Quantitative, 3-D Multivoxel $^{1}$H-MRSI Studies of

*In vivo* Metabolism in a Rhesus Macaque Model of

HIV-Associated Neurocognitive Disorders

by

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Oded Gonen, Ph.D.
DEDICATION

For my parents, for their support over the years.
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I would like to thank my thesis advisor, Dr. Oded Gonen, without whom none of this would have been possible, for his role in inspiring this project, as well as his commitment to inspection and research integrity. I would also like to acknowledge my thesis committee, Drs. Riccardo Lattanzi, Sungheon (Gene) Kim, Lidia Glodzik and Eva-Maria Ratai, for their patience and understanding through the meetings and manuscript updates, past and current members of the laboratory who provided valuable advice and feedback during my nearly six years spent at New York University (NYU). Special thanks go to Drs. Ivan Kirov and Assaf Tal for their mentorship and graduate support, as well as Ke Zhang for his technical assistance. Lastly, I would like to thank my wonderful girlfriend, Magda, for her kindness and support through the final stages of writing this thesis.
PREFACE

During the summer of my first year in college, in 2001, I started a research fellowship as an undergraduate in the laboratory of Eric R. Kandel, M.D. – at that time a recent (2000) Nobel Prize laureate in Physiology or Medicine at Columbia University – and from there began to explore one of the “great” remaining frontiers in science: the human brain. It was while working amongst the many fine researchers and faculty there that I gained an understanding of science and how its impact could benefit mankind. It was also at Columbia where my interest in studying neurological disorders first grew.

After graduating from Columbia in 2004 with a B.A. in neuroscience and behavior, I began working on “functional” magnetic resonance imaging (MRI) approaches applicable to humans, nonhuman primates and mouse models in the laboratory of Scott A. Small, M.D. From 2004 to 2007, I worked on a project aimed at detecting MRI correlates of cerebral metabolism in nondemented elderly and early Alzheimer’s Disease patients. Realizing that I enjoyed studying imaging as a way to probe human disease and their animal models, I began a Ph.D. program in the fall of 2007 at NYU, where I would meet and work with Oded Gonen, Ph.D. Informed by his prior experience studying neuronal physiology using advanced quantitative neuroimaging techniques, I began a research thesis devoted to understanding the long-term neurocognitive disorders associated with HIV-
infection in a nonhuman primate model system. Utilizing three-dimensional proton-MR spectroscopic imaging, I took a “top-down” approach to identify novel biomarkers in, first, the global tissue, gray and white matter compartments, and second, in regional sub-structures important for cognition, to help localize where brain dysfunction begins and how it progresses over time.

Special thanks go to my research adviser, Oded, current lab members, Ivan Kirov and Assaf Tal who were helpful in the preparation of this work, my girlfriend, Magda, whose supportive company I have cherished, as well as friends and family. Finally, the members of my thesis committee deserve a great amount of gratitude for their patience and helpful feedback during the final stages of this dissertation. In 2013, I was awarded an International Society for Magnetic Resonance in Medicine Magna Cum Laude Merit Award for my work entitled, “Multivoxel Proton MR Spectroscopy Reveals Subcortical Glial Response to SIV-Infection in Rhesus Macaques.” I currently live in New York City.
ABSTRACT

Due to rising costs of sophisticated animal model systems, preclinical studies of neurological disorders often utilize nondestructive imaging modalities, such as MRI and proton MR spectroscopic imaging (\(^1\)H-MRSI), for evaluation of tissue injury and response-to-therapy. Previous \(^1\)H-MRS studies have typically used low spatial resolution (~3 cm\(^3\)) single voxels in select brain regions that also suffer partial volume averaging; consequently, these may incur greater quantification error and lower sensitivity to pathologic changes. To overcome these, we utilize high spatial resolution (0.125 cm\(^3\)) three-dimensional (3-D) multivoxel \(^1\)H-MRSI, localizing multiple tissue regions simultaneously, and collect spectra from hundreds of voxels, while also correcting for their partial volume effects. We first demonstrate this approach is highly reproducible in our chosen model of study, and that compared with its predecessor, single-voxel studies, multivoxel \(^1\)H-MRSI offers distinct advantages in both signal-to-noise ratio and spectral resolution.

Second, we apply this approach to a simian immunodeficiency virus-infected rhesus macaque model system of long-term HIV-associated neurocognitive disorders (HAND) that affect approximately 500,000 Americans infected with HIV. Prior histology has demonstrated neuronal injury and glial activation in brain gray and white matter (GM, WM). To assess the virus’ metabolic effects, we test the hypotheses that decreased \(N\)-acetylaspartate (NAA), the \(^1\)H-MRSI-observed
marker for neuronal integrity, and increased glial markers: myo-inositol, choline and creatine (mI, Cho and Cr), can be detected using multivoxel $^1$H-MRSI. We show that metabolic abnormalities occur both: $i$) diffusely, by averaging data from a large (~35% of total brain) volume-of-interest, and $ii$) regionally within subcortical structures important for cognition. We found evidence for indirect brain injury, through glial activation (as reflected by increases in markers mI, Cho and Cr), which may precede neuropathology, since global GM and the deep GM structures’ neuronal cell bodies were relatively spared (as reflected by their unchanged NAA). Global WM NAA declined, however, suggesting diffuse axonal injury consistent with prior studies. Taken together, these findings suggest treatment regimens should target gliosis as a possible strategy against HAND. In conclusion, $^1$H-MRSI may provide a highly reproducible alternative to costly and destructive histopathology for serial disease monitoring and aid in the design of therapeutic strategies.
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INTRODUCTION

HIV-associated neurocognitive disorders

Although the success of combination antiretroviral therapy (CART) has produced considerable delays in human immunodeficiency virus type 1 (HIV-1) progression to acquired immune deficiency syndrome (AIDS), and reduced incidence of severe dementia, a substantial ≥50% of the 40 million individuals infected worldwide [1] – of which approximately 500,000 are American [2] – will have their quality of life diminished by milder, long-term neurological complications, collectively known as HIV-associated neurocognitive disorders (HAND) [3]. Unlike other infectious neurotropic viruses, HIV is distinctly neurovirulent, producing neurocognitive impairment (NCI) in 50% or more of all HIV-infected persons, regardless of whether CART was taken or not [4]; this suggests NCI may be completely spared from the influence of CART [5]. NCI symptoms can range from subtle, minor cognitive impairment to severe dementia. Although HIV is a systemic infection, it particularly affects those parts of the brain that govern executive functions, including higher aspects of attention, information-processing speed and task-dependent behavior [6, 7]. Moreover, HIV-infected persons with NCI still suffer from decreases in quality-of-life and work productivity [7].
Moreover, mounting evidence [8, 9] suggests that even while virus is maximally suppressed in cerebrospinal fluid (CSF) and blood plasma, it may still facilitate development of dementia. This is thought to be due to CART’s poor penetration into the central nervous system (CNS), allowing viral “reservoirs” to accumulate inside infected monocytes, macrophages and microglia [10]. Over time (up to 20+ years), these ultimately produce a toxic cascade that results in neuronal death [11]. In those surviving patients CART response has appeared to decline [12], which is perhaps not surprising given the highly variable nature of HIV – a single mutation may become resistant to standard treatment regimens the longer they are used [9]. These suggest global health burden may only increase as patients live longer. The longer life expectancy of patients combined with the sustained prevalence of neurodegenerative causes emphasizes the need for longitudinal in vivo methods of detection and monitoring.

Limitations of human HIV studies

Unfortunately, in practice, longitudinal HAND studies are often challenging, for (at least) three reasons: (i) unknown date-of-infection, making disease change as a function-of-time studies prone to error. Indeed, given the observation that late HIV+ diagnosis is common – approximately 33% of all cases were within one year of subsequent diagnosis of AIDS – it is suspected infected persons may have had the disease for as many as 10 years before [13]; (ii) comorbid conditions such as
drug abuse, hepatitis C infection, and aging [14] can often preclude diagnosis of
dementia in patients with HIV; (iii) medication and interventional therapies can
also have effects that make understanding of disease neuropathology more
complicated. These factors combine to make studying HAND in humans difficult
to do in practice.

Moreover, (iv) issues of pathologic sensitivity and specificity hobble the current
de facto standard diagnosis of HAND, comprehensive neuropsychological testing,
which despite the ability to reveal deficits in neurocognitive functioning, offer no
insight into the cause or pathogenesis that precedes cognitive decline. In addition,
although much has been learned about HAND from histological exam of HIV-
infected brains, (v) acquiring such data is invasive, and, if received post mortem,
does not afford the patient opportunity to intervene and possibly delay, or even
reverse, progression of disease. Lastly, (vi) postmortem studies of HAND are now
rare due to the sporadic nature of postmortem cases owing to the success of CART
in dramatically reducing the lethality of HIV-infection. Nonhuman primate models,
therefore, may provide an alternative to all of these for studying neuropathogenesis.

**Nonhuman primate models of HAND**

It is a general axiom of comparative biology that when investigating the
processes involved in disease progression, or, for that matter, its prevention or
treatment, the more closely related the animal model is to humans, the more closely
the animal model’s biology will mimic human biology, and the more relevant the findings will be for human disease [15]. In principle then, the most relevant animal model for HAND research would be the chimpanzee (Pan troglodyte), or the bonobo (Pan paniscus), equally close relatives of humans [16]. However, there are major drawbacks to the use of these large (almost equivalent in size and weight to humans) primates, including their prohibitive acquisition and high maintenance costs, their status as endangered species [17], and their (>50 years) longevity in captivity [16], which inexorably make studies of chronic disease such as HAND lengthy, costly and potentially underpowered. It has therefore come to pass that the rhesus macaque (Macaca mulatta; shown in Fig. i-1) has largely been used for animal research as a compromise between evolutionary relatedness and practical concerns. Macaques and humans share both morphological and functional characteristics [17] (e.g., body composition comprised of fat and lean body mass, characteristically slow life history, delayed breeding, high survivorship, etc.), idiosyncratic traits (e.g., a menstrual cycle), as well as chronic diseases that affect cognitive function [18, 19] and cerebrovascular health [20-22]. Rhesus monkeys have been used to study perplexing human health problems and are a valuable step before substances/interventions can be tested in Phase I human studies [23]. Some well-known examples include the rabies, smallpox and polio vaccines as well as AIDS advancements, all facilitated by the use of rhesus macaques [24].
Rhesus macaques are also remarkably able to replicate the clinical symptoms of AIDS after infection with strains of the simian immunodeficiency virus (SIV), e.g., severe weight loss, opportunistic infections, lymphoid depletion and CNS lymphoma [25]. The SIV-infected rhesus macaque also develops neuroAIDS and provides an excellent model of HAND in humans [26], enabling studies that investigate brain injury and repair as a function of time post-infection while also excluding secondary conditions (e.g., drug abuse, opportunistic infections, CART).

Fig. i-1. A nonhuman primate model of neuroAIDS: the simian immunodeficiency virus-infected rhesus macaque (*Macaca mulatta*).

Because of their genomic, morphologic and biologic similarities, SIVs are the closest known relatives of the HIVs (types 1/2) [25]. All SIV/HIVs belong to a
subfamily of lentiviruses that share a common origin, genomic organization and pathogenesis [25]. The virus isolated from captive macaques, SIVmac, has sequence homology most similar to HIV type 2 (HIV-2) [27], but particular virulent strains, notably SIVmac251 and SIVmac239, have demonstrated virologic patterns similar to HIV-1 infection at the early, late, and intervening asymptomatic periods, with considerable similarity in host survival (e.g., a ~50% survival rate at two to four years) [28]. Importantly, SIV also expresses CD4+ cell tropism, inducing CD4+ T-lymphocyte loss and progression to terminal AIDS at a rate and frequency similar to that observed for HIV-1 infection in humans [25, 29-31]. SIV-infection of rhesus macaques appears to bear the closest similarity in pathogenesis (more than even the chimpanzee [32]) to HIV-infection and terminal AIDS in humans [32-34]. Similarities include invasion of the CNS through infected macrophages, development of encephalitis with characteristic multinucleated giant cells, perivascular macrophages, white matter lesions, and subtle white matter astrocytosis [35]. Cognitive and psychomotor disturbances in both monkey and human have also been described with disease progression [36-38]. Moreover, SIV-infected macaques develop SIV encephalitis (SIVE) at the same prevalence as HIV-infected humans, ~25%, and within the same 1 to 3 years timeframe [39, 40].

In a rapid-progression model, CD8+ T-lymphocytes, an important regulator of neuroimmune-activated function [41], are selectively eliminated using a mouse-human chimeric monoclonal antibody, as demonstrated by Schmitz et al. [41].
Prior histopathology studies in a CD8+ lymphocyte-depleted macaque model have confirmed that as early as 21 days after SIV-infection with persistent depletion, a rapid neuropathogenic and immunologic CNS response (perivascular macrophage accumulation) can be detected, and a high incidence of SIVE (80% of infected animals) by three months post-infection [42]. These animals showed increases in virus-laden perivascular macrophages and multinucleated giant cells in the CNS, astrogliosis, microgliosis and neuronal injury. In one histopathological study, the research “gold standard” for diagnosis, SIVE between the non-CD8-depleted and depleted models at comparable infection stages was observed to be “essentially identical [42].”

While examining SIV-infected macaques without CD8 depletion may perhaps be useful in studying the milder forms of encephalitis and neurocognitive decline, they can be prohibitively restrictive. First, similar to the human disease, a low percentage (~25%) of those inoculated go on to develop SIV encephalitis [43]. Second, if it does occur, prolonged progression (1 to 3 years) to terminal AIDS makes study of the relevant CNS changes too long and potentially underpowered. Therefore, to address these obstacles, we utilize an accelerated model [44, 45] that yields both: i) rapid disease progression; and ii) a high incidence of SIV encephalitis (developed by 80% of CD8-depleted animals three months post-inoculation [42]).
Costs of animal research

Disadvantages to using rhesus macaques as practical models of human pathogenesis and immunity can be summarized as follows: *i* the costs for purchasing and maintaining monkeys, although cheaper than chimpanzees, are still significant and must be multiplied 20+ times over their 20 to 30 year – longer for “pathogen-free” varieties – life span in captivity [17, 46]; *ii* their relatively long (3 to 5 years) developmental period from birth to sexual maturity; *iii* their low reproductive output of one offspring per year and seasonal breeding that occurs only during the fall [47, 48]; and *iv* a poor or inadequate record of captive breeding at National Primate Research Centers in the United States [24] – an important consideration for building colony size, half of which is kept for breeding [17, 24]. Additionally, the supply of captive-bred monkeys is limited due to high demand from bio-medical/defense companies for models of host pathogenesis/immunity and for vaccine development (e.g., AIDS vaccines) [24]. Furthermore, a legal ban on monkeys exported from India (a major natural habitat of the rhesus macaque) since 1978 [49] has further limited supply and driven up cost. Consequently, the cost per animal has increased from $1,000 in 1990 to $5,000 to $12,000 in 2003 [24]. Specific pathogen-free varieties can cost even more [24, 25].

Adding to the difficulty of obtaining animals (even if supply were not limited by biological or economic realities) are the maintenance constraints set by bioethical
policy – the Animal Welfare Act [50] and federal regulations (Institute for Laboratory Animal Resources’s Guide for the Care and Use of Laboratory Animals [51]) that mandate special housing and handling requirements. Criticism and media attention from The Humane Society for the United States [52] and other animal rights advocacy groups, such as People for the Ethical Treatment of Animals, who argue “not enough is being done in the way of finding alternatives [that minimize the use of primates in research] [24],” have also aimed to restrict how and when animals are used for research. All of the above factors combine to produce high cost (not to mention legal and ethical) barriers to doing experiments on monkeys in the same (destructive) fashion they are done on cheaper, more readily available animal models, such as mice.

Although studies of murine models of HIV infection would benefit from their rapid development, ease of handling and genetic modification, and prolific reproduction rates [53], to date, there is no mouse/rat model in which persistent HIV-1 infection takes place in vivo [53-55]. There are several major obstacles to efficient HIV replication in a murine model, described as follows: (i) murine cells lack HIV-1 cell receptor molecules, such as human forms of CD4 and chemokine receptor, CCR5, both of which are necessary for permissive HIV-1 entry [56, 57]; (ii) murine cells do not support efficient viral gene expression and lack factors necessary for assembly and release of virions [56, 58]; and (iii) mouse cells express a range of identified host factors that actively interfere with HIV
infection/replication [53]. These include cyclin T1 [59], defective Rev function [60], certain restriction factors (as reviewed in [61-64]), such as apolipoprotein B mRNA-editing catalytic enzyme, polypeptide-like 3G, TRIM5α, Lv1 and the murine splicing inhibitor, mp32 [65]. Although various transgenic mice/rats with human CD4 and CCR5 receptors, and splicing-associated factors have been developed [55, 65], these have found limited success due to the myriad of blocks at multiple stages in the viral life cycle, and therefore have only been used to study partially or in some specific aspects the early pathogenesis/antiviral immunogenicity [66]. Moreover, as yet unknown species-specific barriers are likely to be discovered and will also hinder development of a fully permissive HIV-1 infection murine model [53].

Feline immunodeficiency virus (FIV) models have also shown progressive immunodeficiency and neurological impairment similar to humans infected with HIV-1 [67, 68]. Studies on FIV-infected domestic cats have demonstrated a similar temporal course of infection, chronicity and severity to HIV-1 induced neuroAIDS [68-70]. However, concerns about the domestic cat’s lesser phylogenetic relevance as an animal model, as well as FIV’s related, but different, genomic sequence (e.g., FIV lacks the Nef, Vpr and Vpu proteins present in HIV [71]) and antigenicity (unlike HIV, FIV enters cells primarily through the CD134 receptor [72]; only chemokine co-receptor CXCR4 is shared with HIV [73]), have perhaps limited
interest in developing this model as a candidate for HIV infection/treatment strategies [74].

Lastly, all small animal models have historically been met with general skepticism as it remains unclear how relevant they are to HIV-1 infection in humans with an intact immune system [55]. Consequently, nonhuman primates, the closest known relatives (with 92.5 to 95% genetic homology [75]) to humans, have remained the animal models of choice, especially the rhesus macaque which is cheaper and more readily available than either the chimpanzee or bonobo, equally close relatives of humans [17, 75]. Moreover, rhesus macaques are similar to humans at the cellular and physiological levels – an important consideration for the treatment of diseases [15]. Indeed, Ward et al.’s survey of the literature [76] comparing the relative pharmacokinetics of 56 drug compounds cross-species in the rat, dog, monkey (a combination of rhesus and cynomolgus monkeys) and human found that oral exposure levels of the monkey was most similar to humans with the lowest absolute mean error (1.7-fold better than the dog and 6.2-fold better than the rat) and with the least bias. The authors concluded that the monkey was categorically and quantitatively more predictive of human pharmacokinetics than either the rat or dog, highlighting the continued importance of using macaque models for preclinical in vivo studies.
Thus, due to the costly nature of a destructive macaque study (especially if it involves serial observations), noninvasive, nondestructive modalities – in particular magnetic resonance imaging (MRI) for morphology and proton magnetic resonance spectroscopy ($^1$H-MRS) for physiology and metabolism – are often used as the probes of choice. Both MRI and $^1$H-MRS have proven useful in detecting brain abnormalities [77-80] and have provided critical knowledge of the dynamics of HIV-associated cerebral injury [81-84].

**Limitations of MRI studies**

In previous studies, MRI was used to detect structural abnormalities including atrophy, or white matter hyperintensities [85-87] and lesions, including toxoplasmosis and lymphoma, caused by the virus in the CNS [88, 89]. However, these structural abnormalities often appear too late in the clinical disease course, after irreversible tissue damage has taken place due to chronic disease conditions. Although MRI typically shows atrophy and diffuse WM hyperintensities at the chronic stages of infection, several $^1$H-MRS studies [84, 90] have reported early metabolic abnormalities in patients with HAND despite normal MRI appearance, suggesting $^1$H-MRS methods may be more sensitive in assessing CNS involvement.

**What is $^1$H-MRS?**

$^1$H-MRS is a noninvasive, nondestructive imaging technique that has been increasingly used to investigate a wide range of biochemical processes and systems
in the human body. It has shown both flexibility and versatility to provide specific, \textit{in vivo} biochemical information about a wide range of physiologic conditions, including changes brought about during brain disease, and restoration following treatment [91-93]. It has also enhanced the translation of biomedical research by using the same basic techniques to study various biological systems from the cell, to animals, to humans. These information can be evaluated both qualitatively and quantitatively to increase the specificity of diagnoses, especially since conventional MRI, while exquisitely sensitive to structural, soft-tissue changes in many neurological disorders, lacks high specificity valuable in making a diagnosis. Furthermore, $^1$H-MRS and MRI can be combined, making possible the evaluation of metabolic, functional and anatomical data from a single experiment [94].

$^1$H-MRS is well-suited for clinical applications and readily incorporated into a routine clinical MR protocol. It uses the same standard hardware as MRI and all major MR manufacturers now provide software to facilitate $^1$H-MRS exams [95]. Despite its early origins in nuclear magnetic resonance, $^1$H-MRS has only recently (within the last twenty-five years or so) entered the realm of medicine, where it was first employed clinically to study \textit{in vivo} biochemical processes in the human brain during the late 1980s and early 1990s [96, 97] to detect and quantify brain metabolites. Shortly thereafter, in 1995, $^1$H-MRS for brain examination received United States Food and Drug Administration approval and a medical billing code, allowing $^1$H-MRS to be interleaved with MRI sequences [98].
**Major resonances in a (rhesus macaque) brain spectrum**

A typical spectrum obtained from a healthy rhesus macaque brain (shown in Fig. i-2) at 3 T observes several key metabolites: N-acetyl aspartate (NAA) at 2.02 ppm, creatine (Cr) at 3.02 ppm, choline (Cho) at 3.21 ppm and myo-inositol (mI), with a single peak typically resonating at around 3.6 ppm. Of these, none has yielded more diagnostic information than NAA, an amino acid derivative synthesized only in neurons and transported down axons; it is therefore virtually 100% specific to the neuron and its axon, as shown in several immunohistochemical studies [99, 100]. Nearly all diagnostic applications of neurospectroscopy involve measurement of NAA, as it has been shown to be a marker for disease in virtually all brain disorders [101]. Its concentration decline is reflective of neuro-axonal dysfunction, and/or irreversible neuro-axonal loss.

The primary resonance of creatine (Cr) also includes phosphocreatine, which is in constant rapid enzymatic chemical exchange with creatine; phosphocreatine is important in adenosine triphosphate synthesis, and is therefore considered an “energy marker” of both neurons and astrocytes [95]. Choline (Cho)-containing components within the brain include myelin and fluid cell membranes that include phosphocholine and glycerolphosphocholine, as well as free Cho. An important caveat when interpreting Cho, however, is that the majority of choline-containing components are not normally soluble [95], but during pathologic conditions,
aque brain spectrum, with $TE/TR = 33/1440$ ms PRESS sequence used on a Magnetom TIM Trio 3 T MR imager (Siemens AG, Erlangen, Germany). Note that marked above with “·”-s are the exchangeable protons belonging to each metabolite.

membrane turnover (e.g., in tumor, leukodystrophy, multiple sclerosis, axonal degeneration) results in a massive increase in $^1$H-MRS-visible products containing Cho, providing important diagnostic information. Myo-inositol ($mI$), though not diagnostic per se, is considered a diagnostic “modifier” in diseases that affect Cho (tumor, multiple sclerosis, etc.). $MI$ is considered a marker of glial cells [102-104], which undergo “activation” – a process normally characterized by proliferation.

Fig. i-2. Major resonances in a rhesus macaque brain spectrum, with $TE/TR = 33/1440$ ms PRESS sequence used on a Magnetom TIM Trio 3 T MR imager (Siemens AG, Erlangen, Germany). Note that marked above with “·”-s are the exchangeable protons belonging to each metabolite.
Each of the above metabolites has a “normal” ratio that generates a signature pattern of resonances that is the same for each person, unless there is underlying pathology. $^1$H-MRS can therefore be evaluated either by comparing the numeric metabolite values against reference “normal” values, or by examining the resonance patterns for abnormal features. A caveat with the use of ratios involves assigning a metabolite as an internal reference in the denominator of a ratio (e.g., creatine is often used as an internal reference since its concentration is relatively stable in healthy subjects); although this is common in practice and provides certain advantages in quantifying results since the effects of different scanners and other instrumental factors (e.g., coil loading, receiver gain) are internally controlled for, it is less reliable during pathologic conditions (e.g., during epilepsy [105]) when metabolite changes are known to affect both the numerator and denominator.

Changes in the ratio do not necessarily reflect a change in the numerator (as is commonly supposed), since the denominator, or both, could have changed. These are reasons why absolute quantification methods are favored, although these have their own disadvantages, key amongst them being the time-consuming nature of acquiring $T_1$ and $T_2$ relaxation maps for each metabolite of interest for each patient at the same resolution. Post-processing data also becomes more complex.

**Localization**

Spatial localization is necessary when examining specific regions. Data needs to
be collected from a well-defined, spatially dependent region-of-interest (ROI) while excluding signal from unwanted surrounding tissue. Two common approaches for localization, Stimulated Echo Acquisition Mode (STEAM) and Point-Resolved Spectroscopy (PRESS), are described below:

(i) A volume is selected in STEAM by three consecutive slice-selective 90° pulses that generate a stimulated echo from the ROI with full localization achieved in a single scan. Advantages of the STEAM technique include a well-delineated ROI because of the good slice profile achieved by the frequency-selective 90° pulses, minor signal loss due to \( T_2 \) relaxation, excellent water suppression, localized signal that is less dependent on radiofrequency (RF) inhomogeneities, and reduced peak RF power requirements. The disadvantage of STEAM is that, compared to PRESS, its localization suffers up to 50% more signal loss [95].

(ii) The volume in PRESS is selected following application of a slice-selective 90° pulse followed by two slice-selective 180° pulses that generate a spin echo from the ROI. The principal advantage of PRESS over STEAM localization is the additional (2\( \times \)) signal-to-noise ratio (SNR) gained in the collection of a spin echo over a stimulated echo. One disadvantage is increased peak RF power is required to retain the same chemical shift displacement error when compared to STEAM localization [95].

**Single Voxel Spectroscopy (SVS)**
All SVS methods use modulated, frequency-selective RF pulses applied in the presence of a pulsed gradient field. To select a volume, three selective pulses are applied, one after another, in the presence of mutually orthogonal field gradients. The intersection of these three excited planes defines the ROI. *In vivo* studies typically use 3 to 8 cm³ ROIs. If voxel size is decreased, the amount of tissue present in the ROI is also reduced, and thus measurement time must be increased to maintain a consistent SNR. In order to optimize reproducibility, voxel placement must be the same every time. All other parameters can be automatically set as part of the protocol, leaving voxel position as the only factor that can affect reproducibility. This can prove problematic in certain focal diseases, such as brain tumors, where voxel position and size can vary, making reproducibility more dependent on operator skill. Small(er) voxel size also contributes to repositioning error, especially for serial studies. For nonfocal diseases, the size of the ROI typically neglects >95% of the brain, forcing the need for multiple exams to acquire sufficient brain coverage.

**Chemical Shift Imaging (CSI)**

Unlike SVS which acquires signal from a single ROI, the CSI technique can acquire signal from multiple voxels from a single plane. Phase-encoding gradients are employed to encode the spatial dimensions, and the MR signal is collected in the absence of any gradient in order to maintain spectroscopic information [106].
Each acquired voxel contains a MR spectrum that allows for assessment of a metabolic profile for a specific location and the visualization of the spatial distribution of metabolites [106]. CSI also allows for the acquisition of smaller volumes than in SVS techniques (as small as 0.4 cm³ at higher field strengths). Though typically more time-consuming than SVS techniques, CSI can examine a larger area of interest [106]. However, CSI suffers from the disadvantage that the shape of individual ROIs is less well-defined than in SVS. This can result in adjacent ROIs being contaminated by large amplitude signals from surrounding voxels [107], an effect known as “voxel bleed.”

**Three-dimensional proton MR Spectroscopic Imaging (3-D ¹H-MRSI)**

Similar to CSI, our proposed 3-D ¹H-MRSI further subdivides a volume excited by STEAM or PRESS into multiple voxels, with Fourier phase-encoding steps for a single plane. Unlike CSI, our MRSI also acquires a third dimension with Hadamard-encoded steps. While SVS is popular because it is widely available and does not require lengthy acquisition and post-processing, it suffers from a number of major limitations. First, only a small number of pre-selected brain regions can be covered in a single exam. This results in the inability to detect patterns of spectral heterogeneities in global diseases, such as HAND. Second, in order to achieve a high SNR, large, 3 to 20 cm³ [108], voxels are used. This can cause gray and white matter (GM, WM) partial volume effects, especially in deep GM structures, whose
volume is on the order of 2 to 7 cm³. In general, however, per given spatial resolution and measurement time, 3-D ¹H-MRSI yields the same sensitivity as SVS, but with the added advantage of spatial coverage [109, 110]. For example, in the time it takes SVS to obtain one, 1 cm³ voxel, our optimized 3-D ¹H-MRSI method obtains two-hundred-and-twenty-four. The resulting spatial resolution is high even for standard MRSI (1 to 4 cm³ [108]), and allows minimal partial volume with substantial, 28 cm³, macaque brain coverage.

A disadvantage of MRSI (and CSI) compared with SVS is the presence of signal contamination from neighboring voxels, which is intrinsic to Fourier encoding. Both quantification and localization may be affected by the estimated 13% inter-voxel signal leakage [111]. Importantly, multi-slice definition in the proposed 3-D technique is achieved with Hadamard as opposed to Fourier encoding. While intrinsic to the latter (CSI), voxel bleed can be controlled in the former (3-D ¹H-MRSI). This has enabled us to reduce inter-slice signal contamination to below 5% [112]. In addition, the smaller, 0.125 cm³, individual voxel size is advantageous because if a set of voxels is entirely within a structure, their “cross-talk” would be within identical tissue.

**Metabolic quantification**

The quantification of spectral peaks is of fundamental importance to ¹H-MRS. As opposed to clinical MRI, which relies on the detection of spatial or signal
abnormalities, $^1$H-MRS data is rarely assessed solely by visual inspection. The reason for this is that spectroscopic peaks reflect the concentrations of metabolites, and while there are guidelines for visual normal pattern recognition [113], $^1$H-MRS is usually quantified before interpretation.

After peak areas have been calculated by computer algorithms the resulting arbitrary units need to be converted into metabolite concentrations. Since the magnitude of the nuclear magnetization is directly proportional to the number of nuclei from which it originates, the relationship between the voltage induced in the spectrometer receiver coil (i.e., metabolite peaks) and the nuclear (i.e., molecular) concentration in the volume of interest can be described as:

$$S_m \propto \sigma_m \propto c_m \times n_s,$$

with $S_m$ the MR signal of metabolite “m”, $\sigma_m$ the total number of observed spins, $c_m$ the metabolite concentration, and $n_s$ the number of spins contributing to the resonance per molecule. The amplitude of the detected signal, however, is affected by a number of additional factors [Eq. (i.2)], some of which are unknown, preventing the direct calculation of molecular concentrations. Therefore, unlike clinical MRI, all approaches to spectroscopic quantification require the comparison of the detected signal with that of a reference.

The complete expression which describes the voltage signal from a sample containing a metabolite (m) can be expressed by:
\[ S_m \propto \beta \times c_m \times v \times n_s \times f(T_{1m}, T_{2m}, TR, TE, B_1). \]  

where \( v \) is voxel size, \( f(\cdot) \) is a "pulse sequence modulation" factor which depends on the pulse sequence used, its repetition and echo times (\( TR \) and \( TE \)), the metabolite \( T_1 \) and \( T_2 \), relaxation times, and \( B_1 \), the strength of the radiofrequency field within the voxel. \( \beta \) is a scaling factor that can be expressed as

\[ \beta \propto NS \times G \times f \times Q \times nxV_c/a, \]  

where \( NS \) is the number of scans (averages) performed, \( G \) is the receiver gain, \( f \) is the spectrometer operating frequency and \( Q, n, x, V_c, \) and \( a \) are all factors related to the geometry and quality of the radiofrequency receiver coil [114]. The proportionality constant for Eq. (i.3) is unknown, prohibiting the calculation of \( c_m \) without additional calibration measurements, hence the need for a reference. Using a "phantom replacement" method discussed in detail in the next section, a reference signal is acquired, ideally under identical conditions, yielding:

\[ c_m = c_{\text{reference}} \times S_m / S_{\text{reference}}. \]  

While the ratio in Eq. (i.4) cancels out \( \beta, v, n_s, TE \) and \( TR \) [Eq. (i.2)], accurate quantification requires correcting for metabolite relaxation times and in some cases
for $B_1$ errors due to $B_1$ inhomogeneities that can occur if the brain and reference scans are from different locations (i.e., “phantom replacement”). Note that these can occur even if the coil is highly homogenous. In such instances, a correction factor can be obtained by field mapping [115], or by measuring coil sensitivity profiles [116]. Sophisticated line-fitting software can also account for this instrumental factor [117, 118]. Importantly, quantification also requires correction for molecular environment factors reflected in the longitudinal, $T_1$, and transverse, $T_2$, relaxation times [119]. While concentration errors from $T_1$ variations can be reduced to under 5% by using a sequence with a flip-angle $\sim 90^\circ$ and recycle time, $TR \sim 1.2T_1$ [120], $T_2$ errors can be minimized (to under 5%) only in very short $TE$ acquisition schemes ($TE \lesssim 20$ ms) [119]. However, intermediate- ($TE \lessgtr 50$ ms) and long- ($TE \gtrsim 130$ ms) $TE$ spectra are usually preferred for their flatter baseline (due to signal reduction from components with short $T_2$ such as water and macromolecules), improved water suppression, less lipid (i.e., macromolecule) contamination and fewer peaks, leading to simpler interpretation [119]. Absolute quantification in such $T_2$-weighted acquisitions requires accurate estimation of metabolite $T_2$s. These (along with the metabolite $T_1$s) have been estimated previously in this model species at 3 T using the same experimental setup [121, 122], and will be corrected for using values obtained from those studies.

**Absolute quantification with phantom replacement**
The quantification strategy of choice for multivoxel $^1$H-MRSI in the context of these studies is the phantom replacement technique [123]: After a spectrum is recorded from the animal, a standard sample of known concentration is scanned under identical conditions. There is no additional scan time required, and on stable MR systems reference scans are not needed after every animal. Relative levels of the $i=$NAA, Cr, Cho, $m$I metabolites in the $j=1…224$ VOI voxels of the $k=1..5$ animals are estimated from their peak areas, $S_{ijk}$-s, using parametric spectral modeling software (SITools-FITT) developed by Dr. Brian J. Soher [117] using Cho, Cr, $m$I, NAA, glutamate and glutamine full model functions. The $S_{ijk}$-s are then scaled into absolute amounts, $Q_{ijk}$, relative to a 0.5 L sphere of known concentrations, $C_i^{vitr}=12.5$, 10.0, 3.0 and 7.5 mM NAA, Cr, Cho and $m$I (in water at physiological ionic strength to load the coil), with similar VOI size and position used in order to sample the $B_1$ profile as closely as possible:

$$Q_{ijk} = \frac{C_i^{vitr}}{V} \cdot \frac{S_{ijk}}{S_{iR}} \cdot \left(\frac{P_{j}^{180°}}{P_{R}^{180°}}\right)^{1/2} \text{ millimoles}, \quad \text{(i.5)}$$

where $V$ is the voxel volume, the $S_R$ is the sphere’s voxels’ metabolites’ signal, $P_{j}^{180°}$ and $P_{R}^{180°}$ the RF power for a nonselective 1 ms $180°$ inversion pulse on the animal and reference.

This method, however, is sensitive to magnetic field inhomogeneities as well as errors due to $T_1$ and $T_2$ relaxation times. To account for different relaxation times in...
vivo \( T_1^{vivo}, T_2^{vivo} \) and in the reference phantom \( T_1^{vitra}, T_2^{vitra} \), the \( Q_{ijk} \) in Eq. (i.5) are corrected with a factor for each metabolite, \( i \):

\[
f_i = \frac{\exp\left(-TE/T_2^{vitra}\right) \cdot 1 - \exp\left(-TR/T_1^{vitra}\right)}{\exp\left(-TE/T_2^{vivo}\right) \cdot 1 - \exp\left(-TR/T_1^{vivo}\right)},
\]

(1.6)

with macaque 3 T \( T_2^{vivo} \) and \( T_1^{vivo} \) values for NAA, Cr, Cho and mI taken from previous studies \[121, 122, 124\]. For \( T_2^{vivo} \) of mI, we used the human \( T_2^{vivo}=200 \) ms value reported by Posse et al. \[124\]. No age-related \( T_1/T_2 \)s differences were anticipated in this cohort of animals of similar ages as those in a previous report \[121, 122\]. The corresponding values measured in the phantom were \( T_2^{vitra}=483, 288, 200, 233 \) ms and \( T_1^{vitra}=605, 336, 235, 280 \) ms.

**Need for quantitative noninvasive biomarkers: HAND prediction**

\(^1\)H-MRS, by its biochemical nature, affords a way of identifying patho-specific changes associated with disease. For example, NAA is established as a reliable marker of neuronal integrity, and its decline has been associated with virtually all neurological disorders \[125\]. Previous studies in humans using \(^1\)H-MRS have demonstrated its ability to detect CNS involvement, as reflected by studies in neurocognitively-impaired patients showing elevated changes in mI and Cho (suggesting increased glial proliferation) in frontal WM and basal ganglia and decreased NAA (suggesting neuronal injury/loss) in frontal lobe \[126-128\]. Similar

findings have also been reported with use of the SIV-macaque model of
neuroAIDS during the acute stage of infection [129, 130]. This suggests ¹H-MRS
is both more specific (i.e., metabolite changes correspond to pathologic processes)
and more sensitive (i.e., changes occur before clinical symptoms or appearance of
atrophy/lesion) than MRI, and is therefore the preferred modality for neurologic
assessment.

Moreover, NAA decreases are consistent with neuronal loss described in
histopathological reports in HIV patients [131, 132], especially in the frontal
cortex, where widespread ~40% reductions were observed in groups with
dementia[90]. Significant NAA/Cr reductions were also found in subcortical WM
and mesial cortex of patients with mild to moderate cognitive impairment
compared to controls [82]. Increased Cho/Cr levels have also been found [133,
134], and may reflect astrocytosis or microglial proliferation, since glial cells have
three times the amount of Cho as neurons [135]. In this context, $^1$H-MRSI
provides a nondestructive – and noninvasive – tool for detecting brain metabolite
abnormalities, which could serve as biomarkers for dysfunctional cellular
processes (i.e. neuronal death/glial activation as shown in Fig. i-3) in intact
animals, facilitating longitudinal studies that histopathology does not. In vivo $^1$H-
MR spectra are similar in macaque and human brains and both post mortem and in
vivo $^1$H-MRS studies of SIV-infected macaques reveal metabolic abnormalities
[129, 136, 137] similar to those observed in HIV-infected human brains [84, 138].
For example, monkeys with acute SIV-infection exhibit similar in vivo $^1$H-MRS
patterns as those of acutely-infected HIV patients [137].

Unfortunately, SVS $^1$H-MRS studies in the “traditional” macaque model of
SIV-infection (i.e., inoculation followed by natural timeline development of two
years or until terminal AIDS) were insensitive to changes at the chronic infection
period, as reflected by the lack of significant change in all three of the examined
brain areas, despite histopathology revealing evidence of severe encephalitis and
major neuronal loss [139]. Although surprising at first, it should perhaps not be
unexpected that large partial volume contributions from GM, WM and CSF could
have masked changes, explaining why no changes were observed during a period marked by extensive morphological and tissue changes [42]. Further, variable brain injury amongst the animals may have also contributed to masking the temporal metabolite effects. Nonetheless, as the authors point out, there were still correlations between metabolite ratios and plasma viral loads during both the acute and chronic stages of infection [42].

**Limitations of SVS $^1$H-MRS**

Although several $^1$H-MRS studies have reported metabolite concentrations in the diseased and healthy macaque brain [130, 140, 141], all used single 1 to 4 cm$^3$ voxels placed over specific brain regions. This can produce MRS results that are equivocal in that a single voxel’s large size nearly guarantees in each voxel an admixture of GM, WM, and CSF, each of which contains different concentrations of metabolites. For instance, GM has been estimated to contain 1.5× higher NAA concentration than WM [142], and CSF contains very low concentrations of metabolite [143]. Therefore, metabolite concentrations that are uncorrected for the contribution of CSF are generally underestimated, while those uncorrected for the relative contributions of GM and WM are generally overestimated. Such varying tissue compositions within each voxel can make comparisons between animals difficult, and since pathologies often affect tissue types differently, these can compound the errors to reduce the statistical power needed to detect true
metabolite changes [119]. Indeed, Tal et al. recently quantified that a five to 10% absolute metabolite quantification error (more for relative quantifications) can occur when GM and WM partial volumes are not accounted for [144]. The use of a single, small (in comparison to the total brain volume) voxel also makes VOI misregistration errors in longitudinal or cross-sectional studies more likely, thereby reducing metabolite quantification precision and, consequently, reproducibility.

Since many studies do not account for how partial volume fractions from GM, WM, and CSF contribute to the total MRS signal, these have led not surprisingly to conflicting reports. In the basal ganglia, for example, although several studies report neuronal damage early on in AIDS [145-147], one does not [148]. Authors of this study believe this may be due to partial volume effects, notably contamination of GM by both CSF and WM, making reliable quantification of metabolite levels difficult. Since SIVE, especially in its later stages, is diffusely occurring throughout the CNS [42], single-voxels invariably suffer unknown partial volume contamination and miss >95% of this small, ~85 cm³, brain [149].

Unfortunately, addressing these issues by voxel placement alone is difficult since the 1 to 4 mm thickness and tortuous path of the GM cortical ribbon [150] make it notoriously difficult to place the 1.5 × 1.5 × 1.5 cm³ typical voxel in “pure” GM (or WM for that matter). Moreover, VOI misregistration would still be
a concern. Increasing the size of the VOI to reduce misregistration error would increase SNR but come at the expense of larger partial volumes, and decreasing the size would have the opposite effects.

**Advantages of combining 3-D $^1$H-MRSI with MRI segmentation**

The misregistration, SNR and partial volume issues can be corrected by combining $^1$H-MRSI quantification (as described above) with semi-automated high-resolution (1 mm$^3$) MRI segmentation. Due to the relatively small changes that occur during the course of HAND, it is especially important to accurately apply necessary corrections for the known variations in tissue composition that occur due to the spectroscopic acquisition. Using freely available segmentation software [151], WM/GM/CSF masks can be overlaid on top the $^1$H-MRSI grid to yield their respective contents in each voxel [152, 153]. This information can yield the global GM and WM metabolite concentrations by modeling the $^1$H-MRSI signal in each voxel as a linear combination of GM and WM concentration contributions. In this way, sensitivity to global GM and WM metabolic change is increased and variance due to tissue composition is decreased. Also, there is minimal signal spread to adjacent voxels due to voxel bleed (for reference, see section on “3-D $^1$H-MRSI”), and also less repositioning error, due to the much larger VOI (3.5 ×4.0 ×2.0 =28 cm$^3$) used to cover the brain.

**Reproducibility of metabolite concentrations in the rhesus macaque brain**
As 3-D $^1$H-MRSI becomes more prevalent in animal research, it becomes increasingly important to quantify its reproducibility, as this is necessary for adequately-powered study design (which often reduces the minimum number of animals required for statistical power, especially important given the increasing costs of animals like nonhuman primates; refer to previous section: “Costs of animal research”), and to distinguish true metabolic changes from instrumental noise. Due perhaps to cost and complexity constraints, only one study [154] is known to us to have assessed cross-sectional (inter-animal) and longitudinal (intra-animal) metabolite ratios in (just) several regions of the rhesus macaque brain, and none so far have assessed absolute metabolite concentrations for a large volume in this model species at 3 T.

The partial volume and coverage issues can be addressed with 3-D $^1$H-MRSI that can cover extensive (~35 percent) portions of the brain at well below 1 cm$^3$ spatial resolution [155, 156]. The variable $T_1$- and $T_2$-weighting incurred at various $TE>0$ and $TR<5T_1$ used by different $^1$H-MRS sequences, can be accounted for using the metabolites’ $T_1$ and $T_2$s that have been reported for this species at various magnetic field ($B_0$) strengths [121, 122, 157]. Differences due to specific imager and coil combinations can be addressed by absolute quantification against a reference [119, 158], leaving the inter- and intra-animal “biological” variations as the remaining confounds. Unfortunately, since MRS is intrinsically fraught with
noise, the above fluctuations are always exacerbated by instrumental variations that depend on the experimental setup [159].

To reduce the contribution of repositioning and instrumental noise, we averaged the phased and frequency-aligned spectra from all the voxels in the volume-of-interest (VOI) in post processing. (Note that any specific brain structure(s) or tissue type(s) can also be averaged within the 3-D $^1$H-MRS data set, if necessary depending on the hypotheses tested.) Absent a specific hypothesis, whole-VOI averaging yields a single average spectrum that is relevant to diffuse brain disorders with the substantial SNR improvement that is essential for reproducibility [158], at minimal cost in linewidth (spectral resolution) and repositioning errors. Consequently, Chapter 1 aims to: (i) establish the macaque brain’s absolute NAA, Cr, Cho and mI concentrations and their cross-sectional (inter-animal) and longitudinal (intra-animal) variations; and (ii) establish and demonstrate the method’s sensitivity to distinguish pathological change from biological and instrumental variations.

**Global GM and WM pathology in SIV-infected macaque brain**

One pathology-relevant question (with potential implications for targeted therapies) remains what the relative dysfunction is of the *global* GM versus WM. Prior histopathology of SIV-infected macaques has shown neuro-axonal dysfunction and death in both cortical and subcortical GM and WM regions [136,
developing encephalitis [161, 162]. Based on these findings, we test the hypothesis that decreases in global GM and WM NAA and elevated global GM and WM mI are already detectable in the rhesus macaque model of HAND.

To test this hypothesis and to overcome the spatial coverage restriction of single voxel $^1$H-MRS, in Chapter 2 we employ an approach that combines the data from high spatial resolution (0.125 cm$^3$) 3-D $^1$H-MRSI with tissue segmentation from the MRI [144]. This enables us to analyze hundreds of voxels cooperatively, thereby increasing the overall precision [144] – at the cost of sensitivity to possible regional variations. This approach yields the global GM and WM levels of NAA, mI, Cr and Cho in a large, 28 cm$^3$ (~35%) portion of the rhesus macaque brain. We compare them in five animals before and four-to-six weeks after SIV infection, when animals are persistently CD8$^+$ lymphocyte-depleted [163].

**Regional dysfunction in SIV-infected rhesus macaque brain**

Although $^1$H-MRS has previously identified region-specific metabolic responses to SIV-infection [130], this was confounded by use of low, 1 to 4 cm$^3$, spatial resolution single voxels, which inevitably suffer partial volume contamination from GM, WM and CSF. As described previously, if partial volumes are not accounted for, underestimation errors due to CSF, overestimation
errors due to differential GM:WM fractions, or both, are likely to produce 5 to 10% absolute quantification errors (and more for ratios) in estimates of metabolite concentrations [144].

The partial volume issues can be addressed using 3-D $^1$H-MRSI to cover extensive (~35 percent) portions of the brain at well below 1 cm$^3$ spatial resolution, affording the ability to measure individual substructures of the size and scale (typically 0.5 to 0.8 cm$^3$ for striatal volumes [164]) found in the rhesus macaque brain. To increase spectral and spatial resolutions, a post-processing method of “zero-filling” in the time domain is applied [165, 166]. The increases are accomplished through the artificial adding of zeros to the end of the free induction decay acquisition, thereby increasing the number of acquisition points, and, consequently, the number of overlapping, interpolated voxels (i.e. a finer grid/bin size). Although no new information is being added to the data, this may increase the effective spectral/spatial resolutions after application of the Fast Fourier Transform and has been demonstrated previously to reduce partial volume effects [165, 166]. This may be due to the fact that smaller voxels are more likely to fall entirely within the same tissue moiety. Lastly, to improve SNR and reduce the contributions of VOI repositioning and instrumental noise, phase- and frequency-aligned spectra from all voxels within each ROI to yield a single average spectrum can result in 1.4 to 3.2× SNR improvement.
HIV-1 preferentially targets specific GM regions

Based on prior postmortem histopathologic studies of patients with HIV-infection and nondemented cognitive compromise, HIV-1 infection preferentially targets specific (subcortical) gray matter regions, in particular the basal ganglia (caudate nucleus, globus pallidus, and putamen) [167, 168], thalamus [169], as well as white matter [78] and hippocampus [170-172]. These regions are potential sites of neurotoxic disruption, as reflected by neuropathologic evidence of subcortical neuronal injury/loss, as well as glial activation consistent with a pattern of encephalitis and dendritic loss. Based on these findings, we test, in Chapter 3, the hypothesis that neuronal injury and glial activation occur in basal ganglia and other subcortical regions of SIV-infected rhesus macaque brain. We perform 3-D $^1$H-MRSI at 0.125 cm$^3$ spatial resolution over an extensive, 28 cm$^3$, volume of the macaque brain (centered along the corpus callosum) before and after SIV-infection of five healthy rhesus macaques. We then compare their absolute concentrations of NAA, Cr, Cho, and mI in atlas-based tracings of ROIs suspected in the pathology of HAND, i.e., the putamen, caudate and globus pallidus of the basal ganglia, as well as thalamus, hippocampus and centrum semiovale. If successful, $^1$H-MRSI detectable changes indicative of underlying neuro-axonal injury and/or glial activation in areas crucial for normal cognitive-motor function may explain the neurocognitive impairment often observed later in the disease, and possibly serve as their surrogate marker.
Thesis Central Premise

The thesis central premise is that 3-D $^1$H-MRSI may be able to detect metabolic changes reflective of SIV-related disease activity both \textit{i)} diffusely and \textit{ii)} regionally – at the level of substructures important for cognition – that may be indicative of early neurodegeneration and perhaps explain the associated neurocognitive impairments.

This central premise leads us to formulate the following three testable hypotheses, \textbf{H1} – \textbf{H3}:

\textbf{H1:} \textit{3-D $^1$H-MRSI is sufficiently sensitive to detect global brain abnormality during SIV-infection in as few as 5 animals.}

\textbf{H2:} \textit{Global gray matter and white matter neuro-axonal injury and glial activation occur in a live rhesus macaque model of HAND.}

\textbf{H3:} \textit{Neuronal injury and glial activation occur in basal ganglia and subcortical regions of SIV-infected rhesus macaque brain}

It has been difficult to test such hypotheses due to the diffuse nature of HAND and use of mostly single-voxel MRS that limited studies to MRI-visible pathology and very few foci, missing most of the tissue. These limitations will be addressed with: \textit{(i)} our 3-D $^1$H-MRSI that covers much (~35\%) of the brain, to test \textbf{H1}; and \textit{(ii)} combining this data with tissue segmentation methods to estimate, using a novel approach, the NAA, Cr, Cho, and $m$I level in \textit{all} the GM and WM, to test \textbf{H2}. Lastly, we will apply \textit{(iii)} 3-D $^1$H-MRSI and regional analyses of specific
regions susceptible to HAND pathology in order to test \textbf{H3}. To this end we monitored five healthy rhesus macaques \textit{pre}-infection and four-to-six weeks \textit{post}-infection in order to test \textbf{H1} – \textbf{H3} as described in the following chapters.
CHAPTER 1

Cross-sectional and Longitudinal Reproducibility of Rhesus Macaque Brain Metabolites: A Proton MR Spectroscopy Study at 3 T

ABSTRACT

Nonhuman primates are often used as preclinical model systems for (mostly diffuse or multi-focal) neurological disorders and their experimental treatment. Due to cost considerations, such studies frequently utilize nondestructive imaging modalities, MRI and proton MR spectroscopy (\(^1\)H-MRS). Cost may explain why the inter- and intra-animal reproducibility of the \(^1\)H-MRS-observed brain metabolites, are not reported. To this end, we performed test-retest three-dimensional brain \(^1\)H-MRS in five healthy rhesus macaques at 3 T. Spectra were acquired from 224 isotropic (0.5 cm)\(^3\)=125 \(\mu\)L voxels, over 28 cm\(^3\) (~35\%) of the brain, then individually phased, frequency aligned and averaged into a spectrum representative of the entire volume of interest. This dramatically increases the metabolites’ signal-to-noise

ratios, while maintaining the (narrow) voxel linewidth. The results show that the average $N$-acetylaspartate, creatine, choline and $\text{myo}$-inositol concentrations in the macaque brain are: $7.7 \pm 0.5$, $7.0 \pm 0.5$, $1.2 \pm 0.1$ and $4.0 \pm 0.6$ mM/g wet weight (mean±standard deviation). Their inter-animal coefficients of variation (CV) are 4%, 4%, 6% and 15%; and the longitudinal (intra-animal) CVs are lower still: 4%, 5%, 5% and 4%, much better than the 22%, 33%, 36% and 45% intra-voxel CVs, demonstrating the advantage of the approach and its utility for preclinical studies of diffuse neurological diseases in rhesus macaques.
INTRODUCTION

Due to similarities in physiology, anatomy and cellular function to its human counterpart, the rhesus macaque brain is used as a “pre-clinical” model system for various diseases, such as ischemic stroke [173, 174], Parkinson’s disease [175], schizophrenia [176], multiple sclerosis [177], and neuroAIDS [38, 130]. Due to the cost of such studies, especially if they involve serial observations, noninvasive imaging methods, such as MRI for morphology and proton MR spectroscopy (1H-MRS) for physiology and metabolism, are often the modalities of choice. Indeed, at the TE<40 ms that are often used, in vivo 1H-MRS can monitor the N-acetylaspartate (NAA), creatine (Cr), choline (Cho) and myo-inositol (mI) signals, the putative markers for neuronal integrity, cellular energy status, membrane turnover rates and glial proliferation, respectively [101, 102, 178-180].

Although several 1H-MRS studies have reported metabolite concentrations in the healthy macaque brain [130, 140, 141, 154], all used just single 1 to 3 cm³ voxels placed over specific isolated brain regions. Since most of the above diseases are diffuse throughout the CNS, such single-voxels invariably suffer partial volume contamination and miss over 95% of this small, ~85 cm³, brain [149]. Due perhaps to cost and complexity constraints, only one study is known to us to have assessed cross-sectional (inter-animal) and longitudinal (intra-animal) metabolite ratios in (just) several regions of the rhesus macaque brain. Although
the reproducibility metric is essential for adequately powered study design and to
distinguish real changes from biological and instrumental noise [154], its global
average values and their range has not been reported.

The partial volume and coverage issues can be addressed with three
dimensional (3-D) MRS that can cover extensive (tens percent) portions of the
brain at well below 1 cm³ spatial resolution [155, 156]. The variable $T_1$- and $T_2$-
weighting incurred at various $TE>0$ and $TR<5T_1$ used by different $^1$H-MRS
sequences, can be accounted for using the metabolites’ $T_1$ and $T_2$s that have been
reported for this species at various magnetic field ($B_0$) strengths [121, 122, 157].
Differences due to specific imager and coil combinations can be addressed by
absolute quantification against a reference [119, 158], leaving the inter- and intra-
animal “biological” variations as the remaining confounds. Unfortunately, since
MRS is intrinsically fraught with noise, the above fluctuations are always
exacerbated by instrumental variations that depend on the experimental setup
[159].

To reduce the contribution of repositioning and instrumental noise, we
averaged the phased and frequency-aligned spectra from all the voxels in the
volume of interest (VOI) in post processing. (Note, that any specific brain
structure(s) or tissue type(s) can also be averaged within the 3-D $^1$H-MRS data
set, if necessary depending on the hypotheses tested.) Absent a specific

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hypothesis, whole-VOI averaging yields a single average spectrum that is relevant to diffuse brain disorders with the substantial signal-to-noise improvement that is essential for reproducibility [158], at minimal cost in linewidth (spectral resolution) and repositioning errors. Our two primary goals, consequently, are: 

(i) to establish the macaque brain’s average NAA, Cr, Cho and mI concentrations and their cross-sectional (inter-animal) and longitudinal (intra-animal) variations; and (ii) establish and demonstrate the method’s sensitivity to distinguish pathological change from biological and instrumental variations. Towards these ends we applied test-retest 3-D multivoxel $^1$H-MRS to the brain of five healthy rhesus macaques.

**MATERIALS AND METHODS**

**Nonhuman primates**

Five (3 females, 2 males; 4.3 to 5.6 kg weight) healthy 3 years old adult rhesus macaques (*Macaca mulatta*) were scanned under constant veterinary supervision. Each was tranquilized with 15 to 20 mg/kg intramuscular ketamine hydrochloride and intubated to ensure a patent airway during the experiment (no mechanical ventilation was needed). Intravenous injection of 0.4 mg/kg atropine was used to prevent bradycardia. Continuous infusion of 0.25 mg/kg/minute propofol was maintained via a catheter in a saphenous vein. Heart and respiratory rates, oxygen saturation and end-tidal CO$_2$ were monitored continuously and a water blanket

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was used to prevent hypothermia. One macaque underwent CD8⁺ lymphocyte depletion (to facilitate rapid progression to AIDS and SIV encephalitis [163]) with an antibody (cM-T807) targeted against CD8 at 6, 8 and 12 days post-inoculation, was subsequently infected by intravenous injection with SIV₃mac251 virus (10 ng SIVp27) and then rescanned 6 weeks later. The protocol was approved by both the Harvard Medical School and Massachusetts General Hospital Institutional Animal Care and Utilization Committees.

**MRI**

All experiments were done in a 3 T MR whole-body imager (Magnetom TIM Trio, Siemens AG, Erlangen, Germany) with a circularly-polarized transmit-receive human knee coil. To guide the ¹H-MRS VOI and for tissue segmentation, sagittal and axial turbo spin echo (TSE) MRI: TE/TR=16/7430 ms, 140×140 mm² field-of-view (FOV), 512×512 matrix, 2.0 mm sagittal and 1.2 mm axial slice thickness, were acquired.

**VOI tissue fraction (Tf)**

Since the VOI also includes ventricles and sulci (see Fig. 1-1) correction for their CSF partial volume is required. To this end the TSE images were segmented with our FireVoxel package [153], as shown in Fig. 1-2. The process begins with automatic detection of a “WM seed,” (which, in reality, is a GM seed for T₂-weighted images as this option had not been updated at the time of this writing for
$T_2$-weighted images) yielding its average signal intensity, $I_{GM}$, for a GM region. Following selection of all pixels at or above 50% (but below 142.5% to exclude the CSF) of $I_{GM}$, a tissue-mask is constructed per slice in three steps: morphological erosion, recursive region growth retaining pixels connected to the seed; and morphological inflation to reverse the effect of erosion. Pixels of intensity above 142.5% of $I_{GM}$ were classified as CSF. The $T_f$ for each macaque was the tissue volume, $V_T$, (sum of all non-CSF pixel volumes in the VOI) divided by the 28 cm$^3$ nominal VOI.

**VOI GM and WM fractions**

Following a process similar to the above, all pixels at or above 90% (but below 142.5% to exclude CSF) of $I_{GM}$, were classified as GM, as shown in Fig. 1-2c. The GM fraction ($GM_f$) for each macaque was the product of the number of GM pixels × the pixel-volume, divided by $V_T$. Pixels below 90% (but above 50% to exclude air cavities) were classified as WM, as shown in Fig. 1-2d. The WM fraction ($WM_f$) was the product of WM pixels × pixel-volume, divided by $V_T$.

**Multivoxel 3-D $^1$H-MRS**

A 4.0 cm anterior-posterior (AP) × 3.5 cm left-right (LR) × 2.0 cm inferior-superior (IS) =28 cm$^3$ VOI was centered on the corpus callosum of each animal and angled along its genu-splenium line, as shown in Figs. 1-1a and 1-1b. We used the manufacturer’s automatic shim procedure, followed by manual
shimming, to adjust the first- and second-order shims to a consistent 26±1 Hz full-width-at-half-maximum (FWHM) VOI water linewidth. The VOI was then excited using PRESS (TE/TR= 33/1440 ms) with two second-order Hadamard encoded slabs (4 slices) interleaved along the IS direction every TR, as shown in Fig. 1-1b, for optimal SNR and spatial coverage [120]. Interleaving also allowed us to apply very strong, 18 mT/m, Hadamard slice select gradients to reduce the chemical shift displacement between NAA and mI to 0.2 mm, 4% of the slice width [181]. The slices’ planes were encoded with 16×16 2D-Chemical shift imaging over an 8×8 cm² (LR×AP) FOV to yield nominal (0.5 cm)³= 0.125 cm³ voxels. Although the actual voxel size (FWHM of the point spread function for uniform 2-D phase encoding) was actually 0.55×0.55×0.5≈ 0.15 cm³ [182, 183], for consistency reasons we will refer to its nominal size in the ensuing discussions. This localization grid yielded 8×7 voxels per slice in the VOI, as shown in Fig. 1-1, in each of the 4 slices. Given the 4.0×3.5×2.0=28 cm³ (AP×LR×IS) dimensions of the VOI, a total of 224 voxels fell within it. The MRS signal was acquired for 256 ms with 512 points at ±1 kHz bandwidth and each 16×16×4 scan took 12.5 minutes.

The MRS data was processed offline using in-house software. It was voxel-shifted to align the CSI grid with the NAA VOI, Fourier transformed in the time, AP and LR dimensions and Hadamard reconstructed along the IS direction. The 224 VOI spectra were each frequency-aligned and zero-order phased in reference
Fig. 1-1. **Left:** Axial (a) and sagittal (b) $T_2$-weighted TSE MRI of a macaque head shows the location and geometry of the $4.0 \times 3.5 \times 2.0 \text{ cm}^3$ VOI (solid white frame) as well as the location of the two second-order Hadamard slabs (4 slices) in the IS direction (1-1b). **Top right (c):** Real part of the $7 \times 8$ (LR\times AP) cm$^2$ $^1$H spectra matrix from the VOI in a, on common chemical shift and intensity scales. Note the spectral resolution and SNR (10.2±2.1, 5.3±1.9, 4.1±1.5 and 4.5±1.8
for NAA, Cr, Cho and \( mI \) obtained in these 0.125 cm\(^3\) voxels in 12.5 minutes of 3-D \(^1\)H-MRS acquisition. **Bottom right (d):** Metabolic maps for NAA, Cr, Cho, \( mI \) obtained from the spectra in e. Note the reproducibility of the gross anatomical features, e.g., lateral ventricles in the MRI in a, for each of the metabolites, reflecting the SNR and localization performance.

to the NAA peak, then averaged, retaining individual spectra linewidth and improved the SNR by \( 224^{\frac{1}{2}} \approx 15 \), as shown in Fig. 1-3.

Relative levels of the \( i^{th} \) (NAA, Cr, Cho, \( mI \)) metabolite in the \( j^{th} \) subject were estimated from their peak area, \( S_{ij} \), using the SITools-FITT parametric spectral modeling and least-squares optimization software of Soher et al. [117] that also provided the linewidth estimate. Analysis of this baseline modeling showed that for a 5 Hz spectral linewidth the mean fit errors are 3.4\%, 2.3\% and 2.8\% for NAA, Cr and Cho [159]. Seven model functions: NAA, aspartate, glutamate, glutamine, Cr, Cho and \( mI \), were used to fit our data. (Note that aspartate’s model function was included here as an “insurance policy” to prevent its narrower linewidth peak from being part of the baseline. Its signal value was not included for subsequent analysis – see the Results & Discussion.) The \( S_{ij} \)-s were then scaled into absolute concentrations, \( C_{ij} \), relative to signals from a 2 L sphere of \( C_{i}^{\text{vivo}} = 12.5, 10.0, 3.0 \) and 7.5 mM NAA, Cr, Cho and \( mI \) in water of physiological ionic strength (to properly load the coil) as [184]:
Fig. 1-2. Cerebrospinal fluid (CSF) segmentation of $T_2$-weighted MRI. **Top, left:** a: Axial $T_2$-weighted TSE image shows the anatomical coverage of the 4.0×3.5 cm² VOI. **b-d:** same as a, overlaid with the CSF, GM and WM masks generated by the FireVoxel package and used to obtain tissue, GM and WM fractions in the VOI for Eq. (2.2). Note the accurate CSF and each different tissue type differentiation.
\[ C_{ij} = C_{i}^{\text{vitro}} \frac{S_{ij}}{S_{R}} \frac{V_{ij}^{180\circ}}{V_{R}^{180\circ}} \frac{1}{T_{f}}, \]  

(1.1)

where \( S_{R} \) is the sphere’s metabolite signal, \( V_{ij}^{180\circ} \) and \( V_{R}^{180\circ} \) are the RF voltages needed for a non-selective 1 ms 180\(^\circ\) inversion pulse on subject and reference phantom. The \( C_{ij} \)'s were corrected for relaxation time \textit{in vivo} \( (T_{1}^{\text{vivo}}, T_{2}^{\text{vivo}}) \) and \textit{in vitro} \( (T_{1}^{\text{vitro}}, T_{2}^{\text{vitro}}) \) with a factor [185]:

\[ f = \frac{\exp\left(-TE/T_{2}^{\text{vitro}}\right)}{\exp\left(-TE/T_{2}^{\text{vivo}}\right)} \frac{1 - \exp\left(-TR/T_{1}^{\text{vitro}}\right)}{1 - \exp\left(-TR/T_{1}^{\text{vivo}}\right)}, \]  

(1.2)

using \( T_{1}^{\text{vivo}}=1.3, 1.3 \) and 1.1 s, \( T_{2}^{\text{vivo}}=316, 177 \) and 264 ms reported for NAA, Cr and Cho at 3 T [121, 122, 186]; and their \( T_{1}^{\text{vitro}}=605, 336 \) and 235 ms, \( T_{2}^{\text{vitro}}=483, 288 \) and 200 ms measured in the phantom. Since \( J \)-coupling modulation and low SNR for \( mI \) prevented obtaining its \( T_{2} \), we assumed \( f=1 \) for it. Finally, the \( C_{ij} \)'s were divided by \( T_{f} \) to convert into absolute tissue concentration.

**Strategy**

To determine \textit{intra}-animal reproducibility, two macaques each underwent three separate \(^1\)H-MRSI sessions and one macaque underwent two, each followup session two weeks after the previous one. To determine \textit{inter}-animal reproducibility, two more macaques each underwent one session (total of 10 scans from 5 animals). To determine the variance that is due to VOI repositioning and increase the metabolites’ SNRs, four back-to-back scans were acquired at each session with all experimental parameters kept unchanged for a total of 40 full scans.
a  Non-aligned VOI voxels sum

b  Aligned VOI voxels sum

c  Single voxel
Fig. 1-3. **Top, a:** Single 0.125 cm$^3$ voxel spectrum from a single scan in one of the animals. **Center, b:** Average of the 224 spectra in the VOI (equivalent to its single-voxel acquisition). Note the improved SNR but increased linewidths reflecting $B_0$ variations in the VOI. **Bottom, c:** Same as b but the spectra were pre-aligned before averaging. Note the high SNR as in b but linewidth similar to a reflecting the advantage of pre alignment to minimize susceptibility effects in the VOI and retain single-voxel spectral resolution.

16×16×4 3-D $^1$H-MRS data sets. To compare our results with previous single-voxel reports [130], *intra*-voxel coefficients of variation (CV) for each metabolite in each of the 224 voxels in the VOI were also calculated from the four back-to-back scans in every session. Finally, one macaque was infected with SIV, a disease of known widespread diffuse pathology, in order to examine whether the sensitivity gain facilitates identification of metabolic changes that may otherwise be either not statistically significant or obscured by the noise.

**Statistical Analyses**

Restricted maximum likelihood estimation of variance components in a random effects model was used to assess the *intra*-session, *inter*-session and *inter*-animal variance components of each metabolite. This method produces statistically optimal variance component estimates (in the sense of maximizing the likelihood of obtaining the data that were actually observed) subject to the constraint that estimates must be non-negative. Each CV was estimated by taking
the square root of the relevant variance component estimate and dividing this by the mean value over all measurements, as described by Chard et al. and Tedeschi et al. [187, 188]. In addition, the total variation of each metabolite was estimated as the variance among its 40 observations.

The *intra-voxel* variation (the “residual error” term in the model of Chard et al. [187]) is the component of the *intra*-animal variance that measures the variation expected between the four replicate measures derived for each of the 224 voxels in the VOI, the *intra*-session variation (a.k.a. *inter*-scan variation) is the component of the *intra*-animal variance that measures the variation that is expected between the metabolite levels derived from different scans within a single imaging session of a single animal. It was estimated by computing the variance among the four observations derived from the four repeat scans within a single imaging session of a single animal and then averaging these variances over all sessions and all animals. The *inter*-session variance is the component of the *intra*-animal variance that measures the variation expected between results derived from different imaging sessions of the same animal. The *inter*-animal component measures the expected variation between the true mean metabolite levels of two different randomly selected animals.
RESULTS & DISCUSSION

The position and geometry of the VOI for each of the scans are shown in Fig. 1-1. Typical spectra from the VOI and the metabolic maps generated from them (to demonstrate the quality of the spatial localization), are shown in Figs. 1-1e and 1-1d. Similar spectra were obtained from all animals. Spectral modeling produced a consistent voxel FWHM linewidth of 7.6±0.8 Hz (mean ± standard deviation), as shown in Fig. 1-3a. The metabolites’ SNRs for NAA, Cr, Cho and mI from the mean square of noise (measured from two 50 Hz samples at the beginning and end of the full 1024 Hz spectrum and then averaged), were 10.2±2.1, 5.3±1.9, 4.1±1.5 and 4.5±1.8.

The 224 phased spectra from the VOI were averaged to yield a single spectrum, as shown in Fig. 1-3b, equivalent to a single-voxel PRESS from this entire volume. While it exhibits a dramatic, ~224½, SNR gain due to the averaging, it clearly also suffers over 2.5-fold line broadening resulting from $B_0$ inhomogeneities over the entire VOI [189]. If on the other hand the 224 voxel spectra are pre-aligned, these inhomogeneities are corrected for and their sum then benefits from both the improved SNR and the narrow typical single-voxel linewidth, (FWHM of 6 and 8 versus 20 Hz), as seen by comparing Figs. 1-3a and 1-3c with 1-3b.
Fig. 1-4. Real part of the 224 aligned and averaged spectra from the entire 28 cm$^3$ VOI from each of the 5 animals (1, 2…5), serial sessions (up to three per animal:}
a, b, c) and the 4 back-to-back scans in every session (i, ii, ... iv) (thin black line), corresponding to the labels in Table 1-1. Each spectrum is overlaid with its fitted model function (thick gray line). All spectra are on common intensity and chemical shift scales. Note, (i) the higher (174±11, 96±6, 55±6 and 45±7 for the NAA, Cr, Cho and mI) SNRs for the averages compared with Fig. 1-1; (ii) the quality of the spectral fit; and (iii) the similarity of the spectra within scans, sessions and macaques, reflecting inter- and intra-animal reproducibility.

Consequently, the 224 spectra from each of the 4 scans of each of the 10 sessions (2 animals ×3 sessions + 1 animal ×2 sessions + 2 animals ×1 session) were aligned, then averaged. These 40 whole-VOI spectra are shown in Fig. 1-4, superimposed with the model functions used to obtain the $S_y$-s in Eq. (1.1). Their SNRs: 173.5±11.1, 96.1±6.4, 54.5±6.4 and 45.7±6.6 for the NAA, Cr, Cho and mI reflect the (expected) $224^{1/2}$ gain over the single-voxel SNRs and their linewidths are similar to the single-voxel counterparts, as shown in Fig. 1-3. Excellent fit reliability with mean voxel Cramer-Rao lower bounds (CRLBs) below 15% were obtained for NAA, Cr, Cho, and mI. To optimize the analyses’ reliability, VOI aspartate was excluded since its CRLBs were >20%. The resultant absolute concentrations, for each metabolite, scan and animal, are compiled in Table 1-1. The intra-voxel, intra- and inter-animal CVs per metabolite are compiled in Table 1-2 and box plots of their concentrations’ together with single-voxels’ (intra-voxel – computed from variations in each of the 224 voxels in the VOI along its 4 back-to-back scans each measurement) for comparison are shown
<table>
<thead>
<tr>
<th>Animal (session)</th>
<th>Scan</th>
<th>VOI WM fraction</th>
<th>VOI GM fraction</th>
<th>VOI Tissue fraction</th>
<th><strong>MRS</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NAA</td>
<td>Cr</td>
<td>Cho</td>
<td>mI</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean±SD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a)

|      | 1 (a) | 0.43 | 0.57 | 0.96 | 7.8 | 7.4 | 1.1 | 3.6 |
|      | ii    | 8.1  | 7.7  | 1.2  | 4.2 |
|      | iii   | 7.5  | 7.2  | 1.1  | 4.2 |
|      | iv    | 7.6  | 7.5  | 1.2  | 4.0 |

(b)

|      | 0.45 | 0.55 | 0.97 | 7.9 | 7.6 | 1.3 | 4.2 |
|      | ii   | 7.2  | 7.2  | 1.2  | 3.1 |
|      | iii  | 7.1  | 7.1  | 1.1  | 4.0 |
|      | iv   | 7.3  | 7.0  | 1.3  | 3.7 |

(c)

|      | 0.43 | 0.57 | 0.95 | 7.6 | 7.1 | 1.1 | 4.1 |
|      | ii   | 7.7  | 7.2  | 1.1  | 4.0 |
|      | iii  | 7.5  | 7.1  | 1.2  | 4.0 |
|      | iv   | 7.3  | 7.1  | 1.1  | 4.0 |

2 (a)

|      | 0.45 | 0.55 | 0.97 | 8.8 | 7.5 | 1.2 | 4.4 |
|      | ii   | 8.0  | 7.3  | 1.2  | 4.1 |
|      | iii  | 8.7  | 7.8  | 1.1  | 4.2 |
|      | iv   | 8.1  | 7.5  | 1.2  | 4.1 |

(b)

|      | 0.45 | 0.55 | 0.97 | 8.6 | 7.8 | 1.1 | 4.5 |
|      | ii   | 8.7  | 7.5  | 1.1  | 4.1 |
|      | iii  | 8.7  | 7.6  | 1.2  | 4.5 |
|      | iv   | 8.9  | 7.6  | 1.2  | 4.7 |

(c)

|      | 0.40 | 0.60 | 0.95 | 7.4 | 6.6 | 1.3 | 4.3 |
|      | ii   | 7.5  | 6.1  | 1.4  | 5.4 |
|      | iii  | 8.4  | 7.3  | 1.3  | 5.0 |
|      | iv   | 7.5  | 6.4  | 1.2  | 4.0 |

3 (a)

|      | 0.48 | 0.52 | 0.97 | 7.3 | 6.0 | 1.1 | 3.2 |
|      | ii   | 8.0  | 6.9  | 1.1  | 2.7 |
|      | iii  | 7.0  | 6.3  | 1.2  | 2.6 |
|      | iv   | 7.1  | 6.0  | 1.1  | 2.7 |

(b)

|      | 0.44 | 0.56 | 0.97 | 7.4 | 6.6 | 1.2 | 2.5 |
|      | ii   | 7.6  | 6.5  | 1.1  | 3.5 |
|      | iii  | 7.6  | 6.6  | 1.0  | 3.5 |
|      | iv   | 7.3  | 6.4  | 1.2  | 3.3 |

4 (a)

|      | 0.41 | 0.59 | 0.96 | 7.4 | 7.0 | 1.4 | 4.5 |
|      | ii   | 6.9  | 6.6  | 1.3  | 4.4 |
|      | iii  | 7.3  | 6.8  | 1.4  | 4.4 |
|      | iv   | 7.5  | 7.0  | 1.4  | 4.4 |

5 (a)

|      | 0.43 | 0.57 | 0.95 | 8.1 | 7.4 | 1.3 | 4.3 |
|      | ii   | 7.7  | 7.2  | 1.2  | 4.0 |
|      | iii  | 7.6  | 7.0  | 1.2  | 4.2 |
|      | iv   | 7.8  | 7.1  | 1.2  | 4.3 |

Mean±SD

|      | 0.44±0.02 | 0.56±0.02 | 0.96±0.01 | 7.7±0.5 | 7.0±0.5 | 1.2±0.1 | 4.0±0.6 |
Table 1-1. Volumetric (MRI) and metabolic (MRS) data for each of the 5 animals (labeled 1, 2, … 5), in every serial session (up to 3 per animal – labeled a, b…e) and 4 back-to-back scans each session (labeled: i, ii, … iv) in this study. Absolute average concentration in the VOI in millimoles/g wet weight.

in Fig. 1-5. This demonstrates the improved reproducibility of the VOI averages, a consequence of the ×15 improved SNR [158]. Specifically, (i) intra-voxel CVs are all approximately threefold larger than the mean values reported for 3.4 cm³ voxels in healthy macaques [130]. (ii) The inter-animal CVs (reflecting the inherent biologic differences among these animals) are: 4.3%, 4.0%, 5.8% and 14.5% for NAA, Cr, Cho and mI, dominating the total variance, as shown in Table 1-2 and Fig. 1-5. (iii) The intra-animal variance comprise two categories: (iii.a) the inter-session CVs: 4.0%, 4.9%, 4.5% and 3.9%; and (iii.b) the intra-session CVs: 3.9%, 3.7%, 5.6% and 8.5% for NAA, Cr, Cho and mI, both of comparable magnitude, indicating minimal contribution from serial VOI misregistration. As expected, both intra-animal contributions were smaller than the inter-animal contribution to the total variation, as shown in Table 1-2 and respective box plots in Fig. 1-5.

The sensitivity advantage of this approach is demonstrated by comparing the VOI spectrum of one of these macaques (#1 in Table 1-1) at baseline and 6 weeks post SIV infection, as shown in Fig. 1-6. The infection leads to a 30% and 20%
Inter-animal Intra-voxel variance

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Intra-voxel (“Error”)</th>
<th>Inter-animal</th>
<th>Intra-animal</th>
<th>Total variance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-session</td>
<td>Inter-session</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAA</td>
<td>22.0</td>
<td>4.3</td>
<td>3.9</td>
<td>4.0</td>
</tr>
<tr>
<td>Cr</td>
<td>32.8</td>
<td>4.0</td>
<td>3.7</td>
<td>4.9</td>
</tr>
<tr>
<td>Cho</td>
<td>35.9</td>
<td>5.8</td>
<td>5.6</td>
<td>4.5</td>
</tr>
<tr>
<td>mI</td>
<td>44.7</td>
<td>14.5</td>
<td>8.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 1-2. Coefficients of variation (CVs in %) associated with the intra-voxel (“the residual error” term in the model of Chard et al. [187]), inter-animal and intra-animal (inter- and intra-session) reproducibility of all metabolite concentrations in the VOI. *Back-to-back scans.

...decline in NAA and Cho and an 8% increase in mI, all statistically significant based on the intra-animal CVs in Table 1-2. Given the single voxel CVs in Table 1-2, in contrast, these changes would not be significant and possibly not even visible (seen by comparing the 0.125 cm³ voxels spectra in Fig. 1-1 with the whole VOI average in experimental fluctuations. Indeed, CVs of 11%, 8%, 14% and 13% for NAA, Cr, Cho and mI, were reported in the frontal cortex, WM of the centrum semiovale and basal ganglia of rhesus macaques using single 3.4 cm³ voxels and 6.5 minutes acquisition at 1.5 T [130]. Spectra from our 0.125 cm³...
voxels yield *intra*-voxel CVs that are 3 to 4 fold larger (see Table 1-2 and Fig. 1-5). What may have kept the CV disparity to just 3-4 fold despite order-of-magnitude SNR difference [158], may be that these larger single-voxels also suffered comparably larger partial GM/WM volume and *inter-* and *intra*-animal positioning uncertainty.

It is also noteworthy that increasing the VOI by nearly an order-of-magnitude (from 3.4 cm$^3$ to 28 cm$^3$ at 3 T) helps reduce the *inter-*animal CV by (only) slightly less than a factor of 2. This indicates that at this brain coverage *true* biological variability is the dominating factor that cannot be reduced by further experimental refinements, *e.g.*, SNR or VOI size suggesting that more measurements on healthy animals are unlikely to yield significantly different results. This is of particular importance in model systems, since unlike human diseases, every animal enters these studies healthy. The small *inter-*animal CVs reported here, therefore, make every macaque’s healthy “baseline” scan optional, an advantage given the cost and complexity of these experiments. Moreover, if done at baseline, given the CVs reported here, a scan may enable to detect at the onset, metabolic conditions that may escape the veterinarian’s attention but may later confound the outcome of the disease or treatment for which that animal was a model.
Fig. 1-5. Top, right: Box plots displaying the 25th, median, 75th (box) and ±95%-tiles (whiskers) of the NAA, Cr, Cho and mI concentrations distributions for all voxels of a scans (inter-voxel), sessions across all macaques (inter-animal), sessions of an animal (intra-animal) and all scans for a session (intra-scan). Note the ×4 to ×11 fold, improvement in both inter- and intra-animal reproducibility of the averages versus with the single voxels. Insert: Box plots of the GM, WM and tissue fractions (GMf, WMf, and Tf) distributions in the VOIs of all animals. Note the narrow distribution of tissue types, indicating the minimal GM/WM/CSF partial volume repositioning error of the proposed approach.

Our current approach of synthesizing the VOI spectrum from the average of all phased and aligned single 0.125 cm³ elements in the VOI enabled us to address both the reproducibility and precision issues: First it achieves much (15-fold) higher metabolite SNRs and consequently 4 to 11-fold better CVs [158], by exploiting the $B_0$ homogeneity (narrow linewidth) across the individual small voxels [189]; and correction for regions of $B_0$ variations (frequency shifts) on a voxel-by-voxel basis. Second, its order-of-magnitude higher spatial coverage: 28 cm³ (~35%) of the macaque brain, versus 3.4 cm³ (less than 5%) for single-voxel methods, minimizes partial GM/WM/CSF volume variations, therefore, effectively increasing the inter-session VOI placement accuracy, thereby further improving the metabolites’ reproducibility [190].

The improvement is demonstrated by the subtle (but diffuse) changes brought about by SIV infection in the macaque brain. Indeed, while a decrease in NAA
Fig. 1-6. **Left**: Real part of the VOI (aligned and averaged) spectrum from a healthy macaque (black line) superimposed with its spectral fit function (thick gray line). The arrows next to each peak indicate their respective *intra*-animal CVs (±4%, ±5%, ±5%, ±4% for NAA, Cr, Cho and ml). **Right**: The spectrum from same animal after 6 weeks of SIV infection and CD8+ lymphocyte depletion. Both spectra are on common intensity and frequency scales. Note the marked NAA and Cho decline post-SIV infection but that the Cr, although visually lower is still within the CV whereas the ml is elevated. These changes would have most likely not been significant and possibly not even detectable at the much lower sensitivity of single voxels (compare corresponding box plots in Fig. 1-5).
and NAA/Cr at 6 weeks post SIV infection are consistent with previous reports [191], establishing them with statistical significance using 3.4 cm$^3$ single voxel $^1$H-MRS required four animals. While such changes would have not been significant, indeed possibly even detected given the CVs in Table 1-2 and Fig. 1-5, they are both clearly visible and significant with this approach in a single animal.

The <10% inter-animal CVs in Table 1-2 indicate good cross sectional similarity between healthy animals, i.e., metabolite concentrations differences larger than 10% (20% for ml) are unlikely or, alternatively, if occurring they may be indicative of an abnormal animal. The longitudinal inter-animal reproducibility is even better, exhibiting CVs below 10% across the board. Both these findings are encouraging for $^1$H-MRSI studies given past reports in monkeys of NAA declines ›25% occurring within lesions after ischemic stroke [174], in frontal cortex after acute SIV-infection [129, 136], within acute and chronic lesions caused by experimental allergic encephalomyelitis (EAE) [177], in nigrostriatal dopamine neurons in a model of Parkinson’s disease [175], and Cho changes ›20% within simian acute EAE lesions [177].

Since (unlike human subjects participating in HIV-infection studies) all control animals can be presumed to be healthy at baseline, the CVs determined here for intra- and inter-animal reproducibility can be used to compute “power tables”
designed to ascertain the minimum number of animals necessary to detect a 5, 10, 15 or 20% change with 80 or 90% power (as shown in Table 1-3). Specifically, given the mean percentage changes and standard deviations derived from Table 1-1, the investigator would require fewer than five animals to detect metabolic changes of the above mentioned magnitudes with 80 or 90% power (Table 1-3). Moreover, based on the table, fewer than 10 animals would be required for metabolic changes of ±10% to be detected with 80 or 90% power.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>80% Power</th>
<th>90% Power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% 10% 15% 20%</td>
<td>5% 10% 15% 20%</td>
</tr>
<tr>
<td>[NAA]</td>
<td>3 3 3 2</td>
<td>4 3 3 2</td>
</tr>
<tr>
<td>[Cr]</td>
<td>6 3 3 3</td>
<td>7 4 3 3</td>
</tr>
<tr>
<td>[Cho]</td>
<td>18 7 4 4</td>
<td>23 8 5 4</td>
</tr>
<tr>
<td>[mI]</td>
<td>13 5 4 3</td>
<td>16 6 4 4</td>
</tr>
</tbody>
</table>

Table 1-3. The estimated sample size needed to detect a 5%, 10%, 15% or 20% true mean percentage change in each metabolite with either 80% or 90% power.

Admittedly, the study also encountered several limitations due to the methodology used. First, the sensitivity to metabolic changes reported here, derived from the high SNR shown in Fig. 1-4, come at the cost of localization. Any focal or regional differences in the VOI are averaged out. Although the original localized information is still available (see Fig. 1-1), the proposed strategy reflects a choice of maximum sensitivity to monitor the global effects of
diffuse pathogenesis or treatment responses, especially for the weaker mI signal. Second, although our results correct for $T_1$ and $T_2$ relaxation using literature values, possible effects of pathology on either remains unknown. Use of short $TE<40$ ms and of $TR\sim T_1$, however, reduces the sensitivity of metabolic quantification to $T_1$ and $T_2$ variations smaller than 10% of nominal to below the voxel SNR, as shown by Liu et al. and Zaaraoui et al. [121, 186].

CONCLUSION

Both the cross-sectional and longitudinal average metabolite levels in the healthy rhesus macaque brain can be assumed to vary less than $\pm 10\%$ from the nominal values reported here. Since unlike investigations of human neurological disorders, all animals entering these studies are healthy at baseline, these CVs can be used to compute power tables geared to detect (and identify as “real”) predetermined metabolic changes that are due to either disease progression or treatment response. Given the CVs reported here, these tables will then be subject to more mundane cost constraints to determine the number of animals versus that of the number of scans on each needed in order to detect the desired changes.

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CHAPTER 2

Global Gray and White Matter Metabolic Changes following SIV Infection in CD8-Depleted Rhesus Macaques: Proton MR Spectroscopic Imaging at 3 T\textsuperscript{1}

ABSTRACT
To test the hypotheses that global decreased neuro-axonal integrity reflected by decreased $N$-acetylaspartate (NAA) and increased glial activation reflected by an elevation in its marker, the myo-inositol (mI), present in a CD8 T-cell-depleted rhesus macaque model of HIV-associated neurocognitive disorders. To this end we performed quantitative MRI and 16×16×4 multivoxel proton MR spectroscopic imaging (TE/TR=33/1400 ms) in 5 macaques pre- and 4-6 weeks post-simian immunodeficiency virus infection. Absolute NAA, creatine, choline (Cho), and mI concentrations, gray and white matter (GM, WM) and cerebrospinal fluid fractions were obtained. Global GM and WM concentrations were estimated from 224

voxels (at 0.125 cm³ spatial resolution over ~35% of the brain) using linear regression. Pre- to post-infection global WM NAA declined 8%: 6.6±0.4 to 6.0±0.5 mM (p=0.05); GM Cho declined 20%: 1.3±0.2 to 1.0±0.1 mM (p<0.003); global mI increased 11%: 5.7±0.4 to 6.5±0.5 mM (p<0.03). Global, GM and WM brain volume fraction changes were statistically insignificant. These metabolic changes are consistent with global WM (axonal) injury and glial activation, and suggest possible GM host immune response.
INTRODUCTION

HIV-associated neurocognitive disorders (HAND) are a significant problem for the million infected in the US alone [5, 192]. Unfortunately, incomplete understanding of its pathogenesis hinders development of effective therapies. Recent evidence has shown that despite the benefits of highly-active antiretroviral therapy, HAND incidence among advanced HIV patients has not changed and its prevalence continues to climb [193]. Moreover, mounting evidence [8, 9] suggests that even while the virus is maximally suppressed in CSF and blood plasma, HIV may still facilitate dementia. This is thought to be due to patients living significantly (up to 20+ years) longer with the disease [194], allowing infected monocytes, macrophages and microglia to produce a toxic cascade that ultimately results in neuronal death [11]. MRI and proton MR spectroscopy (\(^{1}\)H-MRS) have been useful in detecting brain abnormalities [77-80], and provide critical knowledge of the dynamics of cerebral injury during HIV-infection [81-84].

Due to practical challenges to retrospective HIV studies, e.g., unknown infection date and overlapping secondary conditions, animal models are often used [195]. The simian immunodeficiency virus (SIV)-infected rhesus macaque shares a similar pathology with HIV-infected humans, including development of AIDS, disease of the CNS and cognitive/behavioral deficits [25, 26, 36, 37]. Although several \(^{1}\)H-MRS studies of SIV-infected macaque models are reported, all used low
spatial resolution (>3 cm³) single-voxels in few brain regions that typically suffer CSF, gray and white matter (GM, WM) partial volume [130, 137, 163]. Consequently, one outstanding, therapy-relevant question remains the relative dysfunction of the global GM versus WM.

Indeed, prior histopathology of SIV-infected macaques has shown neuro-axonal dysfunction and death in both GM and WM regions [136, 160], as well as glial activation in subcortical WM and cortical GM suggestive of developing encephalitis [161, 162]. Based on these findings, our goal in this paper was to test the hypothesis that decreases in global GM and WM N-acetylaspertate (NAA), the marker for neuronal integrity, and elevated global GM and WM myo-inositol (mil), the marker for glial activation [95, 104], are already detectable in an accelerated nonhuman primate model of HAND. To overcome the spatial coverage restriction of single voxel ¹H-MRS, we use an approach that combines the data from high, 0.125 cm³, spatial resolution 3-D multivoxel ¹H-MRS imaging (MRSI) with MRI tissue segmentation [144], to analyze hundreds of voxels cooperatively, thereby increasing the overall precision [144] – at the cost of sensitivity to possible regional variations. We use this approach to compare the global GM and WM levels of NAA, mil, creatine (Cr) and choline (Cho) – the markers of cellular energy/density and membrane turnover [95, 104], in a large, 28 cm³ (~35%) portion of 5 rhesus
macaque brains before and 4 to 6 weeks after SIV infection, when they are persistently CD8+ T-lymphocyte-depleted [163].

**Experimental**

**Nonhuman primates**

Five (2 females, 3 males; 5.0 to 8.6 kg weight) healthy 3 to 4 year-old adult rhesus macaques (*Macaca mulatta*) were scanned under constant veterinary supervision. Each was tranquilized with 15 to 20 mg/kg intramuscular ketamine hydrochloride and intubated to ensure a patent airway during the experiment (no mechanical ventilation was needed). Intravenous injection of 0.4 mg/kg atropine was used to prevent bradycardia. Continuous infusion of 0.25 mg/kg/minute propofol was maintained via a catheter in a saphenous vein. Heart and respiratory rates, oxygen saturation and end-tidal CO₂ were monitored continuously and a water blanket used to prevent hypothermia. All animals were then subsequently infected by intravenous injection with SIV_{mac251} virus (10 ng SIVp27) and their CD8⁺ T-lymphocytes persistently depleted (>28 days post-infection) to speed up progression to AIDS and SIV encephalitis, both late-stage events similar to those of HIV-infection [42, 196], with an antibody targeted against CD8 (cM-T807) at 6, 8 and 12 days post-inoculation. CD8+ depletion without SIV infection has been demonstrated to not produce metabolic changes or pathological abnormalities in the rhesus macaque brain [197]. The animals were rescanned 4 or 6 weeks later to
assess disease activity. The protocol was approved by the Harvard Medical School and Massachusetts General Hospital Institutional Animal Care and Utilization Committees.

**MRI**

All experiments were done in a 3 T whole-body MR imager (Magnetom TIM Trio, Siemens AG, Erlangen, Germany), running software version VB13P. It was equipped with the manufacturer’s circularly-polarized transmit-receive knee-coil capable of delivering a peak 2 kHz (45.2 μT) radio-frequency (RF) $B_1$ field with ~1 kW of power. To guide the $^1$H-MRS volume-of-interest (VOI) and for tissue segmentation, sagittal and axial $T_2$-weighted turbo spin echo (TSE) MRI: TE/TR=16/7430 ms, 140×140 mm$^2$ field-of-view (FOV), 512×512 matrix, were acquired. To cover the entire macaque head, 24 sagittal images 2.0 mm thick each and 40 axial 1.2 mm thick slices were obtained, as shown in Fig. 2-1.

**Multivoxel 3-D $^1$H-MRSI**

A 4.0 cm anterior-posterior (AP) ×3.5 cm left-right (LR) ×2.0 cm inferior-superior (IS) =28 cm$^3$ VOI was image-guided, onto the corpus callosum and angled along the genu-splenium line of each animal to maximize the number of brain voxels within it while avoiding air-filled sinuses and skull lipids, as shown in Fig. 2-1. The manufacturer’s automatic procedure adjusted the first- and second-order shims to 26±1 Hz FWHM VOI water line. The VOI was excited using
$TE=33$ ms PRESS with two, 1 cm-thick, second-order Hadamard-encoded slabs (two 0.5 cm thick slices each), interleaved along the IS direction every 720 ms for an effective $TR$ of 1440 ms for each slab (and slice), as shown in Fig. 2-1b. This results in optimal SNR and spatial coverage [120], as well as allows a strong, 9 mT/m Hadamard slice-selection gradient to keep the maximal 1.6 ppm chemical shift displacement between NAA and $mI$ to 0.5 mm, 10% of each slice’s thickness [181]. The four slices’ planes were encoded with $16\times16$ 2-D Chemical shift imaging over an $8\times8$ cm$^2$ (LR×AP) FOV to yield nominal (0.5 cm)$^3=0.125$ cm$^3$ voxels (0.55×0.55×0.5 cm≈0.15 cm$^3$ given the FWHM of the 2-D point spread function [183]). The VOI was defined in their planes by the two 9 ms 4.9 kHz bandwidth 180° RF pulses, under 3.3 and 2.9 mT/m. The localization grid produced $8\times7$ voxels per slice, for a total of 224 in the VOI, as shown in Fig. 2-1. The MRS signal was acquired for 256 ms at ±1 kHz bandwidth. Each $16\times16\times2$ $^1$H-MRSI scan took ~12.5 minutes and the 4 averages ~50 minutes.

**Brain Volumetry**

The TSE images were segmented with our FireVoxel package [153]. It first corrects all images for non-uniform intensities due to the coil’s RF inhomogeneities, using the common histogram devolution technique of Sled et al. [198]. This process “smoothens” any random artifacts in the MRI that may arise due to the MR acquisition as shown in Fig. 2-2 (compared with the MRI in Fig. 1-2
**Fig. 2-1.** Left (pre-infection): Axial (a) and sagittal (b) $T_2$-weighted turbo spin echo (TSE) MRI of a female macaque head showing the location and size of the 4.0×3.5×2.0 cm³ volume-of-interest (VOI) and localization grid (solid white frame) and two second-order Hadamard slabs, $\uparrow 1$ and $\uparrow 2$, in the IS direction (b).

Right (a’, b’): Same as (a, b) six weeks post-SIV infection in that animal. Note similar VOI placement and brain coverage.
without correction). The process begins with automatic detection of a “WM seed,”
(which, in reality, is a GM seed for $T_2$-weighted images as this option had been
updated at the time of this writing for $T_2$-weighted images) yielding its average
signal intensity, $I_{GM}$, for a cortical GM region. Following automatic detection of all
pixels at or above 50% (but below 172.5% to exclude the CSF) of $I_{GM}$, a tissue-
mask is constructed per slice in three steps: morphological erosion, recursive region
growth retaining pixels connected to the seed; and morphological inflation to
reverse the effect of erosion. Pixels of intensity above 172.5% of $I_{GM}$ are classified
as CSF, as shown in Fig. 2-2a. Following a similar process, all pixels above 120%
(but below 172.5%) of $I_{GM}$ are classified as part of the GM mask; and pixels under
120% (but over 50% to exclude air cavities) classified as WM, as shown in Fig. 2-
2b and 2-2c. The masks were co-registered with the $^1$H-MRSI grid using in-house
software (MATLAB, The Mathworks Inc., Natick, MA) estimating their volume in
every $j$-th voxel in the $k$-th animal ($V_{j,k}^{GM}$, $V_{j,k}^{WM}$, $V_{j,k}^{CSF}$).

Metabolic quantification

The $^1$H-MRSI data was processed offline using in-house software written in IDL
(Research Systems Inc., Boulder, CO). The data was voxel-shifted to align the
NAA grid with the VOI, then Fourier-transformed in the time, AP and LR
dimensions and Hadamard-reconstructed along the IS direction with no spectral or
spatial filters. The spectra were each automatically frequency aligned and phased in
reference to the NAA peak. Relative levels of the \(i=\text{NAA, Cr, Cho (choline + phosphorylethanolamine)}\), \(mI\) metabolite in the \(j=1\ldots224\) VOI voxel of the \(k=1..5\) animal were estimated from their peak areas, \(S_{ijk}\), using the SITools-FITT parametric spectral modeling software using Cho, Cr, \(mI\), NAA, glutamate and glutamine full model functions [117]. The \(S_{ijk}\)-s were scaled into absolute amounts, \(Q_{ijk}\), relative to a 0.5 L sphere of \(C_{i}^{\text{vivo}}=12.5, 10.0, 3.0\) and 7.5 mM NAA, Cr, Cho and \(mI\) in water at physiological ionic strength to load the coil and similar VOI size and position in order to sample the \(B_1\) profile as closely as possible:

\[
Q_{ijk} = \frac{C_{i}^{\text{vivo}}}{V} \cdot \frac{S_{ijk}}{S_{R}} \cdot \left( \frac{P_{j}^{180^\circ}}{P_{R}^{180^\circ}} \right)^{1/2} \text{ millimoles ,} \tag{2.1}
\]

where \(V\) is the voxel volume, the \(S_{R}\) is the sphere’s voxels’ metabolites’ signal, \(P_{j}^{180^\circ}\) and \(P_{R}^{180^\circ}\) the RF power for a non-selective 1 ms 180° inversion pulse on the animal and reference.

To account for different relaxation times \(\text{in vivo} (T_{1}^{\text{vivo}}, T_{2}^{\text{vivo}})\) and in the reference phantom \(T_{1}^{\text{vivo}}, T_{2}^{\text{vivo}}\), the \(Q_{ijk}\) in Eq. (2.1) were corrected with a factor for each metabolite, \(i\):

\[
f_i = \frac{\exp(-TE/T_{2}^{\text{vivo}})}{\exp(-TE/T_{1}^{\text{vivo}})} \cdot \frac{1-\exp(-TR/T_{1}^{\text{vivo}})}{1-\exp(-TR/T_{2}^{\text{vivo}})}, \tag{2.2}
\]

with 316, 177 and 264 ms macaque 3 T \(T_{2}^{\text{vivo}}\) values for NAA, Cr and Cho used [122], that represent a 60:40 GM:WM brain fractions in the VOI. For \(mI\), we used

76
the human $T_2^{\text{vivo}}=200$ ms value reported by Posse et al. [124]. We also used the respective macaque 3 T $T_1^{\text{vivo}}$ values of 1232, 1238, 1107 and 1170 ms [121, 124]. No age-related $T_1/T_2$s differences were anticipated in this cohort of animals of similar ages as those in a previous report [121, 122, 157]. The corresponding values measured in the phantom were $T_2^{\text{vitr}}=483, 288, 200, 233$ ms and $T_1^{\text{vitr}}=605, 336, 235, 280$ ms.

The global tissue concentration of each metabolite in the VOI, $C_{ik}$, was obtained as:

$$C_{ik} = \frac{\sum_{j=1}^{224} Q_{ijk}\cdot f_i}{\sum_{j=1}^{224} (V_{jk}^{GM} + V_{jk}^{WM})} \cdot \text{mM/g wet weight}, \quad (2.3)$$

This $C_{ik}$ has the advantage of (number of voxels)$^{\frac{1}{2}} \sim 15$ fold lower variance than the individual voxels’ and, hence, expected to yield proportionally better precision, as described by Kreis [158].

**Global WM and GM concentrations**

Since the CSF does not contribute to the $^1$H-MRS signal, the $i$-th metabolite amount in the $j$-th voxel in the $k$-th animal can be modeled as the sum of two compartments’ (GM, WM) amounts:

$$Q_{ijk} = Q_{ijk}^{GM} \cdot f_i^{GM} + Q_{ijk}^{WM} \cdot f_i^{WM} = C_{ik}^{GM} \cdot V_{jk}^{GM} \cdot f_i^{GM} + C_{ik}^{WM} \cdot V_{jk}^{WM} \cdot f_i^{WM}, \quad (2.4)$$

where $C_{ik}^{GM}$, $C_{ik}^{WM}$ are the (unknown) global GM and WM metabolites’
Fig. 2-2. **Left**: Axial $T_2$-weighted TSE image shows the anatomical coverage of the 4.0×3.5 cm² VOI, overlaid with the: a: CSF, b: GM and c: WM masks generated by the FireVoxel package. Note the tissue type differentiation performance. **Right**: a’-c’: Same as a-c, *post*-infection of the same female macaque.
concentrations and the GM and WM $f_i$s are given by Eq. (2.2). The $T_2^{\text{vivo}}$s used were 325, 178, 274 and 200 ms for NAA, Cr, Cho and $m$I in GM; 311, 181, 255 and 200 ms in WM [122, 124]. Since no significant GM and WM $T_1^{\text{vivo}}$ differences are reported [121], we used the values following Eq. (2.2). Although $C_{ik}^{\text{GM}}$ and $C_{ik}^{\text{WM}}$ cannot both be derived from Eq. (2.4), since the macaque brain’s GM and WM spatial heterogeneity is on a scale smaller than the voxels, each has different, independent $V_{jk}^{\text{GM}}$ and $V_{jk}^{\text{WM}}$ coefficients. The resulting over-determined system of \( j=224 \) equations in $C_{ik}^{\text{GM}}$ and $C_{ik}^{\text{WM}}$ was solved with linear regression. The regression fitting error per voxel was calculated for each metabolite for every animal pre- and post-infection and averaged. The intra-animal coefficient of variation (CV= standard deviation/mean) of this approach was shown to be under 5% for all metabolites [144].

**Statistical Analyses**

The metabolite change for each metabolite and the brain volume change in each compartment (i.e., global VOI tissue, WM and GM) were computed for each animal by taking their pre- minus the post-infection level so that a positive change reflects a decline over time. The lower and upper limits of a 95% confidence interval for the true mean change from the “pre” to “post”-infection scans were estimated for each metabolite in each tissue compartment. The 5-animal sample size was insufficient to permit a nonparametric test of whether there was a change
in any metabolite for any tissue compartment. As a result, paired sample \( t \) tests were used to assess MRS as well as brain volume changes from the “pre-” to “post-” infection scans. Significance was tested at the \( p<0.05 \) level and SAS version 9.0 (SAS Institute, Cary, NC) was used for all calculations.

**RESULTS**

An example of the VOI position and size, pre- and post-infection, is shown in Fig. 2-1. The corresponding GM, WM and CSF VOI masks, segmented from the TSE images for \( V_{GM} \) and \( V_{WM} \), are shown in Fig. 2-2. Spectra from the VOI and metabolic maps generated from them, pre- and post-infection, are shown in Fig. 2-3. Note that the lateral ventricles in the MRI in Fig. 2-1a, a’, can be detected in the metabolic map for each of the metabolites, reflecting SNR and localization performance. Shimming yielded voxel linewidth of 5.9±0.9 Hz FWHM (mean±standard deviation) in the 2240 voxels (224 voxels/scan \( \times \) 2 scans/animal \( \times \) 5 animals) and SNRs of NAA: 25±8, Cr: 16±6, Cho: 10±3 and \( mI \): 10±4, as shown in Fig. 2-3. Excellent fit reliability with mean voxel Cramer-Rao lower bounds (CRLBs) below 15% were obtained for NAA, Cr, Cho, and \( mI \). To optimize the analyses’ reliability, VOI voxels were included only if their CRLBs were <20% for all metabolites (~175 voxels, or ~80% of the total number of VOI voxels). The global regression fitting errors per voxel for GM/WM were: NAA=0.04±0.02, Cr=0.03±0.01, Cho=0.002±0.001, \( mI \)=0.04±0.04 mM amongst the five animals.
Fig. 2-3. **Top:** Real part of the $7 \times 8$ (LR×AP) $^1$H spectra matrices from the VOI in Fig. 2-1a and a’, on common chemical shift and intensity scales *pre-* (a) and six weeks *post-* (a’) SIV infection. Note the SNR in those $(0.5)^3=0.125$ cm$^3$ voxels: NAA: $25\pm8$, Cr: $16\pm6$, Cho: $10\pm3$ and ml: $10\pm4$ and spectral resolution, leading to
excellent fit reliability reflected by voxel CRLBs below 15% for these four metabolites. Bottom (b, b'): NAA, Cr, Cho, and mI metabolic maps from the spectra in a, a'. Note the gross anatomical features reflecting spatial localization.

and two timepoints.

GM and WM tissue volumes in the VOI were 16.0±0.7 cm³ and 10.1±0.6 cm³ pre-infection, and 15.0±1.1 cm³ and 10.6±0.9 cm³ post- infection. None of these changes were significant (p>0.2 for both). Since the % GM and WM volume changes seem to be offsetting, i.e., the total tissue volume in the VOI appears to remain unchanged, these variations probably reflect different tissue sampling from slight VOI misregistration in the follow-up scan of each animal.

The averages of all 224 spectra in the VOI (equivalent to the numerator of Eq. [2.3]) for each animal pre- and post-infection, overlaid with their fits, are shown in Fig. 2-4. They exhibit SNRs of 390±30, 223±12, 151±17 and 144±17 for NAA, Cr, Cho and mI, a dramatic 224½ ~15-fold gain over the source 0.125 cm³ voxels (compare Fig. 2-4 with Fig. 2-3). The single-voxels’ spectral resolution is also maintained in the averages, as reflected by their 8.2±0.8 Hz FWHM; a result of pre-alignment, this further contributes to their SNR increase.

The resultant global [Eq. (2.3)] as well as GM and WM [Eq. (2.4)] concentrations for each metabolite in each animal, pre- and post-SIV-infection, are shown in Fig. 2-5. They reveal a significant global VOI Cho decline (-13%,
\( p<0.03 \), with a 95% confidence interval (CI) of \([-0.25, -0.08 \text{ mM}]\) and \( mI \) increase (11%, \( p<0.03 \), CI=[0.18, 1.38 mM]. A significant Cho decline (-20%, \( p<0.003 \), CI=[-0.39, -0.16 mM] was found only in the GM. Slight increases at the “trend” level (\( p\sim 0.1 \)) were also observed in the WM Cr and \( mI \), perhaps reflecting the small sample size and smaller VOI WM volume (see above; the WM volume was on average \( \frac{2}{3} \) of the GM’s). Since NAA is known to always decline in all adult neuropathologies, we tested at the single-sided level and found a significant global decline in the whole VOI (-9%, \( p<0.04 \), CI=[-1.31, 0.07 mM], and in the WM (-8%, \( p=0.05 \), CI=[-1.23, 0.13 mM], but no significant change in GM. No metabolite differences greater than two standard deviations from the mean of all animals were observed between males and females when the group was divided according to gender.

DISCUSSION

Although several SIV-infected macaque models \(^1\text{H}-\text{MRS} \) studies have been reported to date, these have been limited by low, \( >3 \text{ cm}^3 \), spatial resolution single-voxels that excluded \( >95\% \) of the brain and suffered CSF, GM, and WM partial volume [130, 137, 163]. To overcome such limitations, we combined the data from high spatial resolution (0.125 cm\(^3\) 3-D multivoxel \(^1\text{H}-\text{MRSI} \) with tissue segmentation from the MRI. This enabled us to analyze the metabolic and structural data from hundreds of voxels cooperatively, increasing the overall
precision, at the cost of sensitivity to possible regional or specific structure variations [144, 158].

Due to the sporadic nature and low incidence of HAND and its consequent paucity of postmortem studies, nonhuman primate models provide an alternative to histopathology for studying its neuropathogenesis. In this context, $^1$H-MRS provides a nondestructive tool for detecting brain metabolite abnormalities in intact animals, facilitating longitudinal studies that histopathology does not. Towards this end, this study aims to quantify the global GM and WM changes associated with SIV-infection in order to test the hypothesis that global neuro-axonal injury and glial cell activation develop in an accelerated nonhuman primate model of HAND.

**WM neuronal dysfunction**

Indeed, we find significant NAA decline in WM that appears earlier or is more profound than in GM. Although evidence of focal neuronal damage in frontal cortex was seen with single-voxel $^1$H-MRS [130], it is possible that global GM dysfunction does not occur until later stages of infection, as suggested previously [162]. Nonetheless, a finding of lower WM NAA is consistent with *ex vivo* studies showing NAA/Cr decline in centrum semiovale and frontal WM in acute- and chronically-infected animal and human samples [199]. Several *in vivo* animal studies also found 5-10% WM NAA/Cr decreases 4 weeks post-infection [163, 191, 200], consistent with us, and some even showed WM NAA decline 27 days
Fig. 2-4. Real part of the aligned and averaged $^1$H spectra from all VOI voxels (thin black lines) representing Eq. (4.3), for each of the animals pre- (left) and 4 (animals 4 and 5) or 6 (animals 1-3) weeks post-SIV-infection (right), superimposed with
their fitted model functions (thick gray lines), on common intensity and chemical shift scales. Animals 1 and 2 are female; 3 through 5 male. Note the excellent SNRs (reflected by CRLBs of less than 2% for the four metabolites) and spectral resolution compared with individual voxels.

post-infection was proportional to synaptic and dendritic integrity deterioration and neuronal loss in quantitative immunohistochemistry [163, 200].

**Diffuse GM and WM glial activation**

Increased global mI levels support our hypothesis of diffuse glial activation [95, 104, 201]. Previous HIV studies have also suggested a role for gliosis, reflected by elevated mI in both WM and GM of patients with mild HIV-dementia and clinically asymptomatic patients [81, 134]; these fit well with immunohistochemical studies showing astrocytic and microglial hypertrophy in both the WM and GM of HIV patients with, or without, dementia [135]. In SIV-infected macaque models, elevated mI has been reported in several GM and WM regions using single voxel \(^1\)H-MRS [130]. Evidence seems to suggest a correspondence with increased levels of glial fibrillary acidic protein and ionized calcium binding adaptor molecule-1, immunohistochemistry markers of astrogliosis and microglial activation, at 4 (or 8) weeks post-infection [200].
GM host immune response

The observed 20% global GM Cho decline (see Fig. 2-5) is consistent with a host immunological response to developing SIV encephalitis, reported previously in the basal ganglia [139]. That is, immunopathological evidence has shown that macaques with basal ganglia Cho/Cr near uninfected level or below at 4 weeks post infection (after an initial increase at 2 weeks post infection) revealed no later signs of encephalitis, whereas those whose levels remained elevated did [139]. Moreover, studies of both the traditional [137] as well as accelerated [200] SIV macaque models of HAND have also demonstrated large reductions in Cho and Cho/Cr (after initial elevation) 2 to 4 weeks post-infection, consistent with our findings of decreased Cho at 4 weeks.

Although it has not been established what role this return to pre-infection levels plays in the context of disease progression, it is reasonable to assume that an immune response is involved given the similar Cho/Cr changes reported after neuroprotective treatment in SIV-infected macaques [130, 191], and following antiretroviral treatment in HIV-infected patients [127]. Furthermore, both treatments have been shown to support host immune defense through: (i) downregulation of microglial activation in the former [202]; and (ii) reduction of infected monocyte trafficking into the CNS in the latter [163].
Finally, increased energy demand may also occur due to glial activation, as reflected by slight increases in WM Cr. Evidence for this can be found in a previous study [200] that showed significant Cr elevation at 8-weeks post-compared with pre-infection and a correspondence of this change with greater histopathological evidence of gliosis.

Caveats

This study is also subject to several limitations. First, the decision to maximize the sensitivity to diffuse, global changes, especially for the weaker mI signal, comes at the cost of localization. Although the original MRSI data is available, regional analyses may suffer 10× lower sensitivity to metabolic changes due to 22-45% intra-voxel CVs [203]. Consequently, a ~25% decline in parietal cortex neuron number 4 weeks after SIV-infection [200] would go undetected. Second, due to the air-tissue interfaces (i.e., paranasal sinuses) in the frontal region that cause severe B₀ field inhomogeneity [204] – leading to signal dropout – our VOI placement was limited to exclude most of the frontal brain – an area known to be affected in HAND. However, since our method assesses global GM and WM, exclusion or inclusion of specific region(s) should not affect the findings in diffuse diseases significantly. For example, given their relatively modest, 2.2 cm³, 14% [164] fraction of the VOI’s 16 cm³ GM volume and the fact that changes there are on the order of % (see Fig. 2-3), areas vulnerable to high viral burden, e.g., basal
Fig. 2-5. NAA, Cr, Cho and mI concentrations’ line-plots for all animals, pre- and 4 (animals 4 and 5) or 6 (animals 1-3) weeks post-SIV-infection, for the whole VOI and its GM and WM. Note significant >10% declines in VOI Cho and NAA,
elevated mI, as well as GM Cho and WM NAA declines. Note the fourth monkey’s mI data was excluded due to metabolic Cramer-Rao lower bounds >20%.

ganglia, cannot in of themselves explain the global GM changes observed. Third, although we account for $T_1$ and $T_2$ in healthy macaques, possible pathology effects on either remain unknown. Determining these effects was too costly and time-consuming, especially since pathology-related changes may vary over the 4-6 week infection period. However, use of short, $TE=33$ ms and $TR\sim T_1$ has been shown to reduce the metabolic quantification variations from $T_1$ and $T_2$ variations of up to 10% to below the ~5% voxel SNR [121, 186]. Fourth, because viral and host immune function can vary over a 2-week period [163], use of two post-infection scan times could have obscured some effects. Due to cost constraints and lack of preliminary differences between the two groups, we combined their data to improve statistical power. Logistical constraints notwithstanding, future studies would benefit from more animals scanned at multiple times, including closer to infection, when alterations in cerebral metabolism due to initial viral activity may be more evident.

Our results reveal global tissue injury and recovery while also distinguishing GM- from WM-related dysfunction during the later stages of SIV infection in an accelerated macaque model of HAND. Although host immunological response appears a likely explanation to some of the changes observed during viral infection,
its disparate effects in GM and WM suggest distinct pathological pathways that need additional study. These, for example, might examine how neuroprotective intervention, in conjunction with/without anti-inflammatory agents, affects the GM and WM and whether either alone, or taken together, might be better strategies for treatment of the disease.

ACKNOWLEDGEMENTS

We thank Drs. Andrew A. Maudsley and Brian J. Soher for their SITools-FITT software and Dr. Joanne Morris and Ms. Shannon Luboyeski for animal veterinary care.
CHAPTER 3

Structure-Specific Glial Response in a Macaque Model of NeuroAIDS: Multivoxel Proton MR Spectroscopic Imaging at 3 Tesla

ABSTRACT

Objective: Since ~40% of persons with HIV also suffer neurocognitive decline, we sought to assess metabolic dysfunction in the brains of simian immunodeficiency virus (SIV)-infected rhesus macaques, an advanced animal model, in structures involved in cognitive function. We test the hypothesis that SIV-infection produces proton-MR spectroscopic imaging (H-MRSI)-observed decline in the neuronal marker, N-acetylaspartate (NAA) and elevations in the glial marker, myo-inositol (mI) and associated creatine (Cr) and choline (Cho) in these structures.

Design: Pre- and four-or-six weeks post-SIV infection (with CD8+ lymphocyte depletion) was monitored with T2-weighted quantitative MRI and 16×16×4

multivoxel $^1$H-MRSI ($TE/TR=33/1400$ ms) in the brains of five rhesus macaques. 

Methods: Exploiting the high-resolution $^1$H-MRSI grid, absolute, cerebrospinal fluid partial volume-corrected NAA, Cr, Cho and mI concentrations were obtained from centrum semiovale, caudate nucleus, putamen, thalamus and hippocampus.

Results: Pre- to post-infection mean Cr increased in the thalamus: 7.2±0.4 to 8.0±0.8 mM (+11%, $p<0.05$); mI increased in the centrum semiovale: 5.1±0.8 to 6.6±0.8 mM, caudate: 5.7±0.7 to 7.3±0.5 mM, thalamus: 6.8±0.8 to 8.5±0.8 mM, and hippocampus: 7.7±1.2 to 9.9±0.4 mM (+29%, +27%, +24% and +29%, all $p<0.05$). NAA and Cho changes were not significant.

Conclusions: SIV-infection appears to cause brain injury indirectly, through glial activation, while the deep gray matter structures’ neuronal cell bodies are relatively spared. Treatment regimens to reduce gliosis may, therefore, prevent neuronal damage and its associated neurocognitive impairment.
INTRODUCTION

Although highly-active antiretroviral therapy (HAART) has reduced HIV-associated mortality and dementia, ~40% of the million infected in the US will suffer milder, long-term forms of associated neurocognitive disorders (HAND), e.g., impairments in memory and executive function, that diminish quality of life and productivity [2, 3]. Overall HAND prevalence has increased in chronically-infected older individuals [205], despite years of HAART [193, 206, 207]. Moreover, the attenuated association between plasma RNA viral load and cognitive impairment in treated individuals suggests the underlying pathology may be related to other, metabolic alterations in CNS areas [208, 209].

Indeed, neuroimaging studies using magnetic resonance imaging (MRI) and proton-MR spectroscopy ($^1$H-MRS) have revealed continued structural and metabolic abnormalities in infected individuals even whilst their viral loads were undetectable [206, 210-212]. These often occur subcortically in the basal ganglia, thalamus and white matter (WM), but also in hippocampus -- all areas crucial to executive, cognitive and memory functions, and most (with the exception of putamen), curiously enough, periventricular in location. These abnormalities have also been found to correlate with cognitive deficits [210, 213]. Histopathology from seropositive patient brains has also confirmed that HIV-1 preferentially targets basal ganglia (especially the caudate nucleus and putamen) [167], thalamus [169], WM [78], and hippocampus [170-172].
Although HIV affects different brain regions differently, its specific metabolic effects in these CNS structures thought to underlie cognitive dysfunction(s) remain less well-characterized. Since human studies of CNS involvement can be logistically challenging early on after infection (as most newly infected are unaware of their status) and studies during the advanced stages of neurologic complication can be difficult in a cognitively-impaired group – with potential opportunistic infections exacerbating morbidity to mortality – animal model systems are often used. Simian immunodeficiency virus (SIV)-infected rhesus macaque, in particular, is a well-established model system mimicking HIV’s development of AIDS, CNS disease, cognitive and behavioral deficits [25, 26, 36, 37]. Both the traditional and accelerated (using CD8+ lymphocyte depletion) models of study have provided insight into the nature and dynamics of HIV cerebral injury and both have shown similar histopathology at the advanced stage [137, 139, 200]. However, past 1H-MRS studies were limited by low, 1 to 4 cm³ spatial resolution (relative to the ~80 cm³ brain) and single-voxels that missed >95% of the brain and suffered gray matter (GM), WM and CSF partial volume, reducing metabolite quantification performance [144].

We address these issues with three-dimensional (3-D) multivoxel proton-MR spectroscopic imaging (1H-MRSI) at 0.125 cm³ spatial resolution, over substantial, ~35%, of the brain [149], correcting for CSF partial volume [120]. Using it we found global abnormalities suggestive of diffuse pathology in five animals [214].

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Histopathology, however, has also revealed regional heterogeneity and variable progression, suggesting different disease mechanisms in various regions and that some may be more susceptible than others [191, 200]. Since the high-spatial resolution $^1$H-MRSI grid also facilitates post hoc analyses and irregularly-shaped region-of-interest (ROI) definition [215], in this study we test the hypothesis that SIV-infection may lead to: (i) “early” (i.e., before the onset of neurocognitive decline) neuronal damage, reflected by a decrease in the concentration of their $N$-acetylaspartate (NAA) marker; and (ii) early glial activation, marked by increased myo-inositol ($mI$), choline (Cho) and creatine (Cr) [104]; in brain regions implicated in memory or cognitive function: the centrum semiovale, caudate, putamen, thalamus and hippocampus, in macaques several weeks post-infection.

**METHODS**

**Nonhuman Primates**

Five (two females, three males; 5.0 to 8.6 kg weight) healthy 3 to 4 year-old rhesus macaques (*Macaca mulatta*) were scanned under constant veterinary supervision. Each was tranquilized with 15 to 20 mg/kg intramuscular ketamine hydrochloride and intubated to ensure a patent airway during the experiment (no mechanical ventilation was needed). Intravenous injection of 0.4 mg/kg atropine was used to prevent bradycardia. Continuous infusion of 0.25 mg/kg/minute propofol was maintained via a catheter in a saphenous vein. Heart and respiratory
rates, oxygen saturation and end-tidal CO₂ were monitored continuously and a water blanket used to prevent hypothermia. All were subsequently intravenously infected with SIVₘₐₑ2₅₁ virus (10 ng SIVp27) and their CD₈⁺ T-lymphocytes depleted to: (i) speed up; and (ii) increase the incidences of progression to terminal AIDS and SIV encephalitis [42, 196]. The model yields CNS pathology similar to that of the later, more commonly diagnosed [216] and increasingly more prevalent [2, 192] stages of HIV-infection in weeks-to-months instead of years [42]. CD8 T-cell depletion was done with a mouse-human chimeric monoclonal, cM-T807, antibody targeted against CD8 at 6, 8 and 12 days post-inoculation [44, 45]. Previous histopathological and ¹H-MRS studies comparing the result of the non-CD8-depleted (“traditional”) versus this CD8-depleted (“accelerated”) models have shown similar findings at their comparable infection stages [42, 136, 200]. Two animals were rescanned 4 weeks and three 6 weeks later. The protocol was approved by the Harvard Medical School and Massachusetts General Hospital Institutional Animal Care and Utilization Committees.

MRI Data Acquisition

All experiments were done in a 3 T whole-body MR imager (Magnetom TIM Trio, Siemens AG, Erlangen, Germany), using its circularly-polarized transmit-receive knee-coil capable of producing a peak 2 kHz (45.2 µT) radio-frequency (RF) B₁ field. To guide the ¹H-MRSI volume-of-interest (VOI) and for tissue
segmentation, sagittal and axial $T_2$-weighted turbo spin echo (TSE) MRI:

$TE/TR=16/7430$ ms, $140 \times 140$ mm$^2$ field-of-view (FOV), $512 \times 512$ matrix, 24 slices, 2 mm each; and 40 axial slices, 1.2 mm thick each, were acquired.

**Multivoxel 3-D $^1$H-MRSI**

A 4.0 cm anterior-posterior (AP) $\times 3.5$ cm left-right (LR) $\times 2.0$ cm inferior-superior (IS)=$28$ cm$^3$ VOI was image-guided, as shown in Fig. 3-1 [203]. The VOI was then excited using PRESS ($TE/TR=33/1440$ ms) with two second-order Hadamard encoded slabs (4 slices) interleaved along the IS direction every $TR$. This approach optimizes SNR and spatial coverage [120] and allows strong, 9 mT/m, slice-selection gradient to keep the 1.6 ppm chemical shift displacement from NAA to mI to 0.5 mm, $\leq 10\%$ of the slice thickness [181].

The slices’ planes were encoded with $16 \times 16$ 2D-chemical shift imaging (CSI) over $8 \times 8$ cm$^2$ (LR×AP) FOV, yielding nominal (0.5 cm)$^3=0.125$ cm$^3$ voxels ($0.55 \times 0.55 \times 0.5$ cm$^3 \approx 0.15$ cm$^3$ given the FWHM of the 2D-CSI point spread function [112, 182, 183]). The VOI was defined in the slices’ planes by the two 9 ms PRESS $180^\circ$ RF pulses, under 3.3 and 2.9 mT/m (4.9 kHz bandwidth). The localization grid formed $7 \times 8$ voxels in each of the 4 slices (Fig. 3-1) for 224 total in the VOI. The MRSI signal was acquired for 256 ms with 512 points at $\pm 1$ kHz
Fig. 3-1. Top & middle: Sagittal (a – before, a’ – 4 weeks after SIV infection) and axial (b, b’) $T_2$-weighted turbo spin echo MRI from a female rhesus macaque head showing the location and size of the $3.5 \times 4 \times 2 \text{ cm}^3$ VOI (solid lines), CSI grid and
two second-order Hadamard slabs, “1{“ and “2{“ encoding 1…4 slices in the IS direction (a). The open arrow on a and a’ denotes the level of b and b’. Note the similar VOI placement and brain coverage and the grid resolution that facilitates definition of irregular brain structures. Bottom left, c and c’: Real part of the 7×8 axial ¹H spectra matrix from the VOI on b and b’ (pre- and post-SIV infection). All spectra represent 0.125 cm³ voxels and are on the same frequency and intensity scales. Note the metabolite SNRs and spectral resolution, leading to reliable fits reflected by voxel CRLBs <15% for the four metabolites.

bandwidth. Each 16×16×4 scan took 12.5 minutes and at 4 averages the 3-D ¹H-MRSI was ~50 minutes.

**Metabolic quantification**

The ¹H-MRSI data were processed using in-house software (Research Systems Inc., Boulder, CO). Residual water signal was removed in the time domain

{Marion, 1989, Improved solvent suppression in one- and two-dimensional NMR spectra by convolution of time domain data}; the data were Fourier transformed in the time, AP and LR and Hadamard reconstructed along the IS directions. Spectra were voxel-shifted to align the CSI grid with the VOI NAA, corrected automatically for frequency and zero-order phase shifts in reference to the NAA peak in each voxel [203]. Relative levels of the i=NAA, Cr, Cho, ml metabolite in the j=1…224 VOI voxel of the k=1..5 animal, Sijk, were estimated from their peak areas using parametric spectral modeling software [117]. The Sijk, were scaled into
absolute concentrations, $C_{ijk}^{vivo}$, relative to a 0.5 L sphere of $C_i^{vitro}$=12.5, 10.0, 3.0 and 7.5 mM NAA, Cr, Cho and mI in water at physiological ionic strength to properly load the coil:

$$C_{ijk}^{vivo} = C_i^{vitro} \frac{S_{ijk}}{S_{ijR}} \frac{V_{R}^{180^\circ}}{V_{k}^{180^\circ}} \cdot f_i \text{ mM,}$$

(3.1)

where $S_{ijR}$ is the sphere’s voxels’ metabolites’ signal, $V_{j}^{180^\circ}$, $V_{k}^{180^\circ}$ the radio-frequency voltage for a non-selective 1 ms 180° inversion pulse on the $k$-th subject, sphere, and $f_i$ a correction for in vivo ($T_{1}^{vivo}$, $T_{2}^{vivo}$) and phantom ($T_{1}^{vitro}$, $T_{2}^{vitro}$) relaxation times differences for metabolite, $i$:

$$f_i = \frac{\exp\left(-TE/T_{2}^{vivo}\right)}{\exp\left(-TE/T_{2}^{vivo}\right) \cdot 1 - \exp\left(-TR/T_{1}^{vivo}\right)},$$

(3.2)

where 3 T NAA, Cr, Cho and mI $T_{1}^{vivo}$=1335, 1263, 1147, 1120 ms and $T_{2}^{vivo}$=325, 178, 264, 200 ms were used for GM; 1154, 1224, 1032, 960 ms and 316, 182, 263, 200 ms for WM ROIs [121, 122, 124]. Corresponding values in the phantom were $T_{1}^{vitro}$=605, 336, 235, 280 ms; $T_{2}^{vitro}$=483, 288, 200, 233 ms.

**Correcting for CSF partial volume**

ROIs may also contain CSF (see Figs. 3-2 and 3-3) whose metabolite concentrations are below the $^1$H-MRS detection threshold [217], leading to metabolite concentration underestimation. To correct for it we produced CSF masks from the axial $T_2$-weighted TSE images using our in-house FireVoxel
Fig. 3-2. Left, (a, b, c); middle, (a’, b’, c’): Axial $T_2$-weighted MRI showing the 3.5×4.0 cm$^2$ VOI (thick white frame), pre- (left) and four weeks post-SIV infection in the same animal. Note VOI placement reproducibility and lack of detectable MRI brain lesions, or atrophy. Right, (a’’, b’’, c’’): CSF partial volumes (beige) in
$^1$H-MR spectroscopic slices corresponding to $a^*$, $b^*$, $c^*$. Four FireVoxel-generated CSF masks (from the MRI) that overlap each 0.5 cm thick $^1$H-MRSI slice (cf. Fig. 3-1) are superimposed over the CSI grid. Each voxel’s metabolite values are corrected for CSF partial volume with multiplication by a factor, $1/T_f$.

software segmentation package [153], as described previously [214]. Our software then estimated the CSF fraction, CSF$_f$, within each voxel – as shown in Fig. 3-2 [144]. Finally, the $C_{ijk}$ (from Eq. (3.1)) in each ROI voxel was divided by its tissue fraction, $T_f = 1 - \text{CSF}_f$.

**Regional analyses**

The left centrum semiovale, and the right caudate head, putamen, thalamus and hippocampus were carefully outlined on the axial MRI of every animal, pre-(healthy) and post-SIV infection, as shown in Fig. 3-3. Only one side was chosen for consistency purposes and to eliminate the need to correct for multiple comparisons (i.e., the Bonferroni correction) that is normally applied when multiple tests for significance are performed using the same set of data (in this case, the ROIs, which include both right and left sides). Our in-house IDL-written software then zero-filled the “processed” 16×16 MRSI matrix (i.e., the metabolic maps) to 256×256 and averaged each metabolite’s concentrations in all voxels that fell entirely or partially within the outline. Note that although zero-filling does not add new information to the data, it can increase the effective spatial resolution and
Fig. 3-3. Left: Real part of the $^1$H spectra averages from all voxels fully or partially within the outlined ROIs (thin black line), superimposed with their fits (thick gray line) for animal #5 in Table 3-1, *pre-*SIV infection. Spectra are on the same
frequency and intensity scales. Middle: Axial $T_2$-weighted MRI showing the VOI (thick white frame), 7×8 axial native CSI grid (thin white lines) and ROIs (yellow outlines): (a) centrum semiovale, (b) caudate (head) nucleus, (c) putamen, (d) thalamus, and (e) hippocampus. Note the relationship between the spatial resolution grid and the size(s) of these structures. Right: Same as ‘Left’ except taken four weeks post-SIV infection. Note the improved ROI SNRs (as compared to the single-voxel spectra in Fig. 3-1) that lead to good spectral fits, as reflected by mean CRLBs of <20% for all metabolites in all ROIs examined.

reduce partial volume effects [165, 166].

**Statistical Analyses**

The temporal change in each metabolite for each ROI was computed for each animal as the “pre-” minus the “post-” infection level so that a positive change reflects a decline over time. The five-animal sample size was insufficient to permit a nonparametric test of whether there was a change in any metabolite (or NAA/Cr ratio) in any ROI. As a result, paired sample $t$ tests were used to assess the temporal change in each metabolite within each ROI. Significance was tested at the $p<0.05$ level and SAS version 9.0 (SAS Institute, Cary, NC) was used for all calculations.

**RESULTS**

An example of the VOI position, size and spectra pre- and four weeks post-SIV
infection, is shown in Fig. 3-1. Shimming yielded a consistent voxel FWHM linewidth, determined by the spectral modeling software, of 5.9±0.9 Hz (mean ± standard deviation) over the 2240 voxels (224 voxels/scan ×2 scans/animal ×5 animals). The SNRs, estimated as each metabolite’s peak-height divided by the root-mean-square of the noise, were: NAA=25±8, Cr=16±6, Cho=10±3 and mI=10±4, leading to reliable fits, reflected by mean voxel Cramer-Rao lower bounds (CRLBs) below 15%. To optimize the analyses' reliability, ROI voxels were included only if their CRLBs were <20% for all four metabolites.

An example of each ROI outline is provided in Fig. 3-3 for a female macaque brain at pre-infection (animal #5 on Table 3-1). Metabolite concentrations in every ROI and animal, pre- and post-SIV infection, are compiled in Table 3-1 and shown as line-plots in Fig. 3-4. Pre- to post- mean Cr increased 11% in the thalamus; while mI increased 29%, 27%, 24% and 29% in the centrum semiovale, caudate, thalamus, and hippocampus (all p<0.05). Slight increases at the trend level (p<0.1) were also observed for Cr in the caudate (+17%) and putamen mI (+24%). Neither NAA nor Cho changed significantly. To be consistent with past 1H-MRS studies in this model system we also obtained the NAA/Cr ratio, which is often used for quantification. Pre- to post- mean NAA/Cr declined from 1.02±0.08 to 0.98±0.07 in the centrum semiovale 0.65±0.03 to 0.53±0.07 in the caudate and 0.99±0.12 to 0.87±0.07 in the thalamus (-4%, -18%, -13%, all p<0.05).
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Table 3-1. Metabolic data (mean±standard deviation [SD]) for each region-of-interest (ROI) of animals #1 through 5, “pre-” and “post-” SIV infection, for each metabolite (NAA, Cr, Cho, mI). A Absolute average ROI concentration [millimoles/g wet weight]. *p<0.05. A “-” indicates data was excluded due to metabolic Cramer-Rao lower bounds >20%. “Semi”=Centrum semiovale; “Caud”=Caudate nucleus (head); “Put”=Putamen; “Thal”=Thalamus; “Hipp”=Hippocampus.

DISCUSSION

Resurgence of more neurovirulent HIV strains, increased drug resistance and higher neurotoxicity associated with prolonged HAART [218, 219] underscore the need for noninvasive laboratory markers of HAND pathogenesis for monitoring disease progression. Although an earlier study found global GM and WM $^1$H-MRSI abnormalities in the brains of the same animals [214], that approach was unable to distinguish multi-focal from truly diffuse pathology [214]. Fortunately, the original, high-spatial resolution $^1$H-MRSI data facilitates post hoc analyses of irregularly-shaped ROIs, as shown in Fig. 3-3 [215]. Consequently, we examined the structure-specific regional changes in the same animals pre- and four-or-six weeks post-infection.

Taken together, the findings of both studies suggest that SIV-infection may indeed produce multi-focal pathology. First, while global WM NAA has been shown to decline [214], no NAA decline is seen here in the centrum semiovale (cf. Fig. 3-4). This may be due to the greater sensitivity obtained by averaging a greater
number of voxel spectra (~90 WM voxel spectra were averaged globally to yield an effective SNR increase of approximately nine-to-ten-fold) in the global analysis versus only five or six voxel spectra averaged within the centrum semiovale analysis provided here. Second, although no global GM or WM mI elevation was seen previously [214], it is detected here in several individual brain GM structures and in the centrum semiovale WM (Table 3-1 and Fig. 3-4). Lastly, despite undetected NAA decline in the VOI’s deep GM structures, histopathology in other macaques has shown neuronal dysfunction/loss in cortical regions outside our 1H-MRSI VOI [191, 200]. Moreover, immunohistochemistry markers of astrogliosis and neuronal integrity, glial fibrillary acidic protein (GFAP), synaptophysin, and microtubule-associated protein 2, along with neuronal counts in animals of the same cohort have shown evidence of variable disease activity in frontal and parietal cortices as well as a pathology gradient in frontal cortex between four and eight weeks post-infection, suggesting greater neurodegeneration and gliosis occur later [191, 200].

Several explanations could account for such regional and temporal variations: (i) various mechanisms may cause different types of damage in different regions, e.g., like those in the basal ganglia, WM and parietal cortex [148], i.e., a “multi-hit,” hypothesis; (ii) damage mechanisms may be similar, but more aggressive in some regions, e.g., thalamus, due to heavier viral loads; and (iii) there may be a
Fig. 3-4. Line-plots of the NAA, Cr, Cho and mI concentration changes from ‘pre-’ to ‘post-’ SIV-infection scans in every ROI for each of the five animals (#1-5, corresponding to Table 3-1). Note the statistically significant increase in thalamus Cr, as well as elevations in mI for centrum semiovale, caudate, thalamus and hippocampus (denoted by open arrows).

spatiotemporal dependence, i.e., the injury begins in one area (that sustains the most damage) and spreads over time, e.g., through Wallerian degeneration. None of these hypotheses was tested in a previous ¹H-MRSI study that examined only
global abnormalities [214], nor with histopathology since it cannot follow-up the same animal.

Our study demonstrates that $^1$H-MRSI can identify structure-specific changes; specifically, we found that SIV-infection produced increases in mI and Cr, reflecting glial activation, in the thalamus and caudate and possibly also in the centrum semiovale and hippocampus. Elevated mI in multiple regions is consistent with previous neuropathology in this model [191, 200], showing widespread elevations of GFAP and ionized calcium binding adaptor molecule 1 – an immunohistochemistry marker of microglial activation, at four and eight weeks post-infection. Unchanged NAA in all GM structures, however, suggests that their neuronal cell bodies may (still) be spared. One implication of this is that astrocytic and microglial activation may precede neuropathogenesis, a conclusion consistent with our previous $^1$H-MRSI finding of elevated global mI in the much larger (28 cm$^3$) VOI, along with global WM NAA decline (axonal pathology) without GM NAA (neuronal cell body) change in these SIV-infected macaques [214].

An alternative possibility is that neurons may have been injured earlier [200], but partially recovered due to immune host activation, suggesting a reversible injury coincident with monocyte-associated levels of viremia [163]. It is also noteworthy that although Ratai et al. [200] reported significant declines in the NAA/Cr ratio in parietal and frontal cortices that correlated with histopathological
declines in synaptophysin and neuronal counts, neither NAA nor Cr change by itself was significant at four-or-six weeks.

It is noteworthy that ratios (often to the Cr level) are a common approach to metabolic quantification. Ratios cancel unknown multiplicative factors, e.g., static, B₀, and radiofrequency, B₁, field inhomogeneities, instrumental gain, scanner and localization method differences, as well as CSF partial volume, at the cost of noise propagation from the numerator and denominator [220]. Indeed, consistent with previous reports [163, 200], our data also yields significant NAA/Cr declines in the centrum semiovale, caudate and thalamus. These reflect coherent decreases in the NAA simultaneous with increased Cr concentrations, as shown in Fig. 3-4, that are each not quite statistically significant in and of themselves. It is noteworthy that the previous global WM NAA decline [214], together with the centrum semiovale NAA/Cr finding here, may suggest diffuse axonal pathology imparted by Wallerian degeneration [221, 222], which leads to direct injury to cortical cell bodies that are outside our ¹H-MRSI VOI (see limitations below).

Admittedly, this study is also subject to several limitations: First, due to the proximity of frontal regions to air-tissue interfaces, e.g., the paranasal sinuses, that cause severe B₀ field inhomogeneity [204], our VOI excluded most of the frontal lobes, an area known to be affected in HAND. Second, to avert lipid contamination our VOI was limited to midline cortex, missing ~85-90% of cortical GM. Third,
cost constraints limited follow-up of these animals, which were participating in a longitudinal antiretroviral study, to: (i) just five animals; and (ii) use of an accelerated model to speed disease progression to terminal AIDS and SIV encephalitis in months versus years with the traditional SIV model, which may be preferred for its proven ability to recapitulate HIV cerebral pathology but requires prohibitively long wait periods. The limited number of animals restricted the statistical power reflected by coefficients of variation (=standard deviation/mean) of the ROI metabolite concentrations to 9%, 12%, 12%, and 13% for the NAA, Cr, Cho and mI. Logistics notwithstanding, future studies might benefit from more animals scanned later to reveal how the disease evolves into its terminal phase.

In conclusion, SIV-infection at this “early” stage appears to cause glial activation, while neuron cell bodies in the deep GM structures remain relatively spared, or, alternatively, may have already recovered. Treatment regimens to reduce gliosis, therefore, may be beneficial in preventing downstream neurodegeneration and perhaps ward off neurocognitive impairments. These results also suggest possible relationships between particular regions and progressive brain injury, a link that if substantiated would support the hypothesis that particular regions are ‘hit’ first, or more aggressively and the disease spreads to other areas only subsequently. Such regional disease heterogeneity and spatiotemporal escalation could be monitored with $^1$H-MRSI as a nondestructive alternative to
histopathology. Provided that animal testing demonstrates safety, future HIV treatment studies may benefit from anti-inflammatory regimens geared towards reducing gliosis as a strategy against HAND.
CONCLUSION

Since the introduction and “success” of antiretroviral drugs in the early 1990s, severe HIV encephalitis and dementia have become rare, and AIDS-related deaths and sickness have declined sharply as a result. Still, as the HIV-infected population grows older, long(er)-term neurological complications loom in the coming decades and remain a major health challenge for the 1.2 million+ currently living with HIV in the United States. These come despite (or perhaps because of) the advent of HAART, and have only recently (in the last several years) come into the focus of research devoted to understanding underlying pathways and agents responsible for the milder, yet longer-lived and increasingly more prevalent, forms of neurocognitive decline that currently affect ~40% of those living with HIV.

Although it is generally accepted that HIV does not infect neurons directly, it is still unclear how the virus is able to induce neuronal damage and cell death, while being virtually absent from the plasma and CSF vasculatures. Evidence has shown support for “Trojan horse” cell types, in particular perivascular macrophages and parenchymal microglia, infected by the virus and that may serve as “viral reservoirs.” These are capable of trafficking into the CNS and facilitating the expression of chemokines/cytokines detrimental to neuronal health. While histopathologically validated, there have been only a handful of in vivo human
studies to address the use of noninvasive laboratory markers, e.g., using $^1$H-MRS, to reliably reveal neuropathogenesis and/or track disease progression.

**Original contributions**

Previous $^1$H-MRS studies examining macaque models of HAND were limited by low spatial resolution (>3 cm$^3$) single voxels that missed >95% of the (~85 cm$^3$) macaque brain and that also suffered (unknown) CSF, GM and WM partial volumes. To overcome these limitations, we combined the data from high spatial resolution (0.125 cm$^3$), 3-D (28 cm$^3$ brain coverage), multivoxel (224 voxels) $^1$H-MRSI with MRI tissue segmentation. This enabled us to analyze cooperatively the metabolic and structural data from 224 spectroscopic voxels per exam, thereby increasing the overall precision, and, consequently, the overall reproducibility of VOI metabolite quantifications; hence their greater sensitivity to (diffuse) pathologic changes (see Chapter 1).

We report – to our knowledge – only the second $^1$H-MRS reproducibility study in this model species and the first done at 3 T, establishing the cross-sectional and longitudinal reproducibility of $^1$H-MRSI-detectable brain metabolites. We found that both the cross-sectional and longitudinal NAA, Cr and Cho concentrations for a healthy macaque brain can be assumed to vary by less than ±12% from their nominal values here. Our findings also demonstrate a dramatic, four to 11-fold sensitivity improvement (as reflected by intra- and inter-animal CVs) over
previous single voxel studies [130], allowing subtler, more diffuse changes related to SIV infection in the macaque brain to be significantly detected in fewer animals. Indeed, using our combined $^1$H-MRSI/MRI segmentation approach, NAA change was both clearly visible (see Fig. 1-6) and “significant” – outside ±two intra-animal, inter-session CVs – with (just) a single animal. In contrast, using a single 3.4 cm$^3$ voxel, a significant decrease in NAA or NAA/Cr at six weeks post-SIV infection would have required (at least) four animals [154].

In addition, since – unlike human subjects participating in HIV-infection studies – all control animals can be presumed healthy at baseline, this afforded us the ability to compute “power tables” designed to ascertain the minimum number of animals necessary to detect a 5, 10, 15 or 20% change with 80 or 90% power (Table 1-3). Specifically, based on the table, an investigator would require fewer than five animals to detect metabolic changes of the magnitude typically detected in vivo (±25% [136]) with 80 or 90% power. Fewer than 10 animals would be required for metabolic changes of ±10% to be detected with 80 or 90% power.

Hence $^1$H-MRSI provides a cost-effective, nondestructive tool for detecting metabolic abnormalities in live, intact animal brains, facilitating the longitudinal study of in vivo neuropathology that post mortem histopathology cannot. Towards this end, in Chapter 2, we quantified the “global” GM and WM metabolic changes associated with SIV$^\text{mac251}$ infection at four-to-six weeks post-infection to test the
hypothesis that diffuse neuronal injury and/or glial cell activation develop “early” (i.e. before the onset of neurocognitive decline) in a CD8 T-cell-depleted rhesus macaque model. Our findings of significant diffuse tissue injury (driven primarily by NAA decline in the subcortical WM; see Fig. 2-5) and possible tissue recovery (driven primarily by Cho decrease in the GM; Fig. 2-5), distinguish GM- from WM-dysfunction during this early stage of infection.

Furthermore, the high-resolution 1H-MRSI grid also facilitated post hoc analyses of irregularly-shaped ROIs, which allowed us to revisit the original, localized 1H-MRSI data for regional analyses, as we demonstrated in Chapter 3. Consequently, we examined structure-specific changes within four subcortical structures – including two from the basal ganglia – and one cortical structure (the hippocampus) for regional heterogeneity of SIV activity in the same healthy/SIV-infected animals. Taken together with previous studies, our findings suggest SIV infection produces a multifocal pathology characterized early on by subcortical glial activation, which may precede, or worsen, subsequent neuronal pathology.

Interestingly, we found the neuronal cell bodies within deep GM structures to be relatively spared – or, alternatively, may have already recovered from a prior injury – as reflected by the lack of significant NAA decline(s) in these regions. This finding is consistent with our earlier study reporting no diffuse (VOI) change in GM NAA, which likely shifts the initial site of subcortical neuronal damage to the
WM. Other human studies involving patients with early stages of AIDS dementia complex also lend support to the notion that subcortical damage is characterized early on by glial activation. Treatment regimens targeting early gliosis, therefore, may prove beneficial in preventing downstream neurodegeneration and potentially ward off its clinical sequelae of neurocognitive impairments.

**Future directions**

Taken together, the findings from these animal studies of SIV infection help to elucidate the underlying disease mechanism(s) of SIV-induced neuronal injury and apoptosis, and, potentially, its HIV equivalents in humans suffering from HAND. It is hoped that knowledge from these studies will open new avenues of research that will explore, and provide valuable insight into, the important CNS pathways (both inflammatory and neurodegenerative), target tissues (e.g., subcortical WM, deep and cortical GM) and pathologic agents involved in chronic HAND. For instance, the absolute (and relative) differences in $^1$H-MRSI-observed metabolic changes following SIV infection between GM and WM are suggestive of distinct pathological mechanisms needing further study.

These studies might examine, for example, the differences between GM and WM in response to host and/or adaptive immunity repair mechanism(s), or to administration of neuroprotective intervention(s) and/or anti-inflammatory drug(s). One next step might be to aim at HAND prevention and treatment through the use
of targeted therapies, and to test whether taking any single drug alone, or in some combination as part of a treatment regimen, identifies a more effective treatment strategy for arresting, or possibly even reversing, disease progression. Provided that animal testing demonstrates safety and the potential for drug efficacy, future HIV treatment regimens may also benefit from anti-inflammatory/neuroprotective drugs, as well as host and/or adaptive immunity interventions geared towards reducing gliosis as a strategy against HAND.

Future studies might also examine the possible relationships between particular regions and progressive brain injury, a link that if substantiated would support the hypothesis that more vulnerable regions are ‘hit’ first, or more aggressively, and disease spreads to other areas only subsequently. Such regional disease heterogeneity and spatio-temporal escalation could be monitored with $^1$H-MRSI as a nondestructive alternative to histopathology. Finally, logistics notwithstanding, future studies would also benefit from more animals scanned at later stages to reveal how the disease evolves into its terminal phase.
REFERENCES


42. Williams K, Alvarez X, Lackner AA. Central nervous system perivascular cells are immunoregulatory cells that connect the CNS with the peripheral immune system. Glia 2001,36:156-164.


57. Browning J, Horner JW, Pettoello-Mantovani M, Raker C, Yurasov S, DePinho RA, Goldstein H. Mice transgenic for human CD4 and CCR5 are


66. Goffinet C, Allespach I, Keppler OT. HIV-susceptible transgenic rats allow rapid preclinical testing of antiviral compounds targeting virus entry or


82. Meyerhoff DJ, MacKay S, Bachman L, Poole N, Dillon WP, Weiner MW,


89. Sakaie K, Gonzalez, RG. Imaging of NeuroAIDS. *NeuroAIDs* 1999, 2.


98. Rubaek E. Magnetic resonance spectroscopy diagnosis of neurological


117. Soher BJ, Young K, Govindaraju V, Maudsley AA. Automated spectral analysis III: application to in vivo proton MR spectroscopy and spectroscopic


133. Tracey I, Carr CA, Guimaraes AR, Worth JL, Navia BA, Gonzalez RG. Brain choline-containing compounds are elevated in HIV-positive patients before


170. Kruman, II, Nath A, Mattson MP. HIV-1 protein Tat induces apoptosis of hippocampal neurons by a mechanism involving caspase activation, calcium


177. Richards TL, Alvord EC, Jr., Peterson J, Cosgrove S, Petersen R, Petersen K,


221. Lexa FJ, Grossman RI, Rosenquist AC. MR of wallerian degeneration in the