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Examining a GCPII+/−/rAAV9 Genetic and Epigenetic Mouse Model of Schizophrenia using Magnetic Resonance

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Examining a GCPII\textsuperscript{+/-}/rAAV9 Genetic and Epigenetic Mouse Model of Schizophrenia using Magnetic Resonance

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Abstract

Schizophrenia is a chronic brain disorder that affects approximately 24 million people worldwide, with about 50% of those affected by the disorder not receiving care (World Health Organization, 2013). Individuals with schizophrenia often exhibit abnormalities in behavior, cognition, and sociability. While the exact cause of the disorder remains unknown, environmental, genetic, and epigenetic factors have been implicated in its onset. This thesis seeks to validate the GCPII+/−/rAAV9 genetic and epigenetic mouse model of schizophrenia by observing whether it induces neurochemical, neurostructural, and behavioral abnormalities that mimic those observed in human patients. Previous studies in our laboratory explored a GCPII+/− mouse model of schizophrenia in which the folate 1 gene, necessary for the production of glutamate carboxypeptidase II (GCPII), was knocked out. Thus, these mice were hypothesized to model glutamate receptor dysfunction by exhibiting altered levels of the neurotransmitter glutamate. Past studies using Magnetic Resonance Imaging (MRI) to acquire coronal images of the brain for volumetric analysis, Magnetic Resonance Spectroscopy (MRS) to evaluate neurometabolite levels, and a behavioral paradigm to examine social tendencies demonstrated that the GCPII+/− model exhibited some, but not all, of the abnormalities associated with schizophrenia in humans. In order to induce epigenetic dysregulation and modify the previous model, a recombinant adeno-associated virus 9 (rAAV9) carrying histone deacetylase enzyme 1 cDNA was introduced into postnatal day (PND) 1 GCPII+/− mice. To further characterize this modified model, Diffusion Tensor Imaging (DTI) was used to visualize abnormalities in white matter connectivity in the brain. This thesis offers an initial analysis of the updated genetic and epigenetic mouse model of schizophrenia as well as an overview of the potential for DTI to be used in future studies.

Heterozygote (HET) vs. wild type (WT) male and female mice with viral injections, saline injections, or no injections were examined with MRI and MRS on postnatal days (PND) 35/36, 49/50, and 63/64, while DTI experiments were conducted on PND 28/29, 42/43, and 56/57. MRI data revealed increased cerebellum volumes in HET males compared to WT controls, though few other neuroanatomical changes reflective of schizophrenia in humans (lateral ventricular enlargement and hippocampal reduction) were observed. MRS experiments found neurochemical abnormalities in each metabolite examined, most notably a reduction in Glx/Cr in HET males at later time points, as well as a significant decrease in (NAA+NAAG)/Cr in both HET males and females. These trends may mirror changes observed in human schizophrenia patients. DTI data did not reveal differences reflective of those observed in human patients with schizophrenia, though it did highlight potential shortcomings of neonatal injections. Initial assessment of the GCPII+/−/rAAV9 mouse model of schizophrenia illustrated the methodological limitations of both the use of DTI in mouse models as well as the attempt to induce epigenetic dysregulation via neonatal injection of rAAV9.
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I. Introduction

Schizophrenia

Schizophrenia is a neurological disorder that has historically been of interest due to its debilitating effects and the lack of understanding surrounding its causes and manifestations (van Os et al., 2009). The disorder occurs in up to 1% of the population worldwide, and with a wide array of symptoms prevalent in those with schizophrenia, diagnosis of the disorder is difficult, especially due to the overlap of many types of symptoms with other types of psychoses (Roth et al., 2009). There are two main categories of symptoms that define the disorder. Positive symptoms are characterized by the presentation of abnormal behaviors, including psychosis (delusions and hallucinations), while negative symptoms are defined by the absence of normal behaviors and include altered levels of motivation, depression, lack of spontaneous speech, and generalized social withdrawal (van Os et al., 2009). Other symptoms include cognitive dysfunction, as people with schizophrenia often have difficulty with memory, attention, and general executive functioning (van Os et al., 2009). The manifestation of symptoms often begins in adolescence and leads to social isolation and stigmatization.

Combined with a vast array of symptoms, inheritance of schizophrenia is thought to be multimodal, with environmental, genetic, and epigenetic factors linked to its onset (Roth et al., 2009). This complex web of factors makes understanding, and ultimately treating, the disorder difficult. For example, studies have recently identified 108 schizophrenia-associated genetic loci, 83 of which had never been reported until 2014 (Ripke et al., 2014). Furthermore, existing pharmacological treatments have a low efficacy for many patients living with schizophrenia (Ripke et al., 2014). Thus, developing a mouse model that effectively mimics the disorder in humans has the potential to improve understanding of the disorder and aid in the exploration of potential treatment options.
Development of Hypotheses

Previous research has been motivated by the distinction between two hypotheses on the neurochemical basis of schizophrenia. Initially, one of the most viable hypotheses on the origins of schizophrenia was based on the function of dopamine in the limbic system, which is an area of the brain involved with learning, emotion, and memory. The hypothesis developed in phases and was initially based on the clinical effectiveness of antipsychotic drugs, specifically those that are antagonists for dopamine receptors, in reducing some of the symptoms of schizophrenia by blocking type 2 dopaminergic receptors (Howes et al., 2009). However, while research was focused on how the blockage of hyperactive dopamine receptors reduced psychotic symptoms, it did not explain all aspects of schizophrenia—specifically, the distinction between positive and negative symptoms. Furthermore, the hypothesis was singularly focused on the function of dopamine, without taking into account the potentially complex array of risk factors for the disorder or the origins of the disorder in specific regions of the brain. Through further development of the hypothesis due to the advent of neuroimaging and spectroscopy studies as well as studies with animal models, it became clear that simply focusing on excessive transmission of dopamine by receptors was inadequate. Combined with data from PET scans of human subjects demonstrating low levels of dopamine in the prefrontal cortex, the concept of regionally specific distributions of dopamine emerged, though the exact irregularity in dopaminergic transmission was not known (Howe et al., 2009). Ultimately, however, the limited success of drugs that function by blocking dopamine receptors to combat negative symptoms led to the development of a different hypothesis to explain the neurochemical basis of schizophrenia (Coyle, 2010).

The alternative hypothesis, and the focus of this thesis, is known as the glutamate hypothesis, which is based on the hypoactivity of the glutamate activated N-methyl-D-aspartate
(NMDA) receptor in the frontal lobe of the brain. NMDA receptors are glutamate-gated ion channels that are highly permeable to calcium; these receptors play an essential role in the development of the central nervous system and the neurological processes involved in functions such as learning, memory, as well as neuroplasticity (Blanke and Van Dongen, 2009). The glutamate hypothesis originated in the observation that phencyclidine (PCP), a non-competitive antagonist to NMDA receptors, induced transient schizophrenic psychosis in human patients (Mouri et al., 2007). Thus, the NMDA receptor was implicated in the pathophysiology of schizophrenia. Glutamate is a neurotransmitter involved in emotion and memory, and the hypoactivity of GCPII, an enzyme responsible for the cleavage of N-acetylaspartyl-glutamate (NAAG) into N-acetylaspartate (NAA) and glutamate in the brain (Figure 1), is thought to be involved in the pathophysiology of schizophrenia (Han et al., 2009).

Figure 1. GCPII cleaves NAAG into NAA and glutamate (Van der Schyf et al. 2006).

**The GCPII^{+/-} Model**

The model examined in this thesis is a revised version of the GCPII^{+/-} model developed by Han et al. (2009). Previous postmortem studies have demonstrated reduced expression of glutamate carboxypeptidase II (GCPII) in cortico-limbic regions of humans with schizophrenia (Han et al., 2009). As stated earlier, the limbic system is intricately involved in both behavior and emotional experience, and is ultimately essential for allowing us to develop self-awareness, motivation, and a sense of how we relate to the outside world (White et al., 2007). Abnormalities in the limbic system, as well as its connections to other brain regions, are thus important to
understanding the pathogenesis of schizophrenia. Furthermore, there are two main pathways of interest implicated in the pathology of schizophrenia. The one-carbon metabolism (C1) and glutamatergic transmission pathways are intricately linked; folate serves as a key nutrient for C1 metabolism, and the interaction between GCPII and folate within this pathway modulates glutamatergic neurotransmission (Schaevitz et al., 2012). Current evidence suggests GCPII is involved in the regulation of synaptic concentrations of NAAG (Han et al., 2009). NAAG is one of the most abundant neuropeptides distributed primarily in the neurons located in the central nervous system (Coyle, 1997). It is a neuropeptide with dual functions—both as an NMDA receptor antagonist and an agonist for a receptor that inhibits glutamate release, metabotropic glutamate receptor-3 (Han et al., 2009). As a result, NMDA receptor activation is diminished and glutamate release is reduced.

The most recent study in our laboratory evaluated this purely genetic mouse model of schizophrenia, the GCPII\+/\- model, in which the folate hydrolase 1 gene, necessary for the production of GCPII was knocked out. Our results from a longitudinal study using magnetic resonance techniques to examine neurometabolite levels and conduct volumetric analyses comparing mice heterozygous for the GCPII mutation and those without the mutation (wild-type) indicated that the model mimicked some, but not all of the symptoms of schizophrenia in humans. Thus, a modification of the past model to include epigenetic factors was desired.

**Revised GCPII\+/\-/rAAV9 Mouse Model: Epigenetic Dysregulation using rAAV9**

Despite genetic factors being linked to the onset of schizophrenia, previous studies of monozygotic twins have elucidated that even in identical twins, who share 100% of their genes, the concordance rate of schizophrenia is less than 70% (Akbarian, 2010), which demonstrates that other factors are at play. More specifically, this fact illuminated the need to focus on both
genetic and environmental factors when trying to understand the complex neuropathology of schizophrenia. Neuroepigenetic approaches to studying schizophrenia, which describe the study of the structure and function of chromatin in the nervous system, have the potential to provide huge advances in available knowledge regarding the pathophysiology of schizophrenia. Chromatin is a DNA-protein complex that effectively packages DNA and thus plays an important role in regulating gene transcription (Sharma et al., 2008). More specifically, epigenetic mechanisms, which describe the covalent alterations to DNA bases or enzyme-induced acetylation or deacetylation of histones, are capable of enhancing or suppressing the expression of specific genes, resulting in a new organization of the genetic material—the epigenome (Figure 2).

Figure 2. Schematic of the essential elements of the epigenome (Akbarian, 2014).

Previous research has long implicated the role of epigenetic factors in the neuropathology of schizophrenia (Sharma et al., 2008). In particular, histone deacetylase enzymes (HDAC) influence gene regulation by modifying chromatin structure surrounding promoter regions in
genes; acetyl groups attached specifically to lysine residues on the N-terminal tail of histone 3 and histone 4 proteins are removed (Sharma et al., 2008). While acetylation causes the histone tail to be released from its wrapping in the DNA strand and enhances transcription, HDAC enzymes remove these acetyl groups and cause the DNA strand to wrap tightly around the histones (Figure 3). The tight wrapping of the DNA strand around histones renders the regulatory regions necessary for transcription inaccessible, and thus gene expression is repressed. Studies have demonstrated increased HDAC1 expression in the prefrontal cortex of human schizophrenic patients, which is ultimately suggestive of deacetylated chromatin and repressed gene transcription in schizophrenic subjects (Sharma et al., 2008).

Figure 3. Regulation of transcription using HDAC enzymes. Acetyl groups are removed, thereby causing DNA strands to wrap tightly around histones and repress transcription (Webb, 2013).

In order to reflect the influence of epigenetics on the neuropathology of schizophrenia in our previous GCPII+/− model, we introduced a recombinant adeno-associated virus 9 (rAAV9) containing the cDNA for histone deactylase 1 into the prefrontal cortex of neonatal mice (Mouradian, 2013). By doing so, we hoped to induce epigenetic dysregulation and thus better model observed symptoms in human schizophrenic patients.

In this study, we utilize magnetic resonance techniques to explore an altered mouse model of schizophrenia, the GCPII+/−/rAAV9 model. To characterize this model, we
implemented a longitudinal study in which mice were examined with MRI and MRS on postnatal day 35/36, 49/50, and 63/64. Furthermore, Diffusion Tensor Imaging (DTI) was utilized to assess abnormalities in white matter connectivity on PND 28/29, 42/43, and 56/57. Through analyzing MRI, MRS, and DTI data, we can begin to evaluate the validity of the GCPII+/−/rAAV9 mice in modeling the neurochemical, neurostructural, and behavioral abnormalities prevalent in schizophrenia in humans. The comparison can be made by assessing similarities and differences between mice heterozygous for the knockout of the folate I gene (HET) versus wild-type (WT) mice lacking the genetic mutation. Furthermore, comparisons can be made between mice receiving the injection of the adeno-associated virus to induce epigenetic dysregulation versus mice with a saline (sham) injection or no injection at all. If the GCPII+/−/rAAV9 mouse model adequately models schizophrenia in humans, we would expect the HET mice that received the rAAV9 injection to differ significantly from the WT mice that were not injected with respect to behavioral, neurochemical, and neurostructural changes. The following schematic outlines the relevant variables used in assessing this model (Figure 4).

**Figure 4.** Summary of the relevant variables involved in evaluation of the GCPII+/−/rAAV9 mouse model of schizophrenia.
**Magnetic Resonance Theory (Hashemi, 2010)**

A longitudinal evaluation of the GCPII+/−/rAAV9 model is possible due to noninvasive techniques using magnetic resonance. Any nucleus with a nonzero spin quantum number (I) can be utilized for a variety of magnetic resonance techniques. Magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), and diffusion tensor imaging (DTI) take advantage of the abundance of protons (^1H) in biological systems (in water and fatty tissue) and their behavior when exposed to an external magnetic field. While the principles of magnetic resonance can be applied to any nuclei with an odd number of protons or neutrons, I will focus on the behavior of hydrogen nuclei in this thesis. Even though magnetic resonance imaging has an extensive history in clinical settings, magnetic resonance spectroscopy is not used clinically, and diffusion tensor imaging is a relatively recent advancement in the field of magnetic resonance techniques.

Nuclei are NMR active if they possess unpaired protons or neutrons, thus giving them a nonzero spin quantum number. This results in magnetic dipole moments. As a result, these types of nuclei can be studied using magnetic resonance techniques. Any charged spinning particles, in this case hydrogen nuclei, create an electromagnetic field that behaves similar to a bar magnet. The magnetic dipole moments of these nuclei are randomly oriented, but upon application of an external magnetic field (B₀), will align themselves parallel, or antiparallel, to the magnetic field (Figure 5). The net magnetization is in the direction of the external magnetic field. In the case of magnetic resonance techniques used in this laboratory, the external magnetic field is supplied by a superconducting magnet.
Figure 5. In the presence of an external magnetic field, the protons’ magnetic moments align parallel or antiparallel to \( B_0 \), resulting in net magnetization in the same direction as \( B_0 \) (http://www.umkcradres.org/Spec/RADPAGE/Magnetized%20nuclear%20spin%20systems.htm).

The spin quantum number of particular nuclei determines the number of quantized energy levels available to a particular nucleus. The number of energy levels available to nuclei is given by the following equation, where \( I \) is the spin quantum number:

\[
# \text{ Energy Levels} = 2I + 1 \tag{1}
\]

By equation (1), it can be calculated that there are two possible energy levels for hydrogen nuclei because the hydrogen nucleus has a spin quantum number of \( \frac{1}{2} \). Thus, the two possible energy states of the hydrogen nucleus are characterized by the \( z \)-component of their spin quantum number: -1/2 and +1/2. The level with a higher energy occurs when the spin magnetic moment is aligned against the magnetic field, while the lower energy level occurs when the spin magnetic moment is aligned with the magnetic field. The net magnetization, which consists of the vector sum of all the individual protons’ spin magnetic moments, is known as the longitudinal magnetization, \( M_0 \), and is in the direction of the external magnetic field since that is the orientation that is lower in energy (Figure 6).

Figure 6. The net magnetization, \( M_0 \), is in the direction of external magnetic field (\( B_0 \)) (Hashemi, 2004).
In the classical model of NMR, as the magnetic moments of the protons align parallel or antiparallel to the magnetic field, the magnetic moments begin to precess about the axis of the external magnetic field. The rate at which the protons’ magnetic moments precess about the external magnetic field, known as the Larmor or resonance frequency ($\omega_0$), is dependent on both the intrinsic gyromagnetic ratio of the proton ($\gamma$) and the strength of the magnetic field ($B_0$) and is described by the Larmor equation:

$$\omega_0 = \gamma B_0$$  \hspace{1cm} (2)

Since the magnetic moments of the protons are out of phase as they precess, the $x$ and $y$ components of the precessions cancel out and result in the net longitudinal magnetization along the $z$-axis (Figure 6).

Magnetic resonance instruments possess metallic radiofrequency (RF) coils that can be used to both transmit RF excitation pulses and receive signal. These coils can transmit RF pulses that introduce a new magnetic field ($B_1$) along the $x$-axis (perpendicular to $B_0$) tuned to the resonance frequency of the protons. With the net magnetization vector initially along the $z$-axis, a RF pulse applied along the $x$-axis perpendicular to the net magnetization vector will result in transverse magnetization. Magnetic moments initially aligned along the $z$-axis with the static external magnetic field shift to precession in the $x$-$y$ plane (Figure 7). As this shift in precession occurs, the net magnetization vector spirals from the $z$-axis into the $x$-$y$ plane in a process called nutation. The magnetic moments precess around the new magnetic field, $B_1$, at a frequency equal to $\omega_1 = \gamma B_1$ by equation (2) until the RF pulse is removed, at which time they again precess around the $z$-axis. Because changing magnetic fields cause the movement of charged particles, specifically electrons, the changing magnetic field induces a current in the coil that can be measured as a signal. The magnetic moments of the protons now have a component precessing in
the x-y plane, and because magnetic fields cause movement of electrons in metal, a signal can be detected in the receiver coil by measuring the current induced in the coil.

**Figure 7.** Applying a 90° (π/2) excitation pulse shifts the net magnetization vector from alignment along the z-axis to alignment in the x-y plane (Hashemi, 2004).

Once the RF excitation pulse is turned off, a number of relaxation processes occur as the spins return to their initial equilibrium alignment – either parallel or antiparallel to the static external magnetic field. One of the relaxation processes releases the excess energy gained by the RF pulse and is governed by principles of thermodynamics—the lowest free energy level for a system is the most favorable. During relaxation, the spins will return to the lowest energy state; they also dephase in the process. These are time-dependent processes and are described by two time constants. The rate at which the component along the z-axis recovers its initial magnetization, \( M_0 \), is described by the T1 relaxation time. This is also known as the longitudinal relaxation time, or spin-lattice relaxation time. The time constant that describes the dephasing of the protons’ magnetic moments, or the rate at which the transverse magnetization vector decays, is described by the T2 relaxation time. The magnetic moments are affected by both spin-spin interactions with other nuclei and the homogeneity of the external magnetic field. Inhomogeneities in the magnetic field, as result of deficits inherent to the magnet, are not uniform and are reflected in an additional time constant, T2*. These inhomogeneities are not reflected in T2, as T2 only represents the transverse magnetization decay that arises from the inherent nature of the tissue. During relaxation, the changes in the x and y components of the magnetization vector induce a current in the RF coil that is received and transduced into an
oscillating, decaying signal. This is known as a free induction decay (FID). These principles form the basis for MR imaging and MR spectroscopy.

**Magnetic Resonance Imaging (MRI) to Address Structural Abnormalities**

MRI is possible due to the abundance of water in living tissue samples, so the signal obtained from the protons in the sample produces a FID that must be spatially encoded using gradient coils in order to get spatially-specific information. In order to spatially encode the signal, multiple RF and magnetic field gradient pulses are delivered in a specific sequence in order to obtain multiple FIDs. Different pulse sequences are utilized to gain contrast that reflects differences in relaxation times for water protons in a variety of tissue types. The spin echo pulse sequence consists of an initial 90° RF excitation pulse followed by a 180° RF pulse in order to refocus spins that had begun to dephase and relax. The refocusing pulse ultimately rephases the spins and thus produces a maximal signal detected by the RF coil. Slice selection gradient coils are on during the RF pulses, phase encoding gradients are used before the refocusing pulse, and the readout gradient is used before the refocusing pulse and while collecting data (Figure 8).

**Figure 8.** (A) Schematic of the timing of the spin echo pulse sequence including gradients (BITC, 2005). (B) Spin echo pulse sequence without the gradients displayed (Kay and Spritzer, 2008).
Furthermore, using a spin echo pulse sequence allows one to acquire T1 or T2 weighted images based on the values used for repetition time (TR) and echo time (TE). TR describes the time interval between applications of the 90° RF pulses, while TE describes the time between the delivery of the RF pulse and the time when signal collection from the echo occurs. With a short TR and a short TE, images with T1 weighted contrast are obtained. In these images, water appears dark, and fatty tissue appears light. T2 weighted images can be produced using a long TR and a long TE. Water appears light and fatty tissue appears dark in images with T2 weighted contrast. It is important to note that T1 and T2 relaxation times are inherent in the tissues. Since the brain consists of different types of tissues—namely cerebrospinal fluid (CSF), grey matter, and white matter—images can be obtained with adequate contrast for analysis. These types of tissues also contain differing amounts of water. For tissues containing more water (CSF contains the most, and white matter the least), magnetic moments dephase more slowly in the x-y plane, and thus larger signal can be detected.

As stated previously, MRI requires spatial encoding in order to understand where the signal produced in the tissue is originating. Spatial encoding is achieved through the use of magnetic field gradient coils. There are three main types of gradient coils: the slice-select, readout (or frequency-encoding), and phase-encoding gradients. Each direction (x, y, and z) requires a gradient to obtain signal along that direction, and these gradient coils are known as $G_x$, $G_y$, and $G_z$. Spatial information is obtained by creating temporary inhomogeneities in the magnetic field in a linear manner along each axis. The slice select gradient ($G_z$) is used to select a slice from the sample from where signal can be received. Within each selected slice, the frequency and phase of the signal is encoded. The phase-encoding gradient ($G_y$) is applied multiple times (one in between the 90° and 180° RF pulses). The frequency-encoding gradient
is applied during the readout time, which is when the echo is received. Ultimately, the frequency and phase of the signal is encoded uniquely for each slice and stored as a data set that must be Fourier transformed to render a final MR image.

The current experimental protocol for this study obtains T2 weighted coronal images of mouse brains using a fast spin echo sequence with 24 slices. The slices are obtained from the caudal end of the brain, which is closest to the tail, to the rostral end of the brain, which is the closest to the snout (Figure 9). The slices consist of vertical cross sections of the brain along the coronal plane that vertically divides the brain’s ventral (closest to belly) and dorsal (closest to back) sections (Figure 9). From each slice, anatomical information is provided that can be used to examine changes in volumes of important structures implicated in schizophrenia. These structures include the cerebellum, the hippocampus, the lateral ventricles, and the third and fourth ventricles.

![Coronal Slice](Figure 9) (A) Schematic of caudal and rostral directions (Amaral, [www.ib.cnea.gov.ar](http://www.ib.cnea.gov.ar)), as well as a line indicating the direction of a coronal slice. (B) Sample of 24 consecutive coronal slices obtained using MRI (Mu, 2011).

**Magnetic Resonance Spectroscopy (MRS) to Explore Neurochemical Changes (Hashemi, 2010)**

Magnetic resonance spectroscopy (MRS) can be used to examine neurometabolite levels in the brain. The neurometabolites of interest in this study are choline (Cho), creatine (Cr), N-
acetylaspartyl glutamate (NAAG), N-acetylaspartate (NAA), taurine (Tau) and glutamate/glutamine (Glu). These neurometabolites have been implicated in the pathology of schizophrenia due to their importance to general neuronal function. Because hydrogen nuclei are prevalent in living systems, ¹H MRS is used throughout this study. Previous studies employing ¹H MRS on ex vivo samples of the cortical region of the mouse brain have been used to determine the concentration of the neurometabolites essential to glutamatergic neurotransmission and general function (Schaevitz et al., 2012). Prior to obtaining MR spectra, the static magnetic field, B₀, must be made homogeneous via manual shimming. After shimming, the sample of interest is placed in the homogenized B₀ and an RF excitation pulse is transmitted. The resulting signal is then received by the RF coils as a FID.

The magnetic field experienced by the protons in a variety of molecules will be affected to differing degrees based on the amount of electron density surrounding them, ultimately giving protons in molecules distinct chemical shifts in an MR spectrum. Since the interactions between the electrons and the magnetic field can affect B₀ based on the degree of electronegativity, an effective magnetic field specific to a particular nucleus is created, Bₑₓᵣ:

\[ B_{\text{eff}} = B_0 (1 - \sigma) \]  \hspace{1cm} (3)

where \( \sigma \) is the shielding constant and is dependent on the electron density surrounding the nucleus of interest. For nuclei with more electron density, the nucleus is more shielded and experiences less of B₀. Thus, the peak corresponding to this nucleus is shifted upfield. The reverse is also true. Nuclei surrounded by less electron density have a deshielded nucleus, experience more of B₀ and thus have a corresponding peak that is shifted downfield in the MR spectrum. Neurometabolites of interest, therefore, can be identified by their unique chemical
shifts along the x-axis (in units of parts per million) of a MR spectrum (Figure 10). Areas under the peaks are then used to indicate relative concentrations of metabolites.

**Figure 10.** Sample $^1$H MR spectrum of a mouse brain with relevant neurometabolites at different chemical shifts labeled.

In this thesis, a specific pulse sequence is used for *in vivo* spectroscopy on the hippocampal/cortex region of the mouse brain. The pulse sequence is known as point resolved spectroscopy (PRESS), which is a single voxel technique for spatial localization (Barker and Lin, 2006). This study places the voxel in the hippocampal/cortex region of the mouse brain to collect signal from this region (Figure 11). In PRESS, three mutually orthogonal slice selective pulses are utilized such that the signal is selectively collected in the voxel where all three slices intersect (Barker and Lin, 2006).

**Figure 11.** MR image displaying the placement of the voxel over the hippocampal region in the mouse brain (Huang, 2012).

Since the concentration of water within the hippocampal voxel is so much greater than the concentration of neurometabolites of interest, methods must be used to suppress the signal
coming from the protons of water before applying the PRESS excitation RF pulses. Additionally, signal emanating from regions outside the voxel of interest must be suppressed so that only the signal from the voxel is collected. Suppression of water and outer volume signal occurs using crusher gradients. Water protons and protons outside the voxel are excited with an RF pulse, and then their spins are dephased using a crusher gradient such that virtually no signal is detected prior to the subsequent RF pulse (Barker and Lin, 2006). In the PRESS sequence, 3 RF pulses with flip angles of 90°, 180° and 180° are applied in the direction of the x, y, and z axes respectively (Figure 12).

![Figure 12. Schematic of the PRESS sequence used to obtain MR spectra in the hippocampal region of the mouse brain (Barker and Lin, 2006).](image)

As stated previously, this study places the voxel in the hippocampal/cortex region of the mouse brain because this is a region of the brain thought to be implicated in the pathology of schizophrenia (Shenton et al., 2001). A brief review of the neurometabolites potentially involved in the neuropathology of schizophrenia is necessary to understand the importance of this aspect of assessment of the GCPII+/−/rAAV9 model.

**Choline**: Choline is essential to normal cell function, and studies have shown it to have beneficial influences on brain structure and function during development (Zeisel and Blusztajn, 1994). Thus, levels of choline are essential to overall neuronal health. Choline is a precursor to acetylcholine, a neurotransmitter (Hyde and Crook, 2000). Choline is therefore an important
biomolecule, and disrupted cholinergic pathways have been implicated in the pathophysiology of schizophrenia (Hydre and Crook, 2000). As a result, levels of choline in the brain can be indicative of neuronal dysfunction and characteristic of the pathophysiology of schizophrenia.  

**Creatine:** Creatine is considered a marker of phosphate metabolism (Kraguljac et al., 2013). $^1$H MRS spectra quantify the amount of total creatine, which is a combination of creatine and a phosphorylated version of creatine, phosphocreatine (Öngür et al., 2009). While creatine is frequently used as an internal standard for human MRS studies since its concentration is maintained within a narrow range in the brain, some studies have observed altered creatine levels in patients with a variety of psychotic illnesses (Öngür et al., 2009). Many studies have positively correlated creatine levels with working memory skills and overall performance on cognitive tasks (Grošić et al., 2014). Thus, a reduction in creatine levels in dorsolateral prefrontal cortex, hippocampus, and basal ganglia has been correlated with diminished performance on certain cognitive tasks (Kraguljac et al., 2013). Regardless, so long as the creatine concentration is assumed to not vary systematically across groups within a study, creatine can be used to normalize spectra and reduce sources of error specific to an individual subject (Öngür et al., 2009). For this reason, the creatine level is used to normalize spectra in this thesis.

**N-acetylaspartyl glutamate and N-acetylaspartate:** NAA is the product of the hydrolysis of NAAG, and is an important indicator of neuronal loss or dysfunction. Many studies have observed significantly reduced levels of NAA in the frontal lobe and hippocampal regions in patients with schizophrenia (Grošić et al., 2014). Some studies have positively correlated NAA with working memory skills (Yeo et al., 2000, as cited in Grošić et al., 2014). Ultimately, these reductions in NAA are related to structural abnormalities on the molecular level, as many studies have implicated neuronal or axonal loss in schizophrenia (Kraguljac et al., 2013). Furthermore,
due to the diminished rates of NAAG hydrolysis, the concentration of NAAG is increased in schizophrenic patients. Since NAAG acts as an agonist for at metabotropic glutamate receptor 3, glutamate release is reduced (Schaevitz et al., 2012).

**Taurine:** Taurine is a semi-essential amino acid synthesized from cysteine. It is an inhibitory neurotransmitter that has been observed to have significantly higher levels in human patients with schizophrenia (De Luca et al., 2008).

**Glutamate and glutamine:** Glutamate is a nonessential, excitatory amino acid in the central nervous system that plays an important role in learning and memory. Studies have shown elevated levels of glutamate in patients with schizophrenia, and antipsychotics are used to enhance glutamatergic transmission (De Luca et al., 2008). While *ex vivo* MRS studies on the mouse brain have allowed significant resolution of glutamate and glutamine levels, previous *in vivo* studies in this laboratory have not shown changes in glutamate levels, perhaps due to the lack in resolution of acquired spectra (Schaevitz et al., 2012). Another potential limitation for the use of *in vivo* MRS is that the technique cannot distinguish between glutamate originating due to metabolic processes and glutamate involved in neurotransmission (Schaevitz et al., 2012).

**Diffusion Tensor Imaging (DTI) to Assess White Matter Connectivity**

Diffusion tensor imaging takes advantage of the movement of water molecules within tissues (Dong et al., 2003). The focus of this thesis has been on the implementation of DTI to better assess the GCPII+/−/rAAV9 model. DTI is a powerful tool in understanding the neuropathology of a variety of psychiatric disorders and brain injuries due to its ability to quantify the degree of diffusion in different types of tissue in the brain. By analyzing DTI data, brain tissues can be characterized and potential pathologies identified.
The contrast between isotropic diffusion, which describes unconstrained diffusion in a pure liquid environment and anisotropic diffusion, which describes directionally-dependent diffusion is essential to the utility of DTI (Figure 13). In brain tissue, the existence of structural barriers to diffusion—myelin sheaths, cell membranes, and white matter tracts, to name a few examples—results in regionally-dependent differences in the anisotropy of the diffusion.

Diffusion in different regions of the brain can be quantified with three main factors. Most generally, the mean diffusivity (MD) values describe the average diffusion of water molecules without accounting for directionality. The apparent diffusion coefficient (ADC) describes the degree of restriction of water molecules. The fractional anisotropy (FA) value describes the degree of directionality of diffusion.

**Figure 13.** Representation of isotropic diffusion (spherical) versus anisotropic diffusion (elliptical) (Beaulieu, 2002). In isotropic diffusion, there are very few barriers to diffusion and diffusion of water molecules is equal in each direction. Isotropic diffusion is a better representation of the degree of diffusion occurring in CSF filled structures in the brain. In anisotropic diffusion, molecules are restricted to diffusion along a principle axis. This type of diffusion is characteristic of diffusion in structures such as white matter tracts.

Furthermore, the diffusion in each three-dimensional voxel can be represented in tensor form as a diagonally symmetric 3x3 matrix (Figure 14), where the principal diffusion directions for a specific three-dimensional tract of the sample can be described. Through diagonalization of the 3x3 matrix, the three eigenvalues and three eigenvectors of the matrix can be determined. The largest of the three eigenvalues describes the value of maximal diffusion, and its corresponding
eigenvector points in the direction of maximal diffusion—the direction parallel to the boundaries present in the system (Dong et al., 2003). By quantifying the diffusion in a collection of voxels within a sample, the nature of the tissue can be characterized because the major axis of diffusion will align in the direction of white matter tracts (Kubicki et al., 2005).

![Diffusion Tensor Matrix](image)

**Figure 14.** The 3x3 matrix representing the diffusion tensor and its corresponding ellipsoid representation (Alexander et al. 2007). The diagonal elements of the tensor are used to determine the mean diffusivity of the sample. The eigenvalues and eigenvectors of the tensor can be used to determine the fractional anisotropy of a specific 3D tract of sample.

In DTI, diffusion is encoded in the MRI signal using magnetic field gradient pulses (Kubicki et al., 2005). Diffusion information is acquired through a collection of diffusion-weighted images along each of the principal gradient directions, allowing water diffusion to be tracked in any direction by using differing combinations of $G_x, G_y, \text{and } G_z$ (Figure 15). In order to determine the six independent elements of the diffusion tensor, a minimum of 6 non-collinear and non-coplanar gradients must be applied (Dong et al., 2003). In our study, a spin echo pulse sequence utilizing a single-shot, echo planar imaging readout is used (Figure 15).
Figure 15. Timing of spin echo-planar imaging pulse sequence (Alexander et al., 2007).

First, a gradient pulse dephases the magnetization in each voxel, while a second pulse rephases the magnetization. Since the phases of non-diffusing molecules will essentially cancel after these two gradient pulses, there will be no signal attenuation from diffusion after application of the RF pulses (Alexander et al., 2007). In contrast, when water molecules are diffusing along a direction within a particular tissue due to the direction of the applied gradient, the signal phase will change by differing amounts during each RF pulse. After the application of the initial RF pulse, the sample has been dephased. Once the second gradient pulse is applied, molecules that have diffused since the initial pulse will not be rephased. Since the signal measured by the receiver coil represents a sum of the signals from all of the water molecules in a particular voxel, the net phase difference due to the translational movement of water molecules results in measurable signal attenuation (Alexander et al., 2007, Mori and Zhang, 2006).

Diffusion measurements are made by determining signal intensity as a function of the b-value, also known as the diffusion sensitizing gradient factor (Beaulieu, 2001). This can be summarized in an equation for the intensity of the signal (S):

\[ S = S_0 e^{-bD} \]  (4)
where $S_0$ represents the signal value with no gradients, $b$ represents the b-value (set at 1000 s/mm$^2$), and $D$ represents the diffusion coefficient (Figure 16). The b-value is dependent on the nature (both strength and timing) of the two gradient pulses and is described by the following equation, where $g$ is the gradient strength, $\delta$ is the duration of the gradient, and $\Delta$ is the time separation from the start of the first gradient to the start of the second gradient (Güllmar et al., 2005):

$$b = \gamma^2 g^2 \delta^2 (\Delta - \delta/3)$$

(5)

Figure 16. (A) Signal strength as a function of the b-value. (B) Anatomical images of human brains obtained using various b-values (http://ej.iop.org/images/0034-4885/76/9/096601/Full/rpp339755f05_online.jpg).

The advent of neuroimaging and post-mortem studies have proved invaluable to advancing understanding of a variety of neurological disorders (Shenton et al., 2001), and diffusion tensor imaging is a fairly new imaging technique that has the potential to provide unique information. Variability in findings between diffusion-weighted imaging studies (including neurocircuitry studies) has made determining the exact nature of white matter abnormalities difficult (Wheeler and Voineskos, 2014). In the past two decades, the hypothesis that the pathology of schizophrenia is rooted in abnormalities in cerebral connectivity has gained credibility with the use of DTI studies, though the small sample sizes and focus on chronic schizophrenic patients have limited the wider application of the findings from such studies (Wheeler and Voineskos, 2014).
The value of DTI in understanding white matter connectivity abnormalities exists largely because while white matter appears homogeneous in conventional MRI, which is insensitive to the organization of fiber tracts and their organization within the brain, DTI is sensitive to these factors. The properties of diffusion in white matter fiber tracts are affected by fiber density, the average fiber diameter, the integrity of myelin sheaths, and the directionality of the fibers within designated voxels (Kubicki et al., 2005). Thus, abnormalities in white matter tracts can be detected with DTI and hopefully linked to observed clinical symptoms.

Many studies in humans with chronic schizophrenia have found decreased FA and increased MD in the prefrontal and temporal lobes, as well as abnormalities in fiber bundles between different regions of the brain (Figure 17), specifically the uncinate fasciculus (involved in decision-making, social behavior, and some types of memory), the corpus callosum (main tract that connects the two hemispheres), the cingulum bundle (connects structures in limbic system) and the internal capsule (connects the cerebral cortex to the spinal cord and brain stem, Wheeler and Voineskos, 2014).

**Figure 17.** White matter tracts thought to be involved in the neuropathology of schizophrenia from a lateral (left) and frontal view (right) of the human brain. The uncinate fasciculus (red), cingulum bundle (purple), corpus callosum (blue) and internal capsule (green) are shown (Wheeler and Voineskos, 2014).

Meta-analyses have revealed two broad, significant areas of reduction in FA in humans—the left frontal deep white matter and the left temporal deep white matter (Ellison-Wright and Bullmore,
2009, as cited in Wheeler and Voineskos, 2014). In terms of connecting white matter abnormalities to clinical symptoms, a handful of studies have correlated anisotropy in white matter in the prefrontal cortex with a variety of negative symptoms, impulsiveness, and aggressiveness (Kubicki et al., 2005). Furthermore, several studies have correlated specific values of fractional anisotropy with errors in tasks related to executive functioning (Kubicki et al., 2005). At the same time as specific white matter tract abnormalities have been implicated in schizophrenia, several studies have also found more generalized abnormalities in diffusion throughout the brain (Wheeler and Voineskos, 2014). However, one overarching Achilles’ heel to the use of DTI in a clinical setting for diagnostic purposes rests in its methodological limitations; there are few accepted parameters for data acquisition, analysis, and post-processing techniques, and studies are plagued by biased sample populations (chronic schizophrenics compared with first-episode schizophrenics) and small sample sizes (Kubicki et al., 2005). Thus, while studies over the past two decades have confirmed some abnormalities in white matter tracts, it remains unclear whether the abnormalities are localized to specific tracts or are characteristic of structures throughout the entire brain; entire networks throughout the brain may be implicated in the neuropathology of the disorder (Wheeler and Voineskos, 2014). Taken together, these suggest that perhaps network-based analyses will become necessary for characterizing the neuropathology of the disorder.

The implementation of DTI in this thesis to assess the GCPII^{+/−}/rAAV9 model is unique. DTI was used with the hope of mirroring observed decreases in FA within regions of white matter thought to be involved in the neuropathology of schizophrenia—namely the cingulate bundle (connects limbic structures), uncinate fasciculus (involved in decision-making, social behavior, and some types of memory), arcuate fasciculus (connects areas involved in language
processing), and corpus callosum (Kubicki et al., 2005, Wheeler and Voineskos, 2014). In addition to implementing DTI in the evaluation of our model, a technique known as fiber tractography may be used to visualize fiber pathways between regions and quantify diffusion in specific fiber bundles (Kubicki et al., 2005). There are a variety of post-processing techniques that have been developed to allow visualization of white matter tracts using DTI data. Fiber tractography functions by choosing regions of interest for analysis, and then grouping together voxels within the region that have similar diffusion characteristics, thus suggesting that they belong to the same fiber bundle and allowing tract-specific characterization of specific DTI metrics (Wheeler and Voineskos, 2014). Another post-processing technique is known as voxel-based morphometry. In this technique, anatomical images are spatially normalized onto a brain atlas and DTI metrics are calculated in each voxel, which can then be compared between patients and between a variety of regions within the brain (Wheeler and Voineskos, 2014). The most recent technique to emerge is known as tract-based spatial statistics (TBSS); voxel-based diffusion metrics are mapped onto alignment-independent representations of white matter tracts (Wheeler and Voineskos, 2014). Similar to the methodological limitations involved in the acquisition of DTI data, there are few established protocols for the implementation of the variety of post-processing techniques. Overall, DTI will likely benefit from more extensive research in both humans and animal models so that functional deficits can be connected with white matter abnormalities in patients with schizophrenia.

This thesis seeks to offer an initial assessment of the GCPII+/−/rAAV9 mouse model of schizophrenia. Furthermore, it seeks to assess the feasibility and quality of data acquired from MRI, MRS, and DTI. There are two major updates in the model that require assessment. First, the introduction of the adeno-associated virus seeks to better mimic the progression of
schizophrenia in human patients as well as offering a better understanding of the role the epigenome plays in the pathology of the disorder. Additionally, a major portion of this thesis centers around the implementation of an additional mode of assessment of the model—diffusion tensor imaging. This type of imaging has huge diagnostic potential for better characterizing the neuropathology of schizophrenia, because the white matter connections between brain regions are proposed to be significantly altered in schizophrenia.
II. Materials and Methods

**GCPII\(^{+/−}\) Model and Animal Maintenance**

All experiments were conducted on heterozygous (HET) and wild type (WT) GCPII\(^{+/−}\) mice with a C57B1/6J background bred in the Wellesley College Animal Facility. All mice used in experiments descended from a founding colony from McLean Hospital; mice were bred on a C57BL/6 background for more than ten generations (Han et al., 2009). All procedures were approved by the Wellesley College Institutional Animal Care and Use Committee and conformed to the standards outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Schaevitz et al., 2012).

Mice were housed in the Wellesley College Animal Facility and kept on a 12 hour light/dark cycle at a temperature of 21 ± 1°C; food and water were available ad libitum. Pups were weaned at approximately postnatal day (PND) 21 and separated by sex in different cages. Ear clipping was used as an identification method for imaging and behavioral studies, and toe clipping was used as an identification method for those mice that received injection of a sterile saline solution or rAAV9, the adeno-associated virus. Mouse genotyping was carried out by Mouse Genotype (Carlsbad, CA) using ear clipping samples.

**Injection of rAAV9 into Neonatal (PND 1) Mice**

Postnatal day 1 mice (day of birth) were removed from the mother temporarily and anesthetized in a plexiglass induction chamber (Braintree Scientific Inc., Braintree, MA) with 4% isoflurane (Abbott Laboratories, Chicago, IL) in O\(_2\) maintained at a flow rate of approximately 0.4 liters per minute. Once anesthetized, mice were transferred to a stereotactic frame (David Kopf Instruments, Tujunga, CA) that contained a neonatal mouse bed (Figure 18); administration of isoflurane and oxygen was maintained using a nose cone made out of plastic
tubing (U.S. Plastic Corporation, Lima, OH). A syringe (Hamilton Laboratory Products, Reno, NV) was mounted in a probe holder attached to the stereotactic frame; separate syringes were used to deliver either the adeno-associated virus or a sterile saline solution.

![Figure 18. Stereotactic set up for neonatal injection of rAAV9 into neonatal mice. Mice were placed in the labeled bed; tubing delivered a constant mixture of isoflurane in oxygen.](image)

Two bilateral injection sites were obtained using the stereotaxic coordinates of the cingulate cortex: +0.75 mm with respect to the bregma, -0.75 mm deep, and 0.25 mm to the right or left of the central suture (Figure 19). Due to variability in neonatal skull size, some injection sites required slight deviations from the given coordinates. Over a course of approximately two minutes, 1 µL of rAAV9 or sterile saline solution was injected in each site; an additional one to two minutes was taken to pull the syringe out of the skull in order to minimize backflow. After injection, pups were removed from the stereotactic frame and rubbed with nesting material from
the cage to prevent rejection by the mother. In addition, vanilla extract was dropped on the mother’s nose to decrease the chance of rejection of the pup. Pups were then returned to their mother’s cage.

![Figure 19. Stereotaxic coordinates of the mouse brain. Approximate injection points are indicated with red asterisks (Paxinos and Watson, 1998).](image)

Injections were performed on multiple litters of mice. While complications arose initially in the fall of 2014 due to unexpected mortalities in a litter of mice one month after injection, subsequent injections proved to be successful with few mortalities. For the litters in this study, about 60% received viral injections, 30% received a sterile saline solution (sham) injection as a control, and 10% received no injection.

**In Vivo Magnetic Resonance Experiments**

All magnetic resonance experiments (MRI, MRS, and DTI) were performed on a 9.4 Tesla, Bruker Avance DRX 400 MHz vertical bore NMR spectrometer with a MicroMouse 2.5 imaging accessory at a gradient strength of 2.4 G/cm/A (Bruker Biospin, Billerica, MA). Paravision 4.0 and Bruker Topsin 1.5 software (Ettlingen, Germany) were used on a Linux system during data acquisition and spectral analysis. MRI and DTI data were analyzed using the image analysis software, Analyze 11.0 (Mayo Clinic, Rochester, MN), on a Samsung PC.
Animal Care

Prior to imaging, mice were anesthetized in a plexiglass induction chamber with 2% isoflurane in O₂ maintained at a flow rate of approximately 0.2 liters per minute. Once anesthetized, mice were transferred in the prone position to the mouse bed in the imaging probe, where the isoflurane/O₂ mixture was delivered using a nose cone and exhaust was filtered out using a F/AIR Scavenger activated charcoal filter (Paragonmed, Coral Springs, FL). The mouse’s head was positioned such that the front teeth were hooked over the bite bar and the brain was centered on the etching in the plastic (Figure 20). Mice were secured in this position using strips of 3M Micropore™ medical tape. Once in the probe, isoflurane was reduced to 1.5%, where it remained throughout the duration of the experiment.

Figure 20. View of the mouse bed. The mouse was placed in the bed such that the front teeth were positioned on the bite bar. The clear tubing provides the input and output for anesthesia and oxygen; the sensor pad aids in monitoring the respiration rate.

Throughout each magnetic resonance experiment, the magnet was maintained at a temperature of 30-32°C to aid in the mouse’s maintenance of body temperature while anesthetized. Respiration rates were monitored using a respiration sensor pad (SA Instruments, Inc., Stony Brook, NY) positioned beneath the mouse’s abdomen on the mouse bed. Bruker BioTrig Triggering and Monitory acquisition and command modules (scaling factor = 2, gain factor = 4) were utilized to receive the signals from the respiration sensor pad and visualized on a
C840 latitude laptop computer. Isoflurane rates were adjusted throughout each experiment using a VIP Veterinary Vaporizer (Colonial Medical Supply Co., Franconia, NH) to maintain the respiration rate between 30 and 100 breaths per minute.

*Magnetic Resonance Imaging*

Once the probe was inserted into the magnet, the RF circuit was tuned and matched and a saved shim file was loaded. The default ‘steph4’ shim file was used for anatomical imaging, and ‘dtimouse14’ was used for diffusion tensor imaging. For all pulse sequences, an automated procedure was used to adjust transmitter powers and receiver gain. A RARE_tripilot scan was used to obtain three orthogonal T2-weighted images along each axis (TE = 12.5 ms, TR = 2000.000 ms, rare factor = 8, matrix size = 128 x 128, averages = 1). These images were used as a reference for the RARE_8_bas scan to ensure that the entire brain was included and centered within the middle of the gradients. If the brain was not centered, the probe was removed from the magnet in order to reposition the mouse and repeat the RARE_tripilot. The RARE_8_bas pulse sequence was run to acquire 24, T2-weighted coronal slices of the mouse brain (TE = 15 ms, TR = 3000 ms, number of averages = 8, slice thickness = 0.7 mm, number of slices = 24, interslice distance = 0.7 mm, rare factor = 8, matrix size = 256 x 256, FOV = 2.56 cm x 2.56 cm).

*Magnetic Resonance Spectroscopy*

After running the RARE_tripilot and RARE_8_bas pulse sequences on PND 35/36, 49/50, and 63/64, MRS experiments were conducted to analyze levels of relevant neurometabolites in the medial region of the hippocampus. The shimming algorithm used for spectroscopy was the FASTMAP_4mm, which automatically adjusted first and second order shims over a 4 x 4 x 4 mm³ cubic voxel positioned over the hippocampus in a RARE_8_bas image. Next, the voxel size was reduced to its final spectroscopy voxel size, 4 mm x 2 mm x 3
mm, and repositioned to fit more precisely over the hippocampus (Figure 11). After tuning and matching the probe, the PRESS_waterline pulse sequence was applied (TR = 2000 ms, TE = 20 ms, number of averages = 8) to suppress the dominant water signal and thus improve resolution of the neurometabolite peaks. Using Bruker TopSpin 1.5 software, the water spectrum was analyzed. If the linewidth of the water peak was less than or equal to 25 Hz, the metabolite spectrum could be obtained due to appropriate magnetic field homogeneity. However, if the linewidth was greater than 25 Hz, the FASTMAP_4mm had to be run again. The PRESS_1H pulse sequence was applied (with water suppression and outer volume suppression) to obtain the metabolite spectrum (TR = 2000 ms, TE = 20 ms, number of averages = 800).

Diffusion Tensor Imaging

On PND 28/29, 42/43, and 56/57, DTI experiments were conducted. The SINGLEPULSE_1H scan (number of repetitions = 200, Tx0 = 33.9) was used to manually shim and homogenize the magnetic field, resulting in a symmetrical water peak and a gradual free induction decay. Once complete, protocols were followed to run a RARE_tripilot pulse sequence and a RARE_8_bas pulse sequence to obtain anatomical images. Next, using the RARE_tripilot as a reference, the EPI_diffusion_tensor pulse sequence was applied to acquire diffusion weighted images of 26 slices in the brain from the cerebellum to the frontal lobe (TE = 18.78 ms, TR = 3900 ms, 30 diffusion directions, 1 diffusion experiment per direction, 5 A0 images, diffusion gradient duration = 2.00 ms, diffusion gradient separation = 7.77 ms, bandwidth = 1000 s/mm², FOV = 20 mm x 20 mm, slice thickness = 0.6 mm). Acquired diffusion weighted images were transferred to the Samsung PC for image visualization and analysis using Analyze 11.0.
**Data Analysis**

**MRI**

All anatomical images were analyzed with Analyze 11.0. After importing using the DICOM tool, the region of interest (ROI) tool was used to trace the whole brain and various structures of interest (cerebellum, third ventricle, fourth ventricle, left and right lateral ventricles, and left and right hippocampus). A Wacom Cintiq 12WX tablet display (Wacom Technology Corporation, Vancouver, WA) was used for more precise tracing. Using the Sample Images tool in the ROI interface, volume measurements were made using the 2-dimensional traced areas and the slice thickness (0.7 mm). The volumes of all brain regions were normalized by dividing by the volume of the whole brain.

**MRS**

To obtain neurometabolite levels, magnetic resonance spectra were analyzed with Bruker TopSpin 1.5. Neurometabolites of interest were N-acetylaspartate/N-acetylaspartylglutamate, glutamate and glutamine, creatine, choline, and taurine. Prior to Fourier transformation, a linewidth function of 10 Hz was applied to each spectrum to improve the signal-to-noise ratio. Each spectrum was also phased and fit to the baseline by manually selecting troughs in the spectra as baseline points. Lastly, to determine the area under each neurometabolite peak and thus the level of each neurometabolite, a Gaussian fit was used. All spectra were calibrated to the N-acetylaspartate/N-acetylaspartylglutamate (NAA/NAAG) peak at 2.01 ppm. As stated earlier, since levels of creatine are thought to remain fairly stable regardless of diseases state, it is used as an internal control. Thus, the amount of each neurometabolite was normalized to the creatine peak at 3.00 ppm.
All diffusion tensor images were analyzed with Analyze 11.0. Images were imported using the DICOM tool, and one base volume and all 26 slices were loaded as an appended multivolume set into the DTI add-on. After specifying diffusion gradient directions and removing background noise, DTI maps were computed. Maps for the Apparent Diffusion Coefficient (ADC), Fractional Anisotropy (FA), Eigenvectors, Relative Anisotropy (RA), Radial Diffusivity (Dr), Axial Diffusivity, and Volume Ratio were created, though only the FA, ADC, and Dr maps were used in this analysis. Object maps containing regions of interest traced in the ROI tool were loaded to compute and display the length and structure of fibers running through these regions. The fibers were saved and loaded onto the FA, ADC and radial diffusivity maps to calculate mean values for each of these parameters. Alternatively, the object maps were loaded onto the FA, ADC, and Dr maps to obtain mean values for diffusion parameters of interest without computing fibers.

Statistical Analyses

Statistical tests were conducted separately for males and females, and analyses were conducted to assess differences between genotype as well as injection type. Due to small sample sizes, the effect of both genotype and injection type could not be assessed simultaneously.

MRI and DTI

Volumes of brain structures and diffusion parameters were expressed as means with corresponding standard deviations. All analyses were conducted in JMP Pro 11, and \( p < 0.05 \) was the threshold for significant differences. Potential outliers could not be tested and eliminated due to small sample sizes. Genotypic differences were assessed within each time point using an independent two-tailed t-test. Levene’s test for equality of variances was utilized to determine if
equal or unequal variances were assumed for the t-tests. One-way ANOVAs were utilized to assess developmental differences within a genotype or injection type and across each time point. One-way ANOVAs were also used to examine differences between injection types within a time point. Post hoc Tukey-Kramer HSD tests were conducted to determine the nature of significant differences determined by the one-way ANOVA.

**MRS**

Neurometabolite ratios were expressed as means with associated standard deviations. All analyses were conducted using the Statistical Package for the Social Sciences (SPSS), and $p < 0.05$ was considered significant. Outliers were first detected and eliminated using a formulation in SPSS that determined “far out” outliers as any values beyond 2.1 times the interquartile range. Genotypic differences were assessed within each time point using an independent two-tailed t-test, where unequal or equal variance was assumed based on the results of Levene’s test for equality of variance. One-way ANOVAs were utilized to assess the developmental differences within a genotype or injection type and across each time point, as well as the differences between injection types within a time point. Post hoc Tukey-Kramer HSD tests were conducted to determine the nature of significant differences determined by the one-way ANOVA.
III. Results and Discussion

*Magnetic Resonance Imaging Analysis*

MRI data were acquired on PND 35/36, 49/50, and 63/64. While the same three litters of mice were followed over time for this thesis, instrumental difficulties and random sampling resulted in small sample sizes and longitudinal gaps in acquired data (Table 1). MRI data are presented here only for these three litters of mice; results are compared with those for mice studied in our lab by earlier researchers. In all of the MRI, MRS and DTI data presented in this thesis, none of the females received injections, as the sex of mice cannot be determined on PND 1. Thus, the random injection of neonatal pups could not control for sex. MRI data were analyzed from WT and HET males and WT females (Table 1A). Data for GCPII +/- males were also analyzed by injection type: non-injected, saline-injected, and viral-injected (Table 1B). Statistical analyses were conducted by genotype to assess differences between WT and HET mice, by injection type to assess differences between non-injected, saline-injected, and viral-injected mice, and by age to assess developmental differences within a particular genotype or injection type. The lack of HET females or injected females meant that MRI analysis was limited to males; structural abnormalities have been shown to be more numerous and more pronounced in male schizophrenia patients (Buckley, 2005).
### Table 1. Summary of sample sizes (N) for MR imaging at each time point by (A) Sex and genotype, and (B) Injection type.

Only males received injections, and due to small sample sizes, mice could not be separated by genotype.

In this study, the structures examined include the cerebellum (CB), the third ventricle (3VT), the fourth ventricle (4VT), the lateral ventricles (Lat) and the hippocampus (HP) (Figure 21). Each structure was normalized to the whole brain (WB) volume and reported as a ratio. Left to right asymmetry was also assessed in the lateral ventricles and the hippocampus. All ratios were averaged between mice in each group for analysis, and MRI findings are reported by the examined structure.
Figure 21. (A) Traced, T2 weighted, coronal MR images of a mouse brain acquired using the RARE_8_bas pulse sequence and compared to a (B) T2 weighted, coronal human MR image (http://www.surgicalneurologyint.com); (C) Relative size of a mouse and human brain (http://www.med.lu.se/expmed/developmental_and_regenerative_neurobiology2/research/creative_environment_modent_at_lu_faculty_of_medicine). 24 slices were obtained throughout the RARE_8_bas pulse sequence, 15 are shown here as a representative sample (Mouradian, 2013). Tracing in this thesis was completed using Analyze 11.0. Regions shown include WB (red), CB (green), 4VT (yellow), left HP (white), right HP (light pink), left Lat (hot pink), right Lat (turquoise), and the 3VT (dark blue).

Cerebellum

HET males exhibited significantly increased cerebellum volumes on PND 63 compared to PND 49 [F(2,2) = 22.3536, p = 0.0428, Figure 22A]. In contrast, cerebellum volumes appeared to remain essentially constant over time in WT males. While sample sizes were small
and no significant differences were found between injection types in all GCPII$^{+/-}$ mice, trends suggest increased CB/WB ratios in viral mice at all time points compared to saline injected mice (Figure 22B).

**Figure 22.** Average cerebellum (CB) volume to whole brain (WB) volume ratios ($\pm$ SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. The cerebellum volume in each mouse was normalized to the whole brain (WB) volume. One-way ANOVA analysis followed by a post hoc Tukey-Kramer HSD test was conducted to assess developmental differences and differences between injection types on PND 35. $^\wedge$ indicates significant developmental differences ($p < 0.05$). An independent, two-tailed t-test assuming unequal variances (Levene’s test, $p < 0.05$) was utilized to examine all genotypic differences and injection-type differences on PND 49 and 63. * indicates a significant genotypic or injection-type difference within a time point ($p < 0.05$).

The cerebellum is intricately involved in both motor tasks and cognition; its connection to the frontal cortex via the thalamus is thought to be disrupted in humans with schizophrenia, though the nature of structural changes is not yet clear (Parker et al., 2014; Okugawa, 2013; Picard et al., 2008). Furthermore, the relation between cognition and motor control has recently been identified as a marker of schizophrenia known as dysmetria (Parker et al., 2014). The disruption of these connections could be implicated in the hallucinations, disordered thoughts, and flat affect frequently observed in schizophrenic patients. While there is a limited range of studies that exists regarding irregularities in the cerebellum in humans with schizophrenia,
current work suggests changes in cerebral structure could be indicative of altered neurological development (Okugawa, 2013; Picard et al., 2008). Some studies have attributed the decrease in cerebellum volume of first-episode patients to an increase in white matter volume (Rasser et al., 2010), while other studies have reported a more generalized decrease in cerebellar volume (Parket et al., 2014).

Much of the research on human patients with schizophrenia has focused on a region of the cerebellum known as the vermis (responsible for proprioception), which has been significantly implicated in the pathophysiology of schizophrenia (Levitt et al., 1999; Rasser et al., 2010). Previous studies have demonstrated increased overall vermal volume in schizophrenic patients (as well as an increase in vermal white matter), which is parallel to the observed increase in cerebellum to whole brain volume ratio in HET male mice between PND 49 and 63, as well as the non-significantly higher cerebellum to whole brain volume ratios observed in viral-injected mice in this study (Levitt et al., 1999). Furthermore, our finding of slightly increased CB/WB ratios in HET males is consistent with previous findings in this lab (Huang, 2012).

Third Ventricle

There were no significant trends in the third ventricle to whole brain volume ratio in WT or HET males, or GCPII^{+/-} mice in any of the injection conditions (Figure 23). However, there appears to be a slight increase in the third ventricle volume of HET males over time (Figure 23A), though larger sample sizes would be necessary to indicate significance.
Figure 23. Average third ventricle (3VT) volume to whole brain (WB) volume ratios (± SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. The third ventricle volume in each mouse was normalized to the whole brain (WB) volume. One-way ANOVA analysis followed by a post hoc Tukey-Kramer HSD test was conducted to assess developmental differences and differences in injection conditions on PND 35. ^ indicates significant developmental differences (p < 0.05). An independent, two-tailed t-test assuming equal variances (Levene’s test, p > 0.05) was utilized to examine all genotypic differences and injection-type differences on PND 49 and 63. * indicates a significant genotypic or injection-type difference within a time point (p < 0.05).

One of the hallmark characteristics of schizophrenia since the advent of neuroanatomical imaging has been ventricular enlargement (Fannon et al., 2000; Gaser et al., 2004). This enlargement in the third ventricle has been correlated with the reduction of a variety of grey matter structures implicated in the pathophysiology of schizophrenia, most usually the thalamus (sensory perception and motor control), but also the striatum (movement control and executive functioning) and the superior temporal cortex (mostly auditory processing) (Shenton et al., 2001; Gaser et al., 2004). Previous work in our laboratory observed significant increases in the third ventricle to whole brain volume ratio of HET females at PND 35 (Huang, 2012) and of HET males and females at PND 77 (Mu, 2011), however these differences have not been observed in this work or more recent studies (Mouradian, 2013). Despite the effect of small sample sizes and
lack of genotypic data on GCPII^{+/−} females, one potential explanation for the lack of significant trends could be the limitations in imaging time. The last MRI data in this study were obtained on PND 63. Based on the non-significant trend in third ventricular enlargement over time in HET males and the relatively stable 3VT/WB ratio in WT males, it is possible that a genotypic difference would be observed at a later time point, though imaging at this time point is not possible due to size limitations of the imaging probe utilized.

**Fourth Ventricle**

No significant differences between GCPII^{+/−} mice of different genotypes or injection types were observed within a time point or longitudinally (Figure 24), though trends in ventricular enlargement were observed in HET males both developmentally and between genotypes on PND 63 (Figure 24A). At the same time, non-significant increases in fourth ventricle to whole brain volume ratios were observed in viral-injected mice compared to saline-injected mice (Figure 24B).
Figure 24. Average fourth ventricle (4VT) volume to whole brain (WB) volume ratios (+ SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. The fourth ventricle volume in each mouse was normalized to the whole brain (WB) volume. One-way ANOVA analysis followed by post hoc Tukey-Kramer HSD was conducted to assess developmental differences and injection condition differences on PND 35. ^ indicates significant developmental differences ($p < 0.05$). An independent, two-tailed t-test assuming equal variances (Levene’s test, $p > 0.05$) was utilized to examine all genotypic differences and injection-type differences on PND 49 and 63. * indicates a significant genotypic or injection-type difference within a time point ($p < 0.05$).

While ventricular enlargement is one of the most consistent findings in human schizophrenia research, specific findings on the fourth ventricle are both less numerous and less robust than studies on lateral ventricles and the third ventricle. Although most studies have observed no change in the fourth ventricle volume, one study indicated enlargement (Keshavan et al. 1998, as cited in Shenton et al., 2001), which is consistent with the slight, though non-significant, increase in fourth ventricular volume over time in HET males. Otherwise, the lack of significant findings in fourth ventricle to whole brain volume ratios is consistent with current literature, as well as previous studies in this laboratory (Mouradian, 2013; Huang, 2012; Mu, 2011). As stated previously, larger sample sizes would be necessary to better understand the significance of the findings.
Lateral Ventricles and Asymmetry

There were no significant trends in the lateral ventricles to whole brain volume ratios in WT or HET males, or GCPII\textsuperscript{+/−} mice in any of the injection conditions (Figure 25). However, there appears to be a slight increase in the lateral ventricular volume of HET males from PND 35 to PND 63 (Figure 25A), and this trend was approaching significance \([F(2,2) = 6.2889, p = 0.1372]\).

![Figure 25](image)

Figure 25. Average lateral ventricle (Lat) volume to whole brain (WB) ratios (± SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. The lateral ventricle volume in each mouse was normalized to the whole brain (WB) volume. One-way ANOVA analysis followed by a post hoc Tukey-Kramer HSD test was conducted to assess developmental differences and injection-type differences on PND 35. ^ indicates significant developmental differences (\(p < 0.05\)). An independent, two-tailed t-test assuming unequal variances (Levene’s test, \(p < 0.05\)) was utilized to examine all genotypic differences and injection-type differences on PND 49 and 63. * indicates a significant genotypic or injection-type difference within a time point (\(p < 0.05\)).

Lateral ventricular enlargement is a consistent finding in human patients with schizophrenia compared to control patients, as 44 out of 55 MRI studies reviewed by Shenton and his colleagues cited enlarged lateral ventricles (Shenton et al., 2001). While enlarged ventricles are not unique to the pathophysiology of schizophrenia, as they occur in disorders such
as Alzheimer’s disease, hydrocephalus, and Huntington’s Chorea, they may suggest tissue loss in neighboring grey matter structures and are thus useful in studying schizophrenia (Shenton et al., 2001). Though the trends are not significant, especially when compared to WT controls, the slightly increased lateral ventricular volumes over time in HET males could be consistent with studies that find ventricular enlargement in humans. Perhaps with larger sample size, differences would emerge.

Furthermore, lateral ventricular enlargement has been found to exhibit a degree of regional specificity (Buckley, 2005). Studies have suggested an enlargement of the left temporal horn (a region of the lateral ventricle) in humans with schizophrenia, and thus lateral ventricular asymmetry was assessed in this study (Buckley, 2005; Shenton et al., 2001; Figure 26). An enlarged left temporal horn could be a result of a reduction in volume of the left-hippocampal side of the amygdala-hippocampal complex, with a greater reduction in the left-hippocampal side (Shenton et al., 2001). While no significant trends were observed, HET males appeared to exhibit slightly higher left/right asymmetry at each time point compared to WT controls, potentially reflecting findings in previous studies (Shenton et al., 2001; Figure 26A). Our results are consistent with previous studies in our lab that did not observe increased asymmetry or lateral ventricular volume in either HET or WT males (Mouradian, 2013; Huang, 2012). Lastly, out of the few injected GCPII+/− males, no clear trends emerged in terms of ventricular asymmetry, though available data were limited (Figure 26B).

The small number of pixels present in the lateral ventricles (and other ventricles) makes volumetric analysis challenging, and could account for the larger error bars for some averages. The degree of error reflects the influence of volume averaging; if a 3D voxel covers two different types of tissues with different signals, the signal intensity will be a weighted average of the
signals, leading to errors. The effect of volume averaging is more pronounced in smaller structures.

**Figure 26.** Average left to right lateral ventricle volume ratio (+ SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. One-way ANOVA analysis followed by a post hoc Tukey-Kramer HSD test was conducted to assess developmental differences and differences between injection conditions on PND 35. ^ indicates significant developmental differences (p < 0.05). An independent, two-tailed t-test assuming equal variances (Levene’s test, p > 0.05) was utilized to examine all genotypic differences and injection condition differences on PND 49 and 63. * indicates a significant genotypic or injection-type difference within a time point (p < 0.05).

**Hippocampus**

There were no significant trends in the hippocampus to whole brain volume ratios in WT or HET males, or GCPI1^{+/-} mice in any of the injection conditions (Figure 27).
Figure 27. Average hippocampal (HP) to whole brain (WB) volume ratio (+ SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. The hippocampus volume in each mouse was normalized to the whole brain (WB) volume. One-way ANOVA analysis followed by a post hoc Tukey-Kramer HSD test was conducted to assess developmental differences and injection-type differences on PND 35. ^ indicates a significant developmental differences (p < 0.05). An independent, two-tailed t-test assuming equal variances (Levene’s test, p > 0.05) was utilized to examine genotypic differences and injection-type differences on PND 49 and 63. * indicates a significant genotypic or injection condition difference within a time point (p < 0.05).

The hippocampus is an essential medial temporal lobe structure that is intimately involved in memory and learning (Shenton et al., 2001; Adriano et al., 2012). More specifically, the hippocampus is centrally involved in systems of cholinergic neurotransmission, as decreased modulation of cholinergic transmission seems to correlate with reduced declarative memory, a key aspect of the endophenotype of patients with schizophrenia (Adriano et al., 2012). Studies have consistently found reduced hippocampal volume (particularly in the left hippocampus) in human schizophrenic patients compared to healthy controls. This result has been shown to be correlated with the presentation of positive symptoms (Shenton et al., 2001). Furthermore, these observed hippocampal volumetric changes have been linked to increased lateral ventricular volumes in schizophrenic patients (Shenton et al., 2001). Additionally, functional MRI studies...
have indicated abnormal activation of the hippocampal area in humans with schizophrenia when compared to healthy controls (Adriano et al., 2012). While most of the current literature seems to suggest a reduction in hippocampal volume in schizophrenia patients, some studies have produced contradictory results that indicate no difference in hippocampal volume; these contradictions speak to the complexity of understanding the pathophysiology of schizophrenia (Adriano et al., 2012).

Previous work in this laboratory found increases in hippocampal to whole brain volume ratios in WT and HET males and females over time, as is expected during growth and development (Huang, 2012). This work, along with the lack of observed reduction in hippocampal volume in HET compared to WT mice in this study could confirm studies that observe no changes in hippocampal volume. Other work in our laboratory found a significant decrease in hippocampal to whole brain volume ratios on PND 77 in HET males and females compared to controls (Mu, 2011). The latter is consistent with the more frequent finding in the literature of hippocampal volume reduction. This observation is also illustrative of the value in carrying out the longitudinal study further to see if the model mimics the progression of the disorder observed in humans. In this study, the observed increase in hippocampal volume from PND 35 to 49 and then observed slight decrease from PND 49 to 63 indicate the challenges associated with such small sample sizes.

Hippocampal asymmetry is hypothesized to reflect disrupted connections between the hippocampus and structures in the prefrontal cortex (Kim et al., 2005). Much of the literature suggests a specific reduction in the left-hippocampal side of the amygdala-hippocampal complex (Shenton et al., 2001). In this study, no significant hippocampal asymmetries were evident between genotypes or injection types (Figure 28). This finding is in contrast to previous findings.
in our lab that observed significantly greater left to right hippocampus ratios in HET mice compared to WT mice (Huang, 2012; Mu, 2011). While samples sizes were small and results were not significantly different, initial trends may suggest smaller left to right hippocampus ratios in viral-injected males compared to saline-injected males, which could support findings in the literature (Figure 28B). Larger sample sizes would be necessary to better understand the significance of this trend.

![Graph A](image1)

**Figure 28.** Average left to right hippocampal volume ratio (± SD) across (A) WT and HET males, and (B) Non-injected, saline-injected, and viral-injected males. One-way ANOVA analysis followed by a post hoc Tukey-Kramer HSD test was conducted to assess developmental differences and differences between injection conditions on PND 35. ^ indicates a significant developmental differences (p < 0.05). An independent, two-tailed t-test assuming equal variances (Levene’s test, p > 0.05) was utilized to examine genotypic differences and differences between injection type on PND 49 and 63. * indicates a significant genotypic or injection-type difference within a time point (p < 0.05).

**Summary of MRI Findings**

Taken together, findings observed in MRI seem to suggest that the GCPII+/−/rAAV9 mouse model of schizophrenia mimics some, but not all of the neuroanatomical changes associated with the disease in humans. It is important to conceptualize our insignificant findings
within the range of literature available on schizophrenia, where many observed changes are both subtle and inconsistent between studies. Our results can be summarized as follows:

• A significant developmental increase in cerebellum/WB was observed in HET males, though similar developmental trends were exhibited between genotypes and injection types otherwise.
• A non-significant increase in third ventricle volume/WB in HET males over time, though no other significant developmental differences were observed in, or between, any genotype or injection type.
• HET males exhibited a non-significant developmental increase in fourth ventricle/WB volume ratio, with a non-significant increase in fourth ventricle/WB volume ratio compared to WT males observed at PND 63. Viral-injected males exhibited non-significantly increased fourth ventricle/WB volume ratios at each time point.
• Similar trends in lateral ventricle/WB volume ratio were exhibited in HET males over time, and no significant trends were observed developmentally or between males in any of the injection conditions.
• HET males exhibited non-significantly increased left>right lateral ventricular asymmetry at each time point, though no significant trends were observed in males in any of the injection conditions.
• No significant genotypic, injection-type, or developmental trends were observed in hippocampus/WB volume ratios in male GCPII+/- mouse, and no hippocampal asymmetry was observed between genotypes or injection types.

It is essential to emphasize that while sample sizes in this study were small, it does not appear that injection type causes a significant effect on grey matter structural abnormalities. The effect is difficult to assess, however, since small sample sizes disallowed genotype controls. Unfortunately, due to limitations in the size of imaging probe, studies cannot go beyond PND 63, which would be helpful in assessing the neurodevelopmental changes associated with schizophrenia in humans. In addition to small sample sizes, the resolution of acquired images and the small, relative size of some structures in relation to the whole brain may introduce significant error into this study’s findings, particularly in the fourth ventricle and the left and right hippocampi and lateral ventricles. Moving forward, it will be necessary to aggregate the MRI findings from this year with previous findings in this laboratory to better understand the validity of the GCPII+/- mouse model in mimicking the pathophysiology of schizophrenia in
humans. Due to small sample sizes and gaps in data, further studies would be needed to examine whether observed trends were representative of significant differences between mice of different sex, genotype, or injection type.

**Magnetic Resonance Spectroscopy Analysis**

Neurometabolite levels were assessed in all mice on PND 35/36, PND 49/50, and PND 63/64 following magnetic resonance imaging. Levels of choline, glutamate and glutamine, N-acetylaspartate and N-acetylaspartylglutamate, and taurine were assessed in the hippocampal/cortex region from acquired spectra (Figure 29).

![Figure 29](image)

**Figure 29.** (A) MR image displaying the placement of the voxel over the hippocampal/cortex region in the mouse brain. (B) Sample $^1$H MR spectrum of a mouse brain with relevant neurometabolites at different chemical shifts labeled.

In addition to the data collected within the past year, MRS data from studies conducted in the lab within the past three years were aggregated and are included in this analysis. As mentioned previously, analyses were split by sex due to previously observed sexual dimorphisms (Huang, 2012). Data were analyzed from WT and HET males, and WT and HET females (Table 2A). Data for male GCPII$^{+/}$ mice were also analyzed by injection type: no injection, saline injection, and viral injection. Analyses were first conducted to assess differences in neurometabolite concentrations across time points in the longitudinal model. Subsequently, analyses were conducted within each time point to assess the effect of genotype on neurometabolite levels, and
next to examine the effect of injection type on neurometabolite levels. MRS results are reported according to each neurometabolite analyzed.

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Table 2. Summary of sample sizes (N) for MR spectroscopy at each time point by (A) Sex and genotype, and (B) Injection type. Only males received injections, and due to small sample sizes, mice could not be separated by genotype. Outliers are included in these sample sizes.

**Choline to Creatine**

HET females exhibited altered levels of choline over time that were approaching significance \([F(2,12) = 3.769, p = 0.054]\). Cho/Cr ratios were nearing a significant decrease from PND 35 to PND 63 (post hoc Tukey-Kramer HSD, \(p = 0.057\), Figure 30B). Observed levels of choline in HET females were significantly greater compared to WT females at PND 35 (two-tailed t-test assuming equal variance, \(p = 0.009\), Figure 30B), though this trend was reversed at PND 63 (two-tailed t-test assuming equal variance, \(p = 0.058\), Figure 30B). HET males exhibited significantly higher levels of choline at PND 63 (two-tailed t-test assuming equal variance, \(p = 0.056\), Figure 30A).
Figure 30. Average choline/creatine concentration ratio (± SD) between PND 35 and 63 in (A) WT and HET males, (B) WT and HET females, and (C) Non-injected, saline-injected, and viral-injected males. Levels of choline were normalized to the creatine peak at 3.0 ppm. A one-way ANOVA was conducted followed by a post hoc Tukey-Kramer HSD test to assess developmental differences and injection-type differences, while a two-tailed t-test assuming equal variance (Levene’s test, p > 0.05) was conducted to assess genotypic differences. Results were considered significant at the p < 0.05 level. ^ indicates a near-significant developmental difference (0.05 < p < 0.07) within a genotype between time points, while ~ indicates a near-significant genotypic difference (0.05 < p < 0.07) within a time point. Significant differences between injection types are indicated with a * (p < 0.05).

Choline levels in males differed significantly by injection type at PND 35 [F(2,18) = 14.542, p = 0.000, Figure 30C], with viral-injected males exhibiting significantly higher Cho/Cr levels than non-injected males (post hoc Tukey-Kramer HSD, p = 0.000), and saline-injected males exhibiting significantly higher levels than non-injected males (post hoc Tukey-Kramer
Males receiving a viral injection were not found to exhibit significantly different levels than males receiving a saline injection, however. Significant trends were also observed at PND 49, in which choline levels were altered between injection types \[F(2,9) = 5.438, p = 0.028, \text{Figure 30C}\], though no post hoc analysis could be conducted due to small sample sizes. Trends suggest, however, that choline levels in viral-injected males were higher than levels in both non-injected and saline-injected males, which appeared to be similar. The trends between injection types veered away from significance at PND 63, however. No developmental differences were observed in any of the injection conditions.

There is not a consensus in the literature on how choline levels in the hippocampal region are altered in human patients with schizophrenia. Some studies have found no significant differences between healthy controls and schizophrenic patients (Deicken et al. 1998; Marsman et al., 2014), while others have observed increased levels (Lutkenhoff et al., 2010), and still others have found decreased levels of choline in humans with schizophrenia (Maier et al., 1996). The lack of observed changes in choline levels between WT and HET males at PND 35 and PND 49 are in agreement with studies that find no alterations in Cho/Cr levels. Furthermore, GCPII+/− females potentially exhibited the inconsistencies outlined in the literature: significant increases were observed in HET females compared to WT females on PND 35, while levels were found to be nearly-significantly lower in HET females compared to WT females at PND 63 (Figure 30B). This change could also be indicative of neurochemical changes associated with development, particularly as a result of alterations in neural membrane composition or sexual maturation (Huang, 2012; Mouradian, 2013). More specifically, altered phospholipid membrane turnover has been correlated with heightened choline levels and the onset of schizophrenia, though this finding is not reflected by the results in this study (Rae, 2014; Lutkenhoff et al., 2010).
Interestingly, there were significant differences between injection-types at PND 35 and 49. In each case, viral-injected mice exhibited higher levels of choline compared to non-injected and saline-injected males, with the most significant changes observed at PND 49. While sample sizes were small and genotype was not controlled for, results suggest that the rAAV9 injection could contribute to altered choline levels, perhaps through altered neural membrane turnover as noted previously. However, due to the lack of consensus in the literature and the conflicting results found in this study, choline may not be the most effective biomarker of schizophrenia.

**Glutamate and Glutamine to Creatine**

Female WT mice exhibited significant developmental changes in Glx/Cr ratios \( [F(2,29) = 3.962, p = 0.030, \text{Figure 31B}] \). Levels decreased significantly from PND 35 to 63 (post hoc, \( p = 0.034 \)). No significant genotypic differences were observed. HET males exhibited a trend of decreased levels of Glx/Cr that became significant, when compared to WT males, at PND 63 (two-tailed, independent t-test assuming equal variance, \( p = 0.020 \)); no developmental differences in HET or WT males were observed (Figure 31A). No significant differences were observed between injection types, and no developmental differences were observed within any of the injection types (Figure 31C).
Figure 31. Average glutamate and glutamine/creatine concentration ratio (± SD) between PND 35 and 63 in (A) WT and HET males, (B) WT and HET females, and (C) Non-injected, saline-injected, and viral-injected males. Levels of glutamate and glutamine were normalized to the creatine peak at 3.0 ppm. A one-way ANOVA was conducted followed by a post hoc Tukey-Kramer HSD test to assess developmental differences and injection-type differences, while a two-tailed t-test assuming equal variance was conducted to assess genotypic differences. Results were considered significant at the $p < 0.05$ level. ^ indicates a significant developmental difference ($p < 0.05$) within a genotype between time points, while * indicates a significant genotypic difference ($p < 0.05$) within a time point.

One of the most suggestive findings in $^1$H MRS studies of patients with schizophrenia is an observed decrease in glutamate (Glu) and glutamine (Gln) levels, which are reflected and assessed in this study as their summed level representative of glutamatergic transmission, Glx (Lutkenhoff et al., 2010). Decreased levels of Glx are hypothesized to reflect abnormalities in neurotransmission and disturbances in the balance of neuron and glial cells that are essential to
regulating the cycle of interconversion between glutamate and glutamate. Decreased glutamate has been correlated with decreased metabolic activity (Rae, 2014). Furthermore, the GCPII\(^{+/−}\) mouse model, based on the glutamate hypothesis, is predicted to reflect NMDAR hypofunction via a variety of mechanisms. Namely, the reduced hydrolysis of NAAG to NAA and glutamate would result in reduced glutamate levels and elevated NAAG levels. The elevated NAAG levels serve as an antagonist to NMDAR function, and thus glutamate concentrations are further reduced (Schaevitz et al., 2012). In this study, this trend in Glx levels was evident in HET males when compared to WT males. While a significant decrease in Glx levels was not observed in HET males at earlier time points, there was an observed trend towards decrease in Glx levels at PND 63. This finding supports the vast majority of the literature that has observed decreases in glutamate levels in human schizophrenic patients when compared to healthy controls (Lutkenhoff et al., 2010).

At the same time, however, recent studies have reported no significant changes in glutamate levels in schizophrenic patients when compared to healthy controls (Marsman et al., 2014). This hypothesis is reflected by our findings in HET females, where no significant genotypic differences were observed at any time point. Furthermore, a significant developmental decrease was observed in WT females that could be suggestive of normal “neuronal pruning” (Marsman et al., 2014), which is dependent on age.

The effect of injection-type remains unclear. While no significant differences were observed, trends suggest decreased Glx levels in viral-injected and saline-injected males when compared to non-injected males. Since viral-injected and saline-injected males exhibited similar Glx/Cr ratios, it appears that the injection type may not exert a specific neurochemical effect characteristic of schizophrenia in humans.
The nature of observed changes in Glx/Cr ratios are highly complex, however. Our finding of decreased levels of Glx in HET males when compared to WT males contrasts with results acquired in an analysis of ex vivo $^1$H MRS of GCPII mice (Schaevitz et al., 2012). The previous findings of increased Glx/Cr ratios were rationalized on the basis that the glutamate/glutamine ratio increased as a result of a reduction in glutamine. Since glutamine is synthesized from glutamate that is taken up in the synaptic cleft, glutamatergic neurotransmission is reduced (Schaevitz et al., 2012). It is plausible, therefore, that the observed decrease in Glx/Cr in HET males reflected decreased concentrations of glutamate in the synaptic cleft. Taken together, it seems possible that the results obtained in this study may begin to adequately reflect the pathophysiological abnormalities in schizophrenia. Nonetheless, it is necessary to consider that the results may be somewhat inconclusive since in vivo $^1$H MRS is not capable of resolving the glutamate and glutamine peaks, and ultimately cannot differentiate between metabolic glutamate and neurotransmitter glutamate (Schaevitz et al., 2012).

$N$-Acetylaspartate and $N$-acetylaspartylglutamate to Creatine
(NAA+NAAG)/Cr levels in HET males exhibited significant developmental differences across time points [$F(2,14) = 31.109, p = 0.000$, Figure 32A]. Similar to the trends observed in Cho/Cr, a significant increase in (NAA+NAAG)/Cr was observed between PND 35 and PND 49 (post hoc Tukey-Kramer HSD, $p = 0.000$) and a subsequent significant decrease from PND 49 to PND 63 (post hoc Tukey-Kramer HSD, $p = 0.000$). Furthermore, significantly decreased levels in HET males compared to WT males at PND 35 (independent, two-tailed t-test assuming equal variance, $p = 0.003$, Figure 32A) were observed. At the same time, however, developmental differences were also observed in WT males [$F(2,12) = 4.930, p = 0.027$], with a significant increase from PND 35 to PND 49 (post hoc Tukey-Kramer HSD, $p = 0.029$), and a non-
significant decrease from PND 49 to PND 63 (post hoc Tukey-Kramer HSD, \( p = 0.067 \)). In addition, significantly altered levels of \((\text{NAA+NAAG})/\text{Cr}\) were exhibited over time in WT females \([F(2,27) = 9.847, p = 0.001]\), with levels increasing from PND 35 to PND 49 (post hoc Tukey-Kramer HSD, \( p = 0.003 \)) and then decreasing from PND 49 to PND 63 (post hoc Tukey-Kramer HSD, \( p = 0.002 \), Figure 32B). Furthermore, HET females at PND 49 exhibited significantly lower levels of NAA+NAAG/Cr compared to WT females (two-tailed, independent t-test assuming unequal variances, \( p = 0.002 \), Figure 32B).
Figure 32. Average N-Acetylaspartate and N-Acetylaspartylglutamate/creatine concentration ratio (± SD) between PND 35 and 63 in (A) WT and HET males, (B) WT and HET females, and (C) Non-injected, saline-injected, and viral-injected males. Levels of N-Acetylaspartate and N-Acetylaspartylglutamate were normalized to the creatine peak at 3.0 ppm. A one-way ANOVA was conducted followed by a post hoc Tukey’s HSD to assess developmental differences, while a two-tailed t-test assuming unequal variance (as determined by Levene’s Test for Equality of Variance, \( p < 0.05 \)) was conducted to assess genotypic differences. Results were considered significant at the \( p < 0.05 \) level. \(^\wedge\) indicates a significant developmental difference \((p < 0.05)\) within a genotype or injection-type between time point, while \(*\) indicates a significant genotypic difference \((p < 0.05)\) within a time point.

No developmental differences were found in males receiving the saline-injection or the viral-injection, and no differences were found within a time point between injection types (Figure 33C). The developmental differences observed in non-injected males represented a compilation of the differences described earlier in non-injected WT and HET males \(F(2,29) = \)
24.387, \( p = 0.000 \}); no new information regarding differences between injection types was garnered from these differences (Figure 32C).

Meta-analyses of hippocampal neurometabolite levels consistently reveal observed reductions in NAA in patients with schizophrenia (Steen et al., 2005), though other studies have revealed increased levels of NAA in the left hippocampus of schizophrenia patients (Lutkenhoff et al., 2010). The NAAG peak overlaps with its metabolic product, NAA, in this study (i.e. the peaks cannot be resolved) and are reported as (NAA+NAAG). Current literature suggests that the composite peak reflects approximately 90% NAA and 10% NAAG (Jessen et al., 2013). A study that utilized \textit{ex vivo} \(^1\)H MRS to examine neurometabolite levels in the cortices of GCP\(^{\text{I+/-}}\) HET mice observed no genotypic effects on (NAA+NAAG)/Cr compared to WT controls (Schaevitz et al., 2012). NAA is viewed as a marker of neuronal integrity in a variety of diseases; levels can be readily reversed over time with therapy or antipsychotic treatment (Rae, 2014; Jessen et al., 2013; Steen et al., 2005).

Interestingly, we observed conflicting trends in this study. Genotypic differences were only observed at PND 49 in HET females compared to WT controls, where (NAA+NAAG)/Cr ratios were found to be significantly reduced, and at PND 35 in HET males compared to WT controls, where (NAA+NAAG) levels were found to be significantly reduced, though the difference does not appear large. The peak in (NAA+NAAG) levels at PND 49 has been hypothesized to relate to the timing of sexual maturity in mice, which occurs around PND 49 (Mouradian, 2013). The significant developmental differences observed in HET males, where levels significantly increased to a peak at PND 49 and then decreased significantly at PND 63, could be illustrative of the process of sexual maturation and related to altered hormonal levels at this time.
Overall, abnormalities in (NAA+NAAG)/Cr are somewhat difficult to interpret, as NAAG is intimately involved in modulating glutamatergic neurotransmission, while NAA is indirectly involved as a byproduct. The changes observed in this thesis speak to the complexity of assessing these abnormalities. Ultimately, resolution of the NAA and NAAG peaks would prove enormously useful to better understanding the nature of observed changes.

*Taurine to Creatine*

HET males exhibited significantly increased levels of taurine compared to WT males on PND 35 (two-tailed, independent t-test assuming equal variance, $p = 0.001$, Figure 33A), though no other significant genotypic differences were found in males. HET males did exhibit significantly altered Tau/Cr ratios over time [$F(2,14) = 8.156, p = 0.004$, Figure 33A], with a significant decrease observed from PND 35 to PND 63 (post hoc Tukey-Kramer HSD, $p = 0.005$). Significantly altered levels of Tau/Cr were also found over time in WT females [$F(2,29) = 3.747, p = 0.036$, Figure 33B], with levels of Tau decreasing significantly from PND 35 to PND 63 (post hoc Tukey-Kramer HSD, $p = 0.034$). No other genotypic differences were found in HET and WT females at any time point (Figure 33B).
Figure 33. Average taurine/creatine concentration ratio (± SD) between PND 35 and 63 in (A) WT and HET males, (B) WT and HET females and (C) Non-injected, saline-injected, and viral-injected males. Levels of taurine were normalized to the creatine peak at 3.0 ppm. A one-way ANOVA was conducted followed by a post hoc Tukey-Kramer HSD test to assess developmental differences and differences between injection types, while a two-tailed t-test assuming unequal variance (Levene’s test for equality of variance, p > 0.05) was conducted to assess genotypic differences. Results were considered significant at the p < 0.05 level. ^ indicates a significant developmental difference (p < 0.05) within a genotype between time points, while * indicates a significant genotypic difference within a time point, or a significant difference between injection types (p < 0.05).

Males exhibited significantly altered levels of taurine between injection types at all time points: on PND 35 [F(2,18) = 14.542, p = 0.000], PND 49 [F(2, 9) = 4.915, p = 0.036], and PND 63 [F(2,10) = 4.707, p = 0.036, Figure 33C]. A post hoc analysis could only be conducted at PND 35 due to small sample sizes at PND 49 and PND 63, and revealed that viral-injected mice exhibited significantly higher Tau/Cr levels than both non-injected mice (post hoc Tukey-
Kramer HSD, \( p = 0.000 \) and saline-injected mice (post hoc Tukey-Kramer HSD, \( p = 0.027 \)).

Again, while no post hoc analysis could be conducted due to small sample sizes at later time points, trends suggest increased taurine levels in viral-injected males compared to both saline-injected and non-injected males on PND 49, and increased taurine levels in saline-injected and viral-injected males compared to non-injected males on PND 63. No developmental differences were observed within any of the injection conditions.

The potential relationship between taurine levels and the pathophysiology of schizophrenia is an area of limited research. Some studies have indicated that taurine may be higher in patients with schizophrenia when compared to healthy controls (De Luca et al., 2008; Samuelsson et al., 2013). Our finding of significantly higher levels of taurine in HET males compared to WT males at PND 35 could reflect these studies, though this trend is reversed at later time points. Furthermore, our finding of significantly higher levels of taurine at PND 35 and PND 49 in viral-injected mice compared to saline-injected mice and non-injected mice suggest that injection-type may play a role. The neural mechanism behind these changes, however, is less clear. One potential hypothesis relates the neuroinhibitive (both neuroprotector and neuromodulator) properties of taurine as a response to oxidative stress present in schizophrenia. Since prolonged oxidative stress, resulting in oxidative damage, is thought to be implicated in the pathophysiology of schizophrenia, an increase in taurine indicates a neuroprotectant response contingent on the antioxidant properties of taurine (Samuelsson et al., 2013). Thus, an increase in taurine has been correlated with the duration of illness (Shirayama et al., 2010, as cited in Samuelsson et al., 2013).

Our finding of a significant developmental decrease in Tau in WT females as well as HET males could be indicative of a reduced need for neuronal maintenance as the mice reach
maturation (Mouradian, 2013; Huang, 2012; Mu, 2011). Since HET females were not observed to be significantly different from WT females, this negates available studies found in the literature, and supports the hypothesis of reduced need for neuronal upkeep. Ultimately, while findings are not conclusive, the results of this thesis suggest that abnormalities in levels of Tau may provide subtle insight into the pathophysiological changes associated with schizophrenia, though the lack of genotypic differences could reflect normal developmental processes.

**Summary of MRS Findings**

The aggregation of MRS data from the GCPII\(^{+/−}\) model indicate the following results, which mimic some of the relevant neurochemical changes observed in human schizophrenia patients:

- HET females exhibited a non-significant developmental decrease in Cho/Cr ratio from PND 35 to 63, with levels starting significantly higher than WT females and ultimately ending lower than WT females. HET and WT males exhibited relatively similar development in Cho/Cr ratios until PND 63, when there was a significant genotypic difference.
- Viral-injected males exhibited significantly higher Cho/Cr ratios compared to non-injected, and potentially saline-injected males on PND 35 and PND 49.
- WT and HET males exhibited opposite developmental trends in Glx/Cr ratios that culminated in a significant reduction in Glx/Cr in HET males compared to WT males at PND 63.
- HET and WT females, as well as males in each injection type, exhibited similar developmental trends in Glx/Cr ratios.
- WT males, HET males, WT females, and males in each injection condition exhibited similar developmental trends in (NAA+NAAG)/Cr ratios, while there was a significant reduction in (NAA+NAAG)/Cr in HET males compared to WT males at PND 35, and HET females compared to WT females at PND 49.
- HET males exhibited significantly increased levels of Tau/Cr on PND 35, though these levels significantly decreased over time. HET and WT females appeared to exhibit similar developmental patterns in Tau/Cr.
- There appeared to be significant differences between males in different injection conditions on PND 49 and 63, though the results are not conclusive.

Taken together, the developmental differences, despite few genotypic differences, may point to the efficacy of the model in exhibiting reduced GCPII function, and ultimately NMDAR
The subtle nature of the observed trends could be suggestive of a correlation between neurometabolite transmission in the glutamatergic pathway and potentially reduced grey matter volumes (Marsman et al., 2014). However, standard deviations in some cases may be large due to small sample sizes and the small region that was examined. Thus, the placement of the voxel is essential and may account for significant errors exhibited in this study.

**Diffusion Tensor Imaging Analysis**

White matter abnormalities were assessed with diffusion tensor imaging in all mice on PND 28/29, PND 42/43, and PND 56/57 following magnetic resonance imaging. As stated earlier, acquired DTI data were analyzed using the Region of Interest tool in Analyze 11.0. The corpus callosum was traced using the eigenvector color maps (Figure 34A), and these traced regions were subsequently mapped onto maps of various diffusion parameters. The diffusion parameters assessed in this study were fractional anisotropy, the apparent diffusion coefficient, and radial diffusivity (Figure 34B-D).
Figure 34. (A) Representative sample of coronal slices containing the traced corpus callosum on eigenvector color maps, and (B) a fractional anisotropy (FA) map, (C) an apparent diffusion coefficient (ADC) map, and (D) a radial diffusivity (Dr) map. Eigenvector color maps were utilized to trace the corpus callosum, as indicated by the red tracing lines. The corpus callosum was identified and traced using the Waxholm Space Delineated mouse brain atlas (2012) as a guide. Traced regions were saved as object maps and superimposed on each map to obtain values for FA, ADC, and Dr, respectively.

All data were collected from three litters of mice over the past year. Sample sizes were small due to instrumental challenges and the long acquisition time necessary for diffusion tensor imaging data. Ultimately, data were analyzed from HET males, WT males, and WT females (Table 3). Analyses were first conducted to assess differences within each genotype and injection type across time points in the longitudinal model. Consequently, analyses were conducted within each time point to assess the effect of genotype on selected diffusion parameters, and next to examine the effect of injection type on the diffusion parameters. While statistical analyses were conducted, the discussion of the results will focus instead on the feasibility of diffusion tensor imaging in this model. In particular, much of the discussion of results will focus on the challenges inherent in the method.
Table 3. Summary of sample sizes (N) for diffusion tensor imaging at each time point by (A) Sex and genotype, and (B) Injection type. Only males received injections, and due to small sample sizes, mice could not be separated by genotype.

The corpus callosum was selected as a structure to assess both the validity of the GCPII+/−/rAAV9 mouse model of schizophrenia as well as the feasibility of the implementation of diffusion tensor imaging to detect white matter abnormalities in the model. One of the hallmarks of schizophrenia in human patients is disordered processing of both internal and external experiences (Innocenti et al., 2003). The corpus callosum is the largest white matter tract in the brain (located centrally in the cerebrum, Figure 34) and is intricately involved in processing and responding to experiences within the environment because it is responsible for most communication between brain hemispheres (Lungu and Stip, 2012; Patel et al., 2011). Its large size relative to the whole brain as well as its crucial role in connectivity made it an effective structure to test the implementation of DTI in our model. Connectivity is expected to increase with age as a result of an increase in the size of the structure over time (Patel et al., 2011).
Furthermore, many post-mortem morphological studies of human schizophrenic patients have demonstrated abnormalities in the structure of the corpus callosum, particularly decreased thickness and cross-sectional area, as well as decreased fiber density (Lungu and Stip, 2012). These abnormalities in the corpus callosum have been associated with many symptoms in schizophrenia, especially deficits in tasks requiring information transfer, visual-motor skills, and auditory tasks requiring selective attention (Lungu and Stip, 2012; Patel et al., 2011). In this study, DTI data were acquired to analyze changes in a variety of diffusion parameters with the goal of shedding light on potential abnormalities in white matter tracts in the corpus callosum. While sample sizes are small and analysis is essentially limited to males, valuable information regarding the utility of DTI and its potential to be used in future work are assessed. DTI findings are reported according to each assessed parameter—fractional anisotropy, the apparent diffusion coefficient, and radial diffusivity.

**Fractional Anisotropy**

As discussed previously, fractional anisotropy (FA) is a measure of the degree of directionality of water diffusion along a particular axis. Diffusion that is more restricted along an axis is described as anisotropic (closer to 1) and has a higher FA value, while unrestricted diffusion in all directions is isotropic and has a lower FA value (closer to 0). Thus, a decrease in FA value may correlate with white matter integrity, as loss of integrity via demyelination, reduced axonal membrane density, or attenuated fiber coherence could enhance isotropic diffusion of water molecules within a region (Patel et al., 2001).

There were no significant genotypic or developmental differences in FA values for WT males, HET males, or WT females (Figure 35A-B). Furthermore, no significant differences in
FA values were observed between injection types and small sample sizes limited the robustness of statistical tests (Figure 35C).

**Figure 35.** Average fractional anisotropy (FA) values in the corpus callosum (± SD) between PND 28 and 56 in (A) WT and HET males, (B) WT females, and (C) Non-injected, saline-injected, and viral-injected males. A one-way ANOVA was conducted followed by a post hoc Tukey-Kramer HSD test to assess developmental differences and differences between injection types at PND 56, while an independent, two-tailed t-test assuming unequal variance (as determined by Levene’s Test for Equality of Variance, $p < 0.05$) was conducted to assess genotypic differences and differences between injection types at PND 42. Results were considered significant at the $p < 0.05$ level, and no * or ^ indicate no differences were found to be statistically significant.

A meta-analysis of DTI studies on the corpus callosum in schizophrenia patients has revealed that changes in corpus callosum structure could be most pronounced within the areas of the genu (located at anterior and connects left and right prefrontal cortex) and splenium (connects posterior cortices) (Patel et al., 2011). While many studies have found significantly
lower FA values in human schizophrenic patients in both of these regions (Patel et al., 2011), there is not a clear consensus in the literature; many other studies have observed negative findings with regard to a decrease in FA (Patel et al., 2011), and the distinction between first-episode and chronic patients appears to influence findings. In this thesis, the lack of significant difference between HET and WT males, or between viral-injected males and the saline and non-injected controls could be consistent with studies that have negative findings, though much larger sample sizes would be necessary to understand if our data reflect these negative findings. Furthermore, meta-analyses have revealed that these inconsistencies in FA findings within the corpus callosum could be related to differences in acquisition and the resolution of brain structures, as anticipated when deciding to implement DTI in this study (Patel et al., 2011). Thus, the lack of significant findings with regard to FA values could be indicative of the differences in our acquisition and analysis parameters with those in the literature.

In terms of data acquisition, the signal-to-noise ratio of acquired images is proportional to the square root of number of acquisitions. When conducting in vivo imaging of mice, there is a threshold in acquisition time imposed by the amount of time the mice can be anesthetized. Many studies that analyze white matter connections in the mouse brain utilize chemically fixed brains to obtain ex vivo diffusion-weighted images (Mori et al., 2001; Song et al., 2005; Jiang and Johnson, 2010); this seems problematic when trying to examine the microstructure of tissue since DTI depends on water diffusion, and more recent studies have indicated that these types of studies may not be as sensitive to particular white matter injuries as in vivo imaging (Wu et al., 2013). Since white matter abnormalities have been repeatedly implicated in the neuropathology of schizophrenia and assessment of the GCPII+/rAAV9 model was desired longitudinally, in vivo imaging appears more promising. Due to the combination of DTI data acquisition with MRI
data acquisition, and the limited time that mice could be anesthetized, our acquisition time of 95 minutes was significantly lower than that of studies available in the literature; in vivo acquisition times are often two hours, while ex vivo acquisition times can range from 5 to 24 hours for chemically fixed brains (Zhang, accessed April 2015). Research on the use of in vivo DTI of the mouse brain has indicated that many of the cortical and sub-cortical structures of interest in the mouse brain require high-resolution images acquired through 3D high-resolution imaging procedures, and there is an obvious tradeoff between acquisition time and signal-to-noise ratio. The shorter acquisition times necessitated by the parameters in this study resulted in images with poor signal-to-noise ratios. As a result of the poor signal-to-noise ratio, the precision of traced regions was reduced, and ultimately the precision of the DTI parameters calculated for these regions. This factor, in combination with small sample sizes, could explain the large error observed in some of the parameters. Ultimately, tradeoffs between image resolution and acquisition time could factor into the observed lack of significant developmental, genotypic, or injection-type findings in FA values. However, larger sample sizes would be needed to understand this effect.

*Apparent Diffusion Coefficient*

The apparent diffusion coefficient (ADC) describes the degree of restriction of water molecules during diffusion. With fewer boundaries, water can diffuse more freely, and would be characterized by an observed increase in the ADC value. With loss of white matter integrity as has been implicated in the neuropathology of schizophrenia, water is expected to diffuse more freely, and thus we would expect the HET GCPII+/−/rAAV9 mice to mimic the observed increase in ADC values within regions of interest. In this thesis, however, there were no significant trends observed in the ADC values between WT and HET males, or between males of different
injection types (Figure 36A). Furthermore, no significant developmental differences were observed within WT males or females, HET males, or males in any injection type (Figure 36). Again, sample sizes are extremely small, and potential error as a result of inadequate resolution in images could potentially influence results.

Figure 36. Average apparent diffusion coefficient (ADC) values in the corpus callosum (± SD) between PND 28 and 56 in (A) WT and HET males, (B) WT females, and (C) Non-injected, saline-injected, and viral-injected males. A one-way ANOVA was conducted followed by a post hoc Tukey-Kramer HSD to assess developmental differences and differences between injection types at PND 56, while an independent, two-tailed t-test assuming unequal variance (as determined by Levene’s Test for Equality of Variance, $p < 0.05$) was conducted to assess genotypic differences and differences between injection types at PND 42. Results were considered significant at the $p < 0.05$ level, and no * or ^ indicate no differences were found to be statistically significant.

The literature on changes in ADC values in schizophrenia, particularly in the corpus callosum, is much more limited than that on changes in FA values and appears somewhat
inconsistent. Overall, however, a few studies suggest that changes in the apparent diffusion coefficient are less robust, and may not be an adequate marker of white matter abnormalities (Nenadic et al., 2011). Studies such as these have observed no relevant changes in ADC values in a variety of white matter structures in human patients compared to healthy controls (Nenadic et al., 2011). The lack of indicated significant changes between HET and WT males, as well as between males of different injection types, could be consistent with these findings, though larger sample sizes would be necessary to further assess the significance of these results and whether or not the GCPII+/−/rAAV9 emulated the pathophysiological changes observed in humans with schizophrenia.

In contrast to negative findings of ADC changes in schizophrenic patients, Brambilla and his colleagues observed a subtle increase in ADC values in human schizophrenic patients when compared to healthy controls, though the changes are smaller in relative magnitude compared to a marker such as FA (Brambilla et al., 2005). If changes were observed in ADC values, they could be indicative of unique pathologic mechanisms occurring in patients with schizophrenia. While FA values are a more widely-used marker of white matter abnormalities, ADC has the ability to reflect subtle aspects of tissue microstructure that are not reflected in measuring anisotropy; these aspects include diffusion across membranes and the nature of the intracellular and extracellular environments (Nenadic et al., 2011). Perhaps most relevant to the findings in this thesis, the ADC parameter has the potential to reflect properties of neurotransmission, particularly volume transmission (Nenadic et al., 2011). Volume transmission describes the diffusion of neurotransmitters (including acetylcholine, dopamine, and glutamate) through the fluid-filled extracellular space thus modulating slow change in nonsynaptic receptors; this change is a much slower process than the classic, “point-to-point” model of synaptic
transmission (Taber and Hurley, 2014). Thus, ADC values have enormous potential to complement information garnered from FA values by offering insight into the extracellular environment of cells.

While no relevant ADC changes were observed in this thesis, our findings are consistent with the current state of the literature, as the limited number of positive findings have not yet been consistently replicated, and, at times, have negated earlier work (Nenadic et al., 2011). Furthermore, the lack of significant findings sheds light on the inherent challenges regarding the methodology of DTI, specifically voxel size in acquired and analyzed images. In this thesis, the range of structures that could be traced was severely limited due to the large size of the voxels in relation to the whole brain volume (voxel = 0.6 mm depth x 0.15625 height x 0.15625 width, Figure 34A). As a result, error was introduced in tracing regions of interest. Since the corpus callosum borders on grey matter structures, the lack of precision in tracing could have altered ADC values. Due to the subtle nature of changes in ADC that have been observed in some instances in the literature, it is not surprising that no significant changes emerged. While the sample sizes in this thesis are too small to make conclusions, our findings could indicate that ADC is not necessarily the best marker of white matter abnormalities in schizophrenia, or that the methodology requires revision in order to assess its validity as a marker of schizophrenia.

**Radial Diffusivity**

Radial diffusivity (Dr) describes the degree of diffusion perpendicular to axonal fibers, and has been shown to be strongly correlated with demyelination, which is one of the potential mechanisms of decreased white matter integrity in human patients with schizophrenia (Song et al., 2005). Demyelination leads to observable increases in Dr values, while remyelination can result in a decrease in observed Dr values (Song et al., 2005). In this thesis, HET males exhibited
significantly decreased Dr values between PND 42 and 56 (independent, two-tailed t-test assuming equal variance, \( p = 0.0420 \)), though there were no significant genotypic differences between HET and WT males (Figure 37A). WT males, WT females, and viral-injected males did not exhibit any significant developmental changes (Figure 37A-B). There were, however, significant developmental changes observed in saline-injected mice over time [\( F(2,2) = 219.45, p = 0.0045 \), Figure 37C]. An increase in Dr values was observed between PND 28 and 42 (post hoc Tukey-Kramer HSD, \( p = 0.0097 \), Figure 37A), and a decrease in Dr values was observed between PND 42 and 56 (post hoc Tukey-Kramer HSD, \( p = 0.0042 \), Figure 37C).

Figure 37. Average radial diffusivity (Dr) values in the corpus callosum (± SD) between PND 28 and 56 in (A) WT and HET males, (B) WT females, and (C) Non-injected, viral-injected, and saline-injected males. A one-way ANOVA was conducted followed by a post hoc Tukey-Kramer HSD to assess developmental differences and differences between injection types at PND 56, while an independent, two-tailed t-test assuming unequal variance (as determined by Levene’s Test for Equality of Variance, \( p < 0.05 \)) was conducted to assess genotypic differences and differences between injection types at PND 42. Results were considered significant at the \( p < 0.05 \) level, and ^ indicates a significant developmental difference.
While radial diffusivity in schizophrenia has been studied to a lesser extent when compared to parameters such as fractional anisotropy, a growing area of research focuses on the use of cuprizone demyelination animal models to mimic abnormalities found in human schizophrenia patients (Valeiras et al., 2014; Song et al., 2005). These models have been particularly useful in studying the effect of demyelination and remyelination on relevant DTI parameters. Furthermore, recent studies on the use of radial diffusivity in DTI studies have found increased radial diffusivity in first-episode human schizophrenic patients when compared to healthy controls (Lee et al., 2003). The detection of white matter abnormalities in first episode schizophrenic patients is particularly useful in light of the potential indication of neurodevelopmental differences between schizophrenic and healthy patients with few confounding variables (Lee et al., 2003).

The effect of injection-type is difficult to examine in this thesis, as the small sample sizes did not permit a comparison that included controlling for genotype, which represents an essential aspect of the GCPII+/−/rAAV9 model. However, the observed increase in Dr values in saline-injected mice between 28 and 42 mimics the findings of studies in which increased Dr values are observed in schizophrenic patients. This effect is negated, however, between PND 42 and 56, potentially mirroring the observed developmental decrease in Dr values in HET males between these time points. This result suggests that the saline-injection may not be an effective injection-type control, as significant changes were exhibited developmentally. Furthermore, it may suggest that injection of neonatal mice could potentially cause alterations in brain development as evidenced by altered Dr values, though the missing data points in viral-injected mice makes this effect difficult to assess. However, it is plausible that the initial neonatal injection resulted in altered myelination of axons that was subsequently reversed as myelination occurred throughout
development, as evidence in decreased Dr values between PND 42 and 56. Due to the contradiction in effects between time points and the small sample sizes, it is not possible to elucidate the exact mechanism behind these changes, and whether or not they represent significant trends.

In addition to the observed developmental changes in saline-injected mice over time, the decrease in Dr values in HET males between PND 42 and 56, while perhaps not indicative of demyelination, could indicate more generalized axonal damage, as studies have shown decreases in radial diffusivity when axonal damage occurs (Song et al., 2005). However, it is also possible that myelination processes could explain the decrease in radial diffusivity. The sample sizes are far too small to come to any conclusions about the significance of these results; much more data would be required to approach a feasible suggestion of significant trends and a plausible explanation of the mechanism by which trends are observed. Regardless, the ability to obtain values for radial diffusivity in the corpus callosum within the magnitude of current studies suggest it may be useful in ultimately examining the role myelination and axonal damage play in schizophrenia and other neurodevelopmental disorders.

Fiber Tractography in Analyze 11.0

Diffusion tensor images were used primarily for the acquisition of information on alterations in FA, ADC, and Dr. In addition, DTI data were used to explore the feasibility of implementing fiber tractography, which is utilized to visualize white matter fiber tracts between regions of interest. In Analyze 11.0, the fiber tracking algorithm is based on connecting voxels in images that have similar diffusion characteristics (as quantified by DTI parameters such as those examined in this thesis), and consequently displaying these as fibers. In this study, however, the voxels were found to be too large in comparison to the whole brain volume (voxel = 0.6 mm
Correspondence with Analyze Direct indicated that the DTI software was primarily intended for human brains, and thus its applicability was limited in this thesis. This was primarily due to the fact that the acquired images would require higher resolution and higher signal-to-noise in order to better visualize fibers. Attempts at fiber tracking with the DTI data obtained throughout the year resulted in fibers that were clearly not accurate, as they tended to extend outside the brain in many analyzed mice. Thus, it does not appear that the technique will be applicable or useful in analyzing this model.

**Summary of DTI Findings**

Diffusion tensor imaging was employed in analyzing the corpus callosum due to the consensus in the literature that schizophrenia involves some degree of abnormal white matter connectivity in the brain. While fiber tractography was attempted with acquired DTI data, its implementation was unsuccessful due to the relatively large voxel size in relation to the whole brain size; resultant fibers were not representative of those observed in studies with higher resolution images. Overall, the implementation of DTI in the GCPII+/−/rAAV9 model represents a novel technique that has the potential to offer insight into the neuropathological changes associated with schizophrenia, though alterations in the manner in which DTI data were acquired and processed would need to be taken into consideration. In particular, the tradeoff between acquisition time and image resolution must be taken into account in order to obtain DTI images that are potentially useful for analysis of a variety of white matter structures implicated in the pathophysiology of schizophrenia. This study highlighted the methodological challenges associated with the technique, especially in animal models.

In this study, though the sample sizes are small, DTI data illuminated the following:
• No significant genotypic or developmental differences in FA values for WT males, HET males, or WT females. Furthermore, no significant differences were observed in FA values between injection types and across time points.

• No significant trends in ADC observed between WT and HET males, or between males in each injection condition. Furthermore, no developmental differences were observed within WT males or females, HET males, or males in any injection type.

• No significant genotypic differences in males, though HET males exhibited significantly decreased Dr values between PND 42 and 56. There were no significant developmental differences in WT males or females, or viral-injected males. Saline-injected males exhibited significantly increased Dr values between PND 28 and 42, and significantly decreased values between PND 42 and 56.

Many of the reported changes in the literature are fairly subtle, and thus more data are necessary to analyze the extent to which the GCPII\(^{+/−}\)/rAAV9 mouse model could emulate the characteristics of DTI in human patients. Taken together, alterations in FA, ADC or Dr could indicate developmental abnormalities in the corpus callosum in schizophrenic patients, which is the prevailing hypothesis in the literature. The exact nature of developmental abnormalities that lead to poor interhemispheric connections, however, is less clear. Altered development has recently been reduced to two primary mechanisms: reduced axonal density, or demyelination (Brambilla et al., 2005). While FA, ADC, and Dr are hypothesized to provide a more specialized perspective on the type of neurodegeneration that could be occurring, more studies are needed to better understand the exact mechanisms. The results of this thesis are evidence of the inconsistencies previously observed in literature regarding DTI parameters as markers of white matter abnormalities. The DTI findings in this thesis should serve as a framework for future studies with regards to the methodological challenges associated with the technique. Studies that assess more structures implicated in the pathophysiology of the disorder could elucidate further white matter abnormalities implicated in schizophrenia.
IV. Conclusions and Suggestions for Future Work

This study serves as an initial assessment of the GCPII+/−/rAAV9 mouse model of schizophrenia, and represents a continuation of the assessment of the GCPII+/− mouse model. Overall, we found neuroanatomical and neurochemical changes that could reflect relevant changes in human schizophrenia patients, though relevant abnormalities in white matter connectivity as assessed by diffusion tensor imaging were not observed.

The aggregation of MRS data from the past few years offers our first complete picture of the ability of the GCPII+/− model to reflect neurochemical changes in human schizophrenia patients. The GCPII+/− model appears to mimic some, but not all, of the neurochemical changes associated with the disorder in humans. The observed inconsistencies in neurometabolite levels examined in this study, ironically, are consistent with the literature on human schizophrenia patients in which observed changes are often very subtle and inconsistent between studies. One of the methodological limitations of the MR spectroscopy technique in this study is our inability to resolve the overlapping glutamate and glutamine peaks into their component peaks, as well as the individual NAA and NAAG peaks. This resolution would be necessary in order to offer more conclusive information regarding the nature of neurochemical changes.

The lack of significant neuroanatomical changes observed in this study is not reflective of the disorder in humans, especially the lack of volumetric changes in the hippocampal and lateral ventricular volumes. However, aggregation with past data acquired in this lab would be necessary to better understand the GCPII+/− model as a whole. In addition, more data on the GCPII+/−/rAAV9 model would be necessary to assess its efficacy at modeling neurochemical changes associated with schizophrenia in humans. It appears, however, that the process of tracing anatomical images to assess volumetric changes presents challenges due to the small size of
some structures in relation to the whole brain and corresponding large errors due to volume averaging.

The findings of this study are useful due to the thorough exploration of the methodological challenges associated with diffusion tensor imaging of a mouse model. The most noticeable challenge in this technique is the trade-off between acquisition time and signal-to-noise ratio. Due to the fact that acquisition times cannot be lengthened in our imaging protocol, it is not feasible to implement diffusion tensor imaging in future studies.

Initial findings on the GCPII+/−/rAAV9 mouse model suggest that there may be minimal neuroanatomical and neurochemical differences between viral-injected and saline-injected mice. Overall, however, our data were limited to assess the GCPII+/−/rAAV9 mouse model of schizophrenia due to a lack of mice that were both heterozygous for the GCPII mutation as well as injected with the adeno-associated virus. In order to garner a more complete analysis of the model, the acquisition of more data would be necessary. While there were some subtle neurochemical differences between viral-injected mice compared to saline and non-injected mice (namely, in Tau/Cr ratios), diffusion tensor imaging findings on the radial diffusivity parameter, which is generally viewed as a measure of myelination, indicates that the process of neonatal injections may be inducing developmental alterations not associated with the GCPII mutation or the adeno-associated virus. More specifically, the injections may induce axonal demyelination, which, over the course of development, is reversed via remyelination. Thus, the similarity between viral and saline-injected mice is problematic, regardless of small sample sizes and the inability to control for genotype, as the similarity seems to suggest that the epigenetic dysregulation we hoped to induce is not occurring. Rather, the process of neonatal injection
could produce neuroanatomical changes as a side effect. Thus, it is unlikely that epigenetic
dysregulation via rAAV9 injection will be attempted in future studies.

In order to better understand whether or not epigenetic dysregulation is occurring, future
work in this lab will seek to assess whether or not the injection of the cDNA for the histone
deacetylase enzyme 1 (HDAC1) via the adeno-associated virus resulted in expression of the
HDAC 1 protein via immunohistochemistry experiments. This characterization will be necessary
to understand whether or not neonatal injections into the cingulate cortex are successful at
modulating relevant changes observed in human schizophrenia patients. The results of these
experiments would allow us to come to a conclusion about whether or not the injections are a
useful aspect of our model. The final goal of this project will be to aggregate all previous MRI
and MRS data from the GCPII+/− model to more completely understand the feasibility of
modeling schizophrenia using a mouse model.

Overall, our findings in this study are representative of the challenges inherent in
modeling a disorder where the observed changes in humans are often subtle and contradictory
across different subsets of the population with schizophrenia. Due to the relatively young age of
the mice at which we acquire data, our data are most likely analogous to first-episode
schizophrenic patients. Since many of the neurochemical and neuroanatomical changes
associated with schizophrenia are thought to be progressive throughout development, it would be
beneficial to be able to acquire data at later time points, though this is not possible due to the size
of our imaging probe. Instead, perhaps the GCPII+/−/rAAV9 mouse model could prove to be a
promising model of the changes observed in first-episode schizophrenia patients. Regardless, this
study has broadened our understanding of the feasibility of diffusion tensor imaging as well as
the potential problems associated with attempting to induce epigenetic dysregulation via neonatal injections.
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Appendix: Guide to Diffusion Tensor Imaging Acquisition and Post Processing in Analyze 11.0

Diffusion Tensor Imaging using Bruker ParaVision 4.0 (Mouradian, 2014)

1. Follow protocol for setting up mouse in probe for MRI.

2. Tune and match your sample first. Once probe is placed and \(^1\)H cable is connected, go to spectrometer control tool window, click acquire and choose wobble.

3. Load shim file ‘dtimouse14’ from shim tool.

4. Now load the SINGLEPULSE_1H scan by going to ParaVision Scan control tool New Scan \(\rightarrow\) B_Spectroscopy and choosing SINGPULSE_1H scan.

5. To allow for a seamless adjustment over time, edit the number of reps in the scan to 200 by selecting scan \(\rightarrow\) clicking edit method in spectrometer control tool which will bring you to the parameter window.

6. With the shim tool open, select “GSP” to begin manual shimming. To homogenize the magnetic field, you want to adjust the X, Y, Z and Z\(^2\) values. Object of shimming is to obtain a symmetrical water peak and a gradual free induction decay. Water peak should get narrower and longer by adjusting mainly the Z and Z\(^2\) values. If peaks disappear, stop shimming and start again.

7. Begin by adjusting the Z value to left followed by the Z\(^2\), X and Y. Repeat as necessary.

8. Once shimming is completed, allow the scan to finish and run RARE_Tripilot and RARE_8_bas. Established protocols should be followed for these scans (Mu, 2011).

9. Load EPI-diffusion-tensor scan by choosing New Scan and setting Location to B_DIFFUSION.
   - Select scan and go to spectrometer control tool \(\rightarrow\) edit method
   - Click on radio button ‘expand’ next to Diffusion and make sure that diffusion experiment is set to DW_Tensor with 30 diffusion directions, 1 diffusion experiment per direction and 5 A0 images.
   - Optimize the gradients by changing the Diffusion gradient duration to 2.00 msec and the diffusion gradient separation to 7.77 msec.
   - Scroll down and make sure that the b value is set to 1000 s/mm\(^2\), double check that the parameters are correct by making sure that the gradient scaling per channel is now at about 84% (might be around 83.9%).
   - Go up and set echo time (TE) to the minimum 18.78 msec.
   - Gradient Duration \(\rightarrow\) Expand Standard in plane geometry interface and change Field of View (FOV) parameters to 20 mm x 20 mm. Finally expand Standard slice geometry interface and set slice thickness and interslice distance to 0.6 with 26 slices.
Repetition time should now be 3900 msec, and set averages to 10.

10. Tune and match your sample again by choosing wobble from Acquire drop down menu on spectrometer control tool.

11. Select EPI-diffusion-tensor scan and go to geometry editor on Scan control window. Load corresponding RARE_Tripilot scan and set number of slices to 26. Make sure that slices cover entire brain on sagittal image and click accept. Now run the scan by hitting the traffic light icon.

**Loading Diffusion Tensor Images into Analyze 11.0 using the DICOM tool**

1. Go to File⇒ DICOM tool (alternatively, there is a DICOM tool icon on the toolbar).

2. Within DICOM tool, go to File⇒ import DICOM images and select desired folder containing DWI DICOM images (ex. GCP7213p60). Hit OK.

3. Click on desired folder under “Patient Name.” This will display all of the Volume ID’s included in the scan. The first five volumes are $B_0$ images, and only one is necessary for DTI analysis.

4. Starting with one of the $B_0$ base volumes (Select one volume with volume ID’s=1, 2, 3, 4 or 5), select by clicking on the volume and then selecting “Load Volume.” Continue this process until you have loaded every Volume ID in order (ex- Volume ID=5, Volume ID=6, etc.). This will transfer 31 volumes to the main Analyze window.

5. In the main Analyze window, select all 31 images that correspond to the previously loaded volumes. Go to Apps⇒ DTI. This will open up the DTI add-on.

6. In the “Config” tab, you will need to load a .txt file containing the gradient directions used in the DTI scan. To create the text file:
   
   - Follow the ParaVision 4.0 DTI Protocol to obtain the gradient direction file for the DWI/DTI scan (steps 23-26 under the section entitled “Obtaining Gradient Direction File for DTI Post-Processing”)
   - To create the .txt file, open NotePad on the lab computer
   - On the first line of the .txt file, type 0.000000 three times, separating each value with a single space (ex- 0.000000 0.000000 0.000000). While this is not in the gradient direction file, these are the gradients needed for the base volume ($B_0$).
   - Next, copy each line of the gradient direction matrix into the .txt file such that each gradient direction is separated by one space. Example:
     - 0.333333 0.666667 -0.666667
     - 0.666667 0.333333 -0.333333
   - Once you have typed all of the gradient directions within the matrix, add five more lines that match the last line within the gradient direction matrix so that you
have the same number of lines in the .txt file as there are images uploaded into the Analyze window (ex- if you have 35 loaded images, the .txt file should contain 35 lines)

- Save this as a .txt file in the Schizophrenia folder on the desktop.
- Select “load gradient directions” in the main DTI add-on window and hit “apply”
- Check the box next to “Z” along the “Flip Gradient Signs” row.
- Keep b-value at 1000 s/mm².

7. In the “Map Processing” tab, move the sliding bar in the top left box to find an image that best displays the brain.

8. Move the “Background Threshold” sliding bar until the space in the image surrounding the brain is mostly white, while the brain appears almost completely black. It is better to not have any white “holes” in the brain; background noise (i.e. remaining black pixels) outside the brain can be eliminated from subsequent analysis.

9. Select “Compute DTI Maps.”

10. In the DTI add-on window, go to File→ Save DTI Maps. This will save each of the different types of DTI maps to the main Analyze window. Now, minimize the DTI add-on window but do not close out of it.

11. In the main Analyze window, select the Eigenvector Color Map and the FA map that were just saved.

12. With these maps selected, go to the ROI icon and trace the desired regions of interest as outlined in the MRI volumetric analysis protocol.

13. After tracing the ROIs, go to File→ Save Object Map and exit out of the ROI window.

14. Return to the main analyze window. Now, the object map containing the traced region of interest can be loaded onto any of the DTI maps that were previously computed. Select the DTI map of interest in the main Analyze window.

15. Select “Load Object Map” and choose the file containing the traced object map with the desired ROIs.

16. In the Volume Edit window, go to File→ Load Object Map and select the saved folder containing the displayed fibers. This will overlay the fibers in your ROIs onto the FA map.

17. Go to “Generate”→ “Sample Options” to obtain mean values for the desired diffusion parameter in the ROI. Change the following settings:
   - Turn summing on
   - Turn sequence display off
   - Set the number of decimal places to 6
○ Turn log stats on

18. Select “sample images” to obtain values for the DTI parameter of interest. Averages ± standard deviations can be saved in a .stats or .log file and analyzed.