undergraduate work, he conducted neurovascular research on the immunological response to brain aneurysm formation at the University of Florida. After conducting research for 4 years, Daniel entered the Interdisciplinary Program in Biomedical Science program at the University of Florida where he joined the laboratory of Joanna Long. Under the mentorship of Professors Long and Febo, he conducted research on the neurometabolic changes associated with psychoactive drugs and neurodegenerative diseases.