Glutamate Imaging of Mouse Models of Neurodegeneration

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Abstract
Malfunctions in the glutamatergic system of the central nervous system have been implicated in neurodegenerative diseases such as Alzheimer's disease (AD), tauopathies, and Parkinson's disease (PD). A non-invasive measurement of glutamate would enhance our understanding of neurodegenerative processes and potentially facilitate early diagnosis. The current method for measuring glutamate in vivo is proton magnetic resonance spectroscopy (1HMRS) although it has poor spatial resolution and weak sensitivity to glutamate changes. The primary objective of this thesis was to measure pathology induced changes in glutamate levels in mouse models of neurodegeneration using a novel magnetic resonance imaging technique, glutamate chemical exchange saturation transfer (GluCEST) imaging.

Several studies were performed in three mouse models of neurodegeneration: the APP-PS1 transgenic model of amyloid-beta pathology of AD, the PS19 transgenic model of tau pathology, and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin model of PD. Glutamate levels derived from GluCEST imaging were correlated with results from 1HMRS and immunohistochemistry (IHC). The primary IHC antibodies that were investigated include markers of phosphorylated tau protein, synapse density, neuron density, glial cell reactivity, a glutamate transporter, and an NMDA receptor.

GluCEST contrast correlated with 1HMRS-derived glutamate levels in the striatum of APP-PS1 mice (R2=0.91) and the thalamus of PS19 mice (R2=0.64). However, GluCEST detected deficits in PS19 mice four months earlier than 1HMRS, highlighting the method's enhanced sensitivity to glutamate. Demonstrating the advantage of high spatial resolution, GluCEST imaging measured sub-hippocampal dynamics in glutamate levels in the aging PS19 mouse. A gradient in glutamate levels along the mouse hippocampus was also measured in vivo using GluCEST. While hippocampal glutamate levels were significantly decreased in early stages of PS19 tauopathy, glutamate levels in the dentate gyrus (DG) and cornu ammonis (CA1) increased at 9-13 months. Decreased GluCEST was concurrent with synapse loss and occurred before structural volume loss. Elevated GluCEST was associated with glial fibrillary acidic protein (GFAP) immunostaining in late stages of the PS19 tauopathy model and in the striatum of the MPTP PD model.

Results of this work demonstrate the use of GluCEST imaging to study regional and temporal variations in glutamate in different pathologies associated with neurodegeneration.

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GLUTAMATE IMAGING OF MOUSE MODELS OF NEURODEGENERATION

Rachelle L. Crescenzi

A DISSERTATION

In Biochemistry and Molecular Biophysics

Presented to the Faculties of the University of Pennsylvania

In Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2015

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DEDICATION

For my animal friends:

Ellsworth, Walnut, Sherwood, Gibson, Rose, Apple, Dustin, Elmer, and Salem.
ACKNOWLEDGMENT

S.D.G.

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ABSTRACT

GLUTAMATE IMAGING OF MOUSE MODELS OF NEURODEGENERATION

Rachelle L. Crescenzi

Ravinder Reddy, Ph.D.

Malfunctions in the glutamatergic system of the central nervous system have been implicated in neurodegenerative diseases such as Alzheimer's disease (AD), tauopathies, and Parkinson's disease (PD). A non-invasive measurement of glutamate would enhance our understanding of neurodegenerative processes and potentially facilitate early diagnosis. The current method for measuring glutamate in vivo is proton magnetic resonance spectroscopy (1HMRS) although it has poor spatial resolution and weak sensitivity to glutamate changes. The primary objective of this thesis was to measure pathology induced changes in glutamate levels in mouse models of neurodegeneration using a novel magnetic resonance imaging technique, glutamate chemical exchange saturation transfer (GluCEST) imaging.

Several studies were performed in three mouse models of neurodegeneration: the APP-PS1 transgenic model of amyloid-beta pathology of AD, the PS19 transgenic model of tau pathology, and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin model of PD. Glutamate levels derived from GluCEST imaging were correlated with results from 1HMRS and immunohistochemistry (IHC). The primary IHC antibodies that were investigated include markers of phosphorylated tau protein, synapse density, neuron density, glial cell reactivity, a glutamate transporter, and an NMDA receptor.

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Results of this work demonstrate the use of GluCEST imaging to study regional and temporal variations in glutamate in different pathologies associated with neurodegeneration.
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CHAPTER 1: Glutamate as a biomarker of early neurodegeneration

I. Review of Glutamate in the Central Nervous System (CNS)

Glutamate in the CNS resides in several compartments, both intracellular and extracellular, in neurons and in glial cells. Firstly, there are two main sources of neuronal glutamate: as an intermediate of the tricarboxylic acid (TCA cycle), and derived from glutamine from astrocytes as part of the glutamine-glutamate cycle (Figure 1). The TCA cycle uses glucose as an ultimate source of ATP energy for cells via oxidative metabolism. Isotopic labeled carbon (C\(^{13}\)) studies using nuclear magnetic resonance (NMR) spectroscopy have investigated the metabolism of glutamate as an intermediate (Gruetter et al., 1998). This technique has been used to directly measure oxidative metabolism of glucose by isotopic labeling of glucose with C\(^{13}\). Glutamate is synthesized by transamination of α-ketoglutarate (α-KG) with an amino acid such as alanine, and glutamate can be converted back to α-KG via deamination.

![Figure 1. Glutamate-Glutamine Cycle: Glutamate is released from neurons as a neurotransmitter, and is transported into astrocytes to be recycled as glutamine. Energy is required by glutamine synthetase to convert glutamate to glutamine in astrocytes; however, glutamine is passively converted to glutamate in neurons. Glutamate metabolism is altered in neurodegenerative disorders, manifesting in higher or lower concentrations of glutamate, or its receptors, transporters, or enzymes.](image-url)
A portion of neuronal glutamate is packaged into neurotransmitter vesicles to be transported to the pre-synapses via vesicular glutamate transporter (VGlut, Bellocchio, 2000). Once released into the synaptic cleft, post-synapses of other neurons will accept some of the extracellular glutamate by glutamatergic receptors and transporters. There are three main families of neuronal glutamate receptors: N-methyl D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainite receptors.

The remaining free glutamate doesn’t remain long in the extracellular space, or else it would become excitotoxic (Hertz et al., 1999). Glutamate is actively transported into astrocytes by the glutamate transporter GLT-1/EAAT2. Astrocytes are the location of the glutamine-glutamate cycle, by which three quarters of glutamate is ultimately recycled and stored in the form of glutamine (Benjamin and Quastel, 1975; Westergaard et al., 1995). This process of amidation of glutamate into glutamine is facilitated by glutamine synthetase (GS), which is only present in astrocytes and oligodendrocytes (D’Amelio et al., 1990; Norenberg and Martinez-Hernandez, 1979; Tansey et al., 1991). Glutamine is shuttled back into neurons, which would not be possible in the form of glutamate because it would trigger an action potential (Hagberg et al., 1985). Subsequently, neurons as well as astrocytes can form glutamate from glutamine; this reverse process requires no energy (ATP independent) via phosphate-activated glutaminase (PAG) (Hogstad et al., 1988; Kvamme et al., 1982; Schousboe et al., 1979). Current evidence supports the conclusion that only astrocytes can produce glutamine, and without its synthesis in astrocytes the production of neuronal glutamate would be stopped (Tansey et al., 1991).

The concentration of total glutamate by weight of brain tissue is 5-15mM per kg wet weight, which varies with the region of the brain (Schousboe, 1981). While the greatest concentration of glutamate occurs in neurons (10-15mM, 7x greater than that of other
metabolites), there is a substantial contribution from glial cells (6-10mM) (McKenna, 2007). Extracellular space can contain up to 100µM glutamate during neurotransmission with typical resting levels of 3-4µM. CSF contains around 10µM glutamate (Hamberger and Nyström, 1984; Lehmann et al., 1983).

II. Glutamate is implicated in neurodegenerative disorders

Glutamate is implicated in many neurodegenerative disorders (Danbolt, 2001). Glutamate plays a role in the pathogenesis of the three neurodegenerative disorders which will be dealt with in this thesis: Alzheimer's disease (AD), tauopathies, and Parkinson's disease (PD). Glutamate levels may therefore be a good candidate for a biomarker of early neurodegeneration. The following section introduces these neurodegenerative disorders, explains the evidence of altered glutamate metabolism and function as a neurotransmitter in their early stages, and highlights the current radiology techniques for in vivo diagnosis.

a. Glutamate is implicated in AD and tauopathies

Alzheimer's disease is the most common neurodegenerative disorder and the sixth leading cause of death in the USA (James et al., 2014; Murphy et al., 2013). AD is characterized by two hallmark proteins which accumulate in aggregates in brain tissue that are detected at autopsy: extracellular amyloid-beta “plaques” and intracellular neurofibrillary tangles (NFTs). Although AD is the most well-known tauopathy, a variety of neurodegenerative disorders are classified as tauopathies due to the presence of intracellular aggregates of tau protein. Other diseases in which tau pathology is the most dominant feature include frontotemporal dementia with parkinsonism linked to
chromosome 17 (FTDP-17), corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and Pick’s disease (PiD) (Lee et al., 2001; Thibodeau et al., 2009). Recently, research interest in tauopathies has further intensified with the discovery that chronic traumatic encephalopathy (CTE), which particularly affects athletes engaged in contact sports and military personnel exposed to repeated explosions, is a progressive tauopathy (Lakis et al., 2013; McKee et al., 2009).

The major component of NFTs is hyper-phosphorylated tau (h-p-tau) protein (Lee et al., 2001). Tau protein is naturally phosphorylated to regulate its binding to microtubules (Cleveland et al., 1977); in its phosphorylated state, tau dissociates from microtubules, promoting their denucleation (Drechsel et al., 1992). When tau is hyperphosphorylated, tau aggregates into paired helical filaments (PHFs) and straight filaments (together referred to as NFTs) in vitro (Alonso et al., 2001). Presumably, when h-p-tau is unbound from microtubules in vivo, it has a propensity to aggregate into NFTs due to the increased concentration of tau in the cytoplasm.

The presence of NFTs is required for the diagnosis of AD and tauopathies at autopsy (Dubois et al., 2007). NFT formation occurs in stages that are determined semi-quantitatively at autopsy using the Braak scale (Braak and Braak, 1991) and which correlate with the progression of neuropathologic symptoms of tau pathology. Microgliosis is reported in

![Figure 2. NFT pathology of AD.](image)
frontotemporal dementias (FTDs), and glial fibrillary tangles may be present in the same regions where neuron loss and NFT inclusions occur (Komori, 1999). Additionally, synapse loss is the closest histologic correlate to cognitive deficits (Terry et al., 1991) and to the severity of cognitive deficits (DeKosky and Scheff, 1990).

Pathologic tau aggregates accumulate in the dendritic spines of hippocampal neurons and, among other consequences, disrupt the function of neurotransmitters (Drechsel et al., 1992). For instance, VGLut1 (vesicular glutamate transporter protein) is reduced in AD cortical tissue (Kirvell et al., 2006) and correlates with cognitive impairment scores (Kashani et al., 2008). VGLut1 is also reduced in the perforant pathway of patients with cognitive impairment (Robinson et al., 2014); however, in patients a decade younger (mean age 88.8 yrs) with cognitive impairment, VGLut1 is elevated over healthy controls in the mid-frontal gyrus (Bell et al., 2007). The hippocampus and mid-frontal gyrus also show reduced expression and protein levels of another excitatory amino acid transporter protein (EAAT1, EAAT2, Jacob et al., 2007). This may be due to their association with phosphorylated tau localized to NFTs in AD, PSP, and CBD patients (EAAT2, Sasaki et al., 2009).

Glutamate receptors, namely NMDA and AMPA, are also implicated in tauopathies. Early autoradiography and immunohistochemistry (IHC) studies in AD tissue have shown reduced NMDA binding in the cortex (Greenamyre and Young, 1989; Jansen, 1990; Penney et al., 1990), reduced AMPA binding in the hippocampus (Dewar et al., 1991), and reduced AMPA receptors in the entorhinal cortex (Yasuda et al., 1995) and hippocampus (Aronica et al., 1997). Reduced expression levels of genes encoding NMDA and AMPA receptors were measured in the AD hippocampus (Jacob et al., 2007). Direct evidence in cultured cortical neurons shows that NMDA receptor activation is required for
tau-induced cell death, and that reduced expression of NMDA receptors may be protective (Amadoro et al., 2006). In a tauopathy mouse model, reduced levels of AMPA receptor GluR1 were found in post-synaptic dendritic spines where pathologic tau accumulates preferentially over the pre-synapse (Hoover et al., 2010). This occurred at 4.5mo of age before loss of the spine structure itself. Dysfunction of the post-synaptic glutamate pathway may indicate an earlier stage of pathology than synapse loss of dendritic spines.
b. Current challenges and strategies for the diagnosis of AD and tauopathies

The National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) recognizes several non-invasive diagnostic methods for a probable diagnosis of AD: volume changes measured by MRI and cerebral glucose metabolism measured by FDG-PET (Dubois et al., 2007). New biomarkers of earlier stages of AD are being actively sought in the Alzheimer’s Disease Neuroimaging Initiative (ADNI) trials (Weiner et al., 2012). The ADNI I and ADNI II studies are two phases of a multi-center trial which aims to test various novel imaging techniques for their potential as early correlates of AD. This highlights the advanced stage of biomarker research for AD.

The variable clinical symptoms of tauopathies, however, present distinct challenges for diagnosis. While AD is the most prevalent cause of dementia in people over 65 years old, early onset dementia is just as likely to be caused by FTD (Ratnavalli et al., 2002). Diagnosis at such early stages (<65yrs. old) is uniquely difficult for FTD. One study reported that 71% of patients eventually diagnosed as FTD were originally mis-diagnosed as non-dementia (Rosness et al., 2008). This is because early symptoms of FTDs can often be mistaken for other disorders related to semantic difficulties, subtle psychiatric and behavioral changes, and poor motor control (Jicha and Nelson, 2011; Rosness et al., 2008).

Jicha and colleagues concluded that if we could identify the specific neurotransmitter pathways and locations which are affected in an individual, it would be a key step towards personalized medicine for FTD patients, as modulators of these pathways are already
FDA approved (Jicha and Nelson, 2011). There is a vital need for biomarkers that can accurately diagnose early stages of tauopathies.

Proton magnetic resonance spectroscopy ($^1$H MRS) is a non-invasive tool that is regularly used to study metabolic changes in neurodegenerative disorders such as AD. While $^1$H MRS studies typically report reduced N-acetyl-aspartate (NAA) and elevated myo-Inositol (MI) levels in patients with AD (Guevara et al., 2010; Kantarci et al., 2010, 2004; Vion-Dury et al., 2004), few studies report glutamate (Glu) levels. This is likely due to the fact that the glutamate peak is more difficult to measure than NAA or MI because of its low signal-to-noise ratio (SNR). For instance, patients with microtubule-associated protein (MAPT) mutations (including P301L) have increased ratios of MI to creatine (Cr) and decreased NAA/MI ratios while still pre-symptomatic; glutamate ratios were not reported likely because the MRS protocol was not optimized for the SNR of smaller metabolites (Kantarci et al., 2010).

Glu changes have been reported by MRS at higher field strength in a few AD studies. In a study performed at 3T in the posterior cingulate gyrus of AD patients, the Glu and glutamine (Gln) ratio to Cr significantly decreased (Hattori et al., 2002). Using a 4T system, Glu/Cr and NAA/Cr concentration ratios were found to decrease significantly in the hippocampus of AD patients (Rupsingh et al., 2011).

However, the ratio of Glu+Gln to total Cr was not statistically significantly different in an AD population with mild cognitive impairment scores (Kantarci and Jack Jr., 2003).
Only NAA/MI was significantly different in a converter MCI population which develops AD (Kantarci et al., 2007). These early stages of pathology may be detectable using a more sensitive measurement tool of glutamate levels.

The $^1$H MRS signatures of various mouse models of neurodegeneration have also been reported. Longitudinal $^1$H MRS studies in a variety of mouse models of AD have been reviewed (Mlynárik et al., 2012). These models reported significant reductions in Glu and NAA roughly 6-10 months after initial amyloid deposition and later elevation of MI (Mlynárik et al., 2012). A longitudinal study in the APP-PS1 mouse model of amyloid-beta plaques reported a significant decrease in Glu/Cr and NAA/Cr with age, as well as between control and transgenic mice (Marjanska et al., 2005). Transgenic mouse models of tauopathy have been only sparsely studied using $^1$H MRS. An inducible model with the P301L mutation has shown decreased glutamate levels in the hippocampus (Yang et al., 2011) and in hippocampal extracts (Nilsen et al., 2013) by $^1$H MRS. This evidence from $^1$H MRS in mouse models supports the occurrence of reduced glutamate in AD and tau pathologies and demonstrates the promise of using pre-clinical models for the development of more sensitive biomarkers.

Another imaging modality for early detection of tauopathies is positron emission tomography (PET). Several PET radiotracers for tau imaging are under development. Radiotracers for pathologic tau primarily target PHF tau, the pathologic structure found in AD brains (Fodero-Tavoletti et al., 2014). However, PHF tau is not specific for the form of aggregated tau found in pure tauopathies (Ballatore et al., 2007). For instance, the radiotracer 18F-THK523 demonstrated specificity for PHF tau in AD patients but not in other tauopathies; 18F-T807 for PHF tau likewise was only demonstrated in the AD brain (Villemagne et al., 2008). Another tracer is nonspecific for NFT and amyloid-beta plaques,
A promising agent is 11C-PBB3, which binds to aggregated tau in AD and non-AD brains (Maruyama et al., 2013). Despite its high specificity, the major shortcomings of PET include radiation exposure, low resolution, imaging logistics as a result of the short half-lives of radioligands, and limited applicability to functional studies. These findings underscore the critical need for development of non-invasive and high-resolution imaging techniques for the detection of tau pathology.

c. Glutamate is implicated in PD

Parkinson’s disease (PD) is the second highest diagnosed neurodegenerative disorder after AD (de Lau and Breteler, 2006). Only 10% of the cases are caused by genetic mutation while the majority are idiopathic. However, the common pathogenesis of PD ends with the degeneration of the nigrostriatal dopaminergic pathway, which connects the substantia nigra to the striatum as part of the basal ganglia motor loop (Alexander, 1994). Due to unknown causes, dopaminergic neuron loss is found in the substantia nigra pars compacta (SNc) of PD patients (Alexander, 1994; Obeso et al., 2000). The nigrostriatal dopaminergic pathway breaks down causing a dopamine deficiency in the striatum (see pathways in Figure 4). As dopamine is depleted from the striatum in PD, the motor cortex is unregulated and over-stimulated. Therefore, the primary symptom of PD is poor motor control including tremors while resting, slow voluntary movement or freezing when attempting voluntary movements (Vercruysse et al., 2014), rigidity and altered gait, and eventually difficulty walking and performing normal tasks (Gazewood et al., 2013; Obeso et al., 2000).
Figure 4. Cortico-striatal pathway. Glutamatergic pathways are shown in green, dopaminergic pathways in blue, and GABAergic pathways in red. (+) and (-) symbols indicate excitatory and inhibitory pathways. With the loss of dopaminergic input to the striatum from the substantia nigra pars compacta in PD pathology, excessive excitatory inputs to the striatum cause excitotoxic damage. Illustration created by Mikael Häggström, based on images by Andrew Gillies/User:Anaru and Patrick J. Lynch [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)], via Wikimedia Commons.

Our primary knowledge of early PD before excessive neuron loss is derived from in vitro studies and key animal models. In particular, 1-methyl-4-phenyl1,2,3,6-tetrahydropyridin (MPTP) is a well characterized neurotoxin that selectively targets
dopaminergic neurons in the SNc (Blesa et al., 2012; Meredith and Rademacher, 2011). When delivered in mice by specific dosing regimens (Jackson-Lewis and Przedborski, 2007), it quickly produces deficits in motor control which resemble those of PD patients. MPTP crosses the blood-brain-barrier through the MAO-B, and is transported into astrocytes (Marini et al., 1992). Astrocytes metabolize MPTP into methyl-4-phenylpyridinium (MPP+) before it is released. MPP+ is a potent inhibitor of complex I in mitochondria (Ramsay et al., 1986) and a specific substrate of the dopamine transporter. Hence, MPTP toxicity is specific to dopaminergic neurons and particularly affects the SNc and striatum of mice (Chan et al., 1991). This mimics the specific location of dopaminergic neuron degeneration found in PD patients.

Glutamatergic excitotoxicity is a secondary effect of PD pathology (Ambrosi et al., 2013; Beal, 2000, 1998). Excitotoxicity is induced when energy production of the mitochondria is impaired (Novelli et al., 1988). As MPTP induces failure of the complex I mechanism in the electron-transport chain of mitochondria, dopaminergic cells cannot produce enough energy; the cell’s membrane potential becomes depolarized, opening glutamatergic Ca2+ dependent NMDA receptor channels (Meredith and Rademacher, 2011; Meredith et al., 2009). This leads to a three-fold increase in extracellular glutamate levels as measured by in vivo microdialysis (Meredith et al., 2009). Such high concentrations of glutamate are toxic to neurons. When treated with NMDA antagonists, MPTP toxicity is prevented in the SNc of rats (Turski et al., 1991) and primates (Lange et al., 1993; Zuddas et al., 1992). Trials of NMDA antagonists in PD patients have likewise shown benefits in motor control, lessening dyskinesia, and improved cognitive scores (reviewed by Stayte and Vissel, 2014).
Glial cells also play a role in glutamatergic excitotoxicity of PD. Impaired mitochondria energy production further causes glutamate to accumulate in astrocytes in vitro (McNaught and Jenner, 2000). This is likely due to the fact that as extracellular glutamate levels are persistently elevated, reduced glutamine synthetase activity is detected, and the clearing mechanism of astrocytes begins to fail (Morales and Rodriguez, 2012). The response of neuroinflammation also causes microglia to become active and release higher levels of glutamate (Barger et al., 2007).

Elevated glutamate levels are localized to the striatum of MPTP mice, which is proposed to simulate early stages of PD before denervation of the striatum. Therefore, PD pathology represents a neurodegenerative disorder that has localized excitotoxicity, synapse loss, and eventual neuron loss. There is, therefore, a great need for a biomarker with high spatial resolution to show specific changes during pre-symptomatic PD pathology.

d. Current challenges and strategies for the diagnosis of PD

When the clinical symptoms of PD become apparent, up to 60% of neurons can already be lost, causing an 80% deficiency in dopamine (DA) levels. Early, in vivo diagnosis before the onset of motor symptoms is critical for the rescue of viable brain from PD pathology.

The glutamate signal measured by $^1$H MRS has been reported to decrease in several studies in the posterior cingulate gyrus (Griffith et al., 2008) and mildly in the substantia nigra (Öz et al., 2006). This is contrary to the excitotoxicity hypothesis of PD pathogenesis. However, it is important to note that the majority of patients recruited in these studies are medicated with cholinesterase inhibitors or L-DOPA, a DA replacement.
molecule. Furthermore, research is not carried out on pre-symptomatic PD patients, who may otherwise exhibit excitotoxicity. These confounds have made it difficult to measure the anticipated excitotoxic effects of PD pathology in a patient population.

Several $^1$H MRS studies have reported glutamate changes in MPTP mouse models. One study reported that glutamate concentration doubled in the dorsal striatum after the onset of MPTP toxicity in mice (Chassain et al., 2010, 2008). When treated with L-DOPA, glutamate concentration returned to baseline levels (Bagga et al., 2013; Chassain et al., 2010). A study in the ventral striatum showed that glutamate levels increased in proportion to the amount of dopamine denervation in the ventral tegmental area (VTA) and dorsal striatum (Chassain et al., 2013).

A PET radiotracer called [11C]-MPEP was developed for an antagonist of the mGluR5 receptor. MGluR5 is a glutamate receptor specific to astrocytes which is active during neuroinflammation and may be regulated by NMDA receptor activity (Ambrosi et al., 2013). In a 6-hydroxydopamine (6-OHDA) neurotoxin rat model of PD, elevated glutamatergic activity was measured using [11C]-MPEP in the striatum where neuron loss had occurred (Pellegrino et al., 2007). In an MPTP model in rhesus macaques, [11C]-MPEP binding increased 10-20% in the striatum, also indicating elevated glutamatergic activity with the decrease in striatal dopamine levels (Sanchez-Pernaute et al., 2008). Other radiotracers for mGluR5 activity are being developed and tested in MPTP models of PD (Mu et al., 2010). A therapeutic effect has been achieved in 6-OHDA mouse models by decreasing mGluR5 levels (Black et al., 2010), and in MPTP mouse models by suppressing excitotoxicity (Aguirre et al., 2005; Battaglia et al., 2004). These studies highlight the importance of monitoring glutamate levels, and the potential utility of a biomarker for glutamate changes in PD pathology.
III. CEST MRI as a potential biomarker of neurodegeneration

Chemical exchange saturation transfer (CEST) MRI has been under development over the last 15 years and provides several key advantages over spectroscopy, PET, and conventional MRI techniques for monitoring specific biomolecular changes during disease. The following section reviews CEST MRI theory, its current applications, and its development for glutamate imaging. This thesis applies glutamate CEST imaging to mouse models of neurodegeneration to demonstrate its utility as a biomarker.

a. CEST MRI theory and applications

The technique of “chemical exchange saturation transfer” MRI exploits the phenomenon of proton exchange between substrate and water. Relevant substrates in the body with exchangeable protons include hydroxyl, amide, and amine side-groups. As protons exchange between substrate and bulk water, the magnetization state of protons is transferred to the solvent (Figure 5). There are several mechanisms by which magnetization is transferred: through chemical exchange and through dipolar cross-relaxation (Van Zijl and Yadav, 2011). The “CEST” experiment aims to measure the chemical exchange mediated magnetization transfer.

The CEST effect is probed using MRI by first applying a radio frequency pulse with a sufficiently narrow bandwidth at a known resonant frequency of the substrate protons (see RF in Figure 5). The pulse acts to selectively saturate the magnetization of the substrate protons. In practice, the duration of the saturation pulse is sufficient to allow for several cycles of exchange to occur during the saturation pulse. When saturated protons exchange with bulk water, their resonant frequency changes to that of their new chemical
environment while maintaining their saturation state. The bulk water signal is effectively decreased in proportion to the concentration of saturated, exchanged protons (depicted in the last bulk water signal in Figure 5).

Figure 5. The principle phenomenon of CEST imaging. The top schematic depicts the population of spins from each pool of protons and their magnetization signal (adapted from Sherry and Woods, 2008. MRI measures the net magnetization, which begins positive as protons are mostly aligned with the applied magnetic field. Shown beneath are the amine protons of glutamate in proximity to bulk water protons. When the frequency selective ($\Delta\omega$) RF saturation pulse begins, the amine protons of glutamate become saturated – in other words, their net magnetization becomes zero as spins are flipped into the transverse plane. As chemical exchange occurs during the duration of the RF pulse, amine protons (blue) exchange chemical environments with bulk water protons (red). Exchanged protons maintain their saturated magnetization state, which effectively reduces the signal from bulk water, and then relax or undergo additional exchange.
Other cross-relaxation and direct saturation effects confound the CEST effect and must be dealt with in the measurement of the CEST signal. One competing effect is magnetization transfer (MT) from protons on semi-solid macromolecules to bulk-water. The CEST effect from amine protons is distinguished from MT from protons on large, slow moving macromolecules in several aspects: chemical exchange occurs on a faster time scale, and the effect is narrow in spectral width (green signal vs. yellow signal in Figure 6). Another competing effect is dipolar cross-relaxation, producing nuclear Overhauser enhancement (NOE). Intermolecular NOE exists especially in tissue, as compared to liquid solutions; the mechanism describes the transfer of magnetization from aliphatic protons on the protein backbone to water protons. This effect has been measured in rat brain tissue around 3.5ppm downfield from water (black signal in Figure 6, Jin et al., 2013; Van Zijl et al., 2003). An additional confounding effect on the CEST signal is that of direct saturation (DS) of protons that resonate close to water. This effect is symmetric with respect to the bulk water resonance.

Therefore the so-called “asymmetry” analysis (Guivel-Scharen et al., 1998) has been employed to measure the CEST effect without contributions from MT and DS. This method relies on the fact that the DS component is symmetric about water, and the broad MT effect can be assumed as a symmetric linear baseline. In practice, a narrow and symmetric water line-width must be achieved through manual shimming (up to second-order shim
gradient directions) before a CEST pulse sequence is applied. CEST asymmetry is calculated as the difference between the water signal after RF saturation at the resonant frequency of exchanging protons (+\(\Delta \omega\), red signal in Figure 7), minus the water signal measured after saturating equidistant downfield from water (-\(\Delta \omega\), blue signal in Figure 7). A CEST asymmetry map is produced from the difference image, where images have been acquired after RF saturation at ±\(\Delta \omega\) (see blue and red outlined images in Figure 7). The difference is reported as a percentage of the negative offset image (Liu et al., 2010). Although NOE effects are asymmetric and exist in the -\(\Delta \omega\) signal, the cross-relaxation signal is slower to develop than chemical exchange so that this contribution is assumed to be minimal (Van Zijl et al., 2003).

\[
\frac{M_{\text{sat}}(-\Delta \omega) - M_{\text{sat}}(\Delta \omega)}{M_{\text{sat}}(-\Delta \omega)} = CEST_{\text{asym}}
\]

Figure 7. CEST asymmetry (%) calculation accounts for contributions to the CEST signal from MT and DS.
The first CEST experiment in the body was applied to diamide groups of urea and was carried out on the healthy human kidney (Dagher et al., 2000). It is remarkable that the CEST signal was obtainable at 1.5T in such a structure. Since this time, the non-invasive CEST imaging technique has been applied in vivo without the use of external contrast agents to an array of specific biological substrates.

CEST imaging of amide protons has been employed to measure pH changes in human tissue. The first application of CEST imaging to detect pH changes utilized chemical exchange of amide protons (APT CEST) at 3.5ppm in the ischemic rat brain (Zhou et al., 2003a, 2003b). The first detection of pH changes in human brain tumors followed in 2006 (Jones et al., 2006). APT CEST may also be sensitive to tumor grade (Wen et al., 2010; Zhou et al., 2008, 2003a).

CEST imaging of hydroxyl protons has been applied to glycogen in the mouse liver (van Zijl et al., 2007) to glucose as a natural contrast agent for tumors (Chan et al., 2012; Walker-Samuel et al., 2013), to glycosaminoglycans in cartilage tissue of the human knee (Cai et al., 2012; Ling et al., 2008; Singh et al., 2012), to myo-inositol in the healthy human brain (Haris et al., 2011) and in a mouse model of AD (Haris et al., 2013b).

CEST imaging of amine protons has been applied to creatine in skeletal muscle (Kogan et al., 2014) and heart tissue (Haris et al., 2014a). Similarly, based on the amine protons of glutamate, glutamate CEST (GluCEST) has been demonstrated (Cai et al., 2012). The major focus of this thesis is concerned with GluCEST applications. Therefore, the following section describes this method in detail.
The development of Glutamate CEST (GluCEST) MRI

In order to observe the CEST effect, the exchange rate (k) of the solute protons should be in the slow-intermediate exchange regime (\( k \leq \Delta \omega \)), where \( \Delta \omega \) is the chemical shift difference between the water resonance and the resonance of exchanging protons on the solute. The exchange rate for glutamate protons under physiologic conditions was estimated using simulations based on Bloch-McConnell equations (McConnell 1958). Amine protons on glutamate resonate 3ppm downfield from water (at 9.4T the glutamate \( \Delta \omega \approx 7200 \text{ rad s}^{-1} \)), with an exchange rate (k \( \sim 3000 \text{ s}^{-1} \)) in the slow to intermediate regime. In developing the GluCEST technique, it was shown that GluCEST asymmetry is directly proportional to glutamate concentration (\( R^2=0.97 \), Figure 8A-B), with a slope of 0.6% GluCEST per mM [Glu] (Cai et al., 2012).

The measurable CEST effect also depends on the amplitude and duration of the saturation pulse (Figure 8C). For human subjects, the B1 power is limited by RF-specific absorption rate (SAR). On the human scanner at 7T, GluCEST is achieved with a \( B_{1\text{rms}} = 3.6\mu\text{T} \) for 1 second duration (Cai et al., 2012). For animal experiments at 9.4T, the \( B_{1\text{rms}} = 6\mu\text{T} \) (250Hz) for 1 second duration is used (limited by the coil hardware).
The CEST effect is also sensitive to changes in pH (Englander and Kallenbach, 1984). The exchange rate catalyzed by hydroxide and hydronium ions depends on pH in a non-linear fashion (Figure 9A, Bai et al. 1993). Data is shown for glutamate dipeptides in 0.5 M KCl and 50mM citric acid or succinic acid buffers, measured at 5°C in D2O at 11.7T; faster exchange from glutamate at higher pH was measured by solvent saturation transfer in water (and 50mM phosphate buffer) and converted to pD scale. For glutamate in solution at low temperature, the exchange rate measured by 1D-NMR is slowest around pH 4, and increases as a parabola towards lower pH and up to physiologic pH. At physiologic temperature, the CEST asymmetry was measured for glutamate using an imaging sequence over a range of pH (Figure 9B-D). Experiments were performed at 37°C at 7T in 10mM glutamate in solutions with various pH using phosphate-buffered saline (PBS). CEST asymmetry peaks around pH 4-5, where the exchange rate more closely meets the slow-intermediate regime compared to pH 7. At physiologic pH from 6-
7.4, the GluCEST effect is linear with a change of -9.5% GluCEST per unit of pH. In Alzheimer’s disease a pH change of at most 0.25 is reported in the hippocampus (Mandal et al. 2012). This would estimate a GluCEST variation of 2.4% CEST asymmetry, which is roughly 10% noise in the typical measured signal in vivo for mice (25% CEST asymmetry).

Figure 9. CEST effect dependence and sensitivity to pH. A) Exchange rate dependence on pH. Reproduced from Bai et al., 1993. B) Z-spectra analysis displays the bulk water signals with and without saturation at various frequency offsets. The CEST effect of glutamate produces decreased water signal at +3 ppm upfield from water, compared to saturation downfield -3 ppm. C) CEST asymmetry curves. Dotted line reports simulated data. D) GluCEST asymmetry at various pH reveals a linear dependence in the physiologic range from 6.0-7.4. Reproduced from Cai et al., 2012.

Other metabolites with exchangeable protons are present in the brain. It is necessary, therefore, to define the contribution to GluCEST from these extraneous metabolites. This was achieved by applying the GluCEST protocol to phantoms of pure metabolites in phosphate-buffered saline (PBS) at physiologic concentrations, pH=7, and temperature=37°C (Figure 10). NAA has an amide proton and showed no contribution to GluCEST at 10mM concentration. Myo-inositol has 6 hydroxyl protons; however, their CEST effect occurs at 0.6 ppm from water, and no GluCEST signal was observed from 10mM concentration. Creatine has amine protons with a CEST effect at 1.8ppm, and
contributed <0.5% GluCEST asymmetry at 6mM concentration. Amine protons exist on aspartate, glutamine, and taurine (sampled at 2mM each); however, no contribution was measured from these metabolites. Gamma-aminobutyric acid (GABA) displayed 1% GluCEST asymmetry at 2mM concentration. This is compared to 6% GluCEST from a glutamate sample of 10mM. In total, ~25% of the GluCEST signal can be attributed to other metabolites. Greater specificity is achieved *in vivo* due to the higher concentration of glutamate compared to GABA and creatine in the brain.

![Figure 10. Contribution to GluCEST asymmetry from other metabolites.](image)

GluCEST asymmetry measures the total endogenous free Glu in the brain, with minor contributions from Cr and GABA. To test the specificity of GluCEST, experiments were performed in solutions immersed in PBS at 7T. Solutions include glutamate (10mM), myo-inositol (MI, 10mM), creatine (Cr, 6mM), glutamine (Gln, 2mM), GABA (2mM), NAA (10mM), aspartate (Asp, 2mM), and taurine (Tau, 2mM). Reproduced from Cai et al. 2012.

The novel GluCEST MRI technique is able to measure total glutamate levels *in vivo* in the healthy human brain (Cai et al., 2012). Since its development and in addition to the studies presented in this thesis, the technique has also shown increased glutamate levels after inducing stroke and thereby producing hypoxic conditions in a rat model (Cai et al., 2012), and in rat brain tumors after administration of glutamate (Cai et al., 2012; Haris et al., 2014b).
c. Thesis Design

This thesis sought to apply the GluCEST imaging technique to mouse models of neurodegeneration in order to determine the potential of GluCEST MRI as a biomarker of early stages of neurodegeneration. There are several advantages of using GluCEST MRI to measure glutamate levels. GluCEST imaging is completely non-invasive as it produces contrast based on endogenous free glutamate without the use of contrast agents. Also, the GluCEST effect has a much larger sensitivity (~100 fold) to glutamate levels than in vivo $^1$H MRS. In addition, exploiting the advantage of an MRI technique, GluCEST can map glutamate levels at a high spatial resolution (156 um$^2$ in-plane) compared to PET and $^1$H MRS with voxels on the order of several mm$^3$.

Chapter 2 of this thesis compares the application of GluCEST to $^1$H MRS in the APP-PS1 mouse model of AD. Chapters 3-5 present GluCEST, $^1$H MRS, and immunohistochemistry studies on mouse models of tauopathy. The final chapter details the application of GluCEST to a model with elevated glutamate levels, the MPTP based mouse model of PD.

Several questions were investigated in this thesis regarding GluCEST: 1) to what extent are glutamate levels measured by GluCEST MRI and $^1$H MRS correlated? 2) What is the earliest stage of tauopathy that can be detected using GluCEST MRI? 3) What is the distribution of glutamate changes throughout the brain of mice with neurodegeneration? 4) Are changes in GluCEST merely reflective of structural changes due to volume loss, or is GluCEST contrast associated with early molecular markers of disease?
Several studies were carried out in which GluCEST and $^1$H MRS were performed in the following mouse models of neurodegeneration: the APP-PS1 transgenic model of the amyloid-beta pathology of AD, the PS19 (P301S) transgenic model of tau pathology, and the MPTP neurotoxin model of PD. Additionally, IHC was performed to determine the pathologic events which accompany changes in glutamate levels. The primary antibodies which were investigated include: AT8 for phosphorylated tau protein, synaptophysin for synapse density, NeuN for neuron density, VGlut1 for vesicular glutamate transporter protein, NR1 for NMDA glutamate receptors, and GFAP for glial cell reactivity.

Results of this work demonstrate the utility of GluCEST imaging to study regional and temporal variations in glutamate in different pathologies associated with neurodegeneration.
CHAPTER 2: Imaging of glutamate changes in a mouse model of Alzheimer’s disease

I. Introduction

$^1$H MRS has been widely used in the study of biochemical changes in the AD brain during disease progression (see Chapter 1, section II.b “Current challenges and strategies for the diagnosis of AD and tauopathies” on page 7). Previous $^1$H MRS research has depicted decreased hippocampal Glu concentration in AD patients compared to healthy controls (Hattori et al., 2002; Rupsingh et al., 2011). However, the clinical utility of $^1$H MRS is limited by poor spatial resolution and a long acquisition time.

This chapter reports the first application of GluCEST MRI in a mouse model of neurodegeneration. Glutamate levels were mapped in the brain of a transgenic mouse model of AD as well as in age-matched wild-type (WT) mice. The correlation between Glu changes measured via GluCEST and $^1$H MRS was investigated. Finally, the potential for GluCEST to detect early AD pathology is discussed.

II. Methods

a. Mouse preparation and monitoring during MRI

Transgenic mice with mutated human amyloid precursor protein (APP) and presenilin-1 (PS1) display amyloid-beta pathology in the hippocampus and spatial memory deficits reminiscent of AD pathology (Puoliväli et al., 2002). Six APP-PS1 mice and six WT mice, aged 18–20 months, were used in this study (from Wyeth Research, Collegeville, Pennsylvania, USA). Mice were housed at the University Laboratory Animal Resources
(ULAR) animal husbandry facility at the University of Pennsylvania. Mice were kept in cages with littermates in a humidity controlled room ~22°C under a 12 hour light/dark cycle with *ad libitum* access to food and water.

All spectroscopy and imaging studies were performed on a 9.4T small-animal spectrometer using a 30 cm horizontal bore magnet fitted with a 12 cm gradient insert and interfaced to a Varian spectrometer (Agilent Technologies Inc., Santa Clara, CA, **Figure 11C**). Mice were anesthetized using isofluorane gas (1.5-2% isofluorane mixed with oxygen at 2 L/min), and secured to a body-bed (**Figure 11A**) inside a 20mm diameter volume coil (M2M Imaging Corp., Cleveland, OH, **Figure 11B**). Body temperature was monitored using an anal temperature probe and maintained at 37 °C using hot air blown inside the bore of the magnet (M2M Imaging Corp., S2PAM software). Breath rate was monitored using a pressure-pillow underneath the chest connected to a fiber-optic readout and maintained between 40-80bpm.
Figure 11. Mouse imaging setup at 9.4T. 
(A) Mouse loaded in a custom made holder constructed by Mr. Allen Bonner at the CMROI to control motion during the MRI scan. (B) Mouse volume coil for transmitting and receiving signal (M2M Imaging Corp., Cleveland, OH). (C) The 9.4T small-animal imaging spectrometer (Agilent Technologies Inc., Santa Clara, CA) located on the ground floor of John Morgan building in the Small Animal Imaging Facility (SAIF). The mouse holder is inserted into the coil, which is inserted into the magnet bore. The MRI scan can last up to 3 hours for a single mouse, during which time they are kept warm using forced hot air and monitored using an anal temperature probe and a respiration pillow.

b. $^1$H MRS method and protocol

Protons exist in biological tissue primarily as a component of water (110M concentration of water protons in the body multiplied by the hydration fraction of tissue, typically 80-90%) but also on small molecules at lower concentrations than water (<1-20mM). Small molecule protons are MR-sensitive with a resonant frequency shifted from water. Magnetic resonance spectroscopy seeks to measure the signal from these small molecules. In order to detect signals from protons bound to small metabolites, the intense water signal will be suppressed by applying a frequency selective saturation pulse (or train
of pulses). Protons from the following metabolites can be detected by spectroscopy at the approximate indicated chemical shifts in the mouse brain at 9.4T (Pfeuffer et al., 1999): lactate (Lac, 1.3ppm), N-acetylaspartate (NAA, 2.0ppm), gamma-aminobutyric acid (GABA) and glutamine (Glx, 2.2ppm), glutamate (Glu, 2.35ppm), glutamine (Gln, 2.45ppm), aspartate (Asp, 2.7ppm), creatine (Cr, 3.0ppm), choline (Cho, 3.2ppm), taurine (TAU, 3.4ppm), and myo-inositol (MI, 3.5-3.6ppm).

Single-voxel spectroscopy is a technique that detects the metabolite signal from a localized region of interest (ROI). These sequences employ gradients applied along 3 perpendicular directions in order to isolate signal from a chosen voxel. There are several sequences developed which can perform single-voxel spectroscopy.

This study used the stimulated echo acquisition mode (STEAM) sequence (supplied by vendor Varian) with the following parameters: voxel size: 3.5mm x 3.5mm x 2mm (voxel volume = 24.5 mL); spectral width: 4 kHz; number of points: 4006; averages: 264; TE = 8 ms; TM = 7 ms; TR = 5 s. Water suppression was achieved using the variable pulse power and optimized relaxation (VAPOR) delay method. Localized shimming was performed to obtain water line widths of 20Hz or less. The unsuppressed water spectrum was also acquired using the same parameters for the purpose of normalization to the water volume in a given voxel. All spectra are displayed normalized to water.

In order to quantify the metabolite concentrations, a spectral range of 0-4.2 ppm was used. Metabolite concentrations, including glutamate, were measured using LCModel software (Provencher, 2001). Concentrations are reported relative to the total creatine (tCr) concentration, which did not vary between genotypes. LC model quantifies data through the recursive fitting of prior knowledge basis sets. During each iteration, a least-
squares algorithm was used to optimize the fit. The quality of the final fit was determined in terms of the Cramer–Rao lower bound (CRLB), a measure of the variance in the error.

c. GluCEST MRI protocol in mice

There are several important factors to consider when designing the GluCEST pulse sequence for small animal imaging. Amine protons on glutamate resonate at 3ppm offset downfield from water. A saturation pulse was implemented with 1 second duration (four Hanning pulses at 250ms each with a 4μs inter-pulse-delay) with an amplitude $B_{1\text{rms}}$ of 5.9 uT, that had been optimized in order to adequately saturate the amine protons on glutamate. A spoiled gradient echo (GRE) readout sequence was used. The time between each saturation pulse was set to 8 s to allow for ample $T_1$ recovery. The final image parameters used in this study are: field of view: 20 x 20 mm$^2$; slice thickness: 2 mm; flip angle: 15°; TR = 6.2 ms; TE = 2.9 ms; matrix size: 128x128.

According to the motivation presented in Chapter 1, section III.a “CEST MRI theory and applications” on page 15, the asymmetry analysis was used to measure the CEST effect. GluCEST asymmetry was calculated as:

$$\text{GluCEST}_{\text{asym}(\Delta\omega=3\text{ppm})} = \frac{M_{\text{sat}(\Delta\omega)} - M_{\text{sat}(-\Delta\omega)}}{M_{\text{sat}(\Delta\omega)}}$$

eqn. (1)

where $M_{\text{sat}} (\pm\Delta\omega)$ are the magnetizations obtained with saturation at a ‘+’ and ‘−’ offset to the water resonance; $\Delta\omega$ is equivalent to the resonance offset of the exchanging spins.

In interpreting the CEST effect, other factors that play a role are $B_0$ inhomogeneities and the suboptimal amplitude and duration of the $B_1$ saturation pulse (Cai et al., 2012). Any local $B_0$ inhomogeneity will cause the saturation pulse to miss the targeted glutamate protons. To account for this, a $B_0$ map is acquired based on differences in phase...
accumulation in GRE images acquired at successive TE = 3.5, 4.0, 4.5 ms (2 signal averages each, Figure 12A). Additionally, several images are acquired with the saturation pulse applied over a range of offset frequencies (Δω = ± 2.4-3.6ppm, steps of 0.2ppm) that spans the variation in B₀ (±0.6ppm) expected from the B₀ map. B₀ corrected CEST weighted images at ± 3 ppm are calculated from the local B₀ value and the acquired CEST weighted images from different offset frequencies using polynomial interpolation.

In addition, B₁ maps were acquired in order to correct for inefficiencies in the saturation pulse. Specifically, B₁ maps were calculated from two images acquired with a rectangular preparation pulse with varying flip angles: 30° and 60° (2 signal averages each, Figure 12B). A linear correction for B₁ was applied to the CEST map using a ratio of the actual B₁ value to the expected value (Singh et al., 2013).
Figure 12. $B_0$ and $B_1$ inhomogeneity maps are acquired to correct GluCEST maps. (A) When the resonant frequency of $B_0$ is intentionally offset ±0.4ppm, $B_0$ maps reveal the proportional inhomogeneity. (B) $B_1$ maps are relatively homogeneous, indicating efficient saturation. (C) The final GluCEST maps are corrected for $B_0$ and $B_1$ inhomogeneities.

For this study, GluCEST, $B_0$ and $B_1$ maps were acquired from two successive brain slices including the striatum (corresponding to the location of the voxel acquired using $^1$H MRS) and the hippocampus. The total imaging acquisition time was around 30 minutes.

For MTR mapping, the same brain slices were imaged after saturation at 20 ppm with a saturation power of 250 Hz and a saturation duration of 1 s. An image with saturation at 100 ppm was also collected and considered as the magnetization off image ($M_0$).
d. Image processing

All image processing and data analysis were performed using software routines written in MATLAB (version 7.5, R2007b, MathWorks, Massachusetts, USA). Acquired images were corrected for $B_0$ and used to generate a GluCEST contrast map using equation eqn. (1) eqn. (1). GluCEST contrast was further corrected for $B_1$ and mapped as false colors (Figure 12C). Z-spectra were obtained from the CEST images by plotting the normal image intensity as a function of the resonance offset of the saturation pulse. CEST asymmetry curves were generated by plotting the relative water signal difference at frequency offsets from 0 to 4.8 ppm.

Similarly, MTR maps were computed by equation eqn. (2):

$$\textit{MTR} = \frac{M_0 - M_{\text{sat}}}{M_0} \times 100\%$$

eqn. (2)

where $M_0$ is the equilibrium magnetization image and $M_{\text{sat}}$ is the magnetization with a saturation pulse applied at 20 ppm.
III. Results

a. GluCEST mapping and $^1$H MRS of wild-type and transgenic mice

AD mice with amyloid-beta pathology display anatomical differences in brain structure compared to WT mice. Dilated ventricles are clearly observable in the proton images displayed in Figure 13A, D. The GluCEST maps illustrate the regional distribution of Glu in the brain of both WT and AD mice (Figure 13B, E). Higher GluCEST contrast was observed in gray matter relative to white matter, which reflects the difference in Glu concentration. The mean GluCEST contrast from the representative WT and APP-PS1 brains over the chosen voxel was 26.41.6% and 19.11.9%, respectively.

To further confirm that the change in GluCEST contrast is a result of a change in Glu concentration, $^1$H MRS was performed for the voxel shown on the anatomical images (Figure 13A, D). This voxel was placed in an area devoid of cerebrospinal fluid. $^1$H MRS spectra showed a decrease in the Glu peak amplitude of APP-PS1 relative to WT mice (arrows, Figure 13C, F). Decreased N-acetyl-aspartate (NAA) peak amplitude and increased myo-inositol (mIns) peak amplitude can also be seen from the $^1$H MRS spectra, which is characteristic of AD pathology.
Figure 13. GluCEST mapping and $^{1}$H MRS of a representative WT and APP-PS1 mouse.
(A, D) The anatomical brain image from a WT and APP-PS1 mouse. (B, E) The corresponding GluCEST maps. The WT GluCEST map shows the regional distribution of Glu in the brain. Higher GluCEST contrast is observed in gray matter relative to white matter. Lower GluCEST contrast is observed throughout the APP-PS1 map. (C, F) The $^{1}$H MRS spectra from the voxel placed in (A) shows the major brain metabolites. The Glu peaks are labeled at 2.3 and 3.75 ppm, where arrows highlight the trend of decreased Glu observed in the spectra of the APP-PS1 mouse compared to WT. tCr, total creatine; Lip, lipid. Figure adapted from (Haris et al., 2013a).

b. Z-spectra and asymmetric magnetization transfer ratio curves

The z-spectra and MTR$_{\text{asymmetry}}$ curves obtained from WT and APP-PS1 mice are shown in Figure 14A, B. The 3 ppm line on the MTR$_{\text{asymmetry}}$ curves corresponds to the location of the GluCEST effect. MTR$_{\text{asymmetry}}$ is observed over a broad spectrum with a maximum CEST contrast at ~2 ppm (Figure 14B). This may be a result of the broad asymmetry of Glu due to its faster exchange rate. Another contribution to the broad
MTR\textit{asymmetry} is the chemical shift averaging effect, which shifts the line to a higher field towards the water resonance. No significant change in MTR contrast was observed in AD (48.3±1.5) relative to WT (48.0±1.1) mice.

Figure 14. Z-spectra and MTR curves from WT and APP-PS1 mice. (A) Z-spectra analysis depicts the normalized magnetization after saturation over a range of frequency offsets from water (Δω = ±5ppm). (B) Asymmetric magnetization transfer ratio (MTR\textit{asymmetry}) curves from WT and APP-PS1 mice for the voxels shown in Figure 13A, D. The dotted line at 3 ppm in the MTR\textit{asymmetry} curves reflects the GluCEST contrast.

c. Reproducibility of GluCEST maps in vivo

In order to evaluate the reproducibility of GluCEST contrast, the GluCEST protocol was performed successively during the same scan session. The GluCEST images were highly reproducible (Figure 15) with the intracovariance being less than 3%.

Figure 15. Reproducibility of GluCEST mapping in a WT mouse.
d. Average GluCEST contrast in the hippocampus

The hippocampus is the primary structure associated with the early loss of pyramidal neurons and their synapses in AD pathology, which control learning and cognitive function (Mu and Gage, 2011). GluCEST maps of brain slices containing the hippocampus were also obtained (Figure 16B, D). There was a significant reduction (31%) in GluCEST in the hippocampus of APP-PS1 mice compared to WT (p<0.01, Figure 16E).

Figure 16. GluCEST maps in the hippocampus region of WT and APP-PS1 mice. (A) Anatomical brain image from a WT mouse. (B) The corresponding GluCEST map. (C, D) Anatomical image and corresponding GluCEST map from an age-matched AD mouse. Significantly decreased GluCEST contrast in the hippocampus was observed in APP-PS1 relative to WT mice. The hippocampus regions are shown in the rectangular boxes in both WT and AD mice (arrows). (E) Bar graph showing the mean hippocampus GluCEST contrast in WT and AD mice.
e. **Correlation of average GluCEST contrast and glutamate concentration**

Comparative analysis showed a decrease of ~28% in GluCEST contrast (p<0.01, Figure 17A) and a decrease of ~29% in Glu/tCr ratio (p<0.01, Figure 17B) in APP-PS1 relative to WT mouse brain over the chosen voxel displayed in Figure 13. The mean Glu/tCr ratios and standard deviations in WT and APP-PS1 mice were 1.58 ± 0.13 and 1.12 ± 0.08, respectively. An excellent positive correlation (R² = 0.91) was observed with a slope of ~15% GluCEST per Glu/tCr ratio.

![Figure 17. Correlation between GluCEST and ^1^H MRS data in WT and APP-PS1 mice.](image)

(A, B) Bar graphs showing the mean GluCEST contrast (%) and spectroscopy-derived glutamate/total creatine (Glu/tCr) ratio from WT and AD mice for the voxels shown in Figure 13. (C) Graph showing the strong positive correlation between GluCEST and Glu/tCr.
IV. Discussion

a. The neurochemical profile and GluCEST in the hippocampus mimics human AD pathology

In the current study, we have shown that high-resolution imaging of Glu alterations in a transgenic mouse model of AD is possible using the GluCEST MRI technique. Significantly decreased GluCEST contrast in APP-PS1 relative to WT mice was observed in the hippocampus and striatum, and the results were further validated through demonstration of comparable changes of Glu/tCr on $^1$H MRS in the striatum.

The hippocampus is the primary region affected in early AD pathology and is associated with learning and memory. A previous $^1$H MRS study reported a progressive decrease in hippocampal Glu/tCr in APP-PS1 mice with advancing age (Marjanska et al., 2005). However, $^1$H MRS can only measure Glu from a single voxel ($8\text{cm}^3$), and thus a heterogeneous distribution of Glu cannot be easily monitored. In the current study, a more profound decrease in GluCEST contrast was observed in the hippocampus relative to other regions clearly suggesting its involvement in the early disease process. In the current study, a decrease in GluCEST contrast of 31% in the hippocampus of APP-PS1 relative to WT mice was measured. This finding is consistent with human AD, for which a decrease in hippocampal Glu of ~35% was observed by $^1$H MRS (Rupsingh et al., 2011). The same study also showed a 15–20% change in the Glu concentration in MCI relative to controls.

As a result of the high reproducibility of GluCEST mapping, it may be feasible to image the change in Glu concentration at high spatial resolution from control to MCI to the full onset of AD. GluCEST MRI, therefore, has the potential to provide a diagnostic marker at the early stage of AD.
b. *Glutamate levels measured by GluCEST correlate with 
1H MRS*

A strong positive correlation between GluCEST and spectroscopy-derived Glu levels was observed (R²=0.91). This suggests that the observed change in GluCEST contrast is caused by a change in Glu concentration. A contributing factor to GluCEST that does not affect 
1H MRS is the magnetization transfer effect from the bound pool of water. A decrease in the magnetization transfer effect would overestimate the GluCEST contrast from AD pathology. However, in the current study, no apparent change in the MTR contrast was observed.

c. *Technical considerations for applying GluCEST to mice in vivo*

The precise saturation of exchangeable protons requires perfect B₀ homogeneity. B₀ inhomogeneity could interfere with the selective saturation frequency and make the two selective frequencies asymmetric relative to the bulk water signal. In this case, the CEST effect would reflect the difference between DS and MT effects. The CEST effect is also highly dependent on the saturation B₁ amplitude (Ward et al., 2000). With fixed saturation duration, a higher B₁ can induce a higher CEST effect (Winter et al., 2006). B₁ homogeneity is a critical requirement for quantitative CEST imaging. Artifacts induced by B₀ and B₁ field inhomogeneity and the corresponding correction algorithms have been discussed previously in detail (Sun et al., 2007). In the current study, we used the methods described previously to correct the B₁- and B₀-induced artifacts caused by small B₀ and B₁ variations.
V. Summary

Using the GluCEST MRI technique, decreased Glu levels in the APP-PS1 transgenic mouse model of AD were imaged at high spatial resolution. Compared with WT controls, AD mice exhibited a notable reduction in GluCEST contrast (~30%) in all areas of the brain. The change in Glu was further validated through $^1$H MRS. A positive correlation was observed between GluCEST contrast and Glu/Cr ratio measured by $^1$H MRS. These results demonstrate the potential of GluCEST as a novel noninvasive biomarker for the diagnosis of early AD, and enable the development of disease-modifying therapies for AD.

- GluCEST contrast is reduced by ~30% in the hippocampus and striatum of the APP-PS1 mouse model of AD.
- GluCEST contrast correlates with the glutamate signal measured by $^1$H MRS in the striatum ($R^2=0.91$) with a slope of 15% GluCEST asymmetry per ratio of Glu/total creatine.
CHAPTER 3: *In vivo* measurement of glutamate loss is associated with synapse loss in a mouse model of tauopathy

I. Introduction

The previous proof-of-principle study in mice with amyloid-beta pathology confirmed that GluCEST MRI is decreased in the hippocampus with AD-like pathology which reflects reduced glutamate concentration. However, many neurodegenerative disorders exist in which tau pathology is the most dominant feature including FTDP-17, CBD, PSP, and PiD (Lee et al., 2001; Thibodeau et al., 2009). This motivates the study of GluCEST as a potential marker of glutamate alterations in the presence of isolated tau pathology.

The transgenic PS19 mouse with mutated human tau at P301S was developed by the Center for Neurodegenerative Disease Research (CNDR) at the University of Pennsylvania (Yasumasa Yoshiyama et al., 2007) and develops several histological symptoms of tau pathology (*Figure 18*). In addition to NFT pathology, which mimics human tauopathy, early symptoms of synapse loss are apparent in the hippocampus of PS19 mice. Synapse loss is the closest histologic correlate to cognitive deficits (Terry et al., 1991) and to the severity of cognitive deficits in AD patients (DeKosky and Scheff, 1990). Therefore, an *in vivo* surrogate marker of synapse loss would be highly valuable as a potentially non-invasive correlate to dementia. As glutamate is the primary excitatory neurotransmitter in the CNS and resides in high concentrations in the healthy synapse, the following study hypothesized that GluCEST imaging would correlate with synapse integrity in the PS19 mouse brain.
Figure 18. Progressive pathologic symptoms of tau pathology in the PS19 transgenic mouse. Histological sections from various brain regions are shown for PS19 (top row) and WT mice (bottom row) at several ages. IHC methods displayed utilized antibodies to the following proteins: synaptophysin for synapse density, glial fibrillary acidic protein (GFAP) for glial cell reactivity, paired helical filament (PHF) tau for h-p-tau, hematoxylin and eosin (HE) for neuronal morphology, and thioflavin S (thio s) and congo red for the beta-sheet structure of NFTs. Reproduced from Yoshiyama et al. 2007.

In this study, GluCEST imaging as well as $^1$H MRS are employed to study the consequence of tau pathology on glutamate in a P301S mouse model with tau pathology. In vivo measures of glutamate are associated regionally with histological measurements of tau burden including the severity of pathological tau, neuron loss, and synapse loss. Given the translational opportunities of GluCEST, these findings in a pre-clinical model of tauopathy have immediate potential application to clinical studies.
II. Methods

a. *PS19 transgenic mouse model of tauopathy*

The mouse model of tauopathy studied here was the PS19 line of the P301S transgenic mouse overexpressing the human P301S mutant tau found in FTDP-17 patients (Yoshiyama et al., 2007). The first sign of tau pathology in this mouse model is defective axonal transport followed by synapse loss and hyper-phosphorylated tau accumulation at presynaptic terminals. As the animals age, pathological tau progresses along the perforant pathway, from the entorhinal cortex into the hippocampus and pre-frontal cortex, while severe neuron loss is apparent at later stages of disease (Hurtado et al., 2010). Behavioral studies show decreased ability in spatial learning with the progression of disease (Brunden et al., 2010). Note that the onset of pathology in this generation of mice is later than originally published (see current generation in Zhang et al., 2012).

This study was approved by the university’s IACUC. Mouse husbandry and handling during imaging was identical to that stated in Chapter 2, section II.a “Mouse preparation and monitoring during MRI” on page 26.

b. $^1$H MRS acquisition in the hippocampus and custom processing

$^1$H MRS was performed on WT (n=8) and PS19 (n=7) mice. The point-resolved spectroscopy (PRESS, Bottomley, 1987) pulse sequence was used with the following parameters: TR/TE = 3000/14 ms, spectral width = 4 kHz, number of points = 4006. The VAPOR water suppression technique was used to acquire a water-suppressed spectrum (averages = 384). An additional spectrum was acquired without water suppression to
obtain the water reference signal for normalization (averages = 16). Unsuppressed water spectra had line widths of 20Hz or less after localized shimming. Total acquisition time for spectroscopy was about 20 minutes. Spectra were acquired from a voxel localized in the hippocampus (2x2.5x3mm³).

Spectra were processed using in-house software (developed in MATLAB v.7.9.0 R2009b). First, manual phasing was performed using the creatine peak (3.0ppm) as a constant phase, and adjusting a variable phase component. Baseline correction was performed to fit the global baseline and local baseline features while maintaining broad macromolecule curves from 0.5-3ppm and from 3-4.2ppm. Metabolite peak locations and widths were identified manually (centered at approximately 1.3 ppm for Lac, 2.0 NAA, 2.2 Glx, 2.35 Glu, 2.45 Gln, 2.7 Asp, 3.0 tCr, 3.2 Cho, 3.4 TAU, 3.5 MI, 3.6 MI-2, 3.75 Glu-2, 3.9 Cr-2, and 4.1 MI-3). A predetermined set of macromolecule peaks were also included (0.9, 1.2, 1.4, 1.7, 1.9, 2.9, 3.9, and 4.3 ppm). The peak-fitting routine (MATLAB “lsqcurvefit”) performs a nonlinear least squares fitting of Lorentzian peaks to the spectra corrected for phase and baseline. The ratio of the integrals of metabolites to the internal standard of total creatine (tCr) is reported.

c. GluCEST imaging and ROI segmentation

GluCEST imaging was performed on aged PS19 mice (n=9, mean age=20.7 months) and their age-matched WT littermates (n=8, mean age=19.0 months). A custom-programmed RF spoiled GRE readout with a frequency selective saturation preparation pulse sequence was used as described previously in Chapter 2, section II.c “GluCEST MRI protocol in mice” on page 30. GluCEST maps were acquired from one slice through
the mid-hippocampus with four signal averages for each saturation frequency. The total scan time for GluCEST, $B_0$, and $B_1$ maps was under 12 minutes.

Regions of interest (ROIs) were segmented by hand from T2-weighted images including the hippocampus, thalamus, and cortex. Sub-regions of the hippocampus cannot be distinguished using the \textit{in vivo} image. Instead, sub-regions were segmented based on a threshold applied to the GluCEST maps: above 30% GluCEST asymmetry was considered the DG region, below 5% as cerebral spinal fluid (CSF) and surrounding voxels with partial volume effects, and the mid-range values as the CA. For comparison, a high-resolution \textit{ex vivo} image of the mouse brain shows that this threshold segments the DG from the CA region adequately (\textbf{Figure 23a}). The high-resolution image was acquired based on the gadolinium enhanced protocol developed by Johnson et al., 2007. The thalamus was segmented excluding the cerebral peduncle, and the cortex was segmented excluding the superior sagittal sinus.

d. General tissue preparation and methods of immunohistochemistry

After imaging, mice were sacrificed using standard methods of transcardial perfusion/fixation as approved by the IACUC of the University of Pennsylvania. The transcardial perfusion procedure was as follows:

i. The mouse was deeply anesthetized to a surgical plane of anesthesia (non-responsive to toe pinch or ear flick). The mouse was anesthetized with an intraperitoneal injection of ketamine/xylazine/acepromazine (100:10:1 mg/kg) according to its weight.

ii. The mouse was restrained in a supine position on dissection block using tape to secure fore and hind limbs.

iii. The thorax was opened to visualize the heart.
iv. A small hole was opened in the right atrium as an exit port for the blood.

v. A small hole was opened in the left ventricle. A blunt needle, connected to a perfusion pump, was inserted into the hole.

vi. Isotonic saline is pumped through the mouse to rinse out the blood. This is necessary to help reduce the background in these tissues. Subsequently, the mouse was perfused with 20mL of fixative solution of 4% paraformaldehyde (PFA) in PBS.

vii. The brain was removed from the skull and stored overnight at 4°C in 4% PFA.

The brain tissue was then prepared for histology. Each brain was bisected into two hemispheres (for some mice both hemispheres were kept). One hemisphere was kept and placed in a steel mold, and sliced into 2mm thick sections using razors (Figure 19). The anatomical sections are described according to the following diagram:

Figure 19. Method of gross-slicing the brain. The dissected brain is placed in a steel mold with grooves for razor blades. Seven slices are made from rostral to caudal, producing 8 slices of tissue that are 2mm thick each. The red lines indicate the edge of the brain slice that is placed on the bottom of the embedding tray. The view from ventral to dorsal (V-D) shows the shape of each slice lying flat in the embedding tray.
The 8 slices were placed in a cassette and stored in a leaching buffer to remove excess fixative for several hours. Cassettes were processed in a paraffin-infusing machine (Shandon Excelsior by Thermo scientific) by the CNDR histology staff. Paraffin-infused slices were placed in an embedding tray and sectioned at 6µm thick using a microtome and hot-water bath (Figure 20). This procedure yields ~250 slices per mouse brain, although typically only the first 150 slides were kept in order to obtain the anatomical sections corresponding to the imaged sections by MRI.

Figure 20. Methods of embedding and sectioning tissue in preparation for IHC. A. The brain slices in the embedding tray are covered in paraffin and set to harden into a block. B. The paraffin block is sectioned using a microtome at 6µm thick. C. Each tissue section is placed on a slide for use with IHC protocols.

Several protocols for IHC were performed using the following general procedure:

i. De-paraffinize slides in xylene and dehydrate in a series of ethanol concentrations.

ii. Block endogenous peroxidase activity by soaking slides in H₂O₂ and methanol.

iii. Apply an optional pre-treatment of boiling in citric acid (Vector Antigen Unmasking Solution, Vector Laboratories) to create more permeable cell membranes.

iv. Apply primary antibody in a buffered TRIS (2-amino-2-hydroxymethyl-propane-1,3-diol) fetal bovine serum (TRIS/FBS) and incubate overnight. This antibody provides specificity for the protein of interest in the tissue.
vi. Apply the secondary antibody, biotinylated anti-immunoglobulinG, which recognizes the primary antibody and is specific for the species in which the primary antibody was raised.

vii. Apply horseradish peroxidase-streptavidin developing system (†BioGenex, Hyderabad, AP India) or (‡Vectastain ABC Kit, Vector Laboratories).

viii. DAB (3,3' diaminobenzidine) is a peroxidase substrate and was used as the chromagen (Vector DAB, Vector Laboratories).

ix. Apply an optional counterstain of hematoxylin to visualize the nuclei of cells.

x. Rehydrate and coverslip slides in the reverse series of ethanol concentrations.

e. **Antibodies applied using IHC on aged PS19 mice**

Three different primary monoclonal antibodies (MAbs) were used in this study. The first MAb is AT8, an antibody specific for phosphorylated tau at residues Ser202 and Thr205 (Goedert et al., 1995) (1:7500 dilution in PBS, Thermo Scientific). This MAb is used to localize pathological tau protein, which is hyper-phosphorylated. Adjacent tissue sections were immunostained with NeuN antibody for neuronal nuclei (1:200 dilution, Millipore), and MAb SY38 for synaptophysin localized to the pre-synaptic terminals (1:2000 dilution, Millipore). Quantification of synaptophysin (WT n=4, PS19 n=7) and NeuN (WT n=5, PS19 n=6) immunostaining in the CA3 was performed on 20x images; synaptophysin in the DG on 4x images, in the thalamus on 20x images, and in the entorhinal cortex on 20x images, after setting an intensity threshold for all images. Percentages reported reflect the area occupied by immunostaining, normalized to the area of the ROI (Image J).
f. Statistical analyses

Statistical analyses were performed using MATLAB (7.9.0 R2009b). Boxplots were generated in MATLAB and represent the median in red, upper and lower quartiles within the box, and the extremes at the whiskers. Outliers were considered those data points beyond one times the interquartile range. Mean and standard deviations of all measures are reported in mean ± std format throughout. Student’s t-test was performed to determine significance of differences between measures obtained in PS19 brains to that from WT animals.

III. Results

a. ¹H MRS from WT and PS19 mouse hippocampus

Representative metabolite spectra from the hippocampus of a WT and PS19 mouse are shown in Figure 21A. Differences in metabolite profiles were evident between WT and PS19 cohorts. The greatest difference was measured in glutamate at 2.35ppm, where the average ratio of glutamate to total creatine [Glu]/[tCr] in PS19 mice was 1.0 ± 0.10 (mean ± std) compared to 1.3 ± 0.13 in the WT cohort (p=0.11). The average concentration of tCr was not different between PS19 and WT mice. The metabolite NAA as a ratio to tCr was also decreased in the PS19 mice (1.1 ± 0.10 vs. 1.2 ± 0.19, p=0.34).
Figure 21. PS19 mice have decreased glutamate in the hippocampus compared to WT according to $^1$H MRS. (A) Example spectra from a PS19 mouse compared to WT shows diminished peaks of N-acetyl-aspartate (NAA, 2.01ppm) and glutamate (Glu, 2.35ppm). (B) The average ratio of glutamate to total creatine $[\text{Glu}] / [\text{tCr}]$ was decreased in the majority of PS19 mice compared to the WT cohort (n=7, 1.0 ± 0.10, mean ± std/2, vs. 1.3 ± 0.13, p=0.11). There was also a slightly lower group average of NAA in PS19 mice (1.1 ± 0.10 vs. 1.2 ± 0.19, p=0.34).

b. GluCEST maps of the whole brain and regions of interest

GluCEST asymmetry in WT mice was higher in grey matter as compared to white matter with very low glutamate levels in the CSF (Figure 22ii). The GluCEST maps from PS19 mice showed distinctively decreased glutamate across the majority of the brain slice (Figure 22bii, Figure 22cii).
a-c i) Anatomical images corresponding to the GluCEST slice through the mid-hippocampus.

a-c ii) GluCEST maps in a WT and two PS19 mice reveal lower glutamate levels throughout the brain of tauopathy mice.

a-c iii) Corresponding slices of brain tissue stained for hyper-phosphorylated tau (AT8) reveal varying severity of pathological tau protein within the PS19 cohort.

a-c iv. & v.) Neuron (NeuN) and synapse (Syn) immunostaining in the CA3 pyramidal cell layer of the hippocampus. While neuron loss in this region is inconsistent, synapse loss is apparent in PS19 mice, for which immunostaining is fainter and reveals a thinning band of synapses.

d) Detailed hippocampal anatomy for reference. CA1-3: *cornu ammonis*, P: pyramidal cell layer of CA, DG: dentate gyrus, G: granule cell layer of DG.

e) Quantified NeuN and Syn staining in the CA3 of the hippocampus. Synapse density is significantly reduced in PS19 mice (24.6 ± 2.1 vs. 33.6 ± 2.6, p≤0.01). Neuron density is less-severely reduced in PS19 mice (55.4 ± 4.4 vs. 61.2 ± 5.7, p=0.18).
Within the hippocampus, two sub-regions of GluCEST contrast are distinguishable (Figure 23b-c). The first region includes the majority of the hippocampus, which has lower GluCEST signal in PS19 mice (15.9% ± 0.76, mean GluCEST asymmetry% ± std) compared to 22.4% ± 1.1 for WT, p≤0.001). This region corresponds to the anatomical sub-region of the hippocampus called the CA and the surrounding axonal layers. The second region corresponds to the anatomical region of the DG, Figure 23c) and has consistently high GluCEST signal in both WT (34.1% ± 0.84) and PS19 (33.0% ± 0.55, p=0.19). Significantly reduced GluCEST levels were also measured in the thalamus region of PS19 mice (23.5% ± 0.89 vs. 27.7% ± 1.4, p≤0.05, Figure 25c). Average GluCEST asymmetry was not statistically different in the cortex of PS19 mice compared to WT (22.8% ± 1.2 vs. 23.3% ± 1.1, p=0.72, Figure 26c).
Figure 23. Average GluCEST asymmetry in sub-regions of the hippocampus.

a) For anatomical comparison, a high-resolution (42 µm isotropic) image of the ex vivo brain was acquired after infusion with gadolinium (Gd) according to the protocol by Johnson et al., 2007 for MR histology.

b-c) The hippocampus is a composite of two distinct sub-regions, the CA and the DG. The DG was segmented based on a GluCEST threshold above 30%, which corresponds to the correct anatomical location as compared to the Gd-enhanced image.

d) In the CA sub-region of the PS19 hippocampus, GluCEST is significantly decreased by 29% (p≤0.001). There is no significant difference between groups in the DG sub-region of the hippocampus (p=0.19).

c. Histological measures of tau burden

We sought a neuropathological correlation to glutamate changes in the tauopathy mouse brain. Brain tissue was processed by IHC for hyper-phosphorylated tau (AT8,
Figure 22iii), for neuron density (NeuN, Figure 22iv), and for synapse density (Syn38, Figure 22v). The severity of tau pathology varied among PS19 mice. An example of a PS19 mouse with mild pathology is shown in Figure 22biii. The majority of PS19 mice demonstrated tau immunostaining in the entire hippocampus and in several layers of entorhinal cortex as in Figure 22ciii.

Neuron density in the CA3 region of the PS19 hippocampus appears to be decreased in some PS19 mice (Figure 22civ) but not in others (Figure 22biv.) compared to the consistently dense band of neurons that are seen in WT mice (Figure 22aiv). Quantified neuron density reflects this inconsistency, whereas no significant difference was measured between WT and PS19 NeuN immunostaining (Figure 22e).

Synapse density is diminished in the CA3 of PS19 mice as evidenced by fainter staining and a thinning band of the pyramidal cell layer compared to WT mice (Figure 22bcv vs. Figure 22av). Quantification reveals significantly lower synapse density in the CA3 of PS19 mice compared to WT (24.6 ± 2.1 vs. 33.6 ± 2.6, p≤0.01, Figure 22e). In the DG, there is no significant difference in synapse immunostaining in PS19 mice relative to WT (74.2 ± 7.5 vs. 79.6 ± 1.2, p=0.25, Figure 24c). The thalamus region has a lower density of punctuate immunostaining under high magnification as depicted in the black and white (BW) insets (Figure 25a-b). Quantification of the intensity in this region shows an 8% lower area occupied in the PS19 thalamus (39.9 ± 2.2 vs. 43.3 ± 1.5, p=0.10, Figure 25d). There was no difference in synapse density in the entorhinal cortex of PS19 mice (49.9 ± 1.5 vs. 50.5 ± 2.2, p=0.37, Figure 26d).
Figure 24. Comparison of GluCEST with synapse immunostaining in sub-regions of the hippocampus.

a-b) Synapse immunostaining and corresponding GluCEST images of the hippocampus region.

c) Quantification of IHC and GluCEST in the DG and CA show similar trends. Synapses are maintained in the PS19 DG, where GluCEST was also high. Both synapse density and GluCEST asymmetry are significantly reduced by 27 and 29%, respectively, in PS19 mice relative to WT.
Figure 25. Comparison of GluCEST with synapse immunostaining in the thalamus.

a-b) Synapse immunostaining and corresponding GluCEST ROIs in the thalamus. The black and white inset shows less punctate staining in the PS19 thalamus.

c) Average GluCEST asymmetry of the thalamus (Thal) region is significantly decreased by 15% \( (p \leq 0.05) \) in PS19 mice compared to WT.

d) Synapse density shows a similar trend, decreasing 8%, although significance was not reached.
Figure 26. Comparison of GluCEST with synapse immunostaining in the cortex.

a-b) GluCEST ROIs in the cortex and corresponding immunostaining in the entorhinal cortex showing no difference in synapse density even though pathological tau is present.

c) Average GluCEST asymmetry was not statistically different in the cortex of PS19 mice (22.8% ± 1.2) compared to WT (23.3% ± 1.1), p=0.72.

d) Quantified synapse density in the entorhinal cortex was not different between the two cohorts.
IV. Discussion

a. Advantages of GluCEST over $^1$H MRS for measuring the distribution of glutamate in the hippocampus

$^1$H MRS measured a decrease in the [Glu]/[tCr] ratio in the hippocampus (Figure 21b). This result is consistent with the trend in glutamate previously observed from APP-PS1 mice (Haris et al., 2013). Glutamate differences were not significant in the PS19 hippocampus likely due to the all-encompassing voxel over distinct sub-regions of the hippocampus. The hippocampus can be divided into two sub-regions, the CA and the DG, which have unique connectivity and function as well as distinct structural changes in AD patients (Greene et al., 2012).

The GluCEST imaging technique was able to distinguish these two sub-regions (Figure 23) as a result of higher spatial resolution compared to conventional $^1$H MRS, as well as increased sensitivity to glutamate. Specifically, using the GluCEST method, the amine protons of glutamate are saturated over a period of one second. During this time, the saturated amine proton magnetization exchanges ~$10^3$ times and accumulates in the water pool. In theory, this amplifies 1 mM glutamate signal to 1M signal, but in practice other experimental parameters such as suboptimal saturation and back exchange preclude the theoretical maximum amplification. As has been shown previously (Cai et al., 2012), in practice GluCEST has at least two orders of magnitude sensitivity advantage over $^1$H MRS.
b. **GluCEST deficits are associated with synapse loss in sub-regions of the hippocampus**

The GluCEST signal in the CA of the hippocampus was significantly reduced by 29%, which is associated with a significant reduction in synapse density by 27% (Figure 24c). This is in contrast to the DG sub-region, where glutamate was maintained even in the PS19 brain, whereas neurodegeneration occurs in the rest of the hippocampus (Figure 23d). Synapse integrity was also intact in the DG of PS19 mice with no significant differences measured from immunostaining (Figure 24c). This is consistent with the fact that there is a continuous turnover of neurons in the DG region compared to the other regions of the brain (Eriksson et al., 1998; Jin et al., 2004a; Spampanato et al., 2012). In the DG of AD patients (Jin et al., 2004b) and in a mouse model of AD (Jin et al., 2004a), there is evidence that neurogenesis is actually increased. Therefore, it is important to distinguish the DG from the rest of the hippocampus of tauopathy brains. To the best of our knowledge, these are the first MRI results that potentially report neurogenesis in vivo.

Tau tangles are most abundant in the hippocampus of PS19 mice, which is where reduced glutamate is expected. However, more widespread glutamate loss was found in regions where pathological tau was not present as in the case of the thalamus (Figure 25a-b). *Vice versa* pathological tau was present in the entorhinal cortex of most PS19 mice, yet GluCEST was not decreased in this region (Figure 26a-b). Therefore, lower glutamate cannot be attributed only to the occurrence of tau pathology.

Rather, synapse loss appears to be the closest neuropathological correlate to GluCEST imaging in the PS19 mouse. A thinning band of synapses was consistently observed in the CA3 region of the hippocampus of PS19 mice, unlike neuron loss which occurred in only a few cases (Figure 22e). Similarly, neuronal dysfunction was only
slightly indicated by a decrease in [NAA]/[tCr] in the PS19 hippocampus (Figure 21b). Also, synapse loss was measured in the thalamus of PS19 mice, where GluCEST decreases were significant and pathological tau was not yet present (Figure 25). Therefore, synapse loss, and not neuron loss or pathological tau, correlates most closely with the location of glutamate loss measured by GluCEST maps in both the CA and thalamus.

Specifically, the mouse in Figure 22b has mild tau pathology and intact neurons, however the synapse band is weak. This case supports the hypothesis that decreased glutamate reflects early stages of neurodegeneration, before neuron loss. In a more severe case in Figure 22c, the GluCEST map is greatly decreased from WT, and associated with severe pathology, thinning neurons, and virtually no synapses in the CA3. This example supports the hypothesis that GluCEST will be decreased further as the severity of tau burden progresses beyond synapse loss.

Synapses are an important location of early neurologic dysfunction (Selkoe, 2002). Synapse loss is also the closest correlate to cognitive deficits in AD patients rather than the amount of tangles (Terry et al., 1991). Glutametergic synapse loss is among the earliest symptoms of disease found in AD brain tissue (Selkoe, 2002). GluCEST imaging, therefore, has the potential to monitor synapse loss in vivo as an early marker of dementia symptoms.

V. Summary

The effect of tau pathology on glutamate levels has been investigated in the brain of a transgenic mouse model using both in vivo MR techniques and IHC. For the first time, in vivo evidence is reported that glutamate is decreased in the CA sub-region of the
hippocampus and in the thalamus where synapse loss also occurs. The DG sub-region maintains glutamate levels in tauopathy mice. Future studies should consider using GluCEST imaging to monitor the health of the DG as a potential region for neurogenic therapy (Chohan et al., 2011). The further development of GluCEST MRI for preclinical applications will be valuable as microtubule-stabilizing therapies are being tested in the PS19 mouse model of tauopathy (Zhang et al., 2012). Highlights from these results are as follows:

- GluCEST imaging has been applied to a P301S mouse model of tauopathy, and shown to significantly decrease in the CA sub-region of the hippocampus and the thalamus.
- Synapse loss is the closest histopathological correlate to GluCEST loss in these regions.
- Glutamate is maintained in the DG sub-region of the hippocampus where neurogenesis is known to occur.
CHAPTER 4: Longitudinal GluCEST imaging correlated with $^1$H MRS in the thalamus of a mouse model of tauopathy

I. Introduction

The following study continued to investigate glutamate deficits in PS19 mice with tauopathy. A longitudinal study was designed to measure the temporal variation in glutamate levels using $^1$H MRS and GluCEST imaging. $^1$H MRS was performed in the thalamus due to the fact that the previous study showed insignificant spectroscopy results in the hippocampus while GluCEST was significantly reduced in the thalamus. Therefore, the thalamus provided a reliable region in which the two modalities could be compared over repeated measures. The goals of this study were 1) to determine whether $^1$H MRS or GluCEST MRI is more sensitive to early metabolic changes in tau pathology in a pre-clinical mouse model, and 2) to evaluate the extent of correlation of glutamate levels measured by the two modalities. The hypothesis is that due to its inherent greater sensitivity, GluCEST will be able to detect earlier changes in glutamate levels in PS19 mice than $^1$H MRS.

II. Methods

a. Experimental design

This study examined the PS19 mouse model of tauopathy, which is a transgenic model with the P301S mutation common in FTDP-17 patients (Yoshiyama et al., 2007). The strain used in this study has been bred on a congenic BL6 background at the CNDR. The effect of a congenic background is to produce a quicker and more consistent
progression of pathology than that characterized on a bigenic background. Two cohorts of PS19 mice and their WT littermates were studied: Cohort 1 including (n=6) WT and (n=6) PS19, and Cohort 2 including (n=3) WT and (n=6) PS19. This study was approved by the university’s IACUC. Mouse husbandry and handling during imaging was identical to that stated in Chapter 2, section II.a. “Mouse preparation and monitoring during MRI” on page 26.

b. **GluCEST imaging**

Imaging and spectroscopy were performed during the same scanning session at several ages: Cohort 1 at 3, 7, and 13 months, and Cohort 2 at 9 and 13 months. A custom-programmed RF spoiled GRE readout with a frequency selective saturation preparation pulse sequence was used as described previously in Chapter 2, section II.c. “GluCEST MRI protocol in mice” on page 30. The following sequence parameters were used in this study: saturation preparation pulse power ($B_{1rms} = 6\text{uT}$ and duration of 1s as four pulses of 250 ms duration each), and GRE readout (effective TR/TE = 6.6/3.3 ms). The entire preparation/acquisition combination was repeated every 8s. GluCEST images were collected with saturation frequencies from ± 2.5 – 3.5 ppm offset from water with a step size of 0.25 ppm.

GluCEST, $B_0$, and $B_1$ maps were acquired from 3 consecutive slices (1mm thick each) spanning the hippocampus from rostral to caudal (Figure 27). Due to changes in the anatomy of PS19 brains as disease progresses, visual estimation of slice positions would be inaccurate. Therefore, the central slice position was determined to be 2.3 mm from the lambda marker viewed in a sagittal scout image. The adjacent slices were acquired 1mm
rostral and caudal to this position. The total imaging time including GluCEST, $B_0$ and $B_1$ mapping for each slice was approximately 15 min.

ROIs were segmented from the $T_2$-weighted anatomical image. The thalamus ROI was segmented manually excluding the white matter region of the cerebral peduncle. The average GluCEST asymmetry % was measured in the thalamus of each slice. Group averages were determined for WT and PS19 mice at each age and for each slice separately.

c. $^1H$ MRS acquisition in the thalamus

Spectroscopy was performed using a PRESS pulse sequence (TR/TE 3000/28ms, 256 averages, sw=4006) while gating to respiration. A voxel was chosen in the thalamus of the right hemisphere of the brain (Figure 27, $2.5 \times 2.5 \times 3 \text{ mm}^3 = 18.75 \text{ mm}^3$ volume). Voxel shimming was performed manually until a water signal line width of at least 20Hz was reached. Water suppression was applied using the WET technique: 4 pulses with 300Hz bandwidth and optimized flip angels of approximately 90° and an inter-pulse delay of 60-80ms chosen in order to keep the acquisition time within a breath period. The water reference peak was also acquired (TR/TE 3000/28ms, 16 averages, sw=4006) with identical water suppression gradients but without RF pulses in order to account for eddy current effects due to water suppression. The total acquisition time for spectroscopy was 19 minutes.

Spectra were processed using in-house software as described in Chapter 3, section II.b. “$^1H$ MRS acquisition in the hippocampus and custom processing” on page 44. All spectra shown are normalized to water. Metabolite concentrations are reported normalized to creatine (3.0 ppm), which did not vary between genotypes or with age.
Figure 27. *In vivo* metabolic data was acquired from a 3mm section of the mouse brain. (a) T2-weighted anatomical images, outlining a voxel (2.5 x 2.5 x 3 mm$^3$) in the thalamus region from which $^1$H MRS was acquired. (b) GluCEST maps corresponding to the anatomical images, spanning three slices (1 mm thick each) through the hippocampus from rostral, middle, to caudal.

**d. Statistical analyses and data handling**

Spectroscopy results are reported with error bars expressing the standard error of the ratio of means (SERM) for ratios of PS19 to WT metabolite concentrations normalized to total creatine concentration:

\[
SERM = \frac{avg^{TG}}{avg^{WT}} \sqrt{\left(\frac{std^{TG}}{\sqrt{N}}/avg^{TG}\right)^2 + \left(\frac{std^{WT}}{\sqrt{N}}/avg^{WT}\right)^2}
\]

where avg$^{TG}$ is the group average [metabolite]/[tCr], and std$^{TG}$ is the group standard deviation of [metabolite]/[Cr] for a given age. N is the number of data points in the group.

Pair-wise student’s one-tailed t-tests were applied to group averages of GluCEST at each age for each slice, assuming unequal variances.

A statistical test for repeated measures was used to determine the significance of longitudinal data. A linear mixed effect model (LMM) was chosen for its flexibility in handling missing data because Cohort 2 was not imaged at 3 months. First-order auto
regressive analysis was used as covariance structure based on Bayesian Information Criterion (IBM SPSS version 22). The linear mixed effect model with time and genotype was fitted for each of the outcome measures with Time, Group (genotype) and Time*Group as fixed effects and intercepts as random effects. A significance level of $p\leq0.05$ was considered statistically significant, and F-values are reported wherever necessary.

For all analyses, GluCEST and $^1$H MRS data from Cohort 1 acquired at 7 months and Cohort 2 at 9 months were combined because the group average values varied less than 2%. Spectroscopy data that could not be accurately analyzed due to large line widths were removed from the study. Due to poor SNR from Glx at 2.2ppm, integrals from 2.0-2.2ppm were combined by a weighted average based on 3 protons from NAA at 2.0ppm and 2 protons from GABA and glutamine at 2.2ppm. GluCEST slices that were corrupted by motion were also removed from the analysis.

In order to correlate GluCEST asymmetry values to $[\text{Glu}]/[\text{Cr}]$ measured by $^1$H MRS, strict inclusion criteria were applied: 1) the spectroscopy voxel must be located entirely inside the thalamus/hypothalamus region, 2) all three GluCEST slices must have been acquired, 3) each GluCEST slice must have the correct anatomy. This is to ensure that a consistent region of tissue is compared between the two modalities. The exact spectroscopy voxel location was mapped onto each slice and segmented from GluCEST maps. The final dataset included at 3 months WT N=5, PS19 N=3; at 7-9 months WT N=9, PS19 N=5; at 13 months WT N=7, PS19 N=3.
III. Results

a. $^1$H MRS reveals a changing neurochemical profile as PS19 mice age

At an early age of 3 months there are subtle differences observed in the neurochemical profile between WT and PS19 mice (Figure 28). Quantification of metabolite concentrations normalized to total creatine levels reveals elevated Glu in PS19 mice at 3 months (Figure 29), as shown in the representative spectrum in Figure 28. A trend in lower levels of NAA, choline (Cho), and taurine (Tau), and elevated lactate (Lac) in PS19 mice is also observed as early as 3 months (Figure 29). By 7-9 months of age, Glu is decreased below WT, and MI is increased above WT levels in PS19 mice. As tauopathy progresses, Glu levels continue to decrease until a significant difference between group averages occurs at 13 months (Table 1). NAA and Cho levels are also significantly lower in PS19 mice compared to WT by 13 months. A significant effect of time was modeled for Glu, Gln, and MI, all normalized to total Cr using an LMM analysis (Table 1). Metabolite levels could not be modeled by genotype, nor the interaction between genotype and time using a LMM analysis.
Figure 28. The neurochemical profile changes as tauopathy progresses in PS19 mice.

$^1$H MRS from the thalamus and corresponding GluCEST images at ages 3, 9, and 13 months. Glutamate levels are elevated in young PS19 mice compared to WT. By 13 months, spectroscopy shows clear loss of NAA (2.0 ppm), and Glu (2.35 ppm) in PS19 mice compared to WT. The corresponding GluCEST maps support the $^1$H MRS results, also showing a decreasing trend in glutamate levels. Group average myo-inositol (MI 3.5-3.6 ppm) levels are increased in PS19 mice by 13 months.
Figure 29. Quantification of $^1$H MRS metabolite levels. Metabolite concentrations are reported as a ratio of PS19 to WT, each normalized to total creatine. Error bars represent the standard error of the ratio of means. Abbreviations: Lactate (Lac, 1.3ppm), N-acetyl-aspartate (NAA, 2.0-2.2ppm), glutamate (Glu, 2.35ppm), glutamine (Gln, 2.45ppm), choline (Cho, 3.25ppm), taurine (Tau, 3.4ppm), and myo-inositol (MI, 3.53ppm). Glu decreases with age in PS19 mice compared to WT. Other decreasing trends were measured in NAA, Cho, and Tau. A significant difference between group averages was measured at 13 months for NAA, Glu, and Cho (*p≤0.05). A trend in elevated levels of Lac and MI was measured in PS19 mice throughout lifetime.
Table 1. Statistical analysis for repeated measures on longitudinal spectroscopy results in PS19 mice.

Metabolite concentrations normalized to total creatine (tCr) measured by $^1$H MRS are listed at each age, mean ± SEM. The absolute concentration of Cr did not vary with age or between genotype. The number of data points included in each group average is listed under #. A linear mixed model (LMM) analysis for repeated measures was applied in order to model the effects of group (genotype), time, and group*time on metabolite levels. A significant effect of time was modeled for Glu, Gln, and MI. Spectroscopy results do not show a significant group effect or interaction between group and time. *p≤0.05

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b. *Longitudinal GluCEST imaging in the thalamus*

GluCEST asymmetry (%) maps from representative WT and PS19 mice, for all three slices, are shown longitudinally in Figure 30. Trends in group-average GluCEST values from the thalamus are graphed below for each slice. In WT mice, an increase in GluCEST as mice age to 7-9 months is observed throughout the brain for all three slices. In PS19 mice, however, GluCEST decreases in the thalamus throughout lifetime. At 7-9 months, GluCEST is significantly lower than WT values in the rostral (22.6 ± 1.7 vs. 27.6 ± 0.71, 18% difference, p≤0.01) and middle thalamus (22.3 ± 1.4 vs. 28.3 ± 1.1, 21% difference, p≤0.01). By 13 months GluCEST is also significantly lower in the PS19 caudal thalamus (22.4 ± 0.85 vs. 24.9 ± 1.2, 10% difference, p≤0.05).
Figure 30. GluCEST asymmetry (%) maps from representative WT and PS19 mice. GluCEST maps are shown for each slice at 3 months (mo.), 7-9mo., and 13mo. Trends in group average GluCEST values from the thalamus are shown for each slice a) rostral, b) middle, and c) caudal. Pair-wise student’s t-tests were performed at each age between WT and PS19 mice (*p≤0.05, ** p≤0.01). In WT mice, GluCEST increases from 3 to 7-9 months in all slices. By 7-9 months of age, GluCEST is significantly reduced in the rostral and middle slices of PS19 mice. The combination of these effects produces the greatest difference in the mid-slice at 7-9 months (21% difference from WT).
c. Correlation of average GluCEST asymmetry and glutamate concentration by $^1$H MRS

Comparatively, there is good agreement between the trends in glutamate levels measured by GluCEST and $^1$H MRS (Figure 31). A linear correlation coefficient of $R^2=0.64$ with a slope of ~25% GluCEST asymmetry per Glu/Cr ratio was measured.

![Figure 31. Correlation of GluCEST and $^1$H MRS in the thalamus. Correlation between group averages of GluCEST and $^1$H MRS results from the same voxel in the thalamus for each time point. Error bars represent the standard error of the mean. There is good agreement between the trends in glutamate levels measured by GluCEST and $^1$H MRS. A linear correlation coefficient of $R^2=0.64$ with a slope of 25% was measured.](image-url)
IV. Discussion

a. Correlation of $^1$H MRS and GluCEST data

One goal of this study was to determine the extent to which the two modalities, $^1$H MRS and GluCEST MRI, correlate in their measurements of neuronal glutamate levels in vivo. Both modalities measured a significant decrease in glutamate levels with the progression of tau pathology. A 21% difference in GluCEST asymmetry was measured in the mid-thalamus of PS19 mice compared to WT at 7-9 months of age (Figure 30, $p\leq0.01$). A significant deficit was measured by spectroscopy at 13 months ($p\leq0.05$, Table 1). Although the two modalities measured the same trend in glutamate, GluCEST was able to detect earlier deficits in PS19 mice.

Two more subtle trends in longitudinal glutamate levels were measured both by $^1$H MRS and GluCEST. First, at 3 months of age there was a slight elevation in glutamate levels in PS19 mice compared to WT: GluCEST measured a 3% elevation in the rostral and middle slices (Figure 30), while $^1$H MRS measured a 2% elevation in the thalamus overall (Figure 29). This effect is potentially an early sign of excitotoxicity. A recent study investigated the excitotoxicity hypothesis in a P301L mouse model of tauopathy at 5 months of age, and found excess glutamate in the extracellular space as well as reduced uptake of glutamate which can lead to excitotoxicity (Hunsberger et al., 2015).

Additionally, glutamate levels were found here to increase in the thalamus of WT mice from 3 months to 7-9 months: GluCEST measured a 5-19% increase compared to 3 month levels depending on slice position (Figure 30), and $^1$H MRS measured a 5% increase (Figure 29). Group averages of Lac, Gln, Cho, and Tau also increased in WT mice from 3 months to 7-9 months, although the differences remained within the range of noise.
(Table 1). This is likely a developmental effect caused by enhanced energetics and metabolism at this age. A complementary study in the cortex of healthy mice from postnatal day 10 up to 3 months of age showed that the trend in glutamate and choline continually increased (Kulak et al., 2010).

The correlation of group average data from GluCEST and $^1$H MRS showed a positive linear correlation coefficient of $R^2=0.64$ (Figure 31). A slope of 25% GluCEST asymmetry per Glu/tCr ratio, and an intercept of 1.6 was measured. A previous study also correlated GluCEST asymmetry to Glu/tCr in the hippocampus region in the APP/PS1 mouse model with amyloid-beta plaques characteristic of Alzheimer’s disease (Haris et al., 2013a). This study reports a correlation coefficient of $R^2=0.91$, a slope of 15%, and an intercept of 2.2. Overall, the range of GluCEST and Glu/tCr values were narrower in the tauopathy mouse model compared to the APP/PS1 model of amyloid pathology, reflected by a shallower correlation slope.

Compared to histopathological events in the PS19 mouse model, hyperphosphorylated tau presents itself in the mid-thalamus region during the latest stages of disease (Hurtado et al., 2010). We have shown here, however, that there are early metabolic changes in the thalamus. In addition to dynamics in glutamate levels, $^1$H MRS also measured an increasing trend in MI as early as 7-9 months. MI is an indicator of gliosis, which is known to occur in the PS19 mouse model (Yoshiyama et al., 2007). In this particular congenic mouse model, we have shown by IHC that enhanced GFAP, as a marker of gliosis, is observed in the thalamus by 6 months (results not shown here).
b. *Longitudinal neurophysiologic changes in the PS19 mouse model of tauopathy*

Currently, we are only aware of two reports of $^1$H MRS in a P301L mouse model. One study at 4.7T on an inducible P301L mouse model at early stages of pathology reports significantly increased $\text{MI/Cr}$ ratio in a voxel containing both hippocampus and thalamus (Yang et al., 2011). NAA/Cr and Glu/Cr ratios were also reduced although insignificant. The second study was performed on the PS19 model in late stage pathology by the author of this thesis (Crescenzi et al., 2014). In a smaller voxel including only hippocampus, significantly lower Glu/Cr and reduced NAA/Cr were measured.

The $^1$H MRS signature of various APP/PS1 mouse models of AD has been reported longitudinally and has been reviewed by (Mlynárik et al., 2012). These models report significant reductions in Glu and NAA roughly 6-10 months after amyloid deposition and later elevation of MI (Mlynárik et al., 2012). In one definitive longitudinal study of the APP/PS1 mouse model (n=27) vs. controls (n=30), a significant decrease in NAA/Cr and Glu/Cr was measured with age, as well as between control and transgenic mice (Marjanska et al., 2005). A significant increase in MI/Cr was also measured, and a significant interaction between group and time was reported based on ANOVA. Effects on other metabolites were not reported. Comparatively, our methodology varied in that the mice in our study were scanned repeatedly. Our findings show a similar significant effect of time on ratios of Glu/Cr and MI/Cr, and significantly lower Glu/Cr in tauopathy mice by 13 months (Table 1). However our metabolite levels did not model a significant effect of time or interaction between time and group by a repeated measures analysis.

Lactate also showed an elevated trend in PS19 mice at every age from 3-13 months (Figure 29). This indicates a hypoxic condition in the brain tissue, perhaps causing...
oxidative stress. Increased levels of lactate can be caused by over-activation of NMDA receptors (Retz and Coyle, 1982), i.e. excitotoxicity, which leads to oxidative stress (Coyle and Puttfarcken, 2014). Acidic conditions in the brain have also been shown to induce gliosis, likely in response to a calcium-dependent protease calpain I (Lee et al., 2000). Calpain I, whose reaction yields reactive oxygen species (McCord, 1985), was discovered to be a necessary mechanistic link between NMDA-excitotoxicity and pathologic tau degradation leading to cell death (Amadoro et al., 2006). These results present in vivo evidence of early excitotoxicity, by $^1$H MRS and GluCEST MRI, and oxidative stress by $^1$H MRS.

c. Advantages of GluCEST over $^1$H MRS for earlier diagnosis

Although both $^1$H MRS and GluCEST-MRI monitor glutamate changes, there are several important advantages of GluCEST highlighted here. For diagnostic purposes, early glutamate elevation and subsequent deficits are the earliest markers of tauopathy investigated here. GluCEST was able to measure glutamate deficits earlier than $^1$H MRS by 4 months. For further applications of GluCEST to mouse models of tauopathy, only one slice through the mid-hippocampus and thalamus would be necessary to measure a 21% deficit in glutamate by 7-9 months of age (Figure 30). Also, a greater dynamic range is available in the GluCEST measurement (Figure 31). Furthermore, GluCEST maps measure glutamate levels at high-resolution (156 µm x 156 µm x 1mm pixel resolution vs. 2.5 x 2.5 x 3 mm$^3$ voxel resolution). In roughly the same total scan time, glutamate levels can be mapped in the whole brain slice rather than in a single voxel. The hippocampus, thalamus, and cortex (Crescenzi et al., 2014), as well as a whole-brain ROI (not published) display lower glutamate levels in mice with tauopathy detected by GluCEST. This is useful
in order to show that glutamate deficits occur throughout the brain as an overall metabolic effect.

V. Summary

The goals of this study were to determine whether $^1$H MRS or GluCEST is more sensitive to longitudinal glutamate changes as tau pathology progresses, and to what extent the two modalities are correlated. $^1$H MRS and GluCEST were performed longitudinally in the thalamus of WT (n=9) and PS19 (n=12) mice at ages 3, 7, 9, and 13 months old. The greatest glutamate deficit in PS19 mice compared to WT was measured in the thalamus by GluCEST at 7-9 months of age (a 21% difference, p≤0.01). A significant deficit was measured by $^1$H MRS at 13 months (p≤0.05). Increasing dynamics in glutamate levels were also measured at 3 months of age when PS19 mice have slightly elevated glutamate levels. An increasing trend in glutamate was measured in WT mice as they age to 7-9 months of age. The following is a highlight of these results:

- Both GluCEST and $^1$H MRS measured the same trend in glutamate levels and are correlated ($R^2=0.64$) in the thalamus of aging PS19 mice.
- GluCEST was able to detect deficits in PS19 mice four months earlier than $^1$H MRS.
CHAPTER 5: Longitudinal imaging of sub-hippocampal dynamics in glutamate levels in a mouse model of tauopathy

I. Introduction

Thus far I have shown that GluCEST is reduced in the hippocampus of a mouse model of AD and in locations of severe synapse loss in the hippocampus of a tauopathy mouse model (Crescenzi et al., 2014). These findings were detected in the late stage of disease when low levels of hippocampal glutamate were measured after synapse loss and volume loss had already occurred.

Evidence from the literature supports a variable trend in glutamate levels during the course of tau pathology and throughout different sub-regions of the hippocampus. Glutamate is a necessary component of long-term potentiation (LTP), the measurable process of learning and memory. Glutamate levels are an indicator of synaptic growth during LTP (Dolphin et al., 1983) and may be an indicator of synaptic dysfunction in neurodegenerative disease (Clare et al., 2011; Gong and Lippa, 2010). LTP is dependent upon NMDA receptors in the hippocampal sub-field CA1 and the DG; however, synaptic transmission does not depend on NMDA receptors in the mossy fiber-CA3 (Lynch et al., 2004; Nicoll and Schmitz, 2005). LTP was found to be impaired in the CA1 and not the CA3 of a mouse model of tauopathy exhibiting cognitive deficits (Chong et al., 2011). In contrast, LTP was enhanced in the DG of young (2-3 months) tauopathy mice (Boekhoorn et al., 2006; Pennanen et al., 2006). An effective in vivo surrogate biomarker of synapse loss would correlate with the sub-hippocampal location and dynamics of synapse loss.

The primary goal of this study was to measure longitudinal changes in sub-hippocampal glutamate levels from early to late-stage pathology in order to determine
when glutamate deficits are observed in relation to synapse and volume loss. Sub-regions of the hippocampus were analyzed in order to associate location-specific deficits in MRI and histological measures. This study was driven by two main hypotheses: 1) decreased GluCEST signal will occur when synapse loss is measurable and before volume loss; and 2) glutamate levels will decrease in specific regions of the hippocampus as disease progresses in PS19 mice compared to WT. Additionally, a histologic basis for changes in glutamate levels was investigated by IHC of age-matched littermates. A variety of cell-type specific antibodies (NeuN and GFAP) and pre-synaptic glutamate transporter (VGlut1) and post-synaptic receptor (NMDA-NR1) antibodies were applied to tissue from age-matched littermates in order to compare longitudinal MRI and histological measures.

II. Methods

a. Experimental design

This study was performed in parallel with that presented in “CHAPTER 4: Longitudinal GluCEST imaging correlated with $^{1}$H MRS in the thalamus of a mouse model of tauopathy” (page 63). A separate analysis was performed on data from the hippocampus driven by the aforementioned hypotheses. Refer to Chapter 4, section II.a. on page 63 for the experimental design. Following imaging, 13 month old WT mice were sacrificed and their brain tissue was processed for histology (n=7). PS19 tissue at 13 months was also processed but not analyzed with histology due to the atrophic hippocampus in aged PS19 mice, so that ROI selection was not possible. In order to correlate histological measures to in vivo imaging data at each age, mouse tissue at younger ages was obtained from
littermates raised by the CNDR under the same housing conditions: 3 months (n=4 WT, n=4 PS19), 6 months (n=6 PS19), and 9 months (n=6 PS19).

This study was approved by the university’s IACUC. Mouse husbandry and handling during imaging was identical to that stated in Chapter 2, section II.a. “Mouse preparation and monitoring during MRI” on page 26.

b. GluCEST Imaging and segmentation by k-means cluster analysis

Refer to Chapter 4, section II.b. on page 64 for the “GluCEST imaging” methods. The GluCEST signal was measured from four anatomical sub-regions of the hippocampus: the DG, the CA1 and CA2,3, and the adjacent fourth ventricle containing CSF. It is not possible to delineate these sub-regions from a T2-weighted *in vivo* MRI (Figure 32i). Although high-resolution atlases exist, both for MRI (Johnson et al., 2010) and histology (Adler et al., 2014), these methods require registration with exact *ex vivo* tissue, which cannot be obtained in a longitudinal study.

In the present study, we first manually segmented the hippocampus proper from the T2-weighted image (Figure 32i). The outline of the hippocampus can be adequately delineated in each slice: rostral, middle (mid), and caudal. The hippocampal mask was applied to the corresponding GluCEST map (Figure 32iii). A secondary segmentation was then applied to each hippocampus using the k-means clustering algorithm (Matlab script by Jose Vicente Manjon Herrera, 2005). The k-means method has been used to segment gray matter, white matter, and CSF voxels from *in vivo* anatomical MRIs of the brain (Leung et al., 2015; Vemuri et al., 2008). Likewise, we used this method to segment three clusters from the hippocampus based on the GluCEST MRI contrast. This algorithm uses
a statistical method to set a threshold between high, medium, and low GluCEST values with respect to each hippocampus (Figure 32iv). The final segmented clusters from all three slices of an example PS19 mouse are shown in Figure 32v.

In total, 9 clusters were segmented from the hippocampus of each mouse. The hippocampus is a curved 3D structure, which was imaged in 2D coronal sections. This presents a problem for labeling ROIs along the functional axis (Strange et al., 2014) or “long” axis of the hippocampus (see Snyder et al., 2011) for more discussion). Rather, the cluster locations within each slice roughly fall along the dorsal-ventral axis. Therefore, clusters are labeled within each slice: dorsal “D” in red, intermediate “I” in green, and ventral “V” in blue. This nomenclature was motivated by the segmentation presented by (Dong et al., 2009) based on gene-expression patterns within the hippocampus. From each cluster the average GluCEST asymmetry value was measured. Additionally, the volume of each cluster was measured as the number of pixels normalized to the total hippocampus volume.

The cluster locations can be qualitatively compared to the location of hippocampal sub-regions of interest, thereby extending the cluster-analysis of GluCEST to specific sub-regions of the hippocampus along the dorsal-ventral axis. In Figure 32vi the sub-regions are outlined on hippocampus atlases and colored according to their corresponding cluster. A final cluster model of hippocampal sub-regions is provided in Figure 32vii for reference.
Figure 3.2. Image acquisition and segmentation pipeline
i. Anatomical T2-weighted MRI acquired from a live mouse brain. The example images shown here are from a 3 month old PS19 mouse. Slice positions were chosen relative to the Lambda marker (yellow) identified on the sagittal scout image. The three slices (1mm thick each) span the hippocampus from rostral to caudal.
ii. GluCEST asymmetry maps represent glutamate levels in each slice. Gray matter regions such as the thalamus have higher glutamate levels, compared to white matter regions like the corpus callosum, where glutamate levels are low.

iii. Hippocampus proper segmented from the T2-weighted MRI and applied to GluCEST maps from each slice.

iv. Histogram of GluCEST values in an example hippocampus. The k-means clustering algorithm sets a threshold between high, medium, and low GluCEST values.

v. Example cluster masks of the hippocampus for each slice. The 3 clusters are anatomically correlated to the dorsal (D, red), intermediate (I, green), and ventral (V, blue) hippocampus.

vi. Three slices of the hippocampus from the Allen Brain Atlas (Johnson et. al., 2010). Hippocampal sub-regions of interest are outlined: dentate gyurs (DG), cornu ammonus (CA1, and CA2-3), and the CSF. Each region is colored to indicate their approximate cluster position. In younger mice the CA1 extends through the caudal-ventral hippocampus, which later becomes CSF as mice age.

vii. Depicted here is an approximate model of the hippocampal sub-regions which contribute to each cluster (dorsal, intermediate, ventral) in each slice (rostral, middle, caudal). The average GluCEST asymmetry and volume were computed for all nine clusters, and displayed in this grid format.

c. IHC protocols and quantification

Mouse brain tissue was prepared for histology following the methods outlined in Chapter 3, section II.d. “General tissue preparation and methods of immunohistochemistry” on page 46. Several antibodies were applied to sectioned brain tissue. AT8 monoclonal antibody was utilized to depict phosphorylated tau at residues Ser202/Thr205 (1:7500), developed with a horseradish peroxidase developing system (BioGenex, India) and DAB (Vector DAB, Vector Laboratories), and counterstained using hematoxylin. Synaptophysin (Milipore Mab38 anti-rabbit, 1:500) was applied for pre-synaptic terminals and developed using the ABC system (Vectastain ABC Kit, Vector Laboratories). Both VGlut1 (pre-synaptic vesicular glutamate transporter protein, Synaptic Systems, Catalog #135304, anti-guinea pig, 1:5000) and NeuN (neuronal cells, Millipore NeuN21, anti-goat, 1:100) received microwave pre-treatment for 15 minutes at 100°C in
citrate buffer (Vector Antigen Unmasking Solution, Vector Laboratories) and were developed using the ABC system. To quantify glial cell proliferation, antibody to GFAP (glial fibrillary acidic protein, generated at the CNDR, 1:2000) was applied, developed using the ABC system, and counterstained with hematoxylin. An antibody for NMDA glutamate receptor sub-unit 1 (NR1, Abcam, 1:5000, with citrate pre-treatment) was applied, developed using the BioGenex system, and counterstained with hematoxylin.

The following antibodies were quantified in the mid-hippocampus: synaptophysin, VGlut1, NeuN, and GFAP. Four successive slides were chosen per mouse, one for each antibody, in the mid-hippocampus based on a visual match to Bregma -2.18 anatomy (see Figure 32vi, middle atlas section). Sections were discarded if damaged or incorrect anatomy was determined after immunostaining. Stained slides were scanned using an automatic slide scanner (Lamina Scanner, PerkinElmer). Scanned slides were viewed using CaseViewer (freeware, v1.3.0.41885), and snapshots were taken at 10x magnification. ROIs were segmented by hand in the dentate gyrus polymorph and granule cell layer, and all laminae of the CA1 and CA2,3 (see Figure 37a for example ROIs). Images were thresholded to the same level for all mice (ImageJ, NIH). The amount of immunostaining was measured as the percentage of area occupied (% AOC) by positive staining within the ROI. GFAP immunostaining was distinguished from hematoxylin by the color deconvolution plug-in (Ruifrok et al., 2001) for ImageJ using the hematoxylin and DAB optical densities (ODs) provided; the hematoxylin image was used for ROI identification, and the chromagen image was thresholded before measuring the %AOC.

Slides immunostained with NR1 were quantified in the caudal-hippocampus due to a greater amount of NR1 positive cells present compared to the mid-hippocampus sections. The caudal-hippocampus was chosen based on anatomical features of Bregma -3.18 (see
Figure 32 (vi third atlas section). ROIs were outlined in the DG, CA1, and CA2,3. Positive chromagen signal was measured in the NR1 stain by identification and averaging of red, blue, green (RGB) OD values from five random NR1 positive neurons for color deconvolution of signal from background and counterstain. A minimum OD threshold was applied to measure the area occupied by NR1 positive neurons and was confirmed for accuracy by visual inspection.

d. Statistical analyses

To determine the effects of time and group (genotype) on GluCEST and volume results, a linear mixed effect model (LMM) for repeated measures was applied to data from each cluster. First-order auto regressive analysis was used as covariance structure based on Bayesian Information Criterion (IBM SPSS version 22). The linear mixed effect model with time and genotype was fitted for each of the outcome measures with Time, Group (genotype) and Time*Group as fixed effects and intercepts as random effects. A significance level of $p \leq 0.05$ was considered statistically significant and F-values are reported wherever necessary. GluCEST data from Cohort 1 acquired at 7 months and Cohort 2 at 9 months were combined to gain statistical power. Data from Cohort 2 at 3 months was not acquired; however, LMM was chosen for its flexibility in handling missing data.

Student’s one-tailed t-test was also performed to determine significant differences between group averages of various data, assuming unequal variance. Error bars express the standard error of the mean (SEM).
III. Results

a. Longitudinal glutamate imaging in sub-regions of the hippocampus

Glutamate levels were measured in vivo using GluCEST imaging from three slices spanning the hippocampus. Representative GluCEST maps from the hippocampus of a WT and PS19 mouse are shown at each age in Figure 33. GluCEST contrast is clearly not homogeneous within the hippocampus. Several obvious features can be observed. The caudal slice generally has higher GluCEST than the rostral slice, and a region of high GluCEST is longitudinally maintained in the dorsal hippocampus. The ventral hippocampus region has reduced GluCEST contrast and is enlarged in the PS19 mouse. GluCEST also appears to increase in various regions as both the WT and PS19 mice age.

Figure 33. Representative GluCEST maps in the hippocampus. Examples are shown here from a congenic BL6-PS19 mouse and a healthy WT mouse as they age from 3-13 months. All three slices are also from the same mice. The exception is at 9 months, where maps from different mice are shown because only Cohort 2 was imaged at this time point.
For a more detailed analysis, GluCEST maps in the hippocampus were segmented into three clusters for each slice. The average GluCEST value in each cluster was averaged across the group of WT and PS19 mice at each age. Group averages from the nine clusters were arranged in a grid and are referenced according to slice position (rostral-R, middle-M, caudal-C) and cluster location (dorsal-D, intermediate-I, ventral-V, see Figure 32vii). Figure 34 illustrates that a spatial gradient is observed in glutamate levels. GluCEST values are highest in the most caudal-dorsal region and are lowest in the more rostral-ventral regions. For instance, the caudal-dorsal (C-D) cluster has the highest GluCEST values, and the rostral-ventral (R-V) cluster has the lowest GluCEST values.

At 3 months of age, the average GluCEST asymmetry is slightly elevated in PS19 mice over WT levels (Figure 34a-b). In all regions of PS19 mice, GluCEST decreases from 3-7 months. In WT mice, however, GluCEST increases in all regions from 3-7 months. The combination of these effects produces the earliest significant differences at 7 months between WT and PS19 mice (Figure 34c). The M-D region at 7 months is the location and age of the greatest measured difference in GluCEST between PS19 and WT mice (30% reduction, p≤0.001, Figure 34c).

After 7 months, GluCEST in WT mice progressively diminishes but remains higher than PS19 mice in all regions except the M-D. At 13 months GluCEST in the PS19 M-D is significantly elevated over WT levels (p≤0.05, Figure 34c). Several other dorsal regions of the PS19 hippocampus show increases in GluCEST compared to earlier time-points: the M-D and R-D regions increase from 7-9 months and 9-13 months, the C-D and M-I region from 9-13 months.

By contrast, the intermediate and ventral regions of the hippocampus progressively decrease from 7-13 months and are significantly reduced from WT in the middle and
caudal slices at each age (Figure 34c). By 13 months, GluCEST is significantly reduced in all ventral regions of PS19 mice compared to WT. The LMM analysis confirms the significant effect of time on GluCEST in the ventral regions and the C-I region (Table 2a). There is also a significant interaction between group*time in the C-I, C-V, and M-V regions.
Figure 34. Glutamate gradient in the hippocampus measured in vivo by GluCEST MRI

a-b) Group average GluCEST asymmetry values from clusters in the hippocampus arranged on an anatomical grid. A gradient in glutamate levels is clearly observed along the rostral-caudal and dorsal-ventral axes of the hippocampus. In WT mice there is a ubiquitous increase in GluCEST levels as mice age to 7 months; glutamate levels decline back to baseline by 13 months. PS19 mice have progressively decreased GluCEST levels throughout lifetime in most regions. An increase in GluCEST is measured among the dorsal regions of the PS19 cohort from 9-13 months.

c) GluCEST asymmetry values were compared for each cluster between WT and PS19 mice using a Student’s t-test. The earliest significant differences between PS19 and WT mice are measured at 7 months in the more caudal, dorsal regions. In later stages of tauopathy, GluCEST values become significantly reduced in ventral regions as hippocampal tissue is lost.
b. Volume changes in sub-regions of the hippocampus

Longitudinal volume changes are represented in bar graphs for each cluster and are arranged in a grid (Figure 35). Interestingly there is an increasing trend in volume of the dorsal PS19 hippocampus until 9 months; the volume is abruptly decreased between 9-13 months (Figure 35a). The volume of the PS19 M-D region becomes significantly reduced compared to WT at 13 months (p≤0.01), while C-D volume remains significantly increased over WT (p≤0.001). Volume is relatively maintained in the dorsal regions of WT mice.

The volume of the C-I cluster decreases at 9 months below WT volume, and reaches significance by 13 months (p≤0.001, Figure 35b). Volume of the M-I cluster shows a more progressive decrease from 3-13 months and is significantly lower than WT at 13 months (p≤0.001). WT mice also show some reduction in M-I volume during the course of the study. The LMM analysis reveals a significant effect of time and group*time interaction on the volume of the C-I, and M-I clusters (Table 2b). The volume of the R-I region is significantly affected by time in the LMM analysis.

C-V volume in PS19 mice follows that of WT mice until 13 months when volume is significantly enlarged (p≤0.01, Figure 35c). There is a significant effect of time on C-V volume by LMM analysis (Table 2b). The greatest change in volume is measured in the M-V cluster at 13 months (58% increase compared to WT, p≤0.001). This region is significantly affected by time and group*time interaction (Table 2b). The volume of the R-V is slightly increased with age in both WT and PS19 mice (Figure 35c), and indeed there is a significant effect of time based on the LMM analysis (Table 2b).
Figure 35. Longitudinal volume changes in sub-regions of the hippocampus
Bar graphs represent the volume of each cluster normalized with the total volume of the hippocampus. Graphs are arranged according to their cluster position (see Figure 32vii).

a) The volume (or proportion of the hippocampus) of dorsal clusters for each slice. Interestingly there is an increasing trend in volume of the dorsal PS19 hippocampus until 9mo.

b) In contrast, the volume of intermediate clusters in the mid and caudal slices progressively decrease in PS19 mice with age, which reaches significance at 13 months compared to WT.

c) The ventricle volume is depicted in the volumes of the ventral clusters. A severe increase in ventricle volume was measured in all 3 slices from 9-13 months in PS19 mice. Ventricle volume is significantly larger than WT at 13 months (also shown in histology sections in Figure 36d).

Significance is reported between WT and PS19 group averages at each age using the Student's t-test. *p≤0.05; ** p≤0.01; ***p≤0.001
Table 2. Effects of group, time, and group*time were fit for GluCEST and volume results using a linear mixed effect model (LMM) for repeated measures.

a) GluCEST in the ventral clusters of all three slices, corresponding primarily to CSF, is significantly affected by time. An interaction between group*time also contributes to ventral GluCEST in the caudal and middle slices. The caudal-intermediate cluster, corresponding to the CA23, is significantly affected by time and the interaction between group*time.

b) Volume of the intermediate and ventral clusters of all three slices is primarily modeled by the effect of time. A group*time interaction exists in the caudal-intermediate, mid-intermediate, and mid-ventral clusters.

c. **Longitudinal pathology in the congenic BL6-PS19 mouse model of tauopathy**

At 3 months of age, mild pathology has already begun to appear in PS19 mice (Figure 36a). Hyper-phosphorylated tau (by AT8 immunostaining) is present in the first layer of the caudal cortex and mossy fibers of the caudal CA3. As PS19 mice age to 6 months, pathology is present in all layers of the rostral-caudal hippocampus and has infiltrated deeper layers of the cortex, the entorhinal cortex, and amygdala. At 9 months tau pathology proliferates through the entire hippocampus, and tissue degeneration is observed (Figure 36c). By 13 months AT8 pathology is present in the thalamus and the frontal cortex in addition to the hippocampus (Figure 36d).
Figure 36. Tau pathology by AT8 IHC in congenic BL6-PS19 mice as disease progresses.

a) AT8 immunostaining in the hippocampus of a 3 month PS19 mouse, from rostral to caudal, shows early pathologic tau in the first layer of the caudal cortex and mossy fibers in the CA3.

b) Pathology spreads by 6 months from the entorhinal cortex and amygdala through all layers of the rostral to caudal hippocampus.

c) Tissue degeneration is apparent in the hippocampus by 9 months and further by 13 months.

d) Pathology is present throughout the thalamus and frontal cortex by 13 months. WT mouse brain shows little degeneration of the hippocampus with age.
Synapse density by synaptophysin immunostaining was quantified in histological sections corresponding to the regions of mid-hippocampus imaged in vivo. The earliest significant difference in synapse density was measured at 6 months in PS19 mice compared to WT mice at 3 months in all sub-regions of the hippocampus (p≤0.01, Figure 37a, Table 3a). Synapse density continues to decrease in all sub-regions of the hippocampus of PS19 mice out to 9 months.

VGlut1, a marker of glutamatergic pre-synapses, reveals interesting dynamics as PS19 mice age (Figure 37b, Table 3b). At 3 months VGlut1 is significantly different than WT in the DG of PS19 mice (p≤0.01). By 6 months VGlut1 immunostaining is increased from that observed at 3-6 months in all sub-regions of PS19 mice. By 9 months VGlut1 presence is significantly reduced from WT in all sub-regions (p≤0.01 in CA2,3). There is a significant reduction in VGlut1 immunostaining in the CA1 and CA2,3 of WT mice by 13 months (Table 3b).

In PS19 mice, both GFAP (Figure 37c) and NeuN (Figure 37d), markers of glial cells and neurons, were significantly different from WT by 9 months NeuN was significantly reduced in all sub-regions (p≤0.05, Table 3d) while GFAP was significantly increased in all sub-regions (p≤0.001, Table 3c). Increased GFAP throughout the hippocampus was the most severe change measured by histology with more than 20x greater presence of glial cells at 9 months throughout the hippocampus.

Positive immunostaining of glutamate receptor NR1 was quantified intracellularly in the caudal hippocampus (Figure 38a). The average %AOC was reduced in all sub-regions of 3 months PS19 mice compared to WT. Significant reductions were measured by 6-9 months in the CA1 and CA2,3 (p≤0.05, Figure 38b-d, Table 3e).
Figure 37. Synaptic and cell-type specific IHC markers in the hippocampus. IHC was performed on sections through the hippocampus corresponding to the mid-slice using various antibodies: a) Synaptophysin for pre-synapse density, b) VGlut for glutamatergic pre-synapse density, c) NeuN for neuronal cells, and d) GFAP for glial cells. ROIs including the dentate gyrus (DG), CA1, and CA23 were segmented from 10x images, and percent area occupied (%AOC) was quantified. All images are displayed at 5x magnification. Graphs report group averages in the CA23. Student’s t-tests were performed between each group average against the WT 3 month average (*p≤0.05; ** p≤0.01; ***p≤0.001). The earliest significant difference was measured at 6mo. in PS19 synaptophysin density compared to WT levels at 3 months in all ROIs (p≤0.001). Glutamatergic synapses by VGlut tend to increase at 6 months throughout the hippocampus, followed by a significant reduction at 9 months compared to WT levels at 3 months (p≤0.01). NeuN is significantly reduced in all ROIs by 9 months (p≤0.05), while GFAP is significantly increased by 9 months (p≤0.001).
Figure 38. Immunostaining of NMDA glutamate receptor NR1 in the caudal CA23.

a) ROIs were manually drawn in the CA1, CA23, and DG.
b-c) Representative bands through the CA23 from a WT mouse at 3 months and a PS19 mouse at 6 months. Positive NR1 immunostaining was considered intracellular. Fewer NR1-positive cells appear in PS19 mice at 3 and 6-9 months in all ROIs.
d) Significantly lower NR1 levels are measured at 6-9 months in PS19 mice (p≤0.05).

Table 3. Quantification of IHC for various antibodies

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#N: number of mice included in each group average.
‡: NeuN group average at 3mo. includes N=2 WT and N=2 PS19 mice.
¥: NR1 group average at 6mo. includes N=4 PS19 6mo. and N=1 PS19 9mo.
*ps0.05; **ps0.01; ***ps0.001

Table 3. Quantification of IHC for various antibodies

Trends from all sub-regions follow that of the CA23, which are graphed in Figure 37 and Figure 38. Student’s t-tests were performed between each group average against the WT 3 month average.
IV. Discussion

a. Reduced GluCEST and synaptic density are measured before volume loss in the PS19 mouse model of tauopathy

The earliest significant differences in GluCEST between PS19 and WT mice were measured at 7 months of age, at which point GluCEST was significantly decreased throughout the entire middle and caudal hippocampus slices. The largest difference was measured in the middle-dorsal region (30% reduced from WT). Synapse density was also significantly reduced in every sub-region of the hippocampus in PS19 mice at 6 months. Volume loss was not significantly different until 13 months between PS19 and WT mice.

Reduced GluCEST occurred not only earlier than volume loss but also in a different location than volume changes within the hippocampus. A shift in location of GluCEST deficits is observed in Figure 34c, where significance shifts are observed from the dorsal-caudal region of the grid at 7 months to the ventral-caudal region by 13 months. Early differences at 7 months seem to be a metabolic deficit in glutamate levels, which is concurrent with reduced synapse density. This is in contrast to lower GluCEST in the ventral regions by 13 months, which is a result of hippocampal volume loss and ventricle volume increase.

b. Interpretation of cluster analysis and glutamate gradient measured in vivo by GluCEST MRI

GluCEST contrast follows a gradient along the dorsal-ventral and caudal-rostral axes of the hippocampus. GluCEST values are highest in the most caudal-dorsal region and decrease towards the rostral-ventral regions. Numerous studies have reported gradients
within the hippocampus in terms of gene expression (Fanselow and Dong, 2010; Thompson et al., 2008), connectivity (Risold and Swanson, 1996), electrophysiology (Staresina et al., 2012), and spatial processing functions (Kjelstrup et al., 2008). These gradients also exist along the dorsal-ventral axis of the hippocampus, and may be better understood in terms of the functional “long” axis (Strange et al., 2014). To facilitate interpretation of our cluster-based analysis in terms of hippocampal sub-regions, an anatomical model is provided in Figure 32vii. According to this model, the dorsal regions correspond anatomically to the DG and may include contributions from CA1, although they remain more dorsal than CA2,3. The intermediate clusters therefore include the hippocampal sub-region CA2,3. In the caudal slice, the CA1 extends from the dorsal to ventral hippocampus in young mice, and may therefore contribute to the GluCEST signal in all caudal clusters. The ventral clusters contain sub-fields of the CA in younger mice, and transition to CSF as tissue is lost. These regions are subject to partial-volume effects between adjacent anatomies.

Gradients in neuron activation and neurogenesis have also been observed within the hippocampus. Neurons in the most dorsal hippocampus are highly active even without a stimulating memory task (Snyder et al., 2011). Additionally, there is a greater density of newborn neurons in the dorsal-DG compared to ventral regions of the DG in young (Snyder et al., 2009) and old rats (Snyder et al., 2011). These findings are consistent with our observations that the highest glutamate levels measured by GluCEST imaging were observed in dorsal regions (rostral to caudal) corresponding to the DG (Figure 34). We also found that neurons, by NeuN immunostaining, appear to be more densely populated in the DG compared to CA1 and CA2,3 (Table 3d). Additionally, NR1 immunostaining increased in WT mice in the DG from 3-13 months (Table 3e).
c. MRI measures increased glutamate levels in vivo during mouse lifetime

Early signs of tau pathology were observed in both the GluCEST measurement and synaptophysin immunostaining. At 3 months there was a trend of higher glutamate levels in every sub-region of the PS19 hippocampus compared to WT (Figure 34a-b). At this age hyper-phosphorylated tau pathology was only present in select layers of the caudal hippocampus, whereas the trend in synapse density was lower in all sub-regions analyzed. A potential explanation for this early state of elevated glutamate, which causes synapse deficits in the absence of phosphorylated tau, would be excitotoxicity. A higher GluCEST signal of 1-2% measured here in the 3 months PS19 hippocampus corresponds to an elevation of glutamate by approximately 2-3 mM/L (Cai et al., 2012), which is more than sufficient to induce excitotoxicity (Nicholls and Budd, 1998). Additional experiments are needed to prove this relationship; however, this demonstrates the potential for GluCEST imaging to measure excitotoxicity in vivo.

Throughout the hippocampus, increased GluCEST levels at 3 months preceded an increase in VGlut1 levels at 6 months in PS19 mice. Another recently published study found a similar trend in VGlut1 at 5 months in a P301L mouse model by immunoblotting of hippocampus tissue (Hunsberger et al., 2015). That finding coincided with decreased expression of GLT-1, a transporter involved in clearing glutamate from the extracellular space. We have also shown a progressive decrease in NR1, an NMDA receptor, which resides on the post-synapse. Together, these effects produce excess glutamate in the extracellular space, which leads to excitotoxicity (Hunsberger et al., 2015). This may be a translatable effect to early stages of neurodegeneration. VGlut was shown to be elevated
in the mid-frontal gyrus of patients with early stages of mild-cognitive impairment (Bell et al., 2007).

Another unexpected finding of this study was a ubiquitous increase in hippocampal GluCEST levels from 3-7mo in WT mice. 7 month old PS19 mice, however, do not show this effect. Instead, the rise in GluCEST levels in PS19 mice occurs at 9 months in the dorsal clusters rostral-mid, and further at 13 months in all dorsal clusters and the mid-intermediate cluster (Figure 34b). These clusters correspond to the DG and CA1 from rostral-caudal, and mid-CA2,3 sub-regions. Indeed the LMM analysis confirms that GluCEST and volume measures in the dorsal regions could not be modeled by time or genotype, but rather show more complex dynamics throughout lifetime.

We suspect there are both developmental effects and pathologic effects creating these dynamics. We propose two potential causes for increased glutamate during lifetime. One process that may elevate glutamate levels is the increased concentration of glial cells throughout lifetime. It is known that GFAP immunostaining increases during healthy aging in mice (Kohama et al., 1995), rhesus macaques (Haley et al., 2010), and humans (David et al., 1997). While the greatest concentration of glutamate occurs in neurons (10-15mM, 7x greater than that of other metabolites), there is a substantial contribution from glial cells (6-10mM) (McKenna, 2007). A proliferation of glial cells around 7 months of age in WT mice could contribute to an increase in GluCEST, given that the GluCEST signal is directly proportional to the amount of total free glutamate in the brain.

The delayed increase in GluCEST measured at 9 months in PS19 mice appears to be correlated with a 20-fold increase in GFAP presence at this age (Figure 37c). Increased GluCEST was only measured in the clusters containing the DG likely due to the greater density of neurons and glial cells here. In contrast, this effect was likely counterbalanced
in the CA1 and CA2,3 regions by a decrease in synapse density (both general and glutamatergic). GluCEST appears to be most closely associated with the combined effects of synaptophysin and GFAP immunostaining.

A second process that could increase the glutamate in dorsal regions of the hippocampus is the phenomenon of neurogenesis. In AD patients (Jin et al., 2004b) and in a mouse model of AD (Jin et al., 2004a), there is evidence that neurogenesis is increased in the DG. In this study, as well as our previous work (Crescenzi et al., 2014), glutamate is elevated in PS19 mice over WT in the DG of the middle slice (cluster M-D here) by 13 months (equivalent to late stage pathology studied previously). While histological confirmation of neurogenesis as the cause for increased GluCEST contrast in the DG requires further investigation, GluCEST is the only technique with potential to show this effect in vivo. This provides an important opportunity for future studies to use GluCEST technology as neurogenic therapies are developed (Chadwick et al., 2011; Chohan et al., 2011; Wang et al., 2010).

d. Considerations for future studies in congenic BL6-PS19 mice with tauopathy

Future studies in this mouse model should consider the time course of disease presented here, and the relevant regions of pathologic symptoms. Immunostaining for tau pathology was indistinguishable between 9 and 13 months in the PS19 hippocampus (Figure 36c-d). By 13 months, immunostaining is difficult to quantify in the hippocampus due to severe tissue loss. Furthermore the greatest significant difference in GluCEST levels between WT and PS19 mice occurred at 7 months, at the same age that significant differences were measurable in synapse density. Therefore, 9 months is a sufficient
endpoint where pathologic events including the formation of pathologic tau, synapse loss, volume loss, and even metabolic dynamics have already occurred.

In this work, mice were studied every 3 months. The interval between 6-9 months appears to be the period of greatest change in pathology including gross morphologic changes of the hippocampus (Figure 36b-c), a sharp reduction in synapse density (Figure 37a), and a shift in location of GluCEST deficits from dorsal to ventral (Figure 34c). It would be interesting to study PS19 mice during this time period more closely. Several remaining questions could be investigated: How sharply does hippocampal volume diminish, and is there a breaking-point of cellular stress which triggers neuronal death? If elevated GluCEST in young PS19 mice is indeed a measure of excitotoxicity, how is glutamate metabolism affected during the 6 month delay before neuronal death? For instance, calpain I is a calcium-dependent protease whose reaction yields reactive oxygen species (McCord, 1985). Calpain I was discovered to be a necessary mechanistic link between NMDA-excitotoxicity and pathologic tau degradation leading to cell death (Amadoro et al., 2006). Subsequently, is the increase in dorsal-hippocampal glutamate protective? GluCEST imaging could aid in in vivo detection of excitotoxicity, and the time course of neuronal death or therapeutic rescue.

V. Summary

The goals of this longitudinal study were to determine the earliest stage at which PS19 mice can be distinguished from WT by GluCEST MRI, and whether GluCEST changes are associated with synapese loss before volume loss occurs in the PS19 hippocampus. Additionally, histological evidence from age-matched littermates supporting MRI-observed changes in glutamate levels was explored using a variety of cell-type specific antibodies.
(NeuN and GFAP), and glutamate transporter (VGlut1) and receptor (NMDA-NR1) antibodies. Sub-regions of the hippocampus were analyzed in order to associate location-specific changes in MRI and histological measures. The most significant difference in GluCEST between WT and PS19 mice was measured at 7 months of age in the CA1 and DG sub-regions (30% reduced from WT, p≤0.001). Synapse density was also significantly reduced in every sub-region of the hippocampus in PS19 mice by 6 months. Volume loss was not significantly different until 13 months between PS19 and WT mice. Further, a gradient in glutamate levels was observed along the dorsal-ventral and rostral-caudal axes within the hippocampus, where the highest GluCEST values were measured in the caudal-dorsal hippocampus. Dynamics in glutamate levels of the WT and PS19 hippocampus were most closely correlated with the combined changes in synapse density by synaptophysin and glial cell reactivity by GFAP IHC. The following are highlights of these results:

- Hippocampal glutamate levels are significantly decreased in early stages of PS19 tauopathy compared to WT, which is concurrent with synapse loss before structural volume loss.
- Glutamate levels increase from 3-7 months in the BL6-WT mouse hippocampus.
- Glutamate levels increase at 9 months and 13 months in the DG and CA1 of the PS19 hippocampus, which is associated temporally with increased GFAP immunostaining.
- GluCEST MRI measured a gradient in glutamate levels in vivo along the dorsal-ventral and rostral-caudal axes of the mouse hippocampus.
CHAPTER 6: Glutamate CEST MRI measures elevated glutamate in vivo in MPTP Mouse Model of Parkinson's Disease

I. Introduction

MPTP toxicity in mice is a well validated model of early glutamatergic excitotoxicity in PD pathology (Jackson-Lewis and Przedborski, 2007). The aim of the current study was to use GluCEST MRI to measure regional changes in glutamate levels in the MPTP mouse model of PD, in order to investigate its potential as a diagnostic tool. MPTP toxicity is spatially selective to dopaminergic neurons in the SNc, thereby increasing glutamate in the striatum and not in regions such as the thalamus. Therefore, GluCEST was hypothesized to be elevated in the striatum and not in an unaffected region such as the thalamus. GluCEST measurements in the striatum were compared with glutamate concentration changes measured by $^1$H MRS in this region. In addition, GluCEST contrast was correlated with two previously established measures of disease in the MPTP model: neurobehavioral measures of motor dysfunction and immunohistochemical markers.

GluCEST imaging is the first technique with potential to show the spatial selectivity of MPTP toxicity which previously has not been reported at high-resolution in vivo. This study is also the first to apply GluCEST to a disease that is marked by increased glutamate levels without the confounding effects of pH changes.
II. Methods

a. MPTP administration and animal care

The IACUC of the University of Pennsylvania approved all the experimental protocols in this study. C57BL6 mice were procured from the Charles River Laboratory, Horsham, PA, USA and kept at the ULAR animal housing facility at the University of Pennsylvania. Mice were housed in a humidity controlled room ~22°C under a 12 hour light/dark cycle with ad libitum access to food and water. Mice aged 3 months old were divided into two groups: control (n = 11) and MPTP (n = 13). Mice in the MPTP group received MPTP (25 mg/kg, via intraperitoneal injection, Sigma/Aldrich, St. Louis, MO, USA) dissolved in normal saline once a day for 7 days, while the control mice received the same volume of normal saline for the same period.

b. Neurobehavioral testing

Forepaw grip strength test was performed on both groups of mice on the 0\textsuperscript{th} and 8\textsuperscript{th} days of treatment using a device described previously (Bagga et al., 2013). Briefly, an apparatus consisting of a steel rod of 1.5 mm diameter and 30 cm long positioned horizontally between two vertical columns 30 cm high. Mice were made to hold the rod using fore limbs and their tail was restrained to prohibit the use of hind limbs. The duration of hanging on the rod was monitored for both groups of animals. The forepaw grip strength was measured as the holding impulse (weight × duration of holding) in order to normalize the effect of different mass of the mice.

The four limb hanging test was performed on both groups of mice on the 8\textsuperscript{th} day of treatment. The testing procedure implemented here was modified from a protocol
previously described (Anandhan et al., 2012). The mice were placed on the top of a
standard wire cage lid and were made to hold for a few seconds before turning the lid
upside down. The latency of mice to fall off the wire lid was measured. The trial was
repeated 3 times (5 minutes apart) and the average value was computed. The four limb
endurance was reported as holding impulse (holding impulse = weight × duration).

c. Acquisition and processing of anatomic MRI, GluCEST MRI, and $^1$H MRS

Mouse husbandry and handling during imaging was identical to that stated in

Axial T$_2$-weighted (TR/TE = 3000/36 ms, matrix = 128 × 128, FOV = 2 cm, NEX = 2,
o. of slices = 16, slice thickness = 1 mm) brain images were acquired. Quantification of
brain ventricle volume was performed using a homemade script in MATLAB (v2009b). The
regions of interest were manually delineated for the third and fourth ventricles and the
cerebral aqueduct combined over the left and right hemispheres. The ventricle volume
was measured as the summed volumes (in voxel units) of the ventricle from multiple slices.
The whole brain volume was also measured as a reference, covering 8 slices from the
rostral end of the forebrain to the caudal end of the cerebellum; the voxel volume of all
slices was summed in order to obtain the whole brain volume. The ventricle volume was
calculated as the ratio of the number of voxels in the ventricles to that in the whole brain.

$^1$H MRS was performed on control (n = 10) and MPTP treated (n = 14) mice using the
PRESS pulse sequence (TR/TE = 3000/28 ms, spectral width = 4 kHz, number of points
= 4006). The WET water suppression technique was used to acquire a water-suppressed
spectrum (averages = 512), and another spectrum was acquired without water
suppression to obtain the water reference signal for normalization (averages = 16).
Unsuppressed water spectra had a line width of 20 Hz or less after localized shimming. Spectra were acquired from a voxel localized in the striatum (2.25 × 2.25 × 2.25 mm^3). All spectra were acquired while gating to respiration. Total acquisition time for spectroscopy was about 20 min.

Metabolite concentrations measured by in vivo \(^{1}\)H MRS were quantified using the LCModel software (Provencher, 2001). A spectral range of 0-4.2 ppm was used for analysis. The following metabolites were included in the basis set: alanine, Asp, Cr, GABA, glucose, Glu, Gln, glycerophosphocholine, phosphocholine, MI, Lac, NAA, N-acetylaspartylglutamate, phosphocreatine, scylo-inositol, and taurine. In addition, 9 pre-simulated macromolecule signals were incorporated in the basis set. The concentration of metabolites was measured using the unsuppressed water peak as a concentration standard. Water content in the brain was assumed to be approximately 80%. Metabolites having a CRLB <20% were considered for further analysis (Srinivasan et al., 2004).

A custom-programmed RF spoiled GRE readout with a frequency selective saturation preparation pulse sequence was used as described previously in Chapter 2, section II.c. “GluCEST MRI protocol in mice” on page 30. The following sequence parameters were used in this study: saturation preparation pulse power (B_{rms} = 6uT and duration of 1s as four pulses of 250 ms duration each), GRE readout (effective TR/TE = 6.2/2.9 ms, number of averages = 4). The entire preparation/acquisition combination was repeated every 8s. GluCEST images were collected with saturation frequencies from ± 2.5 – 3.5 ppm offset from water with a step size of 0.25 ppm.

GluCEST, B_0, and B_1 maps were acquired from 2 slices (2mm thick each). One slice included the striatum where elevated Glu is specifically hypothesized to occur in the MPTP model of PD. The second slice included the thalamus region as a control. Segmentation
of the striatum and thalamus was performed manually on the T2-weighted anatomical MRI, and ROIs were overlaid on the GluCEST maps.

d. **IHC protocols for tyrosine hydroxylase and GFAP**

Mice from the first cohort were utilized for IHC: control (n=5) and MPTP treated (n=7). Mouse brain tissue was prepared for histology following the methods outlined in Chapter 3, section II.d. “General tissue preparation and methods of immunohistochemistry” on page 46.

The primary antibodies used in this study include tyrosine hydroxylase (TH, RbTH UPN88, Millipore, 1:1k dilution, †) which is specific for dopaminergic neurons, and glial fibrillary acidic protein (GFAP, stock prepared at the CNDR, 1:2k dilution, ‡) to monitor the glial cell response. GFAP immunostained slides were counterstained with hematoxylin.

Stained slides were scanned using an automatic slide scanner (Lamina Scanner, PerkinElmer). Scanned slides were viewed using CaseViewer (freeware, v1.3.0.41885). Quantification methods were performed in Image J (NIH) and averaged over the left and right hemispheres for all antibodies. The mean intensity of TH immunostaining in the striatum was quantified from square ROIs segmented from 3.5x images (Figure 43A). ROIs from the color image (without counterstain) were converted to gray-level intensity for quantification. In the SNc, the punctate dark-brown stain was selected using a fixed threshold for all mice (Figure 43F), and the percent area occupied (%AOC) was calculated over a square ROI cropped from 10x images.

In order to quantify GFAP immunostaining, 3.5x images from the striatum and thalamus were analyzed by the color deconvolution plug-in (Ruifrok et al., 2001) using the “H DAB” color optical densities provided. ROIs were selected over the entire striatum and
thalamus from the counter-stained image and applied to the brown image. A consistent threshold was applied, and the %AOC by GFAP immunostaining was calculated.

e. Statistical analyses

For all results, a two-tailed non-equal variance Student’s t-test was used to compare results from MPTP treated with control mice. A significance level of at least $p \leq 0.05$ was determined using the Bonferroni correction. Mean values are reported and graphed, where error bars represent the standard deviation.

III. Results

a. Neurobehavioral observations

Treatment with MPTP did not cause any alteration in the weight of mice ($p > 0.61$). Sub-acute administration of MPTP to mice led to a significant reduction in the fore paw grip strength (Day 0: 1331 ± 167 g.sec, Day 8: 856 ± 211 g.sec; $p \leq 0.001$) while it was unchanged in the control mice (Day 0: 1336 ± 237 g.sec, Day 8: 1313 ± 234 g.sec; $p = 0.80$, Figure 39A). Additionally, four paw hanging test results were found to be significantly reduced in mice treated with MPTP on Day 8 (32.1 ± 11.3 N.sec) compared to control mice on Day 8 (55.8 ± 11.9 N.sec, $p \leq 0.001$, Figure 39B).
Figure 39. Motor function in control and MPTP mice. A. Fore paw grip strength test shows a significant reduction after MPTP treatment (p≤0.001, percent difference: -35.7%). B. Four limb hanging test shows a significant reduction with administration of MPTP (p≤0.001, percent difference: -42.4%).

b. Ventricle volume

The ventricles are enlarged in MPTP treated mice which is qualitatively apparent from T2-weight images (Figure 40A). It was found that sub-acute exposure to MPTP leads to a significant increase of 30.8% in the volume fraction of ventricles in the brain (Control 3.38 ± 0.43 %, MPTP 4.42 ± 0.52 %; p≤0.001, Figure 40B).
A. Ventricle volume is increased in MPTP mice which can be seen from a T2-weighted MRI. B. Significantly larger percent ventricle volume of the whole brain was measured in MPTP 4.42 ± 0.52 % mice versus control 3.38 ± 0.43 %, p≤0.001.

c. Neurometabolite levels measured by $^1$H MRS in the striatum

Qualitative comparison of the localized $^1$H MRS of the striatum showed elevated levels of Glu following treatment with MPTP (Figure 41). LCModel analysis was performed to measure changes in the concentration of metabolites (mmol/g). MPTP treatment led to a significant increase in the concentration of Glu (Control: 12.8 ± 1.9; MPTP: 14.8 ± 2.6 mM, p<0.01), GABA (Control: 2.8 ± 0.5; MPTP: 3.6 ± 0.7 mM, p<0.001), Gln (Control: 5.5 ± 1.4; MPTP: 7.0 ± 2.9 mM, p<0.05), and MI (Control: 5.9 ± 0.9; MPTP: 6.7 ± 1.1 mM, p<0.01, Figure 41B).
Figure 41. Neurometabolite levels measured by $^1$H MRS in the striatum.
A. Spectra from example control and MPTP treated mice. Red horizontal lines mark notable trends of elevated Glu (2.35 ppm) and Glx (3.75 ppm), while NAA levels do not differ. B. Significantly increased concentrations of Glu (Control: 12.8 ± 1.9; MPTP: 14.8 ± 2.6 mM, p<0.01), GABA (Control: 2.8 ± 0.5; MPTP: 3.6 ± 0.7 mM, p<0.001), Gln (Control: 5.5 ± 1.4; MPTP: 7.0 ± 2.9 mM, p<0.05), and MI (Control: 5.9 ± 0.9; MPTP: 6.7 ± 1.1 mM, p<0.01) were measured.

d. GluCEST MRI in the striatum and thalamus

Glutamate CEST (GluCEST) images clearly show the result of elevated glutamate in the striatum following MPTP treatment (Figure 42A). This effect is specific to the slice containing the striatum, whereas the GluCEST contrast in the thalamus is indistinguishable between MPTP and control mice (Figure 42A-C). Indeed the average GluCEST asymmetry (%) in the striatum was significantly elevated in the right (MPTP:
26.1 ± 2.9, Control: 22.9 ± 2.5, p≤0.01), left (MPTP: 25.8 ± 2.8, Control: 22.9 ± 2.6, p≤0.01), and average of both hemispheres (MPTP: 25.9 ± 2.8, Control: 22.9 ± 2.5, p≤0.01, Figure 42B). The average GluCEST asymmetry in the thalamus remained equal in MPTP and control mice (MPTP: 25.8 ± 3.4, Control: 24.8 ± 3.3, p=0.46, Figure 42D).

Figure 42. GluCEST asymmetry in the striatum and thalamus of control and MPTP treated mice.

(A, C) GluCEST maps from control and MPTP treated mice and corresponding ROIs segmented in the striatum and thalamus. (B) Average GluCEST asymmetry (%) in the striatum, including right, left, and combined hemispheres, was significantly elevated in MPTP mice (**p≤0.01). (D) Average GluCEST asymmetry in the thalamus was not significantly different between control and MPTP mice.
e. IHC for dopaminergic neurons and GFAP

In the striatum, the intensity of TH immunostaining was reduced by 22.4% in MPTP treated mice (Control: 106.9 ± 11.8 mean intensity, MPTP: 82.9 ± 11.7, p≤0.001, Figure 43C). The area occupied by TH immunostaining (%AOC) was further reduced by 32.4% in the SNc (Control: 4.39 ± 0.91, MPTP: 2.96 ± 0.99, p≤0.01, Figure 43G). These results indicate a loss of dopaminergic innervations in the striatum due to a loss of dopaminergic neurons in the SNc.

The %AOC by GFAP immunostaining in the striatum was significantly increased in MPTP treated mice (Control: 1.53 ± 2.49, MPTP: 7.75 ± 1.79, p≤0.001, Figure 44C). In the thalamus the %AOC by GFAP was not significantly different between MPTP and control mice (Control: 1.09 ± 0.29, MPTP: 0.87 ± 0.37, p=0.76, Figure 44F). These results indicate the presence of astrogliosis in the striatum and not the thalamus following MPTP treatment.
Figure 43. IHC for tyrosine hydroxylase (TH) reveals a loss of dopaminergic neurons in the striatum and SNC.

A. Images of the striatum where ROIs were selected in the right and left hemispheres. B. The mean intensity was measured over the ROI after conversion to a gray-level image. C. TH was significantly reduced in the MPTP striatum (**p≤0.001). D. The SNC was located from a 2x image of the thalamus (E.) and a snap-shot was taken at 10x. F. A threshold was set to segment the dark-brown punctate stain, and reported as the %AOC. G. A significant reduction in TH was also measured in MPTP mice (**p≤0.01).
Figure 44. IHC for GFAP reveals astrogliosis in the striatum but not in the thalamus. A, D. 3.5x images were taken in the striatum and thalamus. B, E. The DAB image was extracted from the counterstained image using ImageJ color deconvolution. ROIs were drawn around each hemisphere of the striatum and thalamus. C, F. The amount of GFAP positive stain was quantified as the %AOC. A significant elevation was measured in the MPTP striatum (**p≤0.001), while GFAP did not differ in the thalamus.
IV. Discussion

a. GluCEST as a potential biomarker of elevated glutamate in PD

The clinical diagnosis of PD usually occurs after a substantial number of neurons in the SNc have degenerated. A suitable biomarker would allow treatment with putative neuroprotective agents to begin long before the significant and irreversible loss of neurons and would enable the assessment of disease modification. There is a need for PD biomarkers which include (1) prodromal or premotor stage biomarkers, (2) biomarkers of risk or susceptibility, and (3) motor stage biomarkers.

The findings presented in this study provide the first application of GluCEST as a potential biomarker for the early detection of PD in a preclinical model. GluCEST, a novel MRI technique, has been reported to provide high spatial resolution imaging of small decreases in the levels of Glu in the brain (Crescenzi et al., 2014; Haris et al., 2013a). Here we have demonstrated the ability for GluCEST imaging to measure increases in Glu in a location-specific manner in the MPTP mouse model of PD. GluCEST measured in vivo was 13% higher in the MPTP striatum while no difference was measured in the thalamus. This trend corroborates the increased Glu measured by MR spectroscopy in vivo in the striatum.

Increased Glu in the striatum of MPTP mice is associated with two physiologic effects: elevated extracellular Glu and the reactivity of glial cells. First, GluCEST contrast and the Glu signal measured by $^1$H MRS are sensitive to the total concentration of Glu. However, under normal physiologic conditions, the intracellular concentration of Glu (1-10 mmol/L) far exceeds that of the extracellular concentration (up to 100 µmol/L during neurotransmission). The contribution of extracellular Glu to the GluCEST signal is
negligible in healthy mice. Anatomical studies in a rat model of PD and in Parkinson’s patients reveals adaptive changes in striatal glutamatergic synapses suggestive of increased synaptic activity (Anglade, 1996; Ingham et al., 1998; Meshul et al., 1999). This change in glutamatergic synaptic function following DA denervation is confirmed by an increase in the basal extracellular level of striatal Glu as determined by in vivo microdialysis in a rat model of PD (Jonkers et al., 2002; Lindefors and Ungerstedt, 1990; Meshul et al., 1999).

The elevation in GluCEST in the striatum of MPTP mice was found to be associated with elevated GFAP immunostaining in this region; neither marker was increased in the thalamus as a control region. The presence of GFAP indicates the activation or reactivity of glial cells. Glial cells contain 6-10 mmol/L of Glu in healthy brain tissue (McKenna, 2007). Glial cells function in part to clear high concentrations of Glu from the extracellular space in PD (Vila et al., 2001). As glial cells remove Glu from the extracellular space, the glial contribution to total Glu levels would increase. Increased glial Glu concentration in the striatum would be reflected in higher GluCEST values. Glial cells with GFAP reactivity were previously reported to increase in number and size in response to MPTP toxicity (Dervan et al., 2004). Although the glutamatergic transporter function may be compromised in this model (Dervan et al., 2004), the effect of gliosis in the striatum as a response to dopaminergic neuron loss in the SNc would also increase total Glu levels and thereby GluCEST in the striatum.

V. Summary

The goal of this study was to determine the potential for GluCEST MRI as a biomarker of PD in a preclinical mouse model. A PD mouse model was created using the neurotoxin
MPTP. MPTP causes specific loss of dopaminergic neurons in the substantia niagra pars compacta which leads to decreased inhibitory signaling to the striatum and severe increases in the primary excitatory neurotransmitter, glutamate. Motor function is severely impaired in this model after the loss of dopaminergic neurons which was confirmed in this study by the forepaw grip strength test and the four limb hanging test. GluCEST imaging and $^1$H MRS were carried out in vivo in order to measure glutamate levels in the striatum and thalamus. Immunostaining for tyrosine hydroxylase as a marker of dopaminergic neurons and GFAP as a marker of glial cell reactivity was performed in the striatum and thalamus. GluCEST was 13% higher in the MPTP striatum while no difference was measured in the thalamus. This trend corroborates increased Glu measured by MR spectroscopy in vivo in the striatum. GFAP immunostaining in the striatum was also significantly increased in MPTP treated mice. The following are highlights from these results:

- GluCEST is capable of noninvasively measuring elevated glutamate levels in the MPTP neurotoxin model of PD.
- The high spatial resolution of GluCEST is advantageous as a potential biomarker of PD whereas elevated glutamate levels are specific to the striatum.
- Histological quantification of GFAP correlates with elevated glutamate measured by GluCEST in the striatum and not in the thalamus of MPTP mice.
Concluding statement: GluCEST is a promising biomarker of neurodegeneration

This thesis focused on application of the GluCEST MRI method to preclinical models of neurodegeneration. The first study presented in Chapter 2 utilized a well-characterized mouse model of the amyloid-beta plaque pathology of Alzheimer’s disease in order to highlight several advantages of GluCEST over $^1$H MRS. While the measurements of glutamate levels by the two modalities were shown to correlate, the enhanced sensitivity of GluCEST to glutamate changes as well as its ability to map glutamate levels throughout the brain was exploited. Chapters 3-5 discuss a mouse model of tau pathology, and investigate the underlying biochemical correlates to GluCEST contrast. Beginning in a late-stage preliminary study, and continuing in a longitudinal study, synaptophysin immunostaining was found to correlate spatially and temporally with the GluCEST contrast. Before pathologic tau protein can be detected, GluCEST deficits and synapse loss are measurable. This provides a promising early biomarker of tauopathies. Finally, glial cell reactivity (by GFAP immunostaining) may indicate elevated glial glutamate, as detected in vivo by GluCEST. This association was found to be true of later stages of pathology in tauopathy mice and in the MPTP mouse model of Parkinson’s disease. These findings exploit the ability of GluCEST to measure elevated glutamate in vivo, and provide a basis for the hypothesis of alterations in the glutamate-glutamine cycle function in astrocytes.

Results of this work demonstrate the utility of GluCEST imaging to study regional and temporal variations in glutamate in different pathologies associated with neurodegeneration. Translating the GluCEST technique to 7T human brain imaging is already viable.
ABREVIATIONS

\(^1\)H MRS Proton magnetic resonance spectroscopy
AD Alzheimer’s Disease
ADNI Alzheimer’s Disease Neuroimaging Initiative
a-KG alpha-ketoglutarate
AMPA \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP-PS1 amyloid precursor protein, presinilin-1; genes mutated in AD mouse model
ATP adenosine triphosphate
CBD Corticobasal Degeneration
Cho choline
CNS central nervous system
Cr, Cre, tCr total creatine
CSF cerebral spinal fluid
CTE Chronic Traumatic Encephalopathy
DLB Dementia with Lewey bodies
DS direct saturation
FDA Federal drug administration
FDG-PET fludeoxyglucose contrast age for positron emission tomography
FTD Frontotemporal dementia
FTDP-17 Frontotemporal dementia with Parkinsonism linked to chromosome 17
GABA gamma-aminobutyric acid
GDH glutamate dehydrogenase
Gln glutamine
GLT-1/EAA2, EAAT1 glutamate transporter, excitatory amino acid
Glu glutamate
GluR1 glutamate receptor
GRE gradient echo
GS glutamine synthetase
IACUC Institutional Animal Care and Use Committee
IHC immunohistochemistry
L-DOPA L-3,4-dihydroxyphenyalanine
MAO-B monoamine oxidase B enzyme
MAPT microtubule-associated protein
MCI mild-cognitive impairment
mGluR5 metabotropic Glu receptor subtype 5
MI myo-inositol
MPP+ methyl-4-phenylpyridinium
MPTP 1-methyl-4-phenyl1,2,3,6-tetrahydropyridin
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<tr>
<th>Acronym</th>
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<tr>
<td>MT</td>
<td>magnetization transfer</td>
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<tr>
<td>MTR</td>
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<tr>
<td>NAA</td>
<td>N-acetylaspartate</td>
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<tr>
<td>NFT(s)</td>
<td>neurofibrillary tangles</td>
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<td>NINCDS-NRDA</td>
<td>National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association</td>
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<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate glutamate receptor</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>PAG</td>
<td>phosphate-activated glutaminase</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PD</td>
<td>Parkinson's Disease</td>
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<tr>
<td>PET</td>
<td>positron-emission tomography</td>
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<td>PHF(s)</td>
<td>paired helical filaments</td>
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<tr>
<td>PiD</td>
<td>Pick's disease</td>
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<tr>
<td>PSP</td>
<td>Progressive supranuclear palsy</td>
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<tr>
<td>ROI(s)</td>
<td>region of interest</td>
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<tr>
<td>SNc</td>
<td>substantia nigra pars compacta</td>
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<tr>
<td>SNR</td>
<td>signal-to-noise ratio</td>
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<tr>
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<td>tricarboxylic acid cycle</td>
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<td>echo time</td>
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<td>repetition time</td>
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<td>vesicular glutamate transporter</td>
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<td>ventral tegmental area</td>
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