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by

Timothy Michael Shepherd
This document is dedicated to my family (Mom, Dad, Colin, Pat and Monica).
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This dissertation describes experiments using rat hippocampal slices as a model of nervous tissue to evaluate the changes in diffusion-weighted magnetic resonance images (MRI) observed after acute brain injury. A multislice perfusion chamber was designed to acquire diffusion-weighted MRI from multiple rat hippocampal slices simultaneously. These images had signal-to-noise ratios sufficient to analyze the multicomponent diffusion properties of water in rat hippocampal slices. Because the hippocampal slice water diffusion parameters were stable for at least 8 hours after procurement in this chamber, it enabled studies of the temporal evolution of diffusion changes in slices following perturbation with the calcium ionophore A23187. The mean fast diffusing water fraction ($F_{\text{fast}}$) progressively decreased for slices treated with 10 µM A23187 (-20.9 ± 6.3% at 8 hours). Slices treated with 50 µM A23187 had significantly reduced $F_{\text{fast}}$ 80 minutes earlier than slices treated with 10 µM A23187 ($P < 0.05$). These data suggested diffusion-weighted images at high $b$-values and the diffusion parameter $F_{\text{fast}}$ may be...
sensitive correlates of cell swelling in nervous tissue after acute injury. Correlative histology demonstrated selective vulnerability of hippocampal slice neurons to A23187 (CA1 > CA3) and suggested that particular cell populations may contribute disproportionately to the gross water diffusion changes observed after acute nervous tissue injury. Regional analysis of the water diffusion changes after A23187 treatment proved difficult because of microscopic slice movements and partial volume effects. Control data, however, suggested that the distinct cytoarchitecture of hippocampal regions influences the observed diffusion properties of water. To assess the validity of the hippocampal slice model, a final study measured water diffusion in human hippocampal slices procured from epilepsy patients. This human tissue had normal electrophysiological responses and exhibited similar water diffusion properties to rat hippocampal slices.

Data in this dissertation indicate that water diffusion studies in rat hippocampal slices have clinical relevance and provide interesting insights into the diffusion properties of water in normal and acutely injured brain. This work should aid the development of working models of water diffusion in nervous tissue, leading to an improved clinical utility for diffusion MRI.
CHAPTER 1
INTRODUCTION

In this dissertation, the rat hippocampal slice was used to model the effects of calcium-mediated injury on water diffusion and cellular morphology in nervous tissue. Hippocampal slices were treated with the calcium ionophore A23187, which provided a pharmacological model of acute brain injury. Water diffusion in A23187-treated slices was monitored for 8 hours using diffusion-weighted magnetic resonance imaging (chapters 3 and 4). Additional slices treated with A23187 were used for correlative histology studies. Additional studies designed and implemented a multislice perfusion chamber (chapter 2), and measured water diffusion in human hippocampal slices to assess the validity of the rat hippocampal slice model with respect to human applications of cerebral water diffusion measurements (chapter 5). This introduction provides an initial background to the topics relevant to this research and is intended to provide a context for the research described in the following chapters.

Acute Brain Injury

Acute brain injury from ischemic or traumatic events is an important clinical problem in the United States. Ischemic stroke is the third most common cause of death in the United States and affects some 1.5 million new cases each year with approximately 200,000 deaths per year (1). Unlike ischemic stroke, which is most prevalent in the elderly population, traumatic injury to the brain and spinal cord predominately affects males between the ages of 15 and 24. There are two million traumatic brain injuries (TBI) each year in the U.S. and 500,000 of these injuries require hospital admission. Some
100,000 per year die from TBI in the U.S. and another 70,000 - 90,000 survivors will have lifelong disabilities (2). Recent studies have suggested that TBI may significantly increase the risk of developing Alzheimer’s disease in a subsegment of patients (3). There are also an estimated 11,000 new cases each year of spinal cord injury (SCI). The combined cost of TBI and spinal cord injury (SCI) in the U.S. is over $100 billion each year (2).

Unlike ischemic stroke, where brain tissue is deprived of sufficient oxygen and glucose for normal metabolism, TBI involves a mechanical deformation of brain parenchyma that leads to the shearing and tearing of blood vessels and brain tissue (4). This leads to a cascade of molecular and cellular responses similar to secondary injury processes in the ischemic penumbra (2,4). Thus, although acute injuries to the central nervous system (ischemic stroke, TBI and SCI) may differ in the primary injury-producing event, they all share the same “universal response to injury” (5). The molecular and vascular sequelae to traumatic or ischemic injury deprive the brain of sufficient supplies of glucose and oxygen, which disrupts the electrical activity of the brain and rapidly leads to neuronal energy failure (6).

The excitotoxicity hypothesis proposes that depletion of cellular energy reserves after acute injury leads to the loss of neuronal ion homeostasis and excessive depolarization. These changes then lead to excessive synaptic glutamate and overstimulation of neuronal NMDA receptors (7). Although glutamate is not harmful when sequestered at 10 mM concentrations in uninjured nervous tissue, 0.1 mM glutamate for 5 minutes is sufficient to irreversibly injure cultured cortical neurons (8). Most glutamate-mediated toxicity after ischemic or traumatic nervous tissue injury is
mediated by NMDA (N-methyl-D-aspartate) receptors which facilitate the rapid influx of sodium (Na+) and calcium (Ca²⁺) into neurons. Although NMDA-mediated Na⁺ influx leads to rapid cell swelling, the influx of Ca²⁺ ions is responsible for most of the biochemical injury cascades and cellular pathology that follow excitotoxic stimulation (9).

Although NMDA-gated channels may be the principal source for glutamate-induced elevations in intracellular Ca²⁺ concentrations after acute injury, there are several other potential mechanisms. Calcium also may enter cells via voltage-gated Ca²⁺ channels activated by glutamate-induced membrane depolarization, reverse functioning of the Na⁺/Ca²⁺ exchanger due to elevated intracellular [Na⁺] and non-specific membrane leakage due to glutamate-induced cell swelling (9). After traumatic injury, Ca²⁺ may also enter cells due to mechanoporation of membranes (10). The failure of cell functions to extrude, sequester or store Ca²⁺ (e.g. in mitochondria or endoplasmic reticulum) may further increase intracellular Ca²⁺ concentrations (9). In particular, mitochondrial calcium uptake may initially may absorb elevations in intracellular [Ca²⁺], but eventually these organelles will also depolarize. Mitochondrial depolarization then leads to the complete collapse of cellular energy status and the release of additional molecular factors, such as cytochrome C, that further accelerate cell death processes (11).

Calcium-mediated mitochondrial pathology represents one of downstream events following acute brain injury. Disruption of mitochondrial function, along with calcium-mediated induction of nitric oxide synthase, phospholipase A₂, and cyclooxygenase generate excess free radical production (12,13). Free radicals then damage nearby membranes and proteins leading to further cellular dysfunction and triggering
inflammatory responses (5). The subsequent activation of Poly(ADP-ribose) polymerase to repair single-stranded DNA damaged by free radicals further depletes cellular energy stores (12). Elevations in intracellular $[Ca^{2+}]$ also lead to the overactivation of other enzymes involved in membrane turnover, cell signaling and proteolysis. Activation of phospholipases A$_2$ and C leads to the release of free fatty acids, diacylglycerol and arachindonic acid (2). Calcium irreversibly activates calpain, a proteolytic enzyme, that cleaves many cytoskeletal proteins such as spectrin, tubulin, ankryin and neurofilament proteins (14).

The morphologic pattern of cell death and the biochemical cell death cascades triggered may depend on injury severity and the resultant intracellular $Ca^{2+}$ concentration (12). Both apoptosis and oncotic cell death (15) may occur concurrently in the injury penumbra (12). After acute brain injury, inflammation also plays an important role in delayed neuronal cell death (2). As will be discussed further, this dissertation utilizes diffusion-weighted magnetic resonance imaging (MRI) to measure the water diffusion changes that accompany rapid cell swelling and subsequent cell death in the nervous tissue following an acute injury (chapters 3 and 4). To model this, the alteration of calcium homeostasis following acute brain injury can be modeled by the pharmacologic treatment of rat hippocampal slices with the calcium ionophore A23187. This ionphore causes rapid loss of calcium homeostasis and pathological cell swelling in neuronal cultures (16,17).

**The Hippocampus in Acute Brain Injury**

The anatomy of the hippocampus has been described exhaustively elsewhere for both human (18) and rat brain (19). The hippocampal formation is a phylogenetically old structure that consists of two interlocking U-shaped lamina, such that the dentate gyrus
fits into the hippocampus proper (18). The layers in the hippocampal formation are referred to as allocortex and their different histological features and diffusion-weighted MRI appearances are described fully in Appendix A. For this dissertation, the most salient feature of hippocampal connectivity is an excitatory pathway that connects 1) entorhinal neurons to granule cells of the dentate gyrus via the perforant pathway, 2) granules cells to CA3 pyramidal neurons via mossy fibers, 3) CA3 neurons to CA1 pyramidal neurons via Schaffer collaterals, and 4) fibers leaving from the CA3 and CA1 layers to the subiculum and fornix via the alveus and fimbria (19).

Although the hippocampus is a relatively small structure in human and rat brains, the hippocampal neural circuit described above, and others contained within this structure, serve as essential yet poorly understood, components of learning and memory formation (20). The hippocampus is particularly affected by the aging process; in MRI-based volumetric studies, hippocampal atrophy was associated with aging and correlated with memory impairments (21). MRI studies have also demonstrated that traumatic brain injury may accelerate age-related hippocampal atrophy and its associated memory impairments (22). Bilateral hippocampal damage or removal, as occurred in the infamous patient “H.M.”, can prove crippling to normal behavior and participation in society (20). Unfortunately, the human hippocampus is also known to be highly vulnerable to a variety of more common brain insults, including ischemic and traumatic injury (23). Several explanations for the selective vulnerability of the hippocampus to injury have been explored, but it is most likely attributable to the excess of glutamate and NMDA receptors present in the excitatory pathway described above (9,12).
Whereas ischemic or hypoxic injury to the hippocampus selectively damages the CA1 subfield (24), traumatic injury may affect multiple regions of the hippocampus (25,26). In consecutive autopsy cases, Kotapka and colleagues found that 84% of human patients with fatal non-missile traumatic injuries exhibited damage to the hippocampus. These lesions always involved the CA1 region, whereas damage to other regions of the hippocampus was not consistently present (25). Much of this pathology, however, may have been associated with concurrent ischemic injuries to the brain from vascular disruption and increased intracranial pressure (27). This highlights the frequent association of ischemia superimposed on the initial traumatic brain injury. In more controlled TBI animal models, both inertial loading and contusion injury models exhibited significant selective damage to the CA3 and hilar regions of the hippocampus (28,29). Other studies, however, have indicated that damage to the CA1, CA3 and hilar regions of the rat hippocampus can all occur in traumatic injury, even when blood flow is above the ischemic threshold (30). Thus, although it is accepted that the hippocampus is particularly vulnerable to ischemic or traumatic injury, the selective vulnerabilities of the hippocampal regions to particular injury mechanisms remains unclear.

**The Brain Slice Model of Nervous Tissue**

Slices of living nervous tissue from frog brains were first studied by Libbet and Gerard in 1939 (31). Later, pioneering electrophysiology studies in mammalian hippocampal slices were reported by Henry McIlwain and colleagues (32,33). Since this time, the brain slice technique has become increasingly exploited and has become the subject of several books and international meetings (for example, see (34)). Slices from guinea pigs, mice and rats have been used and many brain structures, including cerebral cortex, brainstem, cerebellum, spinal cord and hippocampus, have been studied. The
predominant choice, however, appears to be for hippocampal slices (35). Unlike other brain regions, the laminar anatomy of the hippocampus preserves most functional connections in slices made perpendicular to its septo-temporal axis (34).

The increasing popularity of the brain slice technique can be attributed to its versatility and to its many experimental advantages. Many brain slices can be prepared from one animal and there is complete experimental control of temperature and the extracellular pH and chemical environment. There are no anesthetic requirements that might confound subsequent perturbation studies. Because cardiac and respiratory motions are eliminated, mechanical disturbances to the slices during experiments are minimized. Further, the slices can be visualized during experimentation by various methodologies (e.g. confocal microscopy) (36).

These advantages have encouraged many groups to use rat hippocampal slices to model ischemic injuries in vivo. For example, studies show that hypoxic-ischemic injuries to slices predominately damage the CA1 subfield of the hippocampus, as observed in vivo in animal models and human patients (37). Optical scattering measures in slices indicate that hypoxic-ischemic damage is manifested early by cell swelling in the stratum oriens and radiatum adjacent to the CA1 pyramidal neurons followed by swelling in the dentate gyrus (38). Other studies have used the slice preparation to successfully screen excitatory amino acid agonists and antagonists (39).

To employ slices for these types of studies, however, several methodological concerns must be addressed. First, ischemic and mechanical damage to the brain slice must be minimized during procurement from the rat. A variety of different anecdotal solutions to minimize procurement-induced damage to slices have been proposed, yet few
tested empirically (40). For studies of this dissertation, rats were anesthetized with isofluorane prior to decapitation (41), then hippocampal slices were dissected as rapidly as possible (42) under hypothermic conditions (4°C)(43,44). Low calcium, high magnesium solutions (45) were also incorporated into the study of human hippocampal slices because of the extended time for slice procurement due to the distance between the surgical operating theater and the research lab (see chapter 5 for more details). Ascorbate (46) or proteolysis inhibitor supplements (47) were avoided as these modifications were more likely to alter the subsequent responses of the hippocampal slices to calcium-mediated A23187 treatment (16). After successful procurement, slices provided with sufficient oxygen and glucose can maintain viability for up to 24 hours (40).

Slice perfusion chambers can be interface (the top planar surface of the slice is exposed to air) or submerged (slice is surrounded on all sides by perfusate). Although interface chambers appear to give better neuronal morphology in slice histology (35,40), they are not as conducive to chemical perturbation studies due to the uneven solute distribution (36). Regardless, nuclear magnetic resonance (NMR) experiments require submerged slice designs due to substantial imaging artifacts from an air-water interface. NMR imaging experiments also require relatively thick slices (~ 500 µm) to insure sufficient signal for data analysis.

Unfortunately, submerged chambers may lead to slice hypoxia and subsequent swelling in experiments unless the slice is perfused continuously (36). This problem is made more severe by thick slices that create longer paths for the free diffusion of nutrients into the center of the tissue (43). Supplementing the perfusate with dextran reduces slice swelling due to slice thickness or experiments conducted at higher
temperatures (e.g. at 37°C), but may lead to significant amounts of nuclear pyknosis throughout the slice (48). However, previously published reports clearly demonstrate that the hypothermic conditions employed in the experiments reported herein reduce the submerged slice’s metabolic requirements for oxygen and glucose, and minimize postprocurement swelling (43,44).

The literature suggests that most slice researchers choose preparative methods to achieve slice viability based on preservation of the idiosyncratic experimental phenomenon of interest to their research (e.g. CA1 postsynaptic potentials or granule cell morphology in histology); regrettably, there does not appear to be a common consensus on what constitutes healthy brain slices (35). Most slice researchers share the view that the “model must reliably reproduce the relevant properties of the original”(34). This seems reasonable as long as one recognizes that there are limitations to the validity of these selective models of in vivo nervous tissue, such that slice paradigms are not interchangeable (40). In this dissertation, rat hippocampal slices were used to model both the morphology of heterogeneous cell populations and the water diffusion properties of in vivo nervous tissue. The exquisite sensitivity of MRI to early water diffusion changes after acute brain injury (49) was exploited to monitor the viability of control hippocampal slices. In addition, control slices were checked for morphologic changes using histological sections.

NMR Studies of Brain Slices

Recent studies have demonstrated that hippocampal and cerebrocortical brain slices can be used for NMR investigations (50-52). Brain slices preserve the ultrastructure of nervous tissue and its influences on the properties of water which are the most important features required to model nervous tissue contrast using magnetic resonance imaging
(MRI). There are some distinct advantages to brain slices in MRI not described in the previous section. MRI is extremely susceptible to motion artifacts, yet brain slices eliminate undesired motion from cardiac and respiratory motions. Brain slices also permit NMR experiments on nervous tissue using narrow-bore magnets with significantly higher main static field strengths than possible in human and animal subjects. Further, although some MRI experiments also have been conducted on cell cultures, unlike brain slices, cell cultures fail to model the high cell densities, heterogeneous cell populations and connections of in situ nervous tissue.

Previous studies in this laboratory have indicated that diffusion-weighted images of the hippocampal slice can be obtained by placing rat hippocampal slices into a submerged perfusion chamber lowered into a vertical, wide-bore 600 MHz spectrometer (50). In this design, slices were only perfused with artificial cerebrospinal fluid (ACSF) at room temperature between MRI scans. This method can maintain stable diffusion-weighted signal intensity (at $b = 1000 \text{ s/mm}^2$) for up to 8 hours if perfusion for 5 minutes is repeated at least every 90 minutes (53). Subsequent studies increased the validity of diffusion MRI studies in brain slices by demonstrating that the brain slices exhibited similar responses to perturbations by ouabain (54) and NMDA (55) as rat brain in vivo (56). In these later studies, non-monoexponential water diffusion was observed in the rat hippocampal slices and described using a biexponential model that describes water diffusion as the sum of a fast and a slow diffusing component. Decreases in the fraction of fast diffusing water ($F_f$) after ouabain and NMDA treatment were attributed to cytotoxic edema and relative increases in the size of the intracellular fraction (54,55).
The present dissertation has made several unique contributions to advancing the techniques for brain slice imaging and to advancing our understanding of the water diffusion properties in rat hippocampal slices. Prior studies were only able to image single slices before and after perturbation, and measured changes to the whole slice diffusion-weighted signal intensity (50,54,55). In chapter 2, a new method of simultaneously obtaining diffusion-weighted images from 8 or more slices is described. When perfused periodically, slices in this new chamber maintained stable water diffusion parameters for at least 8 hours after slice procurement. This perfusion chamber design was then employed to characterize the water diffusion changes in rat hippocampal slices treated with the calcium ionophore A23187 (chapter 3). Unlike prior MRI studies of slices, this study characterized the temporal evolution of water diffusion changes in hippocampal slices over multiple timepoints after experimental perturbation. Chapter 4 presents regional analysis of water diffusion in rat hippocampal slices before and after treatment with A23187. These data demonstrate how the unique cytoarchitecture of the rat hippocampal slice can be used to investigate the biophysical basis for water diffusion in nervous tissue. Finally, hypo- and hypertonic perturbation experiments of human hippocampal slices obtained from epilepsy patients are described (chapter 5). Data from this study demonstrate that the water diffusion properties of human nervous tissue were comparable to rat tissue, further validating the use of brain slices as a model to study water diffusion in nervous tissue.

**Diffusion MRI**

The basic concepts of magnetic resonance imaging (MRI) have been reviewed extensively elsewhere (57). For the purposes of this dissertation, the basic concepts and theory behind measuring water diffusion with MRI will be reviewed.
The technique for measuring water diffusion with nuclear magnetic resonance was first described by Stejskal and Tanner (58). A pair of magnetic field gradient pulses were added to a standard spin echo sequence to sensitize the water proton signal to the diffusion of spins. These spatially-oriented gradients dephase spins and then after a certain time, rephase the spins. Spins that remain in the same spatial location rephase completely, whereas spins moved along the direction of the field gradient pulses experience different strength magnetic field strengths and are rephased to different extents. Thus, the nuclear magnetic resonance (NMR) signal intensity is attenuated relative to molecular diffusion. The magnitude of attenuation also depends on the strength and duration of the magnetic gradient pulses. The attenuation of the “diffusion-weighted” signal follows according to the exponential equation;

\[ S(b) = S_0 \cdot \exp(-bD) \]  

[1-1]

where \( S(b) \) is the observed signal intensity, \( S_0 \) is the signal intensity in the absence of the diffusion-weighting gradients, \( b \)-value represents the sensitivity of the sequence to diffusion and \( D \) is the diffusion coefficient of the population of spins observed (water).

For diffusion gradients of equal amplitude and duration, the diffusion-weighting or \( b \)-value is described by the equation;

\[ b = \gamma^2 G^2 \delta^2 \cdot (\Delta - \delta/3) \]  

[1-2]

where \( \gamma \) is the gyromagnetic ratio of the spin, \( G \) and \( \delta \) are the respective amplitude and duration of the magnetic field gradient pulses, and \( \Delta \) is the time interval between the gradient pulses. The term \( (\Delta - \delta/3) \), or the diffusion time \( (T_d) \), represents the effective time interval during which the spins experience between the diffusion gradient pulses (58). Unlike the NMR spectroscopy experiments for which Eqs. [1-1] and [1-2] were originally
described, when the signal is spatially localized for imaging experiments, the $b$-value will be altered by inherent diffusion-weighting in the slice-select, frequency- and phase-encoding gradients required for magnetic resonance imaging (MRI) experiments. Full mathematical descriptions of these imaging gradient cross-terms can be found elsewhere (59), but it should be noted that imaging cross-terms were accounted for in the Paravision software (Bruker, Billerica, MN) employed for the diffusion MRI experiments reported herein.

The diffusion coefficient ($D$) in Eq. [1-1] is describes diffusion caused by the Brownian motion of spins (free diffusion), such as observed in a sample of pure water in an NMR test tube. However, water molecules in biological samples encounter physical barriers and restrictions that impede their random displacements within the diffusion time of the experiment. These physical properties of the biological system will influence the diffusion coefficient measured, so it is more appropriate to describe the water diffusion rates in biological systems calculated from MRI data using the term apparent diffusion coefficient (ADC)(60). This reflects that measured diffusion may be slower than the rate of water molecule movement due their collisions with boundaries. The presence of restriction can be assessed using the Einstein relation, which predicts the mean square displacement ($r^2$) of freely diffusing water in three dimensions;

$$r^2 = 6DT_d$$  \[1-3\]

When $r^2$ does not increase proportionately with increases in $T_d$, restriction is indicated (61).

In early studies, Eq. [1-1] was used to describe the diffusion-weighted water signal attenuation in human and animal nervous tissue (49). Powerful diffusion gradients,
however, allowed researchers to measure water diffusion at \( b \)-values significantly greater than 1000 s/mm\(^2\) with short \( T_d \); data obtained under these conditions indicated that diffusion-weighted water signal attenuation in nervous tissue was non-monoexponential (62,63). This result suggested that water was compartmentalized into at least two environments with different diffusion properties. The water signal attenuation observed depends on the relationship between \( T_d \) and the exchange rates of water between the two compartments (64,65). In most diffusion experiments, the diffusion times employed (13-15 ms in this dissertation, for example) are assumed to be short enough that little exchange of water molecules between compartments occurs (the slow or intermediate exchange regime). This assumption then allows water in nervous tissue to be parameterized by the biexponential equation;

\[
S(b) = S_0 \cdot [ F_f \cdot \exp(-bD_f) + (1- F_f) \cdot \exp(-bD_s) ] \tag{1-4}
\]

where \( F_f \) equals the fraction of fast diffusing water, \( D_f \) is the ADC of the fast component and \( D_s \) is the ADC of the slow component. In the biexponential model described by Eq. [1-4], all water molecules observed are assigned to either the fast or slow compartments such that the fraction of the slow component \( (F_s) \) can be determined;

\[
F_s = 1 - F_f \tag{1-5}
\]

Recent studies have investigated the biexponential model of water diffusion further in erythrocyte ghosts (66). In agreement with previous studies (63), this study demonstrated that changes to the \( F_s \) and \( F_f \) diffusion parameters track with changes in the intracellular fraction, although \( F_s \) does not directly correlate with the intracellular fraction (e.g. an \( F_s \) of 0.30 does not mean 30% of the water is intracellular)(66). This study indicates that the biexponential model is does not fully describe the biphysical properties
of the tissue that influence water diffusion and the biexponential diffusion parameters obtained depend on the diffusion pulse sequence values employed (e.g. \( b \)-values and \( T_d \))(66). A two compartment model of diffusion proposed by Li and colleagues that accounts for restriction, exchange and tortuosity better describes water diffusion in the erythrocyte tissue model (66,67). However, the erythrocyte ghosts study also shows that the biexponential model can be used reasonably when you are aware of its limitations. As reviewed by Norris, there are several other models of water diffusion in biological tissue that parameterize water exchange between compartments, intracellular restriction and extracellular tortuosity (61). These models may offer more complete descriptions of the diffusion properties of water in nervous tissue, but are very data intensive (i.e. requires multiple diffusion measurements at different \( T_d \), as well as T1 and T2 measurements). This makes it difficult to obtain sufficient data for these models in animal and human subjects. For this reason, in the chapters of this dissertation, water diffusion in the rat hippocampal slices will be modeled using Eq. [1-4].

**Diffusion-Weighted MRI of In Vivo Brain Injury**

In acute brain injury, osmotic processes caused by the massive influx of sodium, chloride and calcium ions into cells drives a net flow of water from the extra- to intracellular compartments, leading to cytotoxic edema. Moseley and colleagues were the first to demonstrate that these phenomena caused changes in water diffusion detectable with diffusion-weighted MRI, and show that this technique was particularly adept at detecting ischemic lesions less than an hour after onset (49). Clinical studies have since demonstrated that diffusion-weighted MRI lesion volumes measured within 7 hours of injury correlated well with outcome NIH stroke scale scores and final lesion volumes (68). Several reports have also suggested that diffusion-weighted MRI is superior to
computed tomography (69) and other MRI contrast mechanisms (70,71) for the detection of ischemia in patients. These findings appear to apply equally to traumatic brain injury (72,73), but clinically the focus thus far has been mostly on applications of diffusion-weighted imaging to ischemic injury.

A frequent criticism of previous clinical trials for neuroprotective drugs aimed at treating either ischemic or traumatic brain injury was the failure to select patients appropriately based on brain injury type, severity, location and potential for amelioration (74,75,76). Diffusion-weighted MRI, in combination with other MRI contrast methods, is an ideal method of better stratifying patients for clinical trials of neuroprotective drugs (77,78). By combining diffusion and perfusion MRI techniques, it is possible to recognize regions of brain tissue with abnormal perfusion that have not yet manifest diffusion abnormalities (the “diffusion-perfusion mismatch”) (79). These regions are believed to represent penumbral regions where prompt treatment with drugs, such as tissue plasminogen activator (80), may ameliorate some of the morbidity associated with ischemic injury (77). More recent studies have suggested that the ischemic penumbra includes not just the region of diffusion/perfusion mismatch but also portions of the region with initial diffusion abnormalities (81).

To better understand the value of diffusion-weighted MRI as a surrogate marker of acute nervous tissue injury, several clinical groups have attempted more quantitative interpretations of the diffusion changes observed after ischemic injury. In animal models of brain ischemia, the apparent diffusion coefficient (ADC) of water initially decreases significantly below normal values during the first hours following ischemic infarction in a variety of animal models, then normalizes briefly before developing values near the
diffusion coefficient of freely diffusing water (82). This time course of ADC changes has also been reported for several different animal models of SCI (83,84) and TBI (85-87). In rats with focal ischemia, the duration of insufficient perfusion determines the magnitude of ADC reduction for particular tissue regions (88). Histological ultrastructure studies in ischemic rat brains have shown that ADC normalization in ischemic rat brains actually correlates with pronounced swelling of the perivascular astrocytic endfeet and dark neurons (indicative of cell death) (89). In a different rat model of ischemia, ADC normalization was not attributed to neuronal recovery, but to non-neuronal populations compensating for early fluid imbalances (90). Thus water diffusion reflects a range of biophysical processes that will require more extensive measurements to understand exactly what happens to the ultrastructure of tissue after ischemic injury.

Recent studies have suggested that ADC changes are heterogeneous in human stroke patients and suggests that different tissues may exhibit different rates of temporal evolution to ischemic infarction (91). Intermediate ADC values in humans may indicate penumbral tissue at risk of infraction if left untreated (92,93). Based on such findings, Welch and colleagues have proposed a simplistic tissue signature model to predict outcome for nervous tissue with particular T2 and ADC changes after ischemic injury (94). However, in some studies tissue recovery this does not seem to depend on the magnitude of the initial ADC change, but on the duration and severity of ischemia (95). Clearly, there remains much debate about the clinical meaning of ADC changes and whether these changes can be interpreted in a quantitative fashion. Understanding the predictive value of diffusion or ADC changes in patients suffering acute brain injuries may prove difficult to improve with studies that follow ischemic changes in human or
animal subjects. Experiments to investigate the biophysical basis for water diffusion in normal and acutely-injured nervous tissue (as described in chapters 3 and 4) may be required.
CHAPTER 2
SIMULTANEOUS DIFFUSION MRI MEASUREMENTS FROM MULTIPLE PERFUSED RAT HIPPOCAMPAL SLICES

Introduction

Brain slice experiments have become increasingly popular with neuroscientists because they reduce the complexity of in vivo nervous system models while retaining several key features of normal brain tissue. As with in vitro cell culture models of nervous tissue, brain slices allow complete experimental control of the extracellular environment. However, unlike dissociated cell cultures, brain slices retain most of the in situ functional and anatomical cellular connections between neighboring cell groups, particularly for regions like the hippocampus (96). Since three-dimensional cytoarchitecture is a critical determinant for most MR-based contrast mechanisms in tissue, brain slices provide an excellent model to investigate the biophysical basis of signal changes observed clinically in pathological nervous tissue. When adequately perfused, brain slices can be studied for longer imaging times than are feasible with human and animal subjects. The small size of the slices also permit studies in narrow-bore, high field magnets equipped with very strong gradients, allowing high resolution MR microscopy. Additionally, MR signal changes in slices are not confounded by blood flow artifacts or movements due to breathing. Furthermore, there are no requirements for anaesthetic agents that may confound studies because of neuroprotective effects.

Several groups have recognized these distinct advantages. Blackband and colleagues reported several imaging studies that examined water diffusion changes in
single slices of rat hippocampus after perturbation (53-55). Other researchers have used $^{31}$P and $^1$H NMR spectroscopy to characterize metabolic changes in hypoxic rat cortical brain slices (51,52). These spectroscopy studies employed many brain slices to obtain adequate signal-to-noise ratios. However, no reports exist for the simultaneous imaging of multiple brain slices. Such a method could dramatically reduce the number of rats required per imaging experiment while permitting concurrent measurements of slices under control and perturbed conditions.

The chamber design used by M.T. Espanol et al. (51) was well suited for spectroscopy studies of multiple cerebrocortical slices, but the random oblique slice orientations would be incompatible with most imaging experiments. A chamber that could isolate brain slices in parallel orientations was required. Here, we describe such a novel perfusion chamber designed for MR imaging experiments of multiple rat hippocampal slices inside a vertical bore magnet. We demonstrate the advantages of this device by simultaneously acquiring diffusion-weighted image series for eight hippocampal slices. This acquisition can be repeated for at least 8 h after slice procurement, shows the potential for acute temporal resolution of signal changes after experimental perturbation.

**Methods**

**Multislice Perfusion Chamber**

The multislice perfusion chamber (Fig. 2-1) represents substantial modifications to a previous design (50). The top and bottom support rings were constructed from hollow delrin cylinders with 1 mm thick walls lathed to fit inside a 15-mm NMR tube. Four hollow Delrin spacers (0.80 mm thick) were made to the same dimensions. Polypropylene mesh (0.5 mm pore size) was attached to the underside of each spacer and
the top support ring using cyanoacrylate. A quarter segment of the bottom support ring was removed to allow unhindered perfusate flow from the inflow tube into the chamber. A groove was cut into each support ring and spacer to allow space for the inflow tube (Tygon microbore, 1.0 / 1.7 mm inner / outer diameter), which was attached to the bottom support ring using epoxy resin. Two outflow tubes were used to prevent chamber flooding. The 8 m length perfusion lines consisted of three Tygon tubes inside larger polyvinylchloride tubing. This arrangement allowed the perfusion lines to be used in multiple applications and permitted easy detachment of the chamber.

**Brain Slice Procurement**

The use of laboratory animals for this study was approved by the University of Florida Institutional Animal Care and Use Committee. Rat hippocampal slice procurement has been described previously (40). Briefly, male Long-Evans rats were anesthetized then decapitated. The brain was removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) (120 mM NaCl, 3 mM KCl, 10 mM glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 1.5 mM KH₂PO₄, 1.4 mM MgSO₄) gassed with 95% O₂ / 5% CO₂ to maintain a pH of 7.4. Both hippocampi were dissected and cut into 500 µm thick sections with a McIlwain tissue chopper. Hippocampal slices were immersed in ice-cold ACSF for one hour to minimize procurement-induced ischemic damage (40), then warmed gradually to room temperature for the NMR experiment. Brain slices from several rats (4 total) were used to develop the perfusion chamber. However, in this brief report, results are reported for 8 slices from 1 rat.

**Placement of Brain Slices into Perfusion Chamber**

After the inflow and outflow tubes were connected to the perfusion lines via Luer Locks, the bottom support ring was submerged in a 15-mm NMR tube filled with ACSF.
Working just below the meniscus, a spacer was placed on top of the bottom support ring and two hippocampal slices gently positioned on top of that spacer. This process was repeated, placing slices on the three remaining spacers. After covering the last spacer with the top support ring, the completed perfusion chamber was pushed to the bottom of the NMR tube. The NMR tube was sealed with a slotted cap and parafilm. The chamber was perfused briefly then lowered into the magnet. Although perfusion was discontinued during imaging, the chamber was perfused with ACSF (2 mL/min) for 5 minutes every 35 minutes. This interval does not affect tissue viability during the first 8 hours (53).

**MR Imaging with Perfusion Chamber**

Imaging experiments were conducted at room temperature using a 15-mm birdcage coil interfaced to a vertical Bruker 750-MHz wide-bore spectrometer and console. All MR experiments used multislice imaging sequences. First, pilot T1-weighted images were collected to locate the chamber position and check that air bubbles were not present. Next, the hippocampal slices were localized by sagittal, coronal and axial multislice images using a standard spin-echo imaging sequence with diffusion-weighting gradient pulses placed on either side of the 180° radiofrequency pulse ($b = 1800 \text{ s/mm}^2$). This diffusion-weighted sequence was repeated with four 300-µm thick axial slices optimized so that each slice included both hippocampal slices located within a spacer. A series of eight diffusion-weighted images was then acquired (128 x 64 matrix, 1.5 cm field-of-view, repetition time = 2 s, echo time = 30 ms, 2 averages, diffusion time = 14 ms) employing z-oriented diffusion gradients linearly spaced from 0 to 940 mT/m, resulting in $b$ values between 7 and 8080 s/mm² (including imaging terms). This protocol required 35 minutes for completion. A cycle consisting of this diffusion protocol followed by 5 minutes of perfusion was repeated serially until 8 hours after rat sacrifice (in this report, 8
diffusion protocols were completed). At the conclusion of each perfusion period, additional multislice images were obtained to monitor for slice movement (none was detected).

**Data Analysis**

Four regions of interest (ROI) were drawn on each diffusion-weighted image collected during the first diffusion protocol. An ROI to determine the noise level was placed in the upper corner of the image outside the sample. A second ROI was placed in the perfusate surrounding the brain slices. Two ROI’s were drawn to encompass the two hippocampal slices present in each axial image at the highest $b$ value image. These ROI’s excluded regions where adjacent slices overlapped and regions of slices where pilot images demonstrated significant volume averaging with the perfusate (i.e., absence of signal at high $b$ values). The ROI’s were cloned to all subsequent diffusion protocols, thus providing a sensitive indicator of slice movement. Although it did not occur in this particular experiment, slices that moved would have been rejected from the subsequent analysis.

The signal-to-noise ratio (SNR) for each hippocampal slice was calculated by dividing the mean signal intensity from a slice ROI by the standard deviation of the noise ROI. This calculation was done for every hippocampal slice using both the lowest and highest diffusion-weighted images ($b = 7$ and $8080$ s/mm$^2$).

To check the accuracy of the MR experiments, Eq. [1] was fitted to the mean signal intensity of the perfusate ROI to determine the signal intensity without diffusion weighting ($S_0$) and the apparent diffusion coefficient of perfusate (D).

$$S(b) = S_0 \exp(-bD)$$

[2-1]
Only data from the first four of the eight diffusion-weighted images collected per diffusion protocol were used for this calculation because previous experiments showed signal from perfusate dropped into the noise level at \( b \) values above 1800 s/mm\(^2\) (54). The perfusate diffusion coefficient was determined from each of the four imaging slices obtained per experiment in each of the 35 minute diffusion protocols.

Previous experiments demonstrated that diffusion-weighted signal attenuation in hippocampal slices is non-monoexponential at high \( b \) values. As a first approximation, a biexponential function (54,55) was used to describe the data;

\[
S(b) = S_0 \cdot [f_{\text{fast}} \cdot \exp(-bD_{\text{fast}}) + (1-f_{\text{fast}}) \cdot \exp(-bD_{\text{slow}})]
\]  \[2-2\]

where, \( S_0 \) is signal intensity without diffusion weighting, \( f_{\text{fast}} \) is the fraction of water with fast apparent diffusion coefficient (ADC); and \( D_{\text{fast}} \) and \( D_{\text{slow}} \) represent the ADCs of the fast and slow diffusing water components respectively. Equation [2-2] was fit to the diffusion-dependent attenuation of mean signal intensity in each hippocampal slice ROI using the Levenberg-Marquandt non-linear least squares fitting routine to determine estimates of \( S_0, f_{\text{fast}}, D_{\text{fast}} \) and \( D_{\text{slow}} \). Pixel-by-pixel analysis of diffusion signal attenuation in hippocampal slices was not attempted because of significant signal-to-noise limitations. Although the biexponential fitting may be inadequate to accurately fit the diffusion data (new models are in development), it is useful to compare the biexponential results with previous experiments using a single slice chamber so that reproducibility and tissue stability can be addressed.

**Results**

Eight rat hippocampal slices were imaged simultaneously in the multislice perfusion chamber. Pilot images were used to position the axial MR-defined slices required to image concurrently both rat hippocampal slices located within a spacer (Fig.
The resultant optimized axial images resolved the lamellar anatomy of the rat hippocampus (Fig. 2-3). Also note that minimal overlap of hippocampal slices was achieved. The SNR (mean ± standard deviation) was 103 ± 5 for the lowest diffusion weighted images ($b = 7 \text{s/mm}^2$) and 48 ± 3 for the highest diffusion-weighted images ($b = 8080 \text{s/mm}^2$). The perfusate ADC (mean ± standard deviation) did not vary significantly at different spacer levels of the chamber or for each of the diffusion protocols ($2.01 ± 0.04 \times 10^{-3} \text{mm}^2/\text{s}$).

Eight diffusion experiments were performed within approximately 8 hours of hippocampal slice procurement. Diffusion-weighted signal attenuation in the slices (Fig. 2-4) was well described by the biexponential model described in Eq. [2-2] ($r^2 > 0.99$). Table 2-1 reports the mean parameter estimates $f_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ determined for each of the diffusion experiments. A one-way repeated measures analysis of variance compared the individual slice parameters $f_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ determined over time by the eight diffusion protocols; no statistically significant differences were found.

**Discussion**

This multislice perfusion chamber allowed simultaneous imaging of eight rat hippocampal slices with high SNR. The measured perfusate ADC matched the published values for the water diffusion coefficient at room temperature (97) and was consistent throughout the diffusion protocols. In addition, the perfusate ADC and slice SNR were independent of their vertical position within the perfusion chamber. The mean diffusion parameters $f_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ for hippocampal slices extracted from our measurements (Table 2-1) agree with previously published values (54,55). This suggests that volume averaging due to the complex arrangement of slices in the perfusion chamber was not significant.
Given the sensitivity of diffusion MRI to ischemic damage (49), diffusion measurements were used to monitor the stability of the hippocampal slices in the multislice perfusion chamber. Here, we demonstrated that the slice diffusion parameters $f_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ showed no statistically significant changes ($P > 0.05$) over the 8 hour duration of experiment. The extended stability of slices suggests this perfusion chamber could be used to study the temporal evolution of ADC changes following experimental perturbation.

By altering the number of spacers or slices used, several configurations of the multislice perfusion chamber are possible. However, the number of spacers is limited as samples reach vertical positions in the magnet where imaging gradients become nonlinear or the magnetic field and radiofrequency coil become inhomogeneous. In experiments with phantoms, these problems were shown to be absent ± 8 mm of magnet isocenter for our coil and spectrometer. A drawback to placing more than one hippocampal slice per spacer is that only one image can be acquired per spacer to avoid saturation effects in a multislice image acquisition. This situation requires careful placement of the MR-defined slice based on pilot images in all three orthogonal axes. Despite our success overcoming this problem (Fig. 2-3), in future experiments some slices may need to be excluded because of slice overlap, or substantial volume averaging with perfusate.

There are important advantages to this multislice perfusion chamber over previously reported designs. Since it is feasible to routinely obtain 20+ hippocampal slices from one rat, this design should reduce the number of animals and total time required for particular imaging experiments. This will also be particularly important in studies of human brain slices where sample access is limited. The present chamber
permits simultaneous observation of control and pretreated brain slices. Future modifications may also allow imaging experiments of slices treated separately while inside the perfusion chamber.

Figure 2-1. Expanded schematic of the multislice perfusion chamber in a 15-mm NMR tube. Two 500 µm rat hippocampal slices can fit within each of the 800 µm thick spacers. Open arrows illustrate the flow pattern of the perfusate.
Figure 2-2. Pilot images of rat hippocampal slices inside the perfusion chamber. T<sub>1</sub>-weighted sagittal multislice images (A) verified the position of the chamber and ruled out susceptibility artifacts. The white box outlines the magnified field-of-view for a sagittal diffusion-weighted pilot image (B) used to localize the positions of the hippocampal slices.
Figure 2-3. Diffusion-weighted images ($b = 1838 \text{ s/mm}^2$) of 8 hippocampal slices inside the multislice perfusion chamber (117 x 234 µm in-plane resolution). White circles indicate the inner border of the perfusion chamber spacers.
Figure 2-4. Diffusion-weighted signal attenuation curve of rat hippocampal slices (mean ± standard deviation).
Table 2-1. Rat hippocampal slice biexponential diffusion parameters over time

<table>
<thead>
<tr>
<th>Time diffusion protocol completed&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Diffusion Parameter [mean ± standard deviation] (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$F_{\text{fast}}$ (no units)</td>
</tr>
<tr>
<td>3 h 15 min</td>
<td>0.527 ± 0.041</td>
</tr>
<tr>
<td>3 h 55 min</td>
<td>0.532 ± 0.040</td>
</tr>
<tr>
<td>4 h 35 min</td>
<td>0.529 ± 0.038</td>
</tr>
<tr>
<td>5 h 28 min</td>
<td>0.521 ± 0.034</td>
</tr>
<tr>
<td>6 h 8 min</td>
<td>0.518 ± 0.038</td>
</tr>
<tr>
<td>6 h 48 min</td>
<td>0.528 ± 0.028</td>
</tr>
<tr>
<td>7 h 28 min</td>
<td>0.523 ± 0.023</td>
</tr>
<tr>
<td>8 h 8 min</td>
<td>0.513 ± 0.037</td>
</tr>
<tr>
<td>P value&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.272</td>
</tr>
</tbody>
</table>

<sup>a</sup> This time is calibrated from the time of rat sacrifice.

<sup>b</sup> Change in parameters over time tested for statistical significance using a one-way repeated measures ANOVA.
CHAPTER 3
DIFFUSION MRI STUDIES OF A RAT HIPPOCAMPAL SLICES TREATED WITH A23187

Introduction

Many neuroprotective strategies have proven successful treating animal models of ischemic stroke and traumatic brain injury, yet have failed to demonstrate meaningful benefits to human patients during clinical trials (74). The frequent disappointments in these studies have often been attributed to defects in clinical trial design wherein studies fail to rapidly assess and stratify potential subjects based on the severity, location and complexity of their acute brain injuries (74). Appropriate subject selection is particularly important since brain injury patients represent a far more heterogeneous population compared to their corresponding animal models of disease (75). To address this problem, it has recently proven beneficial to incorporate diffusion-weighted magnetic resonance imaging MRI into clinical trials (76) because of its sensitivity to changes in nervous tissue water diffusion that occur immediately after injury (49). Diffusion-weighted MRI of stroke patients, for example, helps recognize the “perfusion-diffusion mismatch,” which identifies a penumbral volume of nervous tissue that may be rescued from ischemia injury by prompt infusion of tissue plasminogen activator (77).

An improved understanding of water diffusion in nervous tissue and the acute water diffusion changes which follow injury may further increase the sensitivity and specificity of diffusion-weighted MRI as a surrogate marker of acute brain injury. Unfortunately, previous diffusion MRI studies have provided limited data from the first
24 hours following ischemic or traumatic brain injury (for example, (68)). Although this information has been helpful to understand the chronic progression of diffusion changes after brain injury, it is well beyond the theoretical therapeutic windows when experimental interventions have proven beneficial (98). Thus, more studies that better resolve the acute temporal progression of diffusion changes after brain injury are needed to better understand the potential effects of therapeutic interventions early after brain injury.

Also unlike previous clinical studies of acute brain injury, recent studies have demonstrated that diffusion-weighted signal attenuation is non-monoexponential in rat (63) and human brains (99) when measured with very strong diffusion-sensitizing gradients (high $b$-values). As a first approximation, a biexponential function may be used to describe water diffusion in nervous tissue by separating the MRI signal into contributions from fast and slow diffusing components. Shifts between these two components may correlate with changes in the relative size of the intracellular compartment (63,66). Although the biexponential model is underparameterized, it offers a more complete description of multicomponent water diffusion in nervous tissue than the monoexponential fits currently employed clinically.

To obtain water diffusion data for multicomponent analysis throughout the first 8 hours following an acute brain injury would prove extremely difficult in human or animal subjects because of their limited tolerance for long imaging times and because high $b$-value diffusion-weighting is difficult to obtain on relatively large subjects due to current hardware limitations. Thus, to further investigate the value of diffusion MRI as a surrogate marker of acute brain injury, we measured water diffusion in perfused rat
hippocampal slices using a 17.6-T magnet with strong diffusion-sensitizing gradients (1000 mT/m). Unlike human and animal subjects, rat hippocampal slices tolerate long periods of investigation yet maintain the heterogeneous neuronal and glial cell populations (40) that determine in vivo MRI contrast. Further, studies of hippocampal slices are not confounded by anesthesia or movement and perfusion artifacts (40). Previous studies demonstrated diffusion-weighted MRI of rat hippocampal slices (50) and show that slices perturbed by ouabain and NMDA responded similarly (54,55) to the same perturbations of in vivo rat brain (56). Further, human brain slices had comparable water diffusion properties to those reported for human patients and rat brain slices (100).

In this study, water diffusion was monitored in rat hippocampal slices before and after treatment with different doses of A23187. This carboxylic acid calcium ionophore rapidly diminishes calcium gradients across cell membranes (101). Previous studies have demonstrated that neuronal cultures treated with high doses of calcium ionophores, such as A23187, experienced rapid loss of calcium homeostasis and pathological cell swelling (17,102,103). We hypothesized that this cellular response would be detectable in rat hippocampal slices using diffusion-weighted MRI and correlative histology. Unlike previous diffusion studies of nervous tissue injury, using A23187 allows this study to uniquely evaluate calcium overload (16), a downstream integrating event in many forms of acute brain injury (i.e. ischemic stroke or traumatic brain injury) (2,9).

Methods

Brain Slice Procurement

The use of laboratory animals for this study was approved by the University of Florida Institutional Animal Care and Use Committee. Rat hippocampal slice procurement has been described previously (40). Briefly, male Long-Evans rats (250 –
350 g) were anesthetized with isofluorane then decapitated. The brain was removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) (120 mM NaCl, 3 mM KCl, 10 mM glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 1.5 mM KH₂PO₄, 1.4 mM MgSO₄) gassed with 95% O₂ / 5% CO₂ to maintain a pH of 7.4. The ACSF osmolality was 300 ± 1 mOsm/kg as determined by Osmette A freezing point depression osmometer (Precision Systems, Inc.). Both hippocampi were dissected and cut orthogonally to the septo-hippocampal axis into 500-µm thick sections with a McIlwain tissue chopper within 10 min of rat sacrifice. Hippocampal slices remained immersed in ice-cold ACSF for one hour after rat sacrifice to minimize procurement-induced ischemic damage (43,104), then slices were warmed gradually to room temperature (20°C) for the MRI experiment and placed into a perfusion chamber that facilitates diffusion MRI measurements (105). Slices were perfused continuously with ACSF (2 mL/min) while the perfusion chamber was lowered into the magnet and during pilot image acquisition. Although perfusion was discontinued during all diffusion measurements (described below), it has been shown previously that intermittent cessation of flow does not affect slice viability for at least 8 hours following slice procurement (105).

**Diffusion MRI of Rat Hippocampal Slices**

MRI data were obtained at room temperature using a 15-mm birdcage coil interfaced to a Bruker 17.6-T vertical wide-bore magnet and console. Pilot multislice axial, sagittal and coronal T₁ and diffusion-weighted imaging sequences were used to locate the perfusion chamber, then to optimize the positions of axial MR-defined slices through the center of the 500-µm thick rat hippocampal slices. Diffusion-weighted images were acquired using a pulsed gradient spin-echo multislice sequence with a
diffusion time of 14 ms and an echo time of 30 ms. This diffusion measurement consisted of a series of 8 diffusion-weighted images (128 x 64 matrix, 1.5 cm field-of-view, repetition time = 2 s, echo time = 30 ms, 2 averages, δ = 3 ms, Δ = 15 ms) employing diffusion gradients aligned with the B₀-field in linear increments of strength from 0 to 940 mT/m. This resulted in 8 diffusion-weighted images with b-values between 7 and 8080 s/mm² (including imaging terms). Image slice thickness was 300 µm with 117 x 234 µm in-plane resolution and the protocol required 34 minutes for completion. Additional pilot images were acquired between diffusion measurements to monitor for slice movement due to ACSF perfusion; data from hippocampal slices that moved during the experiment were rejected (n = 1).

**Slice Perturbation with A23187**

Before slice procurement, rat hippocampal slices were randomly assigned to treatment by control ACSF, or ACSF containing 10 or 50 µM A23187. Hippocampal slices were exposed to higher doses of A23187 than previous studies in neuronal cultures (16,106) because the slice model was composed of high density, heterogeneous cell populations studied at lower temperatures. Dimethylsulfoxide (DMSO) was required to solubilize A23187 in ACSF, so control ACSF contained 0.1% DMSO. The additions of 0.1% DMSO with or without A23187 did not affect the pH or osmolality of the ACSF solution, and each of the ACSF treatment solutions were gassed with 95% O₂ / 5% CO₂ throughout the experiment. All slices were perfused with standard ACSF during pilot scans and before initial baseline diffusion measurements were acquired. Slices were perfused then with the assigned treatment ACSF for 20 minutes, then perfusion was stopped and a posttreatment diffusion protocol obtained. Preliminary studies
demonstrated that an initial perfusion duration of 20 min with treatment ACSF was sufficient for perfusate exchange. A cycle consisting of 6 minutes perfusion with the assigned treatment ACSF followed by an additional diffusion measurement (34 minutes) then was repeated serially until 12 hours after initial brain slice procurement (each complete cycle required 40 minutes).

The initial treatment period for slices occurred at slightly different times following brain slice procurement due to variations in the time required for hippocampal slice placement and pilot MRI scans (mean time delay from procurement to baseline diffusion measurement was 3.1 ± 0.4 hrs). Therefore diffusion data from rat brain slices required assignment to time-bins for statistical comparisons. The baseline diffusion measurement for each slice was assigned to 0 minutes and subsequent data were assigned to time points increasing in 40-minute increments. Although data were re-assigned to time points relative to the initial MRI measurement, diffusion data acquired more than 12 hours after slice procurement were excluded from the analysis.

**Analysis of Diffusion Data**

To analyze changes in the diffusion-weighted signal intensity in rat hippocampal slices after treatment with the various ACSF solutions, a region-of-interest (ROI) was drawn on the images to enclose the entire hippocampal slice. Previous studies demonstrated that diffusion-weighted water signal attenuation in rat hippocampal slices is non-monoexponential at high \( b \)-values (54). A biexponential equation [3-1] was fitted to the diffusion-weighted signal attenuation in the rat hippocampal slice ROIs using the Levenberg-Marquandt non-linear least squares fitting routine:

\[
S(b) = S_0 [F_{\text{fast}} \exp(-bD_{\text{fast}}) + (1-F_{\text{fast}}) \exp(-bD_{\text{slow}})]
\]  

[3-1]
where \( S_0 \) is signal intensity without diffusion weighting, \( F_{\text{fast}} \) is the fraction of water with fast apparent diffusion coefficient (ADC), and \( D_{\text{fast}} \) and \( D_{\text{slow}} \) represent the ADCs of the fast and slow diffusing water components respectively. Observed changes over time from baseline in the biexponential parameters of the 3 treatment groups (control, 10 \( \mu \text{M} \) A23187 and 50 \( \mu \text{M} \) A23187) were compared statistically using a two-way repeated-measures ANOVA with one factor repeated (Sigma Stat 2.03). If the ANOVA test detected a statistically significant difference between treatment groups at a particular timepoint, a Tukey multiple comparisons test was used to isolate treatments that differed significantly from one another. Statistical significance for all tests was defined as \( P < 0.05 \).

**Histology of Rat Hippocampal Slices**

Because it proved difficult to consistently recover rat hippocampal slices used in the diffusion MRI experiments for subsequent study, an additional 3 rats were sacrificed to provide slices for histological correlation. These slices experienced the same experimental procedure as slices used in the diffusion MRI measurements and were perfused with one of the 3 ACSF treatment solutions (control, 10 \( \mu \text{M} \) A23187 or 50 \( \mu \text{M} \) A23187) 4 hrs postprocurement. At 12 hours postprocurement, the slices were gently removed from the perfusion chambers and immersion-fixed with 4% paraformaldehyde in phosphate-buffered saline. Also, some slices were fixed either immediately after procurement or before perfusion with treatment ACSF (4 hrs into procedure described above) to serve as additional controls. In addition, a few slices that were recovered after the MRI experiments were fixed for histology studies to assess whether differences existed for slices treated in the magnet versus slices treated in the correlative protocol.
The fixed slices were embedded in paraffin, cut into 8-µm thick sections, mounted onto slides and stained with hematoxylin & eosin (H & E). Most slices were sectioned parallel to the plane of the slice (orthogonal to the septo-temporal axis of the rat hippocampus), but in some slices, sections were cut perpendicular to the plane of the tissue (cross-section). These sections were used to determine whether cells at different depths from the tissue surface were affected differently by the various ACSF treatment solutions. Superficial and central planar sections, stained with H & E, were also compared to assess the potential variable effects of tissue depth on tissue response to A23187 treatment.

In all H & E stained sections, morphologic characteristics of granule and pyramidal neurons from the dentate gyrus, CA1, CA2 and CA3 fields were noted. The surrounding neuropil, in such regions as the stratum radiatum, were also inspected for vacuolization and other pathological changes. These features were compared between the slices treated with control, 10 µM A23187 or 50 µM A23187 ACSF solutions for differences in injury-induced cellular morphology and pathology. Then, specific regions were analyzed for nuclear and cytoplasmic changes. Nuclear changes such as pyknotic and eosinophilic nuclei, karyorhexis or karyolysis were noted. The cytoplasm of cells were observed for evidence of eosinophilia, micro-vacuolation and loss of structure or fragmentation.

Results

Diffusion-Weighted MRI of Rat Hippocampal Slices

Twenty-nine of the 35 rat hippocampal slices procured from 17 rats were included for the diffusion MRI aspects of this investigation. The other 6 slices were rejected because of perfusion chamber flooding (n = 3), susceptibility artifacts due to air bubbles
in the chamber (n = 2) and slice movement (n = 1). In comparing the 3 treatment groups (Table 3-1), there were no significant differences for the postprocurement time when the first baseline diffusion measurements were obtained or for the time when slices were subsequently treated. Mean time-to-treatment for all slices (3.8 ± 0.5 hrs) closely approximated the time when correlative rat hippocampal slices were treated outside the magnet for histology studies (4 hrs). Diffusion measurements were obtained on all 29 slices up to 6 hrs after treatment, on 28/29 slices up to 7.3 hrs after treatment and on 20/29 slices 8 hrs after treatment.

High-resolution diffusion-weighted images were obtained of rat hippocampal slices using a vertical, wide-bore 17.6-T magnet to determine achievable SNR and spatial resolution per unit time. Such images (Fig. 3-1) exhibited the detailed laminar anatomy of the hippocampus, but required long acquisition times (4.5 hrs per \( b \)-value measurement) without sufficient perfusion to maintain slice viability (105). For this study, such high spatial resolution was sacrificed for better temporal resolution of the water diffusion changes that accompany slice perturbation with A23187 (4.2 min per \( b \)-value measurement). In figure 3-2, the lower-resolution images demonstrate the signal intensity changes which accompany treatment with A23187. Plotting the signal intensity of the hippocampal slice ROI vs. \( b \)-value gave non-monoexponential diffusion-weighted signal attenuation curves. Figure 3-3 demonstrates the typical changes to an individual hippocampal slice’s diffusion-weighted signal attenuation curve over time following treatment with ACSF containing 10 µM A23187. Similar results to those shown in figures 3-2 and 3-3 were also observed for slices treated with 50 µM A23187.
Biexponential Analysis of Diffusion in Hippocampal Slices Treated with A23187

Diffusion-weighted signal attenuation curves of rat hippocampal slices were well-described by the biexponential function ($R^2 > 0.99$). The biexponential-derived diffusion parameters for all 29 rat hippocampal slices before treatment [mean ± standard deviation] were $0.601 ± 0.057$ for $F_{\text{fast}}$, $1.14 ± 0.14 \times 10^{-3}$ mm$^2$/s for $D_{\text{fast}}$ and $0.067 ± 0.010 \times 10^{-3}$ mm$^2$/s for $D_{\text{slow}}$. These values compare well with previous reports (54,55,105) and were qualitatively similar to biexponential diffusion parameters reported for human subjects (99). There were small, yet statistically significant differences in the baseline biexponential parameters $F_{\text{fast}}$ and $D_{\text{slow}}$ for slices assigned to the 3 different ACSF treatment groups (Table 3-2). Potential explanations for these differences are addressed in the discussion, but it is important to emphasize that slices were assigned to treatment groups before procurement and that this study investigated changes from baseline in the biexponential diffusion parameters. Figure 3-4 depicts the mean percentage change from baseline in the biexponential parameters ($F_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$) for slices treated with ACSF containing 0.1% DMSO, 10 and 50 µM A23187.

For the 3 treatment groups, there were statistically significant interactions between time and treatment in change from baseline for $F_{\text{fast}}$ (ANOVA, $P < 0.001$), but not for $D_{\text{fast}}$ ($P = 0.945$) or $D_{\text{slow}}$ ($P = 0.870$). Compared to vehicle-treated slices, the percentage change in the fraction of fast diffusing water ($F_{\text{fast}}$) for slices treated with 50 µM A23187 was significantly different for all time points after baseline (Tukey, $P < 0.05$). Changes from baseline for slices treated with 10 µM A23187 were significantly different than vehicle-treated slices for all time points after 80 minutes (Tukey, $P < 0.05$). Although the dose of A23187 (10 or 50 µM) predicted when the $F_{\text{fast}}$ diffusion parameter of A23187-
treated slices would first differ statistically from vehicle-treated slices, at no time point were slices treated with 10 or 50 µM A23187 considered statistically distinct from each other.

**Histology of A23187-Induced Pathological Cell Swelling**

Correlative H & E histology of the rat hippocampal slices exhibited clear dose-related differences in particular hippocampal regions (Fig. 3-5). In rat hippocampal slices treated with A23187, susceptible neurons typically manifested pathologic change by intense micro-vacuolation of the neuronal perikaryon and stranded, slightly pyknotic nuclei. These features are typical oncotic changes which frequently lead to the appearance of necrotic cells at later timepoints than observed for this study (15). The described cellular changes were most prominent in aspects of the CA1 cell band (CA1a), but also present in the ends of the CA3 cell band (CA3a and CA3c) and the granule cell layer in the internal blade of the dentate gyrus. The changes were more severe in slices treated with ACSF containing 50 µM A23187 than slices treated with 10 µM A23187. Other regions of the hippocampus, such as CA2, appeared relatively unaffected by A23187, although the neuropil surrounding the affected pyramidal and granule cells did exhibit some staining pallor and vacuolation as well (Fig. 3-5).

Similar to previous studies (43), comparison of H & E stained cross-sections and planar sections from different depths in the hippocampal slices did not suggest different reactions to A23187-treatment based on tissue depth. Additional control slices taken shortly after slice procurement (at 30 min after rat euthanasia) and before perfusion with ACSF treatment solutions (at 4 hrs postprocurement) did not show differences from hippocampal specimens obtained by intracardiac perfusion of normal rats with 4%
paraformaldehyde except for some minor pathology of the internal blade of the dentate gyrus (discussed below). The histological appearance of the few slices recovered from the diffusion MRI studies also did not differ significantly from correlative slices with the same ACSF treatment assignment.

**Discussion**

**A23187-Induced Diffusion Changes**

The interpretation of the $F_{\text{fast}}$ changes observed in rat hippocampal slices treated with A23187 is not trivial since biexponential modeling of nervous tissue water diffusion is underparameterized. Yet, in most cases, changes in the $F_{\text{fast}}$ do track perturbation–induced changes in the relative size of the extracellular compartment (63,66). Previous studies of rat hippocampal slices demonstrated that $F_{\text{fast}}$ changes tracked with cell swelling induced by NMDA and ouabain perturbations (54,55). Thus, $F_{\text{fast}}$ is likely to track cell-swelling in A23187-treated hippocampal slices as well. However, due to the limitations of the biexponential model, it is difficult to determine from the data obtained here how specific biophysical properties of rat hippocampal tissue, such as neuronal size, shape or patency, were altered by cellular swelling induced by A23187 treatment.

Although it is difficult to use the biexponential model to describe the biophysical properties underlying water diffusion in nervous tissue accurately (66), the model provides a more complete description of nervous tissue water diffusion than monoexponential fits. This study suggests the $F_{\text{fast}}$ diffusion parameter could be used as a sensitive correlate of cellular swelling after acute brain injury. Previous studies have demonstrated decreased ADC in acute ischemic brain followed by an increase in ADC beyond normal values several days later (68). These diffusion changes, noted in animal models of both ischemic and traumatic injury, may be due to cellular swelling followed
by cellular and tissue lysis (84,107). Because high doses of A23187 caused a similar yet accelerated pattern of cell swelling and lysis in neuronal cultures (102,103), a biphasic pattern of $F_{\text{fast}}$ changes was hypothesized to be observable for $F_{\text{fast}}$ within the timespan of this experiment on rat hippocampal slices continuously exposed to high concentrations of A23187. This hypothesis assumed that $F_{\text{fast}}$ at least correlates with the relative fractions of intra- and extracellular water, and predicted that $F_{\text{fast}}$ would first be reduced by oncotic cell swelling, but then increase beyond baseline values as substantial numbers of cells within the rat hippocampal slices ruptured. However, high doses of A23187 did not affect all cell populations in hippocampal slices equally as previously observed in neuronal culture studies (16,102). In fact, the non-uniform pathological changes were more consistent with acute hippocampal injury in vivo (25). The correlative histological findings of this study suggest that future experiments that are able to employ regional diffusion analysis of rat hippocampal slices treated with A23187 may provide data sufficient to properly test this interesting hypothesis.

The potential A23187 dose-related diffusion differences and the observed timing of $F_{\text{fast}}$ changes also suggest that diffusion measurements of rat hippocampal slices may be influenced by anatomical or cell-subtype variations in susceptibility to acute injury. Although there appeared to be dose-related differences in the time following A23187 treatment when the $F_{\text{fast}}$ of slices became distinct from control slices, this study was insufficiently statistically powered to characterize these differences. However, the correlative histology clearly suggests that dose-related differences exist. Further, the $F_{\text{fast}}$ for slices was reduced 7-10% from baseline during the first two hours following treatment with either A23187 dose, but it then took an additional 6 hours of A23187
treatment to double this reduction. Since potential dose-related differences in $F_{\text{fast}}$ were only observable during the first 80 minutes following A23187 treatment, these results suggest that there may be a limited number of A23187-sensitive cell populations within the rat hippocampal slices such that reactions from responsive cells become saturated at high doses of A23187. By using the whole-slice ROI method, anatomical distinctions in these populations were not observed, but may be detectable in future studies that employ regional diffusion analysis.

The diffusion data can also be examined without biexponential analysis by simply comparing images of slices at particular diffusion-weightings before and after A23187 exposure. A23187 treatment increased diffusion-weighted signal intensity significantly for all rat hippocampal slices treated with either 10 or 50 $\mu$M A23187 and these changes were noted at all $b$-values (except for the image obtained without diffusion weighting). In fig. 3-2, for example, diffusion-weighted slice signal intensity at $b = 7977$ s/mm$^2$ increased 42% after 8 hours treatment with 10 $\mu$M A23187. Although the SNR is decreased at higher $b$-values, the increases in diffusion-weighted signal intensity between slice MRI images at baseline and at different timepoints after A23187 treatment were better resolved with increasing $b$-value (for example, see Fig. 3-3 boxes A and B). These results support previous reports that have suggested stronger diffusion-weighting (higher $b$-values) may increase the tissue characterization capabilities of diffusion-weighted MRI in human patients when there is sufficient contrast-to-noise (108,109). Future studies will be required to further investigate the clinical merits of high $b$-value diffusion-weighted MRI.
There are some caveats to the diffusion data presented. The comparison of baseline diffusion parameters for slices based on treatment group assignment (Table 3-2) revealed some significant pretreatment differences in the diffusion parameters $F_{\text{fast}}$ and $D_{\text{slow}}$. Because slices were procured in hypothermic conditions, which are neuroprotective (see below), procurement conditions were unlikely to contribute significantly to these baseline differences. Instead, the initial diffusion differences are most likely attributable to differences optimizing the MRI-defined slice through the hippocampal tissue (see methods). The undesired inclusion of perfusate within the MRI-defined slice increases the population of freely diffusing water in the MR-defined ROI, which then alters the biexponential diffusion parameters obtained. The study design, however, controlled for pretreatment differences by assigning slices to treatment groups before procurement and examining diffusion changes in individual slices from baseline after treatment.

Rat hippocampal slices were procured at low temperatures to prevent subsequent altered responses to A23187 treatment due to the procurement process (40,43,104). After the procurement process, water diffusion changes were measured in rat hippocampal slices treated with A23187 at room temperature instead of 37°C because it simplifies slice perfusion chamber design and allows hippocampal slices sufficiently thick (500 µm) for MRI study (40). The potential neuroprotective effects of such hypothermia (110,111) suggest that the measured diffusion responses in this injury model may underestimate the responses of *in vivo* nervous tissue. In addition, the diffusion coefficient of unrestricted water will increase approximately 50% when heated from room temperature to 37°C (97). This may limit direct extrapolation of the data presented, but the study focused on relative changes that should be comparable between the slice model and *in vivo* subjects.
Histological Correlations to Diffusion Findings

In H & E stained sections of correlative rat hippocampal slices treated with A23187, pyramidal neurons and granule cells demonstrated substantial cytoplasmic microvacuolation indicative of pathological cell swelling. These findings are similar to descriptions of early ischemic cell changes (112) and previous reports of rapid cell body swelling and necrosis in cultured neurons treated with the calcium ionophores ionomycin or A23187 (17,102,103). Electron microscopy studies have correlated microvacuolation in the neuronal perikaryon with mitochondrial swelling (113). The neuropil also exhibited dose-related increases in vacuolation (Fig. 3-5).

The cellular pathology observed agrees with previous descriptions of A23187 neurotoxicity, where A23187 creates a rapid intracellular calcium overload beginning in the neuropil and spreading to the cell body at high doses (17) that subsequently leads to mitochondrial permeability transition pore formation and pathological cell swelling (16). Unfortunately, it is difficult to determine whether neurons in the hippocampal slices truly ruptured from oncotic cell swelling since the limited time of swelling may have just led to unstable membranes that ruptured upon fixation (114). Conversely, the correlative histology may underestimate some of the effects of A23187 since non-structural alterations to cellular morphology during the first 8 hours following ionophore treatment would be difficult to observe when employing paraffin embedding and H & E staining.

The anatomically-specific pattern of cellular pathology in the pyramidal cell regions of the rat hippocampal slices after A23187 treatment may be due the selective vulnerability of particular regions previously noted for ischemic or traumatic brain injuries (25). The vulnerability of the CA1 region may be attributed to differences in NMDA receptor concentrations (115). However, because A23187 initially bypasses
neurotransmitter-receptor interactions by increasing calcium concentrations inside the cell, the data presented suggests there may be differences in intracellular calcium-dependent enzyme cascades that contribute to selective vulnerability of particular CA cell bands in the hippocampus. This potential inference, however, assumes that A23187 affects all cells in the rat hippocampal slices equally (as it appears to do so in cell cultures, see (16)), and that selective vulnerability to procurement-induced injury did not predispose particular regions to subsequent injury from A23187 treatment (43). Future non-correlative studies would be required to test these assumptions and further explain this interesting observation.

Granule cell injury was largely limited to the internal blade of the dentate gyrus. Although there were A23187 dose-related increases in the number of cells with pathological cell swelling (Fig. 3-5), this pattern of pathology was unusual and differed from previous reports of perturbation-induced granule cell injury (114). Some minimal cell changes were also noted in a few sections of control rat hippocampal slices at 4 hours postprocurement. A previous report suggested that procurement-induced metabolic injury may increase the subsequent vulnerability of this particular hippocampal region to injury (43).

Unlike correlative histology, the diffusion MRI measurements failed to observe the appearance of substantial dose-related, region-specific pathological changes in the rat hippocampal slices because the present MRI analysis could not examine individual anatomical regions. The histology data, however, suggests that the water diffusion changes noted in the ROI of A23187-treated slices may have originated from a continuous distribution of healthy to pathological cell populations. As such, this study
highlights that potential variations in regional, cell subtype and dose-related responses to acute injury may pass unobserved in diffusion experiments that sacrifice spatial resolution to obtain adequate signal-to-noise in practical scan times, particularly when correlative techniques are not employed. Previous studies in human patients have similarly shown that the ADC changes in ischemic stroke are heterogeneous and may reflect “different temporal rates of tissue evolution toward infarction” (91).

The ROI volume chosen for this study is comparable to typical voxel volumes in clinical diffusion studies (68), and understanding the intravoxel contributions from different regions and cell subtypes could be clinically relevant. Especially since, under certain conditions, cell volume changes are a prerequisite to pathological biochemical changes in apoptotic cell cultures (116). Further, it may be that the therapeutic windows for particular cell populations differ significantly. Unlike studies of rat hippocampal slices, the limitations due to patient tolerance and clinical hardware (i.e. field and gradient strength) make it uncertain that high-resolution MRI experiments capable of resolving such small anatomical cell layers may ever be possible in human subjects. To determine how different cellular regions or subtypes contribute to diffusion changes at different timepoints after acute injury, new methods of acquiring water diffusion data from rat hippocampal slices with high SNR will be required (e.g. smaller radiofrequency coils and continuous perfusion to allow longer acquisition times). Improvements in SNR for hippocampal slice data could then be used for analysis of water diffusion changes at higher spatial resolution. Such data may also prove useful for more complete models of multicomponent water diffusion that are more SNR-intensive (117).
Figure 3-1. Diffusion-weighted image of a rat hippocampal slice with 59-µm in-plane resolution (A) reveals the detailed lamellar anatomy of a 500-µm thick rat hippocampal slice cut perpendicular to the septo-temporal axis [MRI scan parameters; TR/TE = 2000/34 ms, b = 3630 s/mm², matrix = 256 x 256, FOV = 15 x 15 mm, slice thickness = 300 µm, averages = 32, time = 4.5 hrs]. As illustrated in panel B, many anatomical regions of the hippocampus and dentate gyrus can be distinguished in this sample based on differences in diffusion-weighted signal intensity (s = stratum).
Figure 3-2. A23187-induced changes to diffusion-weighted images of rat hippocampal slices (b = 7977 s/mm$^2$). Panels A and B show a control rat hippocampal slice before and after 8 hours of treatment with 0.1% DMSO vehicle respectively. Panel C and D show a different slice before and 8 hours after treatment with 10 µM A23187 respectively (the latex spacer from the perfusion chamber is visible in these 2 images). At this b-value, signal intensity in the A23187-treated slice increased 42% whereas the vehicle-treated slice signal intensity increased 6% over 8 hours. The increased signal intensity in the slices is attributed to increased signal contributions from slowly diffusing water.
Figure 3-3. Typical diffusion-weighted signal attenuation curves of A23187-treated rat hippocampal slices. Each point represents the log signal intensity for the slice ROI in a diffusion-weighted image normalized to the signal intensity of the first image \((b = 7 \text{ s/mm}^2)\). Although diffusion data were collected every 40 min, only alternating data from baseline (0 min), 80, 160, 240, 320 and 400 minutes are shown for clarity. Comparison of boxes labeled A and B illustrates that the signal intensity changes after A23187 treatment are better resolved at higher \(b\)-values given sufficient signal-to-noise. The arrow indicates approximately where data from figure 3-2 \((b = 7977 \text{ s/mm}^2)\) would be plotted in the signal attenuation curve.
Figure 3-4. Mean percentage change from baseline over time for the fraction of fast diffusing water in rat hippocampal slices treated with ACSF containing 0.1% DMSO vehicle (n = 9), 10 µM A23187 (n = 10) or 50 µM A23187 (n = 10) [mean ± SEM]. At early timepoints, slices treated with 50 µM A23187 statistically differed from vehicle-treated slices (#) whereas at later timepoints slices treated with either 10 or 50 µM A23187 differed from vehicle-treated slices (*) (p < 0.05).
Figure 3-5. Correlative histology of A23187-treated rat hippocampal slices 8 hours after treatment with DMSO vehicle (A, B, C, D), 10 μM A23187 (E, F, G, H) and 50 μM A23187 (I, J, K, L). A23187 had dose-related effects on the CA1 subfield, CA3 subfield, stratum radiatum and internal blade of the dentate gyrus. Arrows illustrate cells ruptured from pathological swelling and arrowheads denote artifact from paraffin embedding. [8-μm thick sections stained with hematoxylin and eosin, 630x magnification with oil immersion]
Table 3-1. Time-to-image for rat hippocampal slices in each of the 3 treatment groups [mean ± SD].

<table>
<thead>
<tr>
<th>Group</th>
<th>0.1% DMSO</th>
<th>10 µM A23187</th>
<th>50 µM A23187</th>
<th>ANOVA (P-value)</th>
<th>Total/Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (slices)</td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>N/A</td>
<td>29</td>
</tr>
<tr>
<td>Baseline scan time*</td>
<td>3.2 ± 0.5</td>
<td>3.2 ± 0.4</td>
<td>2.9 ± 0.2</td>
<td>0.265</td>
<td>3.1 ± 0.4</td>
</tr>
<tr>
<td>Treatment time*</td>
<td>3.9 ± 0.8</td>
<td>4.0 ± 0.4</td>
<td>3.7 ± 0.2</td>
<td>0.393</td>
<td>3.8 ± 0.5</td>
</tr>
</tbody>
</table>

* Times refer to hours elapsed since slice procurement.
Table 3-2. Change in biexponential diffusion parameters (fast fraction, fast and slow ADCs) over time for rat hippocampal slices treated with 0.1% DMSO (n = 9), 10 µM A23187 (n = 10) and 50 µM A23187 (n = 10) [mean ± SD].

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Fast Fraction (arbitrary units)</th>
<th>Fast ADC (x 10⁻³ mm²/s)</th>
<th>Slow ADC (x 10⁻³ mm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1 % DMSO</td>
<td>10 µM A23187</td>
<td>50 µM A23187</td>
</tr>
<tr>
<td>0*</td>
<td>0.560 ± 0.040*</td>
<td>0.617 ± 0.070</td>
<td>0.623 ± 0.037*</td>
</tr>
<tr>
<td>40</td>
<td>0.574 ± 0.051</td>
<td>0.602 ± 0.075</td>
<td>0.603 ± 0.026</td>
</tr>
<tr>
<td>80</td>
<td>0.561 ± 0.051</td>
<td>0.590 ± 0.077</td>
<td>0.580 ± 0.045</td>
</tr>
<tr>
<td>120</td>
<td>0.560 ± 0.044</td>
<td>0.574 ± 0.066</td>
<td>0.564 ± 0.024</td>
</tr>
<tr>
<td>160</td>
<td>0.557 ± 0.042</td>
<td>0.571 ± 0.056</td>
<td>0.565 ± 0.036</td>
</tr>
<tr>
<td>200</td>
<td>0.555 ± 0.042</td>
<td>0.570 ± 0.067</td>
<td>0.560 ± 0.028</td>
</tr>
<tr>
<td>240</td>
<td>0.551 ± 0.039</td>
<td>0.564 ± 0.062</td>
<td>0.541 ± 0.046</td>
</tr>
<tr>
<td>280</td>
<td>0.557 ± 0.045</td>
<td>0.544 ± 0.057</td>
<td>0.537 ± 0.037</td>
</tr>
<tr>
<td>320</td>
<td>0.535 ± 0.040</td>
<td>0.542 ± 0.055</td>
<td>0.544 ± 0.038</td>
</tr>
<tr>
<td>360</td>
<td>0.523 ± 0.033</td>
<td>0.538 ± 0.060</td>
<td>0.526 ± 0.038</td>
</tr>
<tr>
<td>400</td>
<td>0.541 ± 0.044</td>
<td>0.530 ± 0.057</td>
<td>0.532 ± 0.025</td>
</tr>
<tr>
<td>440</td>
<td>0.533 ± 0.056</td>
<td>0.526 ± 0.054</td>
<td>0.526 ± 0.030</td>
</tr>
<tr>
<td>480</td>
<td>0.530 ± 0.062</td>
<td>0.488 ± 0.039</td>
<td>0.528 ± 0.028</td>
</tr>
</tbody>
</table>

* In the baseline measurements, groups were tested for statistically significant differences. Groups that were statistically different from one another as determined post-ANOVA by a Tukey multiple comparisons test (P < 0.05) are marked with an asterisk.
CHAPTER 4
REGIONAL ANALYSIS OF WATER DIFFUSION IN RAT HIPPOCAMPAL SLICES

Introduction

The rat hippocampal slice has proven to be a useful model for investigating water diffusion in living nervous tissue using diffusion MRI (54,55). Although the dimensions of the typical slice resemble the voxel dimensions of current \textit{in vivo} diffusion MRI studies of human subjects (68), a frequent criticism of previous brain slice studies was the lack of pixel-by-pixel or regional analysis of the different anatomical lamina of the rat hippocampus. The initial MRI study of rat hippocampal slices, however, did anecdotally report regional analysis of osmotic perturbations that failed to demonstrate any significant differences (50). There are some explanations for why this prior attempt at regional analysis of osmotic perturbations did not yield interesting data. First, regional analysis only employed water diffusion measurements at 1 \textit{b}-value less than 1000 s/mm$^2$, whereas recent studies (e.g. chapter 3) demonstrate that higher \textit{b}-values may significantly increase water diffusion-based tissue contrast and sensitivity to perturbation-induced changes. Further, unlike the calcium ionophore A23187 (16), osmotic manipulations are not pharmacological perturbations and should be expected to affect slices globally.

Several lines of evidence presently suggest that water diffusion is not uniform in rat hippocampal slices and that water diffusion changes in slices after experimental perturbation may also have anatomical dependence. Similar to a previous report (118), Fig. 4-1 illustrates that the various anatomical lamina of the rat hippocampus can be distinguished based on their different signal intensities in high-resolution, diffusion-
weighted images. Further, diffusion-weighted images acquired at increasing \( b \)-value (Fig. 4-2) demonstrate different rates of diffusion-weighted signal attenuation in the various regions of the rat hippocampal slice and suggest that the water diffusion properties of the slice are quite heterogeneous. Potential water diffusion differences can most likely be attributed to the distinguishing cytoarchitectural features of the various regions of the rat hippocampus (19,119). In fact, previous diffusion studies of tetramethylammonium (TMA) ion diffusion have described differing tortuosity and extracellular space volume fractions for some of the lamina of the rat hippocampus (120,121). In addition, beyond indications of regional differences in the normal rat hippocampal slices, previous reports (38,122) and in particular, histological data presented in the previous chapter, suggest that different anatomical regions of the rat hippocampus may also exhibit different magnitudes and patterns of water diffusion changes after experimental perturbation.

In this study, we attempted to measure regional differences for the diffusion properties of water in normal rat hippocampal slices and in slices treated with the calcium ionophore A23187. The hypotheses of this study were 1) that different anatomical regions of the rat hippocampus will have statistically significant differences in the biexponential diffusion parameters \( F_{\text{fast}} \), \( D_{\text{fast}} \) and \( D_{\text{slow}} \) due to their different relative proportions of neuronal cell bodies and neuropil and 2) that subsequent water diffusion changes in hippocampal slices after A23187 treatment will have regionally-dependent magnitudes and temporal profiles.

To investigate these hypotheses, we employed diffusion MRI measurements, similar to those reported in chapter 3, but with improved spatial resolution (117 x 117 µm in-plane) to allow segmentation of the rat hippocampal slice images into separate
anatomical regions. These regions included the CA1 subfield, CA3 subfield, hilus, granule cell layer, molecular layer, stratum (radiatum and lacunosum), stratum oriens and subiculum. Water diffusion in these regions was measured at baseline, then measured serially for 7 hours following treatment with ACSF containing 50 µM A23187. The baseline data obtained demonstrated significant differences in the biexponential diffusion parameters $F_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ for many regions of the rat hippocampal slice. These interesting findings may be explained by well-characterized cytoarchitectural differences in the anatomical regions of the rat hippocampus. Unfortunately, small movements of the slices during MRI acquisition confounded the temporal diffusion data obtained, thus making it difficult to interpret the diffusion changes observed following A23187 treatment.

Methods

Brain Slice Procurement

The use of laboratory animals for this study was approved by the University of Florida Institutional Animal Care and Use Committee. Rat hippocampal slice procurement has been described previously (40,123). Briefly, male Long-Evans rats (250 – 350 g) were anesthetized with isofluorane then decapitated. The brain was removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) (120 mM NaCl, 3 mM KCl, 10 mM glucose, 26 mM NaHCO$_3$, 2 mM CaCl$_2$, 1.5 mM KH$_2$PO$_4$, 1.4 mM MgSO$_4$) gassed with 95% O$_2$ / 5% CO$_2$ to maintain a pH of 7.4. The ACSF osmolality was 300 ± 1 mOsm/kg as determined by Osmette A freezing point depression osmometer (Precision Systems, Inc.). Both hippocampi were dissected and cut orthogonal to the septo-
hippocampal axis into 500-µm thick sections with a McIlwain tissue chopper within 10 min of rat sacrifice.

Hippocampal slices remained immersed in ice-cold ACSF for one hour after rat sacrifice to minimize procurement-induced ischemic damage (43,104), then slices were warmed gradually to room temperature (20°C) for the MRI experiment and placed into a perfusion chamber that facilitates multislice diffusion MRI measurements (105). Slices were perfused continuously with ACSF (2 mL/min) while the perfusion chamber was lowered into the magnet and during pilot image acquisition. Although perfusion was discontinued during all diffusion measurements (described below), it has been shown previously that intermittent cessation of flow does not affect slice viability for at least 8 hours following slice procurement (53).

**Diffusion MRI of Rat Hippocampal Slices**

MRI data were obtained at room temperature using a 10-mm birdcage coil interfaced to a Bruker 17.6-T vertical wide-bore magnet and console. Pilot multislice axial, sagittal and coronal T₁ and diffusion-weighted imaging sequences were used to locate the perfusion chamber, then to optimize the positions of axial MR-defined slices through the center of the 500-µm thick rat hippocampal slices. Diffusion-weighted images were acquired using a pulsed gradient spin-echo multislice sequence with a diffusion time of 14 ms and an echo time of 30 ms. This diffusion protocol consisted of a series of 7 diffusion-weighted images (128 x 128 matrix, 1.5 cm field-of-view, repetition time = 2 s, echo time = 30 ms, 2 averages, δ = 3 ms, Δ = 15 ms) employing diffusion gradients aligned with the B₀-field in linear increments of strength from 0 to 940 mT/m (b-values between 7 and 8085 s/mm²). Image slice thickness was 300 µm with 117 x 117
\(\mu m\) in-plane resolution and the protocol required 60 minutes for completion. Additional pilot images were acquired between diffusion measurements to monitor for slice movement caused by ACSF perfusion.

**Slice Perturbation with A23187**

Rat hippocampal slices were randomly assigned to treatment with control ACSF or 50 \(\mu M\) A23187. Dimethylsulfoxide (DMSO) was required to solubilize A23187 in ACSF so control ACSF also contained 0.1% DMSO. The additions of 0.1% DMSO with or without A23187 did not affect the pH or osmolality of the ACSF solution, and each ACSF solution was gassed with 95% \(O_2\) / 5% \(CO_2\) throughout the experiment. All slices were perfused with standard ACSF during pilot scans and before baseline diffusion measurements were acquired. After the acquisition of baseline diffusion measurements, slices were perfused with the assigned treatment ACSF for 20 minutes then perfusion was stopped and a posttreatment diffusion protocol obtained. A cycle consisting of an additional diffusion protocol (60 minutes) followed by 10 minutes perfusion with the assigned treatment ACSF then was repeated serially until 12 hours after initial brain slice procurement (each complete cycle required 70 minutes).

As described in chapter 3, the initial treatment period for slices occurred at slightly different times following brain slice procurement due to variations in the time required for hippocampal slice placement and pilot MRI scans. Therefore diffusion data from rat brain slices required assignment to time-bins for statistical comparisons. The baseline diffusion measurement for each slice was assigned to 0 minutes and subsequent data were assigned to timepoints increasing in 70-minute increments. Although data were re-
assigned to timepoints relative to the initial MRI measurement, diffusion data acquired more than 12 hours after slice procurement were excluded from the analysis.

**Segmentation of Diffusion-Weighted Images**

To analyze the regional changes in the diffusion-weighted signal intensity in rat hippocampal slices after treatment with the various ACSF solutions, 8 regions-of-interest (ROI) were drawn on the images to individually enclose the CA1 subfield, CA3 subfield, molecular layer, granule cells, hilus, stratum (radiatum and lacunosum), stratum oriens, and subiculum (see Fig. 4-3). The segmentation of these ROIs was done on heavily diffusion-weighted images ($b = 8085 \text{ s/mm}^2$), where contrast between the adjacent anatomical layers was most apparent, then applied to all other diffusion-weighted images in the series. The regions were selected by a single, trained observer based on detailed anatomical descriptions of the rat hippocampus in sections orthogonal to the septo-temporal axis (19,119). A description of the different regions of the hippocampus and guidelines for regional segmentation of images can be found in Appendix A. An ROI of the whole hippocampal slice was drawn for comparison with previous data. Additional ROIs were also drawn to verify correct diffusion-weighting gradient calibrations (perfusate ROI) and to verify that there were sufficient signal-to-noise ratios (SNR) for biexponential fits of signal attenuation data (Noise ROI); these methods have been described in previous chapters and elsewhere (105).

**Analysis of Diffusion Data**

Previous studies demonstrated that diffusion-weighted water signal attenuation in rat hippocampal slices is non-monoexponential at high $b$-values (54). In this study, a biexponential equation [1] was fitted to the diffusion-weighted signal attenuation in the
various ROIs using the Gauss-Newton non-linear least squares fitting method available in Matlab 5.3.1:

$$S(b) = S_0[F_{\text{fast}}\exp(-bD_{\text{fast}}) + (1-F_{\text{fast}})\exp(-bD_{\text{slow}})]$$  \[4-1\]

where $S_0$ is signal intensity without diffusion weighting, $F_{\text{fast}}$ is the fraction of water with fast apparent diffusion coefficient (ADC), and $D_{\text{fast}}$ and $D_{\text{slow}}$ represent the ADCs of the fast and slow diffusing water components respectively. Similar processing was conducted to generate pixel-by-pixel maps of the biexponential diffusion parameters $F_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ for selected datasets using in-house Matlab programs. Baseline biexponential parameters were compared using a one-way ANOVA. To determine the effects of A23187 treatment, observed changes in the water diffusion properties of segmented anatomical regions over time from baseline in the 2 treatment groups (control and 50 µM A23187) were compared statistically using a three-way repeated-measures ANOVA with one factor repeated (Sigma Stat 2.03). If the ANOVA tests detected a statistically significant difference, a Tukey multiple comparisons test was used to isolate groups that differed significantly from one another. Significance for all statistical tests was defined as $P < 0.05$.

**Histology of Rat Hippocampal Slices**

The pathological changes associated with A23187 treatment of rat hippocampal slices were characterized previously (chapter 3), however, additional hippocampal slices were obtained during this study to characterize the cytoarchitecture of the different anatomical regions of healthy, untreated rat hippocampal slices with light microscopy using oil immersion lense at 630x magnification. Untreated control slices were handled as described above for the first 3 - 4 hours after procurement then gently immersion-fixed
with 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The fixed slices were embedded in paraffin, sectioned into 8-µm thick sections parallel to the plane of the slice, mounted onto slides and stained with hematoxylin & eosin (H & E).

**Estimation of Partial Volume Effects**

To estimate the partial volume effects due to the limited spatial resolution of the diffusion-weighted MRI images, optical microscopy images of H & E-stained sections from 5 randomly selected hippocampal slices were segmented in an identical fashion to the diffusion-weighted MRI images (see description above). In addition to area measurements of all regions, the cross-section widths of the CA1, CA3 and granule cell layers were also measured in images of H & E-stained sections for these slices. Slice shrinkage due to tissue processing for histology then was estimated by determining the mean reduction in area for the whole hippocampal slice ROIs from diffusion MRI to histology samples. This ratio was inversely applied to all measurements made in the histology sections to estimate the original areas of different regions of the hippocampal slices prior to processing (e.g. if whole slice area was 5 mm² in diffusion MRI, but reduced to 3 mm² when measured with histology, there would be a 40% reduction in size from processing. Thus, histology area measurements of 60 µm² would be estimated to be 60 / 0.6 = 100 µm² in unprocessed slices). This estimation assumes that rat hippocampal slices and their component anatomical regions (e.g. stratum oriens, CA1 and CA3 subfields) shrink in an equal fashion in all dimensions.

Because the resolution of light microscopy images is submicron (as compared to 117 x 117 µm in-plane resolution for the diffusion MRI images collected), the segmented histology images provide more accurate estimations of the mean areas for particular
segmented anatomical regions of the hippocampal slices. By comparing the diffusion MRI and histology estimates of ROI area, it is possible to quantify the partial volume errors inherent to the MRI images with limited spatial resolution. However, the shrinkage effects of tissue processing must be accounted for first by normalizing mean ROI area for particular segmented regions in the diffusion and histology data using their respective whole slice ROI areas. Thus, a region ROI with an histology area of 100 $\mu m^2$, where the whole hippocampal slice ROI histology area was 500 $\mu m^2$, would have a normalized value of 0.20 (no units). For each region, the mean normalized values for diffusion MRI can be divided by the normalized values for the histology measurements to calculate a “Volume Averaging Ratio.” A resultant ratio greater than 1 indicates that the diffusion MRI segmentation overestimated the area of a particular anatomical region and underestimated the area for ratios below 1.

In a similar fashion, cross-section measurements can be extrapolated. As a first approximation, if regional ROIs are assumed to enclose square areas, it can be shown that cross-section lengths of unprocessed tissue can be estimated by dividing the measured histology cross-section length by the square root of the ratio of whole slice histology ROI to diffusion MRI ROI. This extrapolation is limited, but it can be used to estimate the voxel dimensions necessary to minimize partial volume effects in the data obtained for thin anatomical lamina in the hippocampus (e.g. the CA3 cell band).

**Assessment of Slice Movement**

To assess horizontal slice movement during the experiment time scan, an additional ROI was drawn to encompass all signal above noise contributed by the slice. This ROI differed from the whole slice ROI in that it also enclosed all pixels above noise on the
edges of the hippocampal slice (most of these pixels were not used for quantitative analysis because of partial volume effects with perfusate). This additional ROI was then compared across timepoints to assess slice movement away from its original position due to perfusion. If the slice moved in the horizontal plane, the ROI would no longer encompass all the signal above noise in diffusion-weighted images at $b = 8085 \text{ s/mm}^2$. Slice movements could then be used to adjust the regional ROIs or to reject a slice from study inclusion.

To appreciate how microscopic movements of the slices could affect the biexponential diffusion data, select ROIs for whole slice, stratum, molecular layer and CA1 in 3 randomly chosen hippocampal slices were purposefully moved in each of the 4 cardinal points one voxel away from the desired position. The biexponential parameters from these erroneous ROI locations were then obtained and compared to the original values.

**Results**

Baseline diffusion MRI measurements were achieved in 20 rat hippocampal slices procured for this study; 3 other slices were rejected because of substantial volume averaging between the slice and perfusate in axial-oriented images. During the temporal characterization of diffusion changes after A23187 treatment, 2 additional slices were rejected for gross movements probably due to bulk perfusate flow. Multislice sagittal diffusion scans during ACSF perfusion between diffusion measurements did not indicate any vertical movements of the hippocampal slices. However, twisting or spinning movements in some of the remaining 18 slices precluded acceptable re-orientation of 14 regional ROIs. These rejected ROIs came predominately from the narrow anatomical lamina of the rat hippocampal slice; for example, the CA3 region (5 ROIs), CA1 region
(3 ROIs) and granule cell layer (3 ROIs). It is also important to note that 79 of the 137 remaining regional ROIs (~57.7%) required small adjustments in position at some time during the A23187 experiment due to in-plane translational movements < 2-3 voxels (234 - 351 µm).

Mean times to baseline diffusion measurements (2.8 ± 0.4 hrs) and treatment with ACSF containing 50 µM A23187 or vehicle (4.0 ± 0.4 hrs) for the rat hippocampal slices were similar to times previously reported in chapter 3. After baseline, 6 additional diffusion measurements were obtained in all slices (approximately 7 hours of data obtained after baseline measurements commenced). Segmentation of diffusion-weighted images into ROIs for CA1, CA3, hilus, granule cells, molecular layer, stratum, subiculum, stratum oriens and the whole hippocampal slice was relatively straightforward and is illustrated in Fig. 4-3. Diffusion-weighted signal attenuations in most ROIs were well-described by the biexponential equation, yet 46 out of 959 total fits (~4.8%) were rejected because the nonlinear fitting routine failed to converge or generated erroneous biexponential diffusion parameters (i.e. negative values, F_{fast} > 1.0 or apparent diffusion coefficients with values greater than freely diffusing water at room temperature (97)). An example of the typical diffusion-weighted signal attenuation curves for the various anatomical components of the rat hippocampal slice is shown in Fig. 4-4.

Table 4-1 reports the mean baseline biexponential diffusion parameters for the whole rat hippocampal slice and the component regional ROIs. The table also shows that there was sufficient SNR to make biexponential fits; SNR for each regional ROI at \(b = 8085\) s/mm\(^2\) was 20 or greater. The mean biexponential diffusion parameters of the whole hippocampal slice ROI were similar to previously published values (105). There were
regionally-dependent, statistically significant differences for the mean biexponential parameters $F_{\text{fast}}$, $D_{\text{fast}}$ and $D_{\text{slow}}$ (ANOVA, $P < 0.05$). A Tukey multiple comparisons test (Fig. 4-5) then evaluated individual comparisons between different regions of the hippocampal slice and reports whether the differences in $F_{\text{fast}}$ (A) and $D_{\text{slow}}$ (B) between two particular groups were statistically significant (shaded boxes indicate a $P < 0.05$). For the other biexponential diffusion parameter, $D_{\text{fast}}$ (Tukey test results not shown), the only significant differences noted were that mean $D_{\text{fast}}$ for the hilus ROI was statistically distinct from either subiculum or stratum oriens ROIs.

Histological examination indicates that there is substantial cytoarchitectural heterogeneity of the rat hippocampal slice (Fig. 4-6) such that the regional ROIs may be divided into 3 relative tissue groups (as labeled in Fig. 4-5); group I consists of densely packed neuronal cell bodies (CA1, CA3 and granule cells), group II consists of heterogeneous cell bodies and neuropil (hilus and subiculum) and group III consists of acellular neuropil (molecular layer, stratum, and stratum oriens). The water diffusion properties in these different tissue groups can then be examined before comparing individual regions. For the biexponential diffusion parameter $F_{\text{fast}}$, the three tissue groups of ROIs differ significantly from one another, but there were no statistically significant differences within the tissue groups. The $F_{\text{fast}}$ of the whole hippocampal slice appears most similar to tissue group II (hilus and subiculum) and the stratum ROI. There were less individual statistically significant differences for the diffusion parameter $D_{\text{slow}}$, yet CA3 and the granule cell layer (group I regions) differ from almost all other regions and differ significantly from each other. In contrast, the ADC of the slow diffusing
component \( (D_{\text{slow}}) \) of CA1, the other densely cellular region of the rat hippocampal slice, only differs significantly from the molecular layer and CA3.

To interpret the interesting differences in the baseline biexponential diffusion parameters, it is first important to understand how partial volume effects may alter the data obtained. For the purposes of this report, partial volume effects were defined as the intravoxel mixing of two distinct tissue types in unknown proportions due to the limited spatial resolution of the MRI images obtained (here, ACSF is also considered a tissue type). The volume averaging ratio, described in the methods, and reported in Table 4-2, suggests that MRI-based segmentation of CA1, CA3 and the granule cell layer may have overestimated their “true” areas substantially. As shown in Fig. 4-7, CA1 is a relatively thin anatomical layer relative to the in-plane dimensions of the diffusion MRI voxels. Box 2 in this figure attempts to normalize the MRI voxel dimensions to account for the effects of fixation present in the histological section of CA1 (see methods), yet the CA1 region still appears to occupy only a third of the voxel size (as also indicated by a volume averaging ratio of 2.9 in Table 4-2). This figure is also representative of the CA3 and granule cell layers.

This study also obtained diffusion data from rat hippocampal slices for up to 7 hours after perfusion with 50 \( \mu \text{M} \) A23187 or control (0.1% DMSO) ACSF. In Appendix B, the mean percentage change from baseline over time for the biexponential diffusion parameters \( S_0 \), \( F_{\text{fast}} \), \( D_{\text{fast}} \) and \( D_{\text{slow}} \) are reported for control and A23187-treated slices in each of the regional ROIs. First, it should be noted that the calculated signal intensity without diffusion weighting \( (S_0) \) does not show substantial changes for any of the ROIs. Similar to data presented in the previous chapter, the \( F_{\text{fast}} \) of the whole slice ROI for
A23187-treated slices decreased substantially over 7 hours time compared to controls (-12.3 ± 3.3 % versus –0.7 ± 5.5 % in controls). Diffusion parameters for the whole slice or different regional ROIs of the control-treated slices did not appear as stable as was noted in the previous chapter (see discussion). There were also many changes to the biexponential diffusion parameters of the different regional ROIs for A23187-treated slices, however, it should be noted that there was substantial margins of error for almost all the data reported.

**Discussion**

**Cytoarchitectural Basis for Water Diffusion**

The diffusion-weighted signal attenuation curves (Fig. 4-4) and biexponential diffusion parameters reported (Table 4-1 and Fig. 4-5) indicate that there were many statistically significant differences in the water diffusion properties of the regional ROIs. These differences may be attributed to the cytoarchitectural heterogeneity of the underlying anatomical regions of the rat hippocampal slice (Fig. 4-6).

However, before interpreting these interesting differences, it is first important to understand the potential biophysical underpinnings of the biexponential diffusion parameters and the limitations to their interpretation. The first study to report biexponential parameterization of non-monoexponential water diffusion in nervous tissue noted that the fast and slow components do not correlate exactly with the extra- and intracellular compartments of tissue, but that changes to the fractions of fast and slow diffusing components track changes in the size of the intracellular compartment (63). Thelwall and colleagues have recently indicated that the biexponential model is under-parameterized for studying water diffusion in a red blood cell (rbc) ghosts tissue model...
This study also indicated that when the appropriate data can be obtained, a two-compartment model offers a better model of water diffusion in biological tissue.

In perturbation studies of the rbc tissue model (at fixed diffusion times), changes to $F_{\text{fast}}$ indicate linearly proportional changes to the intracellular compartment size, changes to $D_{\text{fast}}$ also map with changes in the size of the intracellular compartment (or to changes to water exchange rates due to altered membrane permeability) and changes to $D_{\text{slow}}$ indicate changes to intracellular restriction or cell size. The data reported herein may be interpreted in the context of these biophysical assignments. However, it should be cautioned that such interpretation of the water diffusion properties of hippocampal slices is oversimplified since nervous tissues are certain to be more complex than rbc ghosts (e.g. due to heterogeneous cell sizes, shapes and densities).

In the present data, the biexponential diffusion parameter $F_{\text{fast}}$ decreased in 20% increments from tissue type I to type II to type III. Based on the findings of the rbc model, this data would suggest that the intracellular fraction increased as the proportions of neuropil increase, yet preliminary studies using gadolinium-based methods suggest that that the intracellular fraction actually may be higher in the pyramidal neuron layers (type I) than other regions of the hippocampus. The reductions in $F_{\text{fast}}$ (or conversely, increases in the $F_{\text{slow}}$) may instead correlate with increases in the tortuosity of the extracellular space as the proportions of neuropil increase. Unlike the regularly packed, sphere-like packing geometry of the CA1, CA3 and granule cell layers, the neuropil is composed of dense interdigitations of axons, dendrites and synapses. This type of tissue ultrastructure should create a more tortuous path for the diffusion of extracellular water.
This inference is supported by previous experiments that measured TMA (MW 74.1) diffusion in the extracellular space of the rat hippocampus and reported higher tortuosity factors for the stratum radiatum (stratum ROI) than the pyramidal neuron layers (CA1 and CA3 ROIs)(120,121). Further, the partial volume effects detected in ROIs of type I tissues (see Table 4-2) suggest that there may be diffusion-weighted signal contributions of up to 60% from adjacent, cytoarchitecturally different layers that contain water populations with significantly lower F\text{fast} values. Thus, the data suggests that future studies with better spatial resolution may determine that the CA1, CA3 or granule cell layers contain very little, if any, slow diffusing water when analyzed using the biexponential model. Such a finding would further suggest that the F\text{fast} parameter is not a correlate of the extracellular fractions.

It is difficult to directly relate the magnitude of tortuosity factor differences for extracellular TMA diffusion (120,121) to the diffusion MRI observed differences in F\text{fast} for water diffusion in the slices. It is also possible that distinct water exchange rates in the different anatomical regions of the rat hippocampal slice contributed to the F\text{fast} differences noted. In the rbc ghosts tissue model, when the intracellular fraction was held constant, F\text{fast} was reduced by reductions in diffusion time. This finding was attributed to reduced periods for water exchange between the cellular compartments since F\text{fast} was believed to represent water that had sampled the extracellular space for at least some portion of the diffusion time of the experiment (66). Thus, although the diffusion measurements here used a fixed diffusion time, differences in transmembrane water exchange rates within the slices could also contribute to the F\text{fast} differences observed.
Exchange rate heterogeneity in the rat hippocampal slice might be attributed to their different packing geometries or to variations in the membrane properties of the different regions. Different parts of the neuronal membrane have different functional responsibilities that also may be reflected in different molecular and biophysical membrane characteristics (125). In general, type I tissue is largely composed of spherical-shaped neuronal soma, whereas other ROI regions contain increasing proportions of axons, dendrites and synaptic boutons (19). Aquaporin channel blockers that reduce transmembrane water exchange decreased the $F_{\text{fast}}$ for rbc ghosts; if exchange does affect the $F_{\text{fast}}$ values obtained in regions of rat hippocampal slices, this finding suggests that water exchange rates may be highest in the CA1, CA3 and granule cell layers. As discussed below, the present data is incomplete; other models of water diffusion in nervous tissue that parameterize both extracellular tortuosity and transmembrane water exchange rates will be needed to better understand their contributions to the $F_{\text{fast}}$ differences noted in the present data.

Additional studies also will be required to further understand regional heterogeneity for the biexponential diffusion parameter $D_{\text{slow}}$ in the rat hippocampal slices. The mean $D_{\text{slow}}$ for type I tissues was approximately 71-78% higher than the mean $D_{\text{slow}}$ for type II and III tissues in the hippocampal slice. In the rbc ghosts tissue model, the biexponential parameter $D_{\text{slow}}$ depended on intracellular restriction (reductions in rbc size decreased the magnitude of $D_{\text{slow}}$)(66). Increased restriction also seems to lower values for $D_{\text{slow}}$ in the present data for hippocampal slices; regions, like the molecular layer, which contain large proportions of the thin cylindrical geometry of axons and dendrites (i.e. neuropil) had significantly lower $D_{\text{slow}}$ values as compared to regions
containing large pyramidal-shaped neuronal bodies (e.g. the CA3 ROI). In addition, within the type I tissue group, the CA3 cell band ROI had statistically significant increases in $D_{\text{slow}}$ as compared to the CA1 and granule cell layers which have the same relative packing geometry, but contain neurons with smaller diameters (19).

Lastly, a few findings in this study may be due to partial volume effects in the data obtained. The statistically significant, approximately 30% difference between the $D_{\text{fast}}$ of stratum oriens and the $D_{\text{fast}}$ values of the hilus and stratum ROIs may be attributed to partial volume effects with perfusate. Except for an extremely thin layer of inter-hippocampal fibers from CA1 and CA3 (the alveus), stratum oriens is adjacent to ACSF. In contrast, the hilus and stratum ROIs represent the anatomical structures located furthest from ACSF in the present study. Thus, because ACSF has a $D_{\text{fast}}$ of $2.01 \times 10^{-3}$ mm$^2$/s at room temperature (97), intravoxel ACSF may be responsible for the increased $D_{\text{fast}}$ observed. Within tissue group I, the diffusion properties of the CA1 ROI were not as statistically distinct from other regions of the rat hippocampus as the CA3 and granule cell layers. Data in Tables 4-2 and 4-3 suggest that there may be larger partial volume effects in the CA1 ROI from the adjacent anatomical layers of the hippocampus.

The untoward influences of partial volume effects highlight the difficulties encountered in trying to simultaneously obtain diffusion data with sufficient spatial resolution to resolve anatomical differences while also having sufficient SNR for mathematical models of water diffusion in nervous tissue. Pixel-by-pixel maps (for example Fig. 4-8) demand even more SNR for consistent data modeling, yet ultimately may be better for data interpretation since regional ROI analysis imposes a priori groupings of the anatomical diffusion data obtained. In addition, slice movement due to
bulk ACSF perfusion makes image coregistration difficult (as explained in detail below) and may limit future, more data-intensive experiments to characterize diffusion in different regions of the unperturbed hippocampal slices.

To address these concerns, a modified slice perfusion chamber will be built in the near future to acquire diffusion MRI measurements of rat hippocampal slices at higher spatial resolutions using multiple diffusion times. Such data would be compatible with a two-compartment exchange model. Unlike the biexponential model of non-monoexponential water diffusion, this model parameterizes water exchange between compartments, intracellular restriction and extracellular tortuosity (67) and should directly address some of the interesting hypotheses suggested by the present data. The assumptions of the two-compartment model (e.g. compartment geometry), however, may need to be altered to accommodate the complex biophysical properties of different anatomical regions of the rat hippocampal slice. In addition, there are several alternative methods for measuring water diffusion in nervous tissue that may also prove helpful in elucidating the biophysical basis for the water diffusion differences noted in different regions of the rat hippocampal slice (126,127).

**Alternative Explanations for Regional Water Diffusion Differences**

Because the present water diffusion measurements were made exclusively with diffusion gradients aligned with the $B_0$-field, regional variations in diffusion anisotropy were not assessed. Yet there have been some initial, albeit unconvincing reports of anisotropic water diffusion in the hippocampus. Clinical studies have reported minimal anisotropy in the human hippocampus when compared to the corpus callosum (for example, (128)). However, these findings were confounded by limited spatial resolution whereby designated hippocampal voxels were contaminated by known white matter
structures within or apposed to the hippocampus such as the alveus, fimbria and fornix (note, these regions were not included in this study). Mazel and colleagues reported anisotropic tetramethylammonium ion (TMA) diffusion for \textit{in vivo} rat hippocampus (121), but the error of TMA probe positioning (100 – 150 \( \mu \)m) was far greater than the dimensions of some hippocampal regions. A tensor analysis of fixed mouse brain also suggested diffusion anisotropy in the hippocampus (129), yet this study was limited to image interpretation without reporting signal-to-noise ratios or statistical comparisons of quantitative data. Similar to the aforementioned clinical study, the large ROIs employed for this diffusion tensor study, along with its incorporation of substantial zero filling for image processing (129), obscure the purported conclusions.

The uncertainty towards diffusion anisotropy in the hippocampus could have been predicted by comparing the tissue ultrastructure of the hippocampus to classic white matter tracts found in the spinal cord and corpus callosum. In rat spinal cord, there is a highly ordered parallel arrangement of predominately myelinated axons (130); as such, water diffusion is substantially less restricted in orientations parallel to white matter tracts as opposed to transverse orientations (131). Although hippocampal regions such as stratum radiatum also contain parallel axon associations largely oriented orthogonal to the septo-temporal axis (e.g. mossy fibers and Schaffer collaterals), unlike spinal cord white matter, these fibers represent only a small fraction of the nervous tissue cross-section. Axons in these regions are profoundly interdigitated with the dendritic trees of pyramidal neurons in CA1 – CA3, along with a myriad of cell bodies and processes from astrocytes and other neurons (e.g. inhibitory basket cells)(19). This complex anatomy suggests that anisotropy may only be present in the rat hippocampus at length scales
much smaller than those sampled for the diffusion MRI measurements reported here and elsewhere (129).

The extended time required to obtain MRI data amenable to diffusion tensor or trace analysis, even at the present resolution, would have precluded sufficient ACSF perfusion to maintain slice viability (53) and reduced the temporal resolution of diffusion changes after A23187 treatment. It would not be possible with such data to test the leading hypothesis of this study that A23187 affects water diffusion of the rat hippocampus in a temporally and region-specific manner. Preliminary diffusion trace studies of water diffusion anisotropy in fixed rat hippocampal slices employing similar resolution to the image measurements reported herein do not exhibit substantial anisotropic influence. Further, even the studies described above (121,128,129) claim that anisotropy is nearly an order of magnitude smaller than in classic white matter tracts; this suggests that the present baseline data is interpretable with the caveat that anisotropy may also be one contributing factor to the regional water diffusion differences noted. A more thorough characterization of water diffusion anisotropy in slices represents an important future direction of this study.

Additional regional differences that were not fully investigated in this study may also contribute to the water diffusion differences noted.Regional $T_1$ and $T_2$ differences in the rat hippocampal slices were minimized, but not eliminated by the pulse parameters chosen for the present diffusion measurements ($TR = 2$ sec, $TE = 30$ms). The diffusion-images with minimal diffusion-weighting ($b = 7$ s/mm$^2$) showed little if any evidence of regional differences in signal intensity (Fig. 2, panel B), which suggests the contributions from these relaxation processes may be minimal. However, $T_1$ and $T_2$ measurements are
required to fully assess their potential confounding contributions to the diffusion differences noted.

Several methodological refinements incorporated in this study minimize the ischemic injury that occurs during hippocampal slice procurement (40,43). However, the procurement process remains a traumatic event that severs distant neuronal connections of the hippocampus, particularly connections via the fornix and perforant pathway. Some layers of the hippocampus may be affected more detrimentally by the resultant loss of inhibitory and excitatory inputs; these changes might also contribute to the differences observed in the biexponential diffusion parameters. Variations in the iron or myelin content, or capillary density (132) of the hippocampal regions may also contribute to the diffusion differences noted.

Regional Differences after A23187 Treatment

Using the Matlab program, the data obtained in this study may also be presented as map images where signal intensity is determined by scaled values for $S_0$, $F_{\text{fast}}$, $D_{\text{fast}}$ or $D_{\text{slow}}$. Figure 4-8 illustrates a pixel-by-pixel map for $F_{\text{fast}}$ for 2 A23187-treated hippocampal slices. Comparison of these figures suggests a global reduction in $F_{\text{fast}}$ after treatment with A23187. However, such maps may be misleading as they do not convey the error present that largely precludes interpretation of potential A23187-induced changes in the regional anatomy of the rat hippocampus. This is particularly frustrating since the very regions suggested by previous correlative histology of rat hippocampal slices (CA1, CA3 and granule cell layer) to be most affected by A23187 (chapter 3) also appear to be most vulnerable to partial volume effects (Table 4-2) and the confounding influences of microscopic slice movements (Table 4-4).
The measurement errors for the temporal data (described in detail below) also make it difficult to determine how untreated control slices were affected over time. The data may indicate that control hippocampal slices were not as stable as previously reported (53,105). However, in this study, the slices experienced longer time periods without perfusion than recent slice studies (chapters 2, 3 and 5) to obtain spatial resolution sufficient for anatomical segmentation of the diffusion MRI data. This design was based on data from an earlier study that suggested slices remain viable for up to 90 minutes without ACSF perfusion (53). This prior study, though, only characterized diffusion-weighted signal intensity in whole slice ROIs at 1 $b$-value (1000 s/mm$^2$). Temporal changes in particular anatomical areas may have been averaged out by previous studies that employed whole slice ROIs. Certain regions could be more vulnerable to ischemia induced by limited ACSF perfusion or experience pathologic changes due to procurement-induced axotomy.

When potential A23187-induced diffusion changes are then superposed on the potential diffusion changes in control data, it may result in no change from baseline in the biexponential diffusion parameters at particular timepoints in treated slices despite dramatic shifts in tissue water properties. Another difficult confounding problem to note is that diffusion-weighted images were used for anatomical segmentation. This made it difficult at times to distinguish slice or anatomical region movements from diffusion-weighted signal intensity changes introduced by time or treatment with A23187. Regional ROIs were only drawn once and carried forward to all subsequent temporal data to account for this, but future temporal perturbation studies of slices should attempt to identify a means of segmenting images that will not vary with time or treatment.
Partial Volume Effects

Cross-section measurements of anatomical regions with high volume averaging ratios (Table 3) suggested that MRI in-plane spatial resolution below 60 µm would be required to substantially reduce partial volume effects when measuring signal attributed to the CA1, CA3 or granule cell layers. This is particularly true since most voxels are unlikely to be centered on the anatomical regions of interest as drawn in Fig. 4-7. Indices of partial volume effects (Table 4-2 and 4-3) suggest that MRI voxels included in the CA1, CA3 and the granule cell layer ROIs may receive two-thirds of their diffusion-weighted signal intensity from adjacent anatomical regions (i.e. stratum oriens and stratum for CA1 and CA3, and hilus and molecular layer for the granule cell layer). Most of these bordering regions are type III tissue, which is particularly intriguing since the absolute differences for the diffusion parameters $F_{fast}$ and $D_{slow}$ were of the greatest magnitude between tissue type I (CA1, CA3 and granule cells) and tissue type III. Thus, it is likely that the present data significantly underestimated the water diffusion differences between the CA1, CA3 and granule cell layers and other regions of the rat hippocampal slice. In contrast, the 300-µm depth of MRI voxels were unlikely to contribute significantly to partial volume effects since the thickness of individual anatomical lamina changes very gradually through the septo-temporal axis of the rat hippocampus.

Unlike baseline data, segmentation of rat hippocampal slices in posttreatment diffusion measurements was proved difficult because the slices often moved after perfusion with ACSF. Approximately 60% of the regional ROIs needed to be moved at some timepoint during the experiment. To appreciate the potential confounding effects of
sample movement, sample ROIs were purposefully moved to incorrect positions 1 voxel (117 µm) away from the desired location and the biexponential diffusion parameters re-obtained (see methods). The percentage deviation in these values as compared to those obtained from the original, desired ROI location were reported in Table 4-4. These data demonstrate that thinner ROI regions with smaller areas (such as the molecular and CA1 layers) were more susceptible to the confounding influences of subtle slice movements. It should also be noted that movements smaller than the dimensions of a voxel would also confound the data by introducing changes in a voxel’s diffusion-weighted signal intensity.

Other types of slice movement were more difficult to assess. An additional 14 regional ROIs were rejected when the assigned anatomical region twisted or appeared to transform into a shape incompatible with the original ROI dimensions (sometimes this happened independently of gross slice movements). Slices also may move vertically due to the flow pattern of perfusion (chapter 2). Perturbations, such as A23187, that induce swelling may change slice buoyancy (Dr. Blackband, personal communication). Although multislice diffusion-weighted sagittal images acquired after each perfusion period did not indicate vertical movements, it should be noted that most in-plane movements of the slices were less then 1 mm; vertical movements on this scale may have been difficult to recognize through visual inspection of sagittal images. The hippocampal slices were thicker than the MR-defined axial slices (500 µm vs. 300 µm), small vertical movements of the slices may not have been detrimental since the laminar anatomy is consistent in the vertical dimension.
Future Directions

This study has suggested that there are distinct water diffusion regimes within different anatomical regions of the rat hippocampal slice. Future studies will require extended MRI acquisitions with higher spatial resolution to obtain data sufficient for complicated mathematical models of water diffusion in nervous tissue (67). To accommodate such studies, future experiments may employ a custom-made radiofrequency coil with a smaller diameter (~ 5-7 mm) and employ a slice chamber with hollow-fiber perfusion lines that also immobilizes hippocampal slices with agarose gel. These modifications should increase SNR per unit time, minimize slice movements due to the bulk flow of perfusate and eliminate the interruption of scans for slice re-perfusion with fresh, oxygenated ACSF. The implementation of such a design may also facilitate future experiments to test whether there are anatomical differences in the magnitude and temporal pattern of diffusion changes after A23187 treatment.
Figure 4-1. Evidence of the laminar anatomy in diffusion MRI of rat hippocampal slices. Diffusion-weighted images ($b = 3630 \text{ s/mm}^2$) of 4 rat hippocampal slices at 59-μm in-plane resolution. These panels illustrate the consistent laminar anatomy of the rat hippocampus when it is cut orthogonal to the septo-temporal axis.
Figure 4-2. Diffusion-weighted images of rat hippocampal slices at $b$-values from 7 – 8086 s/mm$^2$ (B – H) illustrate anatomical differences in diffusion-weighted signal attenuation. Note that signal attenuation in the CA3 and granule cell (GC) regions are more substantial at high $b$-values, like ACSF, as compared to other anatomical regions of the hippocampal slice [ML = molecular layer].
Figure 4-3. Segmentation in a diffusion-weighted image of the rat hippocampal slice depicting typical ROI dimensions for the different anatomical regions. [GC = granule cells, ML = molecular layer, s-oriens = stratum oriens]
Figure 4-4. Diffusion-weighted signal attenuation curves for different anatomical regions of a typical rat hippocampal slice (Mean ± SEM).
Table 4-1. Baseline SNR and biexponential diffusion parameters [mean ± SD] for segmented regions of rat hippocampal slices

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>SNR at $b = 8085$ s/mm²</th>
<th>$F_{\text{fast}}$ (no units)</th>
<th>$D_{\text{fast}}$ ($10^{-3}$ mm²/s)</th>
<th>$D_{\text{slow}}$ ($10^{-3}$ mm²/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole slice</td>
<td>19</td>
<td>38.3 ± 11.8</td>
<td>0.551 ± 0.770</td>
<td>1.022 ± 0.215</td>
<td>0.055 ± 0.010</td>
</tr>
<tr>
<td>CA1</td>
<td>20</td>
<td>31.5 ± 14.9</td>
<td>0.657 ± 0.100</td>
<td>0.980 ± 0.319</td>
<td>0.066 ± 0.029</td>
</tr>
<tr>
<td>CA3</td>
<td>19</td>
<td>21.8 ± 3.5</td>
<td>0.686 ± 0.038</td>
<td>1.033 ± 0.215</td>
<td>0.106 ± 0.025</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>19</td>
<td>51.2 ± 12.5</td>
<td>0.439 ± 0.102</td>
<td>1.036 ± 0.240</td>
<td>0.046 ± 0.008</td>
</tr>
<tr>
<td>Granule cells</td>
<td>20</td>
<td>20.0 ± 3.1</td>
<td>0.717 ± 0.045</td>
<td>0.978 ± 0.211</td>
<td>0.081 ± 0.034</td>
</tr>
<tr>
<td>Hilus</td>
<td>19</td>
<td>32.8 ± 7.6</td>
<td>0.556 ± 0.054</td>
<td>0.869 ± 0.253</td>
<td>0.051 ± 0.016</td>
</tr>
<tr>
<td>Stratum</td>
<td>20</td>
<td>44.0 ± 18.2</td>
<td>0.500 ± 0.123</td>
<td>0.895 ± 0.290</td>
<td>0.047 ± 0.011</td>
</tr>
<tr>
<td>Subiculum</td>
<td>18</td>
<td>32.6 ± 10.3</td>
<td>0.592 ± 0.059</td>
<td>1.068 ± 0.215</td>
<td>0.054 ± 0.017</td>
</tr>
<tr>
<td>Stratum Oriens</td>
<td>17</td>
<td>44.7 ± 12.4</td>
<td>0.0474 ± 0.081</td>
<td>1.161 ± 0.306</td>
<td>0.053 ± 0.010</td>
</tr>
</tbody>
</table>

1-way ANOVA (P-value)*

<table>
<thead>
<tr>
<th>Region</th>
<th>P-value</th>
<th>P-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole slice</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Diffusion parameters with statistically significant variance (P < 0.05) were subsequently compared for regional differences using a Tukey multiple comparisons test (see Figure 4).
Figure 4-5. Tukey multiple comparisons test P-values used to detect statistical differences between anatomical regions of the rat hippocampal slices for the mean biexponential diffusion parameters $F_{\text{fast}}$ (A) and $D_{\text{slow}}$ (B). The different anatomical regions were categorized into 3 groups; densely packed neuronal bodies (I), mixed cell bodies and neuropil (II) and densely interdigitated neuropil (III). [Symbols for ROIs: Sub = subiculum, Wh = whole slice, Hil = hilus, GC = granule cells, ML = molecular layer, Str = stratum, SRO = stratum oriens]

<table>
<thead>
<tr>
<th></th>
<th>Wh</th>
<th>SRO</th>
<th>Str</th>
<th>ML</th>
<th>GC</th>
<th>CA3</th>
<th>CA1</th>
<th>Sub</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hil</td>
<td>1.000</td>
<td>0.049</td>
<td>0.409</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
<td>0.915</td>
<td></td>
</tr>
<tr>
<td>Sub</td>
<td>0.818</td>
<td>&lt;0.001</td>
<td>0.012</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.011</td>
<td>0.235</td>
<td></td>
</tr>
<tr>
<td>CA1</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.290</td>
<td>0.964</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.952</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML</td>
<td>&lt;0.001</td>
<td>0.927</td>
<td>0.274</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Str</td>
<td>0.567</td>
<td>0.984</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRO</td>
<td>0.091</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-5 continued
Figure 4-6. Comparison of hematoxylin and eosin stained sections (8-µm thick) illustrate the different neuronal sizes, shapes, densities and diversity within the molecular layer (A), granule cell layer (B), hilus (C), stratum radiatum (D) CA1 subfield (E), CA3 subfield (F), stratum oriens (G) and subiculum (H) of the rat hippocampal slices (630x magnification). The stratum radiatum is part of the stratum ROI in these experiments.
Table 4-2. Comparison between ROI area in diffusion MRI images and H & E stained sections for different regions of the rat hippocampal slices. Data [mean ± SD] were obtained from 5 randomly chosen slices (MRI and histology slices did not correspond).

<table>
<thead>
<tr>
<th>Segmented Region</th>
<th># pixels</th>
<th>MRI Area (mm²)*</th>
<th>H &amp; E Area (mm²)</th>
<th>Volume Averaging Ratio†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>16.2 ± 5.9</td>
<td>0.31 ± 0.06</td>
<td>0.05 ± 0.01</td>
<td>2.9</td>
</tr>
<tr>
<td>CA3</td>
<td>17.0 ± 6.7</td>
<td>0.26 ± 0.08</td>
<td>0.07 ± 0.01</td>
<td>1.8</td>
</tr>
<tr>
<td>Molecular layer</td>
<td>55.3 ± 14.4</td>
<td>0.91 ± 0.19</td>
<td>0.42 ± 0.04</td>
<td>1.0</td>
</tr>
<tr>
<td>Granule cells</td>
<td>21.5 ± 6.7</td>
<td>0.42 ± 0.10</td>
<td>0.10 ± 0.01</td>
<td>1.8</td>
</tr>
<tr>
<td>Hilus</td>
<td>15.7 ± 5.0</td>
<td>0.25 ± 0.11</td>
<td>0.13 ± 0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>Stratum</td>
<td>86.8 ± 27.1</td>
<td>1.42 ± 0.51</td>
<td>0.70 ± 0.04</td>
<td>0.9</td>
</tr>
<tr>
<td>Subiculum</td>
<td>114.3 ± 37.1</td>
<td>1.59 ± 0.46</td>
<td>0.89 ± 0.33</td>
<td>0.8</td>
</tr>
<tr>
<td>Statum oriens</td>
<td>50.0 ± 13.0</td>
<td>0.71 ± 0.16</td>
<td>0.33 ± 0.05</td>
<td>1.0</td>
</tr>
<tr>
<td>Whole slice</td>
<td>494.9 ± 78.1</td>
<td>6.94 ± 1.31#</td>
<td>3.12 ± 0.47</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* The diffusion MRI ROI volumes (µL) can be calculated by multiplying the reported areas by 0.30 mm.
† See methods for an explanation of this ratio; ratios greater than 1 represent overestimation of region size in the diffusion MRI segmentation and vice versa.
# The summed area of the segmented regions of the rat hippocampal slice is 6.12 x10⁻² mm² (88% of the whole slice ROI).
Figure 4-7. H & E stained section of the CA1 subfield of a rat hippocampal slice (630x) suggests volume averaging of the thin laminar anatomical regions of the hippocampus. Box 1 represents the in-plane dimensions of an image voxel from the diffusion MRI experiments (117 x 117 \( \mu m \)). Since slices used for histology shrank to 45% of their original MRI-measurement area after fixation (see Table 4-2), box 2 may more accurately represent a potential region enclosed by an MRI image voxel. The double arrow denotes the width of the CA1 cell band and a typical cross-section measurement for Table 4-3.
Table 4-3. Cross-section width for segmented regions of rat hippocampal slices with significant volume averaging ratios (see table 4-2). Note the MRI voxel has 117-µm in-plane resolution.

<table>
<thead>
<tr>
<th>Segmented region</th>
<th>Number of measurements*</th>
<th>Cross-section width in H &amp; E (µm)</th>
<th>Estimated prefixation width (µm)#</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1</td>
<td>22</td>
<td>28.6 ± 6.0</td>
<td>42.6 ± 8.9</td>
</tr>
<tr>
<td>CA3</td>
<td>26</td>
<td>38.0 ± 6.2</td>
<td>56.6 ± 9.2</td>
</tr>
<tr>
<td>Granule cells</td>
<td>31</td>
<td>42.5 ± 11.7</td>
<td>63.3 ± 17.4</td>
</tr>
</tbody>
</table>

* 4 - 6 cross-sections were measured per segmented region from 5 random slices.

# See methods for explanation of how unfixed length estimates were made.
Table 4-4. Influence of slice movement on diffusion parameters obtained. Mean percentage change (± SD) in the biexponential diffusion parameters for selected regions of the rat hippocampal slice when the segmented ROI is translated one voxel up, down, left or right of the original position (3 hippocampal slices were assessed in 4 directions each, N =12).

<table>
<thead>
<tr>
<th>Region</th>
<th>$S_0$</th>
<th>$F_{\text{fast}}$</th>
<th>$D_{\text{fast}}$</th>
<th>$D_{\text{slow}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole slice</td>
<td>1.4 ± 0.4%</td>
<td>1.9 ± 0.1%</td>
<td>4.6 ± 0.6%</td>
<td>3.2 ± 0.3%</td>
</tr>
<tr>
<td>Stratum</td>
<td>1.2 ± 0.5%</td>
<td>4.0 ± 1.2%</td>
<td>4.4 ± 2.4%</td>
<td>4.9 ± 1.3%</td>
</tr>
<tr>
<td>Molecular Layer</td>
<td>4.8 ± 1.5%</td>
<td>24.8 ± 14.5%</td>
<td>30.9 ± 16.8%</td>
<td>12.9 ± 9.7%</td>
</tr>
<tr>
<td>CA1</td>
<td>3.4 ± 1.2%</td>
<td>17.7 ± 6.2%</td>
<td>13.7 ± 1.9%</td>
<td>22.0 ± 5.7%</td>
</tr>
</tbody>
</table>
Figure 4-8. Pixel-by-pixel map of the Ffast diffusion parameter (values from 0 to 1) for 2 rat hippocampal slices treated with 50 mM A23187. Black spaces represent pixels where the biexponential fitting model failed to converge.
CHAPTER 5
WATER DIFFUSION MEASUREMENTS IN PERFUSED HUMAN HIPPOCAMPAL SLICES UNDERGOING TONICITY CHANGES

Introduction

Diffusion-weighted MRI provides image contrast based on differences in the intravoxel incoherent motion of water (60). Over the past decade, diffusion MRI has proven particularly useful clinically because it is capable of distinguishing pathological from normal nervous tissue in the first hours following ischemic brain injury (49). Diffusion MRI can be important to clinical trials of neuroprotective agents (77). In 1996, Niendorf et al. reported that water diffusion-weighted signal attenuation in rat nervous tissue was non-monoexponential when measured at high $b$-values. This multiexponential diffusion of water was mathematically described using a biexponential model that assigns signal attenuation as the sum of two components (slow and fast diffusing) (63). Recently, several groups have reported similar findings in human subjects (99,133,134). Although the biophysical origins for diffusion contrast after ischemic injury and non-monoexponential diffusion-weighted water signal attenuation in nervous tissue remain unclear (61), several studies have shown that analyses of tissue water diffusion correlate with changes in tissue microstructure on perturbation (54,55,105).

Our laboratory has developed a method for the magnetic resonance imaging of rat hippocampal slices to investigate diffusion in a controllable nervous tissue model (50). The use of high-field magnets with gradient strengths up to 1000 mT/m allows diffusion measurements of tissue water in rat hippocampal slices at short diffusion and echo times.
that are not possible in current clinical scanners. Experiments with such short diffusion times allow water diffusion to be studied approaching the limit of slow exchange between the intra- and extracellular compartments, and provide diffusion data appropriate for analytical models that depend on the short gradient pulse approximation (61). The perfused rat hippocampal slices exhibit many aspects of normal in vivo brain physiology and can be interrogated under a variety of perturbations (54,55). With these important advantages, the brain slice model is well-suited for experiments to determine the tissue microstructure changes associated with MR-observed changes in water diffusion. Such experiments should lead to further refinements in the mathematical and physical models that have been developed to describe diffusion in nervous tissue (as reviewed by Norris (61)).

These models may help explain the diffusion characteristics of normal and pathological nervous tissue in human patients and lead to improved clinical decision-making. It is therefore important to determine the validity of diffusion measurements in rat hippocampal slices as a model for the diffusion properties of human nervous tissue. The primary hypothesis of this study was that diffusion in human hippocampal slices at high $b$-values is similar to diffusion in rat hippocampal slices and in vivo human brain. Further, it was hypothesized that osmotic perturbation would alter diffusion in human hippocampal slices in a similar fashion to previous studies of rat hippocampal slices perturbed osmotically (50). Data leading to the acceptance of these two hypotheses would help validate rat hippocampal slices as a useful model for studying diffusion in brain tissue.
Unfortunately, it was impractical to obtain healthy human hippocampal tissue to test these hypotheses. Instead, human hippocampal slices were obtained from patients that underwent anterior temporal lobectomy for the treatment of medically-intractable epilepsy. The epileptic pathology, which renders this tissue available for study, alters the normal neuronal connectivity of the tissue over time and leads to quantitative differences in the diffusion properties of the tissue (128). Despite these changes, the remaining neurons and glia in these hippocampal slices are relatively normal (as demonstrated in this study using electrophysiology) such that the specimens obtained provide a reasonable model of normal tissue for testing the above hypotheses. Diffusion measurements of the human hippocampal slices obtained were made at high \( b \)-values using a procedure developed for prior experiments with healthy rat hippocampal slices using a multislice perfusion apparatus (105) placed inside a 750-MHz spectrometer with 1000-mT/m gradients (50). The technical advantages inherent to the brain slice imaging method also permitted diffusion measurements in living human nervous tissue using echo and diffusion times significantly shorter than previously reported.

**Methods**

**Procurement of Human Tissue**

Human brain tissue procurement was approved by the University of Florida Institutional Review Board. Hippocampal specimens were obtained from 5 patients undergoing anterior temporal lobectomy for the treatment of temporal lobe epilepsy. Specimens were taken from the middle one-third of the hippocampus and immediately immersed in oxygenated ice-cold artificial cerebrospinal fluid (ACSF), then transported to the laboratory in less than 5 minutes. The ACSF contained 124 mM NaCl, 2.5 mM KCl, 10 mM glucose, 26 mM NaHCO\(_3\), 1 mM CaCl\(_2\), 1.25 mM NaH\(_2\)PO\(_4\) and 6 mM
MgCl₂. The ACSF was gassed with 95% O₂ / 5% CO₂ to maintain a pH of 7.4. This ACSF had relatively low calcium and high magnesium concentrations to reduce procurement-induced ischemic damage to the hippocampal tissue (40). Several 400-µm transverse hippocampal slices then were prepared from each specimen using a vibratome. For all samples, adjacent surgical sections were fixed, then stained with hematoxylin and eosin (H & E). These histology samples were analyzed for hippocampal sclerosis, which was defined as neuronal loss and gliosis found predominately in the dentate hilus and CA1 subfields (135). A few slices recovered after the diffusion MRI experiments described below were also fixed and stained with H & E to characterize any effects from the experimental protocol employed.

**Hippocampal Slice Perfusion and Perturbation**

After sectioning, the hippocampal slices were allowed to gradually warm to room temperature (20°C) and placed into an ACSF solution containing lower magnesium and higher calcium concentrations (120 mM NaCl, 3 mM KCl, 10 mM glucose, 26 mM NaHCO₃, 2 mM CaCl₂, 1.5 mM KH₂PO₄ and 1.4 mM MgSO₄) gassed with 95% O₂ / 5% CO₂ to maintain a pH of 7.4. This ACSF solution was defined as isotonic. The slices then were placed into a perfusion chamber (50) and lowered into a 15-mm diameter NMR tube. This chamber held and perfused up to 5 brain slices simultaneously. Slices were perfused with isotonic ACSF (2 mL/min) whilst the perfusion chamber was lowered into the magnet and during pilot image acquisition. Although perfusion was discontinued during all diffusion measurements to avoid flow artifacts, previous reports demonstrated that the slices remained stable for at least the first 8 hours following slice procurement (53).
To make solutions for osmotic perturbations, 20% hypotonic ACSF was made by diluting isotonic ACSF 4:1 with distilled water and 20% hypertonic ACSF was made by adding 60 mM mannitol to isotonic ACSF. The osmolality was $301 \pm 1$ mOsm/kg for isotonic ACSF, $236 \pm 1$ mOsm/kg for 20% hypotonic ACSF and $361 \pm 2$ mOsm/kg for 20% hypertonic ACSF as determined using an Osmette A freezing point depression osmometer (Precision Systems, Inc.). Once the first diffusion protocol was completed (described below), slices were perfused with the 20% hypotonic ACSF for 30 minutes. This time period was sufficient for complete perfusate exchange. After acquisition of a second diffusion protocol measurement, slices were perfused with 20% hypertonic ACSF for 30 minutes, then a final diffusion protocol was obtained. Both the hypotonic and hypertonic ACSF solutions were also gassed with 95% O$_2$ / 5% CO$_2$ to maintain a pH of 7.4. Additional slices followed this same procedure (perfusion for 30 minutes followed by a diffusion protocol), but were perfused with isotonic ACSF throughout (3 cycles). These samples served as controls for the stability of the slices in the magnet.

**MRI Measurements**

MR data were obtained using a 15-mm birdcage coil interfaced to a Bruker 17.6-T vertical wide-bore magnet and console. Pilot multislice axial, sagittal and coronal T$_1$ and diffusion-weighted imaging sequences were used to locate the perfusion chamber, and then define 300-µm thick axial MR-slices through the center of the 400-µm thick human hippocampal slices. Diffusion-weighted images were acquired using a pulsed gradient spin-echo multislice sequence with a diffusion time of 14 ms (diffusion time, $T_d = \Delta - \delta/3$) and an echo time of 30 ms. After optimizing the positions of the axial imaging slices, a “diffusion protocol” was acquired. This protocol consisted of 8 diffusion-weighted
images (128 x 64 matrix, 1.5 cm field-of-view, repetition time = 2 s, echo time = 30 ms, 2 averages, \( \delta = 3 \) ms, \( \Delta = 15 \) ms, slice thickness = 300 \( \mu \)m) employing rectangular-shaped diffusion gradient pulses (100-\( \mu \)s rise time) oriented along the \( B_0 \)-field direction, increasing in magnitude in linearly-spaced steps from 0 to 940 mT/m. This protocol resulted in 8 diffusion-weighted images with \( b \)-values between 7 and 8080 s/mm\(^2\) (including imaging terms). The single diffusion gradient orientation assumes that diffusion in the human hippocampus is relatively isotropic at the image resolution used (128,136,136). Image slice thickness was 300 \( \mu \)m with a 117 x 234 \( \mu \)m in-plane resolution. This protocol took 34 minutes to complete. Additional pilot images were acquired between diffusion protocols to monitor for vertical or horizontal slice movement due to perfusion; no movement of slices was observed.

**MRI Data Processing**

Three regions-of-interest (ROI) were drawn in the diffusion-weighted images; an ROI that circumscribed the entire human hippocampal slice (‘slice’), an ROI in the perfusate adjacent to slices (‘perfusate’) and an ROI in the upper corner of the image outside the sample (‘noise’). Whole slice ROIs were chosen to facilitate comparisons of human brain slice data with previously reported diffusion data from rat hippocampal slices (54,55). Further, pixel-by-pixel analysis of diffusion-weighted signal attenuation in the human hippocampal slices was not attempted because of significant signal-to-noise ratio (SNR) limitations (each voxel represents approximately 8 x 10\(^{-3}\) mm\(^3\) or 8 nL of tissue). The SNR of an image was calculated by dividing the mean signal intensity of a slice ROI by the standard deviation of the noise ROI. This calculation was done for
every diffusion protocol using both the lowest and highest diffusion-weighted images ($b = 7$ and $8080 \text{ s/mm}^2$).

To ensure the accuracy of the diffusion MR measurements, Eq. [5-1] was fit to the mean signal intensity of the perfusate ROI to determine the signal intensity without diffusion weighting ($S_0$) and the apparent diffusion coefficient of perfusate ($D$):

$$S(b) = S_0 \exp(-bD).$$ [5-1]

Only data from the first four diffusion-weighted images collected per diffusion protocol were used for this calculation because previous experiments demonstrated that signal from perfusate dropped to noise levels at $b$-values above $1800 \text{ s/mm}^2$ (54). The perfusate diffusion coefficient was determined for each diffusion protocol and agreed well with published values for pure water at room temperature (97).

Previous studies demonstrated that diffusion-weighted signal attenuation in rat hippocampal slices is non-monoexponential at high $b$-values (54). As a first approximation to describe the diffusion properties of water, a biexponential function was fitted to diffusion-weighted signal attenuation from the human hippocampal slices using the Levenberg-Marquandt non-linear least squares fitting routine:

$$S(b) = S_0 [F_{fast} \exp(-bD_{fast}) + (1-F_{fast})\exp(-bD_{slow})]$$ [5-2]

where $S_0$ is signal intensity without diffusion weighting, $F_{fast}$ is the fraction of fast diffusing water, and $D_{fast}$ and $D_{slow}$ represent the apparent diffusion coefficients (ADCs) of the fast and slow diffusing water components respectively. This biexponential analysis was employed to compare the results with previous experiments in rat brain slices (54,55) and with $in vivo$ human diffusion data (99,133,134). Observed differences between the fast and slow diffusing components of the 3 treatment groups (isotonic, 20% hypotonic
and 20% hypertonic ACSF) were compared statistically using a one-way repeated-measures ANOVA. If the ANOVA test indicated a statistically significant difference (defined as P < 0.05 in this experiment), the 3 groups were further compared using a Tukey multiple comparisons test to isolate treatment groups that differed significantly from one another.

**Slice Electrophysiology**

To characterize the physiological viability of these samples, electrophysiology recordings were conducted on additional human hippocampal slices obtained from the surgical specimens. These experiments elicited action potentials and synaptic responses from neurons found within the slices using methods described previously (137). Briefly, paired hippocampal slices were placed into a submerged recording chamber and perfused with room temperature isotonic ACSF. Granule cells in the dentate gyrus of the hippocampal slices were identified using infrared differential interference contrast videomicroscopy with a fixed-stage microscope. Neurons chosen for recording were more than 50 µm deep in the 400-µm thick hippocampal slices to avoid areas that may have been damaged by the vibratome (40) (note also that the 300-µm thick MR-defined slices were centered in the 400-µm thick hippocampal slices to minimize the potential influence of the cut edges on the diffusion data obtained). Tight-seal (>1 GΩ) whole-cell recordings were obtained from the cell body of the granule cells using patch electrodes with a resistance of 3-5 MΩ. These electrodes were filled with a solution containing 120 mM K-glucuronate, 8 mM NaCl, 10 mM HEPES, 4 mM MgATP, 0.4 mM Na3GTP, 0.2 mM EGTA, and 0.1 % biocytin (pH 7.3, 290 mOsm/kg). Neurons were voltage clamped at –68 mV using an Axopatch 1D amplifier, and access resistance (15-20 MΩ) was
monitored regularly during the recordings. To characterize action potentials in the procured slices, spikes were induced in the granule cells by a 200-ms 15 pA direct current injection while in current-clamp mode. To characterize synaptic responses, an additional glass electrode, filled with ACSF (electrode resistance = 3-5 MΩ), was placed into the inner region of the dentate molecular layer. Synaptic responses were evoked by this electrode with a 5-pulse train stimulation at 20 Hz. All subsequent data were filtered at 2 kHz, digitized at 10 kHz, and stored in a computer using pClamp 8 software (Axon Instruments) for off-line analysis.

Results

Hippocampal specimens were obtained from 5 patients undergoing anterior temporal lobectomy and histologic samples from each patient were diagnosed with hippocampal sclerosis by neuropathological examination (Fig. 5-1). These histological features were present in the hippocampal specimens before and after the diffusion MRI experiments, which indicates that pathological changes were due to the chronic epilepsy and not the MR protocol utilized. The 400-µm thick human hippocampal slices typically had dimensions of 4 x 7 mm and usually included variable portions of Cornu Ammonis (CA1, CA2 and CA3 subfields) with or without the dentate gyrus. For MRI experiments, 20 slices were obtained from the 5 patients; 15 slices were used for experiments with varying tonicity and 5 were used for control experiments. A few additional slices were excluded from these experiments because of substantial non-acute hemorrhagic lesions. These slices were unlikely to be viable and the blood breakdown products present would have created substantial imaging artefacts due to susceptibility effects. Slices included in
the study were prepared promptly and imaging experiments were begun within 2 hours of surgical procurement.

Some hippocampal slices obtained from the surgical specimens were used for electrophysiology recordings. As shown in fig. 5-2, granule cells in the dentate gyrus of these paired slices demonstrated normal-appearing action potentials in terms of amplitude (between 70 – 110 mV) and duration (between 1 – 10 ms) (125). The patch-clamped neurons also showed frequency adaptation to the 200-ms current injection and demonstrated short-term depression in their synaptic responses as would be expected for healthy granule cells (data not shown). Such results were obtainable from 95% of the granule cells present even 8 - 10 hrs after slices were cut from the surgical specimens. This suggests that slices can remain physiologically viable and stable for many hours after procurement.

In MRI experiments, the perfusate water ADC [mean ± standard deviation] did not vary significantly between experiments or diffusion protocols (2.01 ± 0.08 x 10^{-3} mm^2/s), and approximated the previously published value for water at this temperature (2.03 x 10^{-3} mm^2/s at 20°C)(97). The SNR of hippocampal tissue [mean ± standard deviation] for the diffusion protocols was 59.1 ± 21.3 at the lowest b-value and 26.4 ± 8.8 at the highest b-value. A 1-mm thick diffusion-weighted image of a human hippocampal slice is shown in Fig. 5-3 (TR/TE = 2000/30 ms, ∆/δ = 15/3 ms, b = 2749 s/mm^2, 128 x 128 matrix, 1.5 x 1.5 cm FOV, 4 averages). This image is of higher resolution than used for the diffusion protocols, but demonstrates the detailed hippocampal anatomy present in some of the hippocampal slices studied. The lamellar hippocampal organization was less apparent in diffusion images of other slices due to the pathologic changes brought by hippocampal
sclerosis. Quantitative diffusion data did not come from this high-resolution image, but from images with 300-µm thickness and a 128 x 64 matrix. Examples of such images are shown in Fig. 5-4. These images demonstrate the typical signal intensity changes observed in the human hippocampal slices after perturbation with 20% hypotonic and 20% hypertonic ACSF solutions.

Diffusion-weighted signal attenuation curves of the human hippocampal slices were non-monoexponential (Fig. 5-5) and well described by biexponential fits ($R^2 > 0.99$) using Eq. [5-2]. The parameters [mean ± standard deviation] for biexponential analysis of the data obtained from all slices in isotonic ACSF ($n = 20$) were $0.686 ± 0.082$ for $F_{\text{fast}}$, $1.22 ± 0.22 \times 10^{-3}$ mm$^2$/s for $D_{\text{fast}}$ and $0.06 ± 0.02 \times 10^{-3}$ mm$^2$/s for $D_{\text{slow}}$. Diffusion in control slices ($n = 5$), which were repeatedly perfused with isotonic ACSF, did not show statistically significant changes in any of the biexponential parameters over the 4 - 5 hr duration of the MRI experiments ($P > 0.05$, ANOVA). In the remaining 15 slices, successive hypotonic and hypertonic perturbations of the human hippocampal slices caused substantial changes in the diffusion-weighted signal attenuation curves (for example, see Fig. 5-5). The fraction of fast diffusing water demonstrated -8.2 % and +10.1 % mean changes from baseline isotonic values after perfusion with 20% hypotonic and 20% hypertonic ACSF respectively (all groups $P < 0.001$, Tukey test) (Table 5-1). ACSF tonicity, however, did not alter the ADC of the fast diffusing component in any of the treatment groups ($P = 0.311$, ANOVA). The ADC of the slow diffusing component for slices exposed to hypertonic ACSF differed significantly from the slow component ADCs of those same slices in isotonic or hypotonic ACSF ($P < 0.001$ for both comparisons, Tukey test). However, the slow component ADCs of the slices in isotonic
and hypotonic ACSF did not differ significantly from one another (P = 0.833, Tukey test).

**Discussion**

This report demonstrates that it is feasible to measure multiexponential water diffusion in human brain slices obtained from surgical specimens. The preparation of these slices was similar to the preparation of rat hippocampal slices described previously (53). The principal difference was a 5 minute increase in the time interval between brain specimen procurement and tissue slicing. A modified ACSF with high concentrations of magnesium was employed during this period to help minimize any potential damage that could occur during the transport of human brain specimens from the surgical theater to the research laboratory (40).

The data suggest that the human hippocampal slices obtained were viable for at least 4-6 hours after procurement. The histological appearance of the slices did not change between samples fixed immediately after surgical procurement and subsequent slices fixed after the MRI experiments were completed (4 - 7 hours later). Furthermore, electrophysiological studies of paired hippocampal slices at similar timepoints demonstrated that granule cells in the dentate gyrus exhibited normal action potentials and synaptic responses. In addition, the diffusion properties of water, as determined by biexponential analysis, were stable in control human slices perfused serially with isotonic ACSF. This further demonstrates the viability of the human tissue obtained since diffusion has been shown to be acutely sensitive to pathophysiologic and microstructural changes in brain tissue (49).

MRI of the human hippocampal slices was facilitated by use of a high-field vertical spectrometer (750 MHz) and a recently developed multislice perfusion chamber (105).
that permitted simultaneous MR measurements on multiple hippocampal slices obtained from the same patient. Although there was some variability in the appearance of the specimens due to the epileptic pathology, MR images from most slices revealed the detailed lamellar anatomy expected from coronal-sliced hippocampal tissue (as shown in Fig. 5-3). Images for the diffusion protocols also had sufficient SNR for robust biexponential fits (26.4 ± 8.8, for example, at $b = 8080$ s/mm$^2$). Diffusion-weighted signal attenuation in the human hippocampal slices was non-monoexponential (Fig. 5-5) and similar to data from perfused rat brain slices (54,55,105), rat (63,138,139) and human brains in vivo (99,127,133,134). Although there is substantial debate regarding its interpretative value (66,105,138,139), biexponential analysis using Eq. [5-2] modeled the experimental data well ($R^2 > 0.99$) and facilitates a general comparison with previous studies on rat brain slices and in vivo studies as discussed below.

The diffusion-weighted signal attenuation in the human brain slices changed substantially with 20% hypertonic and then 20% hypertonic osmotic perturbations (Figs. 5-4 and 5-5). These results are analogous to changes in diffusion-weighted signal intensity at $b = 975$ s/mm$^2$ previously reported for rat hippocampal slices treated with the same protocol (50). Comparison of the mean estimated parameters from biexponential analysis for human slices in the different ACSF solutions (Table 5-1) showed an 8.2% decrease in the fast diffusing fraction for slices in 20% hypotonic ACSF and an 10.1% increase in the fast fraction for slices in 20% hypertonic ACSF. It has been suggested that the observed diffusion changes with variations in tonicity reflect changes in the relative fraction of the intracellular compartment (54,55,66,105). This is supported by many previous studies of rat hippocampal slices that have employed alternative methods,
such as intrinsic optic signal and electrical impedance measurements, to demonstrate cellular swelling and shrinking in response to hypo- and hyperosmotic perturbations respectively (for example, see (140)). Therefore, in these human slices, it is assumed that perfusion with hypotonic ACSF produces an osmotic gradient that causes cellular swelling, whereas hypertonic ACSF causes the opposite response, cellular shrinkage. It is important to note that the correlation between changes in the fast diffusing fraction and changes in the relative fraction of the intracellular compartment does not require that the two are directly equivalent (i.e. a 10% reduction in $F_{\text{fast}}$ does not represent a 10% equivalent reduction in the extracellular compartment) (63,66,105). As stated, the relationship between the biexponential parameters and the biophysical properties of nervous tissue remains unclear (54,55,61,141,142), and indeed, in most cases, it is likely to be an incomplete description of water diffusion in a biological tissue. It is clear that the biexponential analysis is too simplistic for heterogeneous tissues, and that models incorporating exchange may be more appropriate when the slow exchange limit cannot be assumed (66,105,117).

Both human hippocampal slices taken from epilepsy specimens and normal rat hippocampal slices demonstrated similar diffusion changes in response to osmotic perturbations that induce cellular swelling and shrinkage (50,53-55). The diffusion changes in human brain slices also were comparable to prior experiments that induced cellular swelling with a variety of agents in isolated rat optic nerves (143) and rat brains in vivo (56, 63,144, 145). The similar diffusion properties between rat hippocampal slices and other models used to study diffusion in nervous tissue, along with the reproducibility of rat hippocampal slice diffusion measurements in similarly obtained human
hippocampal slices, supports the validity of future studies that employ rat brain slices to study diffusion in nervous tissue using protocols which are not feasible in human subjects.

It is also interesting to compare the diffusion data from human brain slices with previously published *in vivo* diffusion measurements in human subjects (99,127, 133,134). Selected literature reporting biexponential diffusion parameters for *in vivo* human gray matter are shown in Table 5-2. So far, clinical studies have not reported diffusion measurements at high $b$-values in small anatomical structures such as the hippocampus, so diffusion in hippocampal slices was compared to the values reported for gray matter. Diffusion in human brain slices can be measured at significantly shorter diffusion and echo times than can be achieved on a clinical scanner, thus reducing the influence of exchange and $T_2$ weighting on the diffusion parameters obtained. Given these differences, it is not surprising that the biexponential diffusion parameters obtained for human hippocampal slices at short gradient pulses with short diffusion and echo times are different than previously published values (99,127,133,134). In particular, the slow ADC was significantly lower when measured in human brain slices under these measurement conditions. Although the hippocampus may not be a classic gray matter structure, the slow ADC reported for human hippocampal slices, even with the potential temperature corrections described below, is still lower than even the slow ADCs reported for white matter in human subjects (99,133,134).

The diffusion data obtained for human brain slices at short diffusion and echo times are more suitable for developing models of diffusion in nervous tissue, yet there are several important limitations to direct comparisons of this data with *in vivo* diffusion
measurements in human subjects. Unlike the *in vivo* human brain, the human hippocampal slices were studied at room temperature. This temperature simplifies the requirements for the perfusion chamber and maintains slice viability for longer periods of time (40). Based on a previous report, the self-diffusion coefficient of unrestricted water should increase by approximately 50% from room temperature to 37°C (97). The relationship between diffusion and temperature in nervous tissue is likely to be more complex due to restriction and exchange effects. This will require further investigation, yet the human hippocampal slice diffusion data does suggest the ADC of the slow diffusing water fraction is significantly lower than previously reported for human brain *in vivo*. We attribute this to the shorter diffusion time employed in these experiments (Table 5-2).

Another important caveat to direct comparison of slice diffusion data with healthy human controls is that hippocampal slices were obtained from patients with hippocampal sclerosis from chronic epilepsy. This is an avoidable limitation to this study since it is unlikely that healthy human hippocampal tissue will ever be obtainable for brain slice experiments. Hippocampal sclerosis is characterized by substantial neuronal loss and gliotic scarring in the hippocampus (see Fig. 5-1)(135), which expands the extracellular space, decreases neuronal densities and reduces asymmetric cellular organization in the hippocampus (128). The resulting increase in $T_2$ relaxation times for affected tissue proves useful for MRI assessment of temporal lobe epilepsy (146). The short echo times used here (30 ms) reduce the potentially confounding influence of epilepsy-induced $T_2$ relaxation time prolongation on the diffusion parameters reported.
The changes in tissue structure also led to changes in water diffusion in sclerotic human hippocampi. Several studies in human subjects have demonstrated that hippocampal sclerosis increased the hippocampal ADC 10 - 24% (128,136,147). This suggests that the diffusion parameters reported here differ quantitatively from values that would have been measured in healthy tissue. It is difficult to determine if the fast and slow ADC’s obtained by biexponential analysis were affected equally since these prior studies did not examine diffusion in sclerotic hippocampi at $b$-values greater than 1000 s/mm$^2$. Unfortunately, the lack of hippocampal slices from healthy human specimens precludes this study from contributing further to our understanding of diffusion changes that result from epilepsy. To address this issue, future studies employing similar methods could examine hippocampal slices obtained from a rat model of epilepsy that exhibits similar neuropathological changes to those noted in these human hippocampal samples (148).

Figure 5-1. Histological sections of the dentate gyrus from a normal human hippocampus autopsy specimen (A) and from one of the temporal lobectomy specimens used for the diffusion MRI experiments presented (B) (hematoxylin & eosin stain, 400x magnification). Panel B demonstrates marked reductions in granule cell density due to cell death. In addition, the nuclei of surviving granule cells show less intense basophilic staining and the neuronal bodies are dispersed by excess mossy fiber sprouting. These pathological changes are characteristic of hippocampal sclerosis.
Figure 5-2. Whole-cell recording of granule cells in a human hippocampal slice for action potentials evoked by a 200-ms duration, 15-pA DC current injection into a granule cell located in the dentate gyrus of a human hippocampal slice. These data were obtained 4 hrs after procurement in a hippocampal slice from the same surgical specimen as other slices used for the diffusion MRI studies presented. The action potentials have normal amplitude and duration, and the action potentials decrease in frequency over time as would be expected for healthy granule cells (frequency adaptation).
Figure 5-3. Diffusion-weighted MRI of a perfused human hippocampal slice (117-µm in-plane resolution, $b = 2749$ s/mm$^2$). This particular sample consisted mostly of dentate gyrus from the hippocampal body. The lines enclose the CA3 region and molecular layer (ML). The arrows point to the granule cell layer (GCL).
Figure 5-4. Serial diffusion-weighted images ($b = 8080 \text{ s/mm}^2$) of a human hippocampal slice in isotonic ACSF (A), 20% hypotonic ACSF (B) and 20% hypertonic ACSF (C). In contrast to the previous figure, this particular sample consisted of the Cornu Ammonis regions of the hippocampus (CA1, CA2 and CA3 subfields). Signal intensity is significantly increased after hypotonic perturbation and significantly decreased after hypertonic perturbation.
Figure 5-5. Representative diffusion-weighted water signal attenuation plot for a human hippocampal slice perfused with isotonic, 20% hypotonic and 20% hypertonic ACSF solutions. The semilog plot has been normalized to the signal intensity of the initial diffusion-weighting for each tonicity. The arrow depicts where data obtained from Fig. 5-4 would be plotted. Mean signal-to-noise for all slices (n = 20) was 26.4 ± 8.8 at b = 8080 s/mm².
Table 5-1. Biexponential diffusion parameters [mean ± SD, n = 15] from human hippocampal slices perturbed by isotonic, 20% hypotonic and 20% hypertonic ACSF solutions.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>$F_{\text{fast}}$</th>
<th>$D_{\text{fast}}$ (10$^{-3}$ mm$^2$/s)</th>
<th>$D_{\text{slow}}$ (10$^{-3}$ mm$^2$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic</td>
<td>0.711 ± 0.073*</td>
<td>1.256 ± 0.246</td>
<td>0.067 ± 0.021</td>
</tr>
<tr>
<td>20% Hypotonic</td>
<td>0.653 ± 0.076*</td>
<td>1.266 ± 0.260</td>
<td>0.065 ± 0.015</td>
</tr>
<tr>
<td>20% Hypertonic</td>
<td>0.783 ± 0.063*</td>
<td>1.299 ± 0.223</td>
<td>0.077 ± 0.021*</td>
</tr>
<tr>
<td>ANOVA</td>
<td>&lt; 0.001</td>
<td>0.311</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

* ANOVA results with P < 0.05 were subsequently evaluated with a Tukey multiple comparisons test. Groups that were statistically distinct are labeled (*).
CHAPTER 6
CONCLUSIONS

Rat hippocampal slices provide a controllable tissue model to investigate the biophysical basis of diffusion-weighted magnetic resonance signal changes observed clinically in nervous tissue after ischemic or traumatic injury. Previous MRI studies of brain slices demonstrated that brain slices responded to osmotic and pharmacological perturbations in a similar fashion to *in vivo* nervous tissue (50,54,55). These prior studies also were limited by low-throughput. In chapter 2, a new multislice perfusion chamber was described that allowed for the simultaneous acquisition of diffusion-weighted magnetic resonance images from multiple perfused rat hippocampal slices. These images had signal-to-noise ratios sufficient to analyze the multicomponent diffusion properties of water. The tissue water diffusion parameters were similar to previously reported values (54) and were stable for at least 8 hours after slice procurement. This created the ability to study diffusion changes over time in multiple brain slices following experimental perturbation.

Chapter 3 successfully employed the advantages of the multislice perfusion chamber to study the acute temporal evolution of multicomponent water diffusion changes in rat hippocampal slices after treatment with the calcium ionophore, A23187. Data obtained from this novel tissue model of acute brain injury suggests that the biexponential diffusion parameter $F_f$ may be a sensitive correlate of cellular swelling in nervous tissue and that diffusion changes after acute brain injury may be intravoxel volume-averaged summations of responses from anatomically or temporally distinct
healthy and pathologically-injured cell populations. Diffusion data from this study concurs with previous clinical studies that diffusion-weighted images at high \( b \)-values may offer improved characterization of normal and pathological nervous tissue under certain conditions. Ultimately, these results suggested that regional analysis of A23187-treated hippocampal slices may improve our understanding of the unique region-specific morphological processes that follow acute cellular injury and contribute to a better understanding of clinical measurements of diffusion in human stroke and traumatic brain injury patients.

In chapter 4, the first systematic regional analysis of water diffusion in rat hippocampal slices (chapter 4) indicated that the diffusion properties of water were significantly influenced by a region’s cytoarchitectural composition. In particular, water diffusion properties in the rat hippocampal slice were distinct for 3 unique nervous tissue types; densely packed neuronal bodies (CA1, CA3 and the granule cell layer), neuropil (the molecular layer, stratum radiatum, lacunosum and oriens) and regions of heterogenous cell bodies combined with neuropil (subiculum and hilum). Further characterization of water diffusion in the different anatomical regions of the rat hippocampal slice may improve our understanding of the biophysical basis for water diffusion in nervous tissue. Unfortunately, it proved difficult with the current perfusion chamber design to investigate whether A23187 affects different regions of the rat hippocampal slice at different magnitudes or temporal patterns. Future modifications to the perfusion chamber design may limit the confounding influences of microscopic slice movement and partial volume effects due to limited spatial resolution.
The data presented in chapter 5 demonstrated that the diffusion properties of water in human hippocampal slices were comparable to those observed in previous studies of rat hippocampal slices. Furthermore, the human slices responded to osmotic perturbations in a similar fashion to that observed in rat hippocampal slices (50,54,55). These results help validate the use of rat brain slices for studying water diffusion in nervous tissue. The diffusion data obtained from human hippocampal slices using short echo and diffusion times also suggested that water diffusion measurements of \textit{in vivo} human brain using clinical scanners may be insufficient to adequately characterize slow diffusing water components.

The brain slice methodology presented here provides a unique opportunity to further explore the biophysical basis of diffusion in human nervous tissue by maintaining a viable nervous tissue model in a controllable environment. The hardware limitations inherent to current clinical studies of diffusion make it difficult to repeat these studies in human subjects at short diffusion times. However, the data obtained on rat hippocampal slices may be capable of reconciling complex, data-intensive mathematical models of nervous tissue water diffusion with diffusion MRI data that is clinically feasible to obtain from human patients with acute brain injuries.
APPENDIX A
ANATOMICAL LAYERS OF THE RAT HIPPOCAMPAL SLICE

For the purposes of this work, “segmentation” is defined as the drawing of ROIs such that they contain voxels exclusively assigned as representative of a particular anatomical region. Segmentation was done using heavily diffusion-weighted MRI images. In the descriptions below, the terms “proximal” and “distal” are used to describe hippocampal anatomy in relation to an imaginary line that runs from the subiculum to CA1 to CA3 to the dentate gyrus. Thus, CA3 is distal to CA1 and CA1 is proximal to the dentate gyrus. Note also that voxels assigned to particular “anatomical” layers of the hippocampus in this study may contain other anatomical regions that cannot be fully distinguished by segmentation based on diffusion-weighted MRI. Examples include CA1 and CA3 (which both may contain CA2), stratum and subiculum (which may contain each other) and stratum oriens (which may contain alveus and/or fimbria). These descriptions were prepared from more detailed explanation (19,119).

CA1

These pyramidal neurons are smaller than those found in the CA3 region and have a more spherical shape. The neurons are packed densely 4-5 neurons deep distally (towards CA3), but the deeper neurons become more loosely packed as this neuronal layer approaches the subiculum. The entire pyramidal neuron layer exhibits low signal intensity in diffusion-weighted MRI. In this study, the proximal end of the CA1 layer was taken as a point across from the apex of the dentate gyrus where signal intensity begins to become diffuse and increase in intensity. The border with CA3 is described below.
CA3

These large pyramidal neurons have elongated cell bodies with ovoid nuclei. The CA3 neurons are packed tightly 4-5 cells deep along radial orientations (perpendicular to the gross curvilinear line of this cell layer). For MRI segmentation, the distal end of the CA3 region was defined as the opening of the dentate gyrus. Histologically, CA3 and CA1 neurons are separated by a short transition zone (approximately 100 µm long) of mixed neuronal phenotypes referred to as CA2. This region cannot be distinguished with MRI, but a bulbous enlargement of the low signal intensity line marking pyramidal neurons found as the pyramidal layer begins a gradual 180° turn into the dentate gyrus was chosen as the CA1-CA3 division point for this study. This selection is not arbitrary and chosen based on prior experience locating this histological feature in hematoxylin and eosin-stained sections.

Molecular Layer

This U-shaped layer is composed axons from the perforant pathway and other incoming pathways forming synapses on the dendrites and dendritic spines of granule cells. There are few cell bodies. The outer borders of the molecular layer on MRI are the residual hippocampal sulcus along the internal blade and ACSF perfusate on the external blade. The inner border apposes the dark signal intensity of the dentate gyrus granule cell layer. In diffusion-weighted images, the two ends of the molecular layer taper at the same position that the dark band of granule cells tapers.

Granule Cell Layer

This layer contains the smallest neuronal cell bodies with highest density packing (119). The granule cells are stacked 8-15 cells deep in radial strands (119). The thin
polymorphic layer is adjacent to the hilar border of the granule cell layer and is not resolvable by MRI. In diffusion-weighted images, the granule cell layer appears dark and is bordered by the hilus and molecular layer.

**Hilus**

The hilus is composed of large, loosely packed mossy and basket cells uniformly distributed in a crescent-shaped area between the internal and external blades of the dentate gyrus. Mossy fiber outputs from the granule cell layer run through this region towards the CA3 pyramidal neurons. This tissue region has intermediate diffusion-weighted MRI signal intensity as compared to darker appearance of the adjacent CA3 and granule cell layers and does not extend past the U-shaped patterns of the granule cell and molecular layers. It also does not include the dark signal intensity band that extends from the CA3 cell band into the dentate gyrus.

**Stratum**

This region is composed of 3 different anatomical lamina of the rat hippocampus; stratum lacunosum, lucidum and radiatum. The regions were combined into the region “stratum” because they were barely distinguished with MRI even in heavily diffusion-weighted images with high signal-to-noise ratios (see Fig. 4-1). All 3 regions share many traits with each other and the molecular layer described above. There are very few cells bodies, but instead the interdigitations of pyramidal neuron dendrites and axons from mossy fibers, Schaffer collaterals and other axonal pathways. In diffusion MRI the stratum is bordered by the low signal intensity pyramidal neuron layer, the residual hippocampal sulcus and the molecular layer. The region begins in the proximal
hippocampus at the same point that the CA1 region begins (low signal intensity band) and tends to have slightly higher signal intensity than the adjacent subiculum.

**Subiculum**

The subiculum is composed of heterogeneous collection of neuronal and glial cell bodies with additional axonal pathways and dendrites. The trapezoid-shaped subiculum can be divided into many sublayers, but these cannot be distinguished using diffusion MRI. Due to the procurement process the subiculum in rat hippocampal slices is most often bordered by perfusate on 3 sides and the distal border forms against the ends of the stratum, CA1 cell band and stratum oriens. Some slices may also include a small section of entorhinal cortex (see upper right panel of Fig. 5-1), where the additional bend of tissue extending from the hippocampus would not be included in the subiculum ROI. In addition in a few slices, the almost the entire subiculum was removed during procurement.

**Stratum Oriens**

A narrow, relatively cell-free area of tissue outside the pyramidal neurons of CA1 – CA3. Stratum oriens is largely composed of pyramidal neuron basal dendrites. On diffusion MRI images, this layer is between the dark, low signal intensity call bands of CA1 and CA3, and the perfusate. It begins proximally at the same point as CA1 and stratum and ends at the opening of the dentate gyrus.
APPENDIX B
PERCENTAGE CHANGES FROM BASELINE TO BIEXPONENTIAL DIFFUSION
PARAMETERS FOR DIFFERENT HIPPOCAMPAL REGIONS AFTER A23187
TREATMENT
<table>
<thead>
<tr>
<th>Biexponential Diffusion Parameter</th>
<th>Control (0.1 % DMSO) (N = 7)</th>
<th>50 µM A23187 (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S₀</td>
<td>F₁</td>
</tr>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>0.8 ± 10.6 %</td>
<td>3.0 ± 3.3 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>1.4 ± 9.8 %</td>
<td>1.4 ± 3.9 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>0.3 ± 11.2 %</td>
<td>1.5 ± 4.9 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>0.6 ± 12.1 %</td>
<td>2.1 ± 5.9 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>2.3 ± 12.4 %</td>
<td>0.1 ± 5.7 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>3.0 ± 10.7 %</td>
<td>-0.7 ± 5.5 %</td>
</tr>
</tbody>
</table>
### Table B-2. Treatment Changes to Water Diffusion in the CA1 ROI

<table>
<thead>
<tr>
<th>Biexponential Diffusion Parameter</th>
<th>Control (0.1 % DMSO) (N = 6)</th>
<th>50 µM A23187 (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>S₀ 0.0 ± 0.0 %</td>
<td>F&lt;sub&gt;fast&lt;/sub&gt; 0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>1.3 ± 7.0 %</td>
<td>2.7 ± 5.0 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>1.3 ± 7.4 %</td>
<td>4.3 ± 4.9 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>2.2 ± 4.0 %</td>
<td>2.5 ± 8.4 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>4.8 ± 7.2 %</td>
<td>4.4 ± 9.2 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>3.1 ± 5.9 %</td>
<td>3.2 ± 10.2 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>3.2 ± 6.8 %</td>
<td>1.4 ± 8.9 %</td>
</tr>
</tbody>
</table>

### Table B-3. Treatment Changes to Water Diffusion in the CA3 ROI

<table>
<thead>
<tr>
<th>Biexponential Diffusion Parameter</th>
<th>Control (0.1 % DMSO) (N = 3)</th>
<th>50 µM A23187 (N = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>S₀ 0.0 ± 0.0 %</td>
<td>F&lt;sub&gt;fast&lt;/sub&gt; 0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>2.1 ± 0.4 %</td>
<td>-6.9 ± 4.0 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>-0.7 ± 2.2 %</td>
<td>-7.1 ± 1.4 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>1.3 ± 2.0 %</td>
<td>-8.1 ± 9.5 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>0.6 ± 3.7 %</td>
<td>-7.5 ± 5.0 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>-3.3 ± 2.0 %</td>
<td>-10.9 ± 7.4 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>-1.1 ± 3.2 %</td>
<td>-11.9 ± 7.7 %</td>
</tr>
</tbody>
</table>
### Table B-4. Treatment Changes to Water Diffusion in the Molecular Layer ROI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (0.1 % DMSO) (N = 7)</th>
<th>50 µM A23187 (N = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biexponential Diffusion Parameter</td>
<td>S₀</td>
<td>F_fast</td>
</tr>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>9.6 ± 9.5 %</td>
<td>5.0 ± 19.5 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>8.2 ± 6.8 %</td>
<td>4.1 ± 11.4 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>8.1 ± 10.4 %</td>
<td>5.0 ± 10.4 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>6.7 ± 11.1 %</td>
<td>11.0 ± 17.8 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>8.0 ± 11.1 %</td>
<td>5.9 ± 10.0 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>10.9 ± 11.1 %</td>
<td>7.9 ± 15.1 %</td>
</tr>
</tbody>
</table>

### Table B-5. Treatment Changes to Water Diffusion in the Granule cell ROI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (0.1 % DMSO) (N = 5)</th>
<th>50 µM A23187 (N = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biexponential Diffusion Parameter</td>
<td>S₀</td>
<td>F_fast</td>
</tr>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>-1.0 ± 7.5 %</td>
<td>0.2 ± 3.3 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>0.9 ± 4.9 %</td>
<td>-1.4 ± 3.0 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>0.3 ± 1.9 %</td>
<td>-4.5 ± 2.9 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>0.3 ± 4.4 %</td>
<td>-5.7 ± 3.4 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>-0.8 ± 3.8 %</td>
<td>-6.4 ± 7.9 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>0.5 ± 6.0 %</td>
<td>-8.4 ± 5.6 %</td>
</tr>
</tbody>
</table>
Table B-6. Treatment Changes to Water Diffusion in the Hilus ROI

<table>
<thead>
<tr>
<th>Biexponential Diffusion Parameter</th>
<th>Control (0.1 % DMSO) (N = 6)</th>
<th>50 µM A23187 (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>1.0 ± 3.9 %</td>
<td>1.5 ± 13.4 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>0.6 ± 4.3 %</td>
<td>36.4 ± 72.2 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>-0.8 ± 4.8 %</td>
<td>-2.4 ± 10.4 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>-0.7 ± 4.5 %</td>
<td>-10.5 ± 33.0 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>0.6 ± 6.0 %</td>
<td>2.2 ± 50.7 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>-0.9 ± 5.1 %</td>
<td>12.3 ± 68.4 %</td>
</tr>
</tbody>
</table>

Table B-7. Treatment Changes to Water Diffusion in the Stratum ROI

<table>
<thead>
<tr>
<th>Biexponential Diffusion Parameter</th>
<th>Control (0.1 % DMSO) (N = 7)</th>
<th>50 µM A23187 (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>1.4 ± 4.7 %</td>
<td>17.8 ± 24.9 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>1.1 ± 4.9 %</td>
<td>12.3 ± 27.1 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>0.5 ± 3.4 %</td>
<td>12.3 ± 27.1 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>-0.6 ± 6.6 %</td>
<td>18.2 ± 29.5 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>-0.2 ± 3.4 %</td>
<td>36.4 ± 35.4 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>-1.0 ± 7.2 %</td>
<td>28.2 ± 32.8 %</td>
</tr>
</tbody>
</table>
Table B-8. Treatment Changes to Water Diffusion in the Subiculum ROI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (0.1% DMSO) (N = 6)</th>
<th>50 µM A23187 (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biexponential Diffusion Parameter</td>
<td>S₀</td>
<td>F&lt;sub&gt;fast&lt;/sub&gt;</td>
</tr>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>-1.7 ± 12.4 %</td>
<td>-1.5 ± 3.4 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>-3.3 ± 10.2 %</td>
<td>-3.6 ± 4.8 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>-2.7 ± 10.9 %</td>
<td>-5.5 ± 6.9 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>-3.9 ± 10.8 %</td>
<td>-5.9 ± 5.6 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>-2.8 ± 13.7 %</td>
<td>-9.5 ± 8.1 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>-0.6 ± 11.6 %</td>
<td>-10.8 ± 6.2 %</td>
</tr>
</tbody>
</table>

Table B-9. Treatment Changes to Water Diffusion in the Stratum Oriens ROI

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control (0.1% DMSO) (N = 7)</th>
<th>50 µM A23187 (N = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biexponential Diffusion Parameter</td>
<td>S₀</td>
<td>F&lt;sub&gt;fast&lt;/sub&gt;</td>
</tr>
<tr>
<td>0 min</td>
<td>0.0 ± 0.0 %</td>
<td>0.0 ± 0.0 %</td>
</tr>
<tr>
<td>1 hr 10 min</td>
<td>-0.3 ± 4.5 %</td>
<td>4.2 ± 8.2 %</td>
</tr>
<tr>
<td>2 hr 20 min</td>
<td>-0.3 ± 5.4 %</td>
<td>6.5 ± 10.8 %</td>
</tr>
<tr>
<td>3 hr 30 min</td>
<td>-3.1 ± 7.8 %</td>
<td>9.0 ± 13.2 %</td>
</tr>
<tr>
<td>4 hr 40 min</td>
<td>-5.0 ± 7.9 %</td>
<td>10.4 ± 12.1 %</td>
</tr>
<tr>
<td>5 hr 50 min</td>
<td>-6.1 ± 10.4 %</td>
<td>9.3 ± 18.3 %</td>
</tr>
<tr>
<td>7 hr</td>
<td>-7.1 ± 9.5 %</td>
<td>8.9 ± 13.1 %</td>
</tr>
</tbody>
</table>


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BIOGRAPHICAL SKETCH

Timothy M. Shepherd was born in Lexington, Kentucky in November 1974. During his early childhood, he attended a Head Start program in Hazard, Kentucky and first grade at 4 separate schools. Since 1981, he has lived and attended school mostly in Florida. From 1989-1991 he lived in Helston, Cornwall, England with his family and attended Truro school. After completion of his high school degree at Stanton College Preparatory School in Jacksonville, Florida (1992), he attended Florida State University and graduated in 1996 with a B.S. in Biochemistry. Mr. Shepherd is presently enrolled in the M.D./Ph.D. program at the University of Florida.

After completing both of his degrees, Mr. Shepherd plans to pursue a career in academic radiology research.