Structural Mechanisms of Bidirectional Synaptic Plasticity in the Dentate Gyrus of Freely Behaving Rats

A thesis presented to the University of Otago
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Abstract

This research thesis is part of a collaborative study between the laboratories of Kristen M. Harris and Wickliffe C. Abraham; its purpose: to elucidate the structural mechanisms responsible for the expression of memory within the brain. Bidirectional plasticity is a term which delineates the two principal states of cellular memory, and is characterized by persistent increases (long-term potentiation, LTP) and decreases (long-term depression, LTD) in synaptic transmission.

In chapter one, we used chronically implanted electrodes to investigate parameters that may regulate bidirectional synaptic plasticity within the dentate gyrus of freely moving rats. The induction and persistence of bidirectional synaptic plasticity were compared across two different rat strains, two different phases of the circadian cycle, and three patterns of high-frequency stimulation. Results indicated that LTP was larger and more persistent in Long-Evans than Sprague-Dawley rats. Additional testing in Long-Evans rats revealed that short-term potentiation but not LTP was greater when experiments took place during dark rather than light cycles of the circadian rhythm. Input-output testing demonstrated that these effects were associated with significant differences in dentate granule cell excitability. Bidirectional synaptic plasticity was also strongly dependent on the pattern of afferent stimulation: conventional theta-burst stimulation induced negligible amounts of plasticity, while trains utilizing a 400 Hz pulse frequency yielded consistently robust effects. The results from chapter one were used to optimize the procedures used in subsequent experiments.
In chapter two we used serial section transmission electron microscopy (ssTEM) to examine features of anatomical ultrastructure within the dentate gyrus. Results from chapter two served to illustrate that dendrite caliber, microtubule number and spine density were systematically distributed throughout the granule cell dendritic tree. Knowledge of these systematic distributions was used to inform the sampling scheme of subsequent experiments.

In chapter three, bidirectional synaptic plasticity was induced within the dentate gyrus of freely behaving rats; animals were sacrificed 30 min after induction and prepared for ssTEM. Results indicated that LTP was associated with systematic increases in spine head volume and post-synaptic density (PSD) area within the middle molecular layer of the granule cell dendritic tree, and systematic decreases in the inner molecular layer. Interestingly, these structural changes appeared to balance between adjacent layers. This finding indicated that excitatory synaptic structures could be homeostatically coordinated within the dentate granule cell dendritic tree.

The experimenter concludes by discussing the implications of these findings, and suggests directions for future investigation.
Acknowledgments

To my supervisors, Kristen Harris and Cliff Abraham: I owe you both a tremendous debt of gratitude. Thank you for helping me realize my interest in the field of neuroscience, and providing me with the opportunity to pursue my goals. You have been amazing role models in both science and life.

To my friends and colleagues: science is a team effort, and not all of it takes place in a laboratory. Participation in this research project has afforded me the chance to travel to new places, and forge new friendships. A heartfelt “thank you” goes out to members of the Abraham and Harris labs (and the non-science friends I met along the way).

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To my family: We are American born Kiwis. There have been times when we are together, and times when we are a world apart. Home has always been where you are.

Finally
this thesis is dedicated to my grandmother, Grams
who taught me to always make the most of my opportunities
“I am a collection of water, calcium and organic molecules called Carl Sagan”

-Carl Sagan, *Cosmos*
# Table of Contents

Abstract ........................................................................................................................................ iii

Acknowledgments ......................................................................................................................... v

List of Tables and Figures ........................................................................................................... x

General Introduction ..................................................................................................................... 1

References .................................................................................................................................... 5

Chapter One: Parameterization Of Bidirectional Synaptic Plasticity In The Dentate Gyrus
Of Freely Moving Rats .................................................................................................................. 6

1.2 Methods .................................................................................................................................. 13

1.2.1 Surgery .................................................................................................................................. 13

1.2.2 Recording procedures ........................................................................................................... 14

1.2.3 Electrophysiology ................................................................................................................ 15

1.2.4 Statistics ............................................................................................................................... 17

1.3 Results ..................................................................................................................................... 19

1.3.1 Effect of strain on bidirectional synaptic plasticity .............................................................. 20

1.3.2 Effect of circadian rhythm on bidirectional synaptic plasticity ........................................... 24

1.3.3 Effect of stimulation protocol on bidirectional synaptic plasticity ................................. 28

1.4 Conclusions ............................................................................................................................ 32

1.5 References .............................................................................................................................. 33

Chapter Two: Allometry Of The Granule Cell Dendritic Tree ....................................................... 40

2.1 Introduction .............................................................................................................................. 41

2.2 Method .................................................................................................................................... 47

2.2.1 Animals ............................................................................................................................... 47

2.2.2 Perfusion ............................................................................................................................ 47

2.2.3 Tissue processing ............................................................................................................... 48

2.2.4 Ulrassectioning .................................................................................................................. 48

2.2.5 Serial Section transmission electron microscopy (ssTEM) .............................................. 50

2.2.6 Measured variables ............................................................................................................. 50
2.2.7 Statistics ........................................................................................................................................53
2.3 Results ...............................................................................................................................................53
  2.3.1 Allometry of the granule cell dendritic tree .................................................................................53
  2.3.2 A quantitative method for unbiased region selection .................................................................56
2.4 Conclusions .......................................................................................................................................61
2.5 References .........................................................................................................................................62

Chapter Three: Structural Mechanisms Of Bidirectional Synaptic Plasticity In The
Dentate Gyrus Of Freely Behaving Rats .........................................................................................66
  3.1 Introduction ........................................................................................................................................67
  3.2 Methods ............................................................................................................................................72
    3.2.1 Animals ......................................................................................................................................72
    3.2.2 Surgery .....................................................................................................................................73
    3.2.3 Recording procedures ..............................................................................................................74
    3.2.4 Electrophysiology ....................................................................................................................75
    3.2.5 Perfusion ..................................................................................................................................76
    3.2.6 Tissue processing .....................................................................................................................76
    3.2.7 Ultracectioning ........................................................................................................................76
    3.2.8 Serial section transmission electron microscopy (ssTEM) ......................................................77
    3.2.9 Measured variables ...................................................................................................................77
    3.2.10 Statistics ................................................................................................................................78
  3.3 Results ..............................................................................................................................................79
    3.3.1 Induction of bidirectional synaptic plasticity ..............................................................................79
    3.3.2 Systematic changes in spine volume accompany the induction of bidirectional synaptic plasticity .................................................................................................................................82
    3.3.3 Volume changes associated with bidirectional plasticity are most pronounced in the spine head ...............................................................................................................................................86
    3.3.4 Spine head changes associated with bidirectional synaptic plasticity are accompanied by changes in PSD area .......................................................................................................................88
    3.3.5 PSD area correlates with spine head volume ..........................................................................90
    3.3.6 Changes in spine head volume and PSD area are balanced across MML and IML layers of the granule cell dendritic tree ...........................................................................................................93
  3.4 Conclusions .....................................................................................................................................95
List of Tables and Figures

FIGURE 1.1 Schematic illustration of electrode positions........................................ 14
FIGURE 1.2 Schematic illustration of the HFS protocols........................................ 18
TABLE 1.1 Summary of baseline parameters......................................................... 19
FIGURE 1.3 Effect of strain on I/O electrophysiology............................................ 21
FIGURE 1.4 Effect of strain on bidirectional synaptic plasticity................................. 23
FIGURE 1.5 Effect of circadian cycle on I/O electrophysiology................................. 25
FIGURE 1.6 Effect of circadian cycle on bidirectional synaptic plasticity..................... 27
FIGURE 1.7 Effect of stimulation protocol on bidirectional synaptic plasticity............. 30
FIGURE 2.1 Serial section transmission electron microscopy (ssTEM)....................... 49
FIGURE 2.2 Measured variables............................................................................... 52
FIGURE 2.3 Allometry of the granule cell dendritic tree........................................... 55
FIGURE 2.4 A quantitative method for unbiased region selection............................... 60
TABLE 3.1 Summary of electrophysiological baseline parameters........................... 80
FIGURE 3.2 Induction of bidirectional synaptic plasticity......................................... 81
FIGURE 3.3 Systematic changes in spine volume accompany the induction of
tbidirectional synaptic plasticity. ........................................................................... 83
FIGURE 3.4 Representative micrographs illustrating spine changes associated with
bidirectional synaptic plasticity. ............................................................................. 85
FIGURE 3.5 Volume changes associated with bidirectional plasticity were most
pronounced in the spine head.............................................................................. 87
FIGURE 3.6 Spine head changes associated with bidirectional synaptic plasticity were
accompanied by changes in PSD area. ................................................................. 89
TABLE 3.2 Summary of three-dimensional reconstruction data. ........................................... 90

FIGURE 3.7 PSD area correlates with spine head volume. ................................................. 91

FIGURE 3.8 Summary of structural changes associated with the induction of bidirectional synaptic plasticity. .............................................................................................................. 94
General Introduction

Evolution has shaped the circuits of the brain to respond adaptively to dimensions of the physical environment. Some of these circuits are strictly orchestrated by the genetic code, and reflect lessons which have been learned over millions of years. Other circuits are “plastic”, and provide brain tissue with the ability to acquire information that has not come pre-installed. The acquisition and retention of new information is called memory.

This research thesis is part of a collaborative study between the laboratories of Kristen M. Harris and Wickliffe C. Abraham; its purpose: to elucidate the structural mechanisms responsible for the expression of memory within the brain. The techniques applied to address this aim reflect the combined expertise of the Harris and Abraham labs.

Abraham lab (University of Otago, NZ): In vivo electrophysiological recording

Neurons communicate across specialized junctions called synapses. The Abraham lab has pioneered techniques to stimulate and record synaptic communication within the neural architecture of freely behaving rats. Previous studies have shown that synaptic communication can exhibit persistent changes in efficacy. Long-term potentiation (LTP) is a state characterized by persistent increases in synaptic communication (Bliss and Lomo, 1973; Douglas and Goddard, 1975). Long-term depression (LTD) is a state characterized by persistent decreases in synaptic communication (Bear and Abraham, 1996). Collectively, these changes in synaptic communication are known as bidirectional synaptic plasticity, and are thought to represent the cellular correlate of memory within the brain (Bliss and Collingridge, 1993; Lee et al., 2000). The dentate gyrus is a sub-
region of the brain which has been implicated in the processes of learning and memory (Barnes et al., 1994). In the present investigation, bidirectional synaptic plasticity was induced within the dentate gyrus of freely moving rats.

**Harris lab (University of Texas, Austin, USA): serial section transmission electron microscopy**

Most excitatory synaptic contacts within the brain exist on tiny anatomical protrusions known as dendritic spines (Bourne and Harris, 2007). Individual dendritic spines can be resolved with conventional methods of light microscopy; however, high magnification imaging is required for accurate quantification (Harris et al., 2006). The Harris lab has refined a technique known as serial section transmission electron microscopy (ssTEM). In the process of ssTEM, a tissue sample is cut into hundreds of ultrathin (45 nm) sections, and photographed at high magnification with a transmission electron microscope. Resulting micrographs are aligned and calibrated with specialized software, then arranged in a consecutive stack. Anatomical structures present within this stack can be measured, traced, and reconstructed into three-dimensional models. ssTEM is exceptional in its ability to visualize and quantify the fine structures of the brain, including dendritic spines. In the present investigation, ssTEM was used to analyze the structures of the dentate gyrus at a baseline state, and following the induction of bidirectional synaptic plasticity. The principal interest of this study was to establish how dendritic spines might change to support the expression of LTP and LTD.
The body of this thesis consists of three chapters:

Chapter one

In chapter one we endeavored to parameterize bidirectional synaptic plasticity within the dentate gyrus of freely moving rats. Induction and persistence characteristics of bidirectional synaptic plasticity were compared across two different rat strains, and two different phases of the circadian cycle. Furthermore, we sought to establish a pattern of high frequency stimulation that would induce a robust form of bidirectional synaptic plasticity within the dentate gyrus. Results from chapter one revealed that the induction and persistence characteristics of bidirectional synaptic plasticity varied significantly according to the rat strain used, and the circadian cycle during which experimentation took place. Subsequent testing was used to investigate the mechanisms of these differences. Stimulation experiments revealed that bidirectional synaptic plasticity within the dentate gyrus was frequency sensitive. Testing indicated that induction and persistence effects were maximal when stimulation pulses were administered at a specific frequency. The results from chapter one were used to optimize the procedures used in subsequent experiments.

Chapter two

In chapter two we used ssTEM to examine baseline features of anatomical ultrastructure within the dentate gyrus. Tissue segments were prepared from inner molecular (IML), middle molecular (MML), and outer molecular (OML) layers of the dentate granule cell dendritic tree. The experimenter found that the anatomical
dimensions of the granule cell tree correlated with the distribution of specific subcellular organelles; crucially, these relationships described the distribution of dendritic spines. Results from chapter two served to illustrate that synaptic resources were systematically distributed throughout the granule cell dendritic tree. Knowledge of these systematic distributions was used to inform the sampling scheme of subsequent experiments.

Chapter three
In chapter three, bidirectional synaptic plasticity was induced within the dentate gyrus of freely behaving rats. In the experimental hemisphere, LTP was induced within the afferents projecting to the MML layer of the granule cell dendritic tree. Simultaneously, LTD was induced within the afferents projecting to the OML. In the control hemisphere, baseline stimulation was applied to the afferents projecting to the MML. Animals were sacrificed 30 min after induction and prepared for ssTEM. Tissue samples were excised from IML, MML and OML regions of the control and experimental hemisphere granule cell dendritic tree, processed, and analyzed with three-dimensional quantitative reconstructions. Results indicated that bidirectional synaptic plasticity was associated with systematic changes in spine head volume and post-synaptic density (PSD) area. Interestingly, these structural changes appeared to balance between adjacent regions. This finding indicated that excitatory synaptic structures could be homeostatically maintained within the dentate granule cell dendritic tree.

The experiments conclude by discussing the implications of these findings, and suggest directions for future investigation.
References


Chapter One: Parameterization Of Bidirectional Synaptic Plasticity In The Dentate Gyrus Of Freely Moving Rats

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Formatted for Hippocampus

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1.1 Introduction

Memory is proposed to exist through distributed adjustments of synaptic transmission within neural networks (Martin et al., 2000; Lynch, 2004). Bidirectional plasticity is an experimental phenomenon whereby the efficacy of synaptic transmission may be persistently increased or decreased. Changes in synaptic transmission may be expressed at the level of individual synaptic contacts (Matsuzaki et al., 1994), and scaled across multiple regions to maintain a homeostatic level of cell activation (Turrigiano et al., 1998; Turrigiano, 2008). The mechanisms of bidirectional plasticity are conserved across several vertebrate and invertebrate species, and may be observed through any number of contemporary methodological preparations (Bliss and Collingridge, 1993; Malenka and Bear, 2004). Interestingly, however, the strength and persistence characteristics of bidirectional plasticity are highly dependent upon the network in which they are expressed (Abraham et al., 1995; Abraham and Williams, 2003; Parvez et al., 2010). The dentate gyrus is a subregion of the brain which has been implicated in the processes of learning and memory (Barnes et al., 1994) Contemporary evidence suggests that changes in synaptic transmission within the dentate gyrus may serve to "tune" the expression of memory-related representations in hippocampal area CA3; furthermore, this tuning may be exerted through the expression of bidirectional synaptic plasticity (Alme et al., 2010). In the following study, we address variables which modulate the induction and persistence of bidirectional synaptic plasticity within the dentate gyrus of freely moving rats.
The perforant path input of the dentate gyrus consists of medial and lateral fiber bundles; these fiber groups project to adjacent regions of the granule cell dendritic tree and possess electrophysiologically dissociable characteristics (Abraham et al., 2001). When recorded by an extracellular electrode positioned in the dentate hilus, activation of medial fibers results in a waveform characterized by a steep field excitatory postsynaptic potential (fEPSP) slope, and a prominent population spike (PS). Activation of lateral fibers results in a waveform characterized by a shallow fEPSP and minimal PS (Fig. 1.3A; Abraham and Goddard, 1983). fEPSP slope provides a measure of synaptic transmission, whereas population spike amplitude is indicative of synchronized cell firing. Patterned stimulation of medial or lateral fibers has been shown to induce persistent changes in synaptic transmission. High frequency stimulation (HFS) is capable of inducing persistent increases in fEPSP slope, a state referred to as long-term potentiation (LTP; Bliss and Lomo, 1973a; Douglas and Goddard 1975). Low frequency stimulation is capable of inducing persistent decreases in fEPSP slope, a state referred to as long-term depression (LTD; Levy and Steward, 1979; Abraham and Goddard, 1983). Collectively, these changes constitute the properties of bidirectional plasticity. Intriguingly, experimental evidence indicates that bidirectional synaptic plasticity is coordinated between medial and lateral inputs of the dentate network; HFS induced potentiation of one fiber pathway results in the simultaneous depression of the opposing pathway (Abraham and Goddard, 1983; Doyere et al., 1997; Abraham et al., 2001). This effect was once thought to be "heterosynaptic", whereby synaptic contacts adjacent to the stimulated pathway are depressed via an indirect chain of cellular connections (Abraham et al., 1985). However, recent evidence has demonstrated that both medial and lateral fibers of the perforant path
must be co-active at the time of stimulation for bidirectional synaptic plasticity to be coordinated within the dentate gyrus (Abraham et al., 2007). The expression of this effect is likely attributable to the structure of the granule cell dendritic tree, the proximity of its principal inputs, and the spontaneous activity which takes place within intact neural networks (Benuskova and Abraham, 2007).

The field of learning and memory has been predominantly informed by studies conducted on the rat species, Rattus norvegicus (Lockard, 1968; Andrews, 1996; Manger et al., 2008). Problematically, however, comparative research has demonstrated that numerous empirical differences exist between common Rattus norvegicus strains (Diana et al., 1994; van der Staay and Blokland, 1996, Dhabhar et al., 1997). Long-Evans rats, for example, expend significantly more time engaged in walking, rearing, and exploration-related behaviors than Sprague-Dawley rats. Interestingly, this disposition of Long-Evans animals is correlated with significant increases in hippocampal EEG activity (van Lier et al., 2003). In vivo electrophysiological testing has demonstrated that LTD in hippocampal area CA1 is more readily induced in Sprague-Dawley and Wistar rats than Hooded Listers, and this effect may be attributable to inherent differences in Group III metabotropic glutamate receptor activation (Manahan-Vaughan and Braunewell, 1999; Manahan-Vaughan, 2000a; b). Rat strain differences are also evident in the expression of LTP, with Sprague-Dawley rats exhibiting less LTP in area CA1 than Wistar or Hooded Lister rats (Manahan-Vaughan, 2000b). Because Long-Evans rats are frequently used for behavioral studies, but their ability to show bidirectional synaptic plasticity in vivo has not yet been compared against any other commonly used strain, we used chronically
implanted electrodes to compare the induction and persistence of LTP and LTD in the
dentate gyrus of freely moving Long-Evans and Sprague-Dawley rats, the latter of which
we have studied extensively for their LTP/LTD induction and persistence capability
(Abraham and Goddard, 1983; Abraham et al., 1995; Abraham and Huggett, 1997;
Abraham et al., 2002).

Circadian rhythm influences several physiological systems of the brain and body
(Benington and Heller, 1995; Siegel, 2005; Granados-Fuentes et al., 2006; Eckel-Mahan
and Storm, 2009). Disruption of the circadian cycle has been shown to have an adverse
effect on hippocampal dependent memory, and significantly attenuates the induction of
LTP (Campbell et al., 2002; Marks and Wayner, 2005; Craig and McDonald, 2008).
Interestingly, excitability of granule cell fEPSP slope and PS amplitude has been
observed to fluctuate in time with the circadian cycle. In diurnal animals such as
monkeys, granule cell excitability is observed to peak during the light phase of the
circadian cycle; in nocturnal animals such as rats, excitability peaks during the dark phase
of the circadian cycle (Barnes et al., 1977). When working with rats, in vitro hippocampal
slice experiments have revealed that dentate granule cells exhibit greater LTP in tissue
that was extracted during the dark cycle rather than light cycle of the animal’s circadian
cycle (Harris and Teyler, 1983; Chaudhury et al., 2005). Similar studies conducted in
cortical preparations have revealed that LTD is more readily induced during the light
cycle of the animal’s circadian rhythm (Vyasovskiy et al., 2008). As a necessary
consideration, evidence suggests that the mechanisms of circadian modulation involve
periodic and widespread alterations in synaptic transmission across both cortical and sub-
cortical cell networks (Molle et al., 2006). For this reason, it is unknown if in vitro slice preparations provide the scope necessary to evaluate circadian modulation within a relevant context. Until the mechanisms of circadian oscillation can be isolated, in vivo recording from the intact brain presents the most physiologically relevant experimental procedure by which these changes can be investigated.

Experimentally induced persistent modification of synaptic transmission was first demonstrated in an anesthetized in vivo preparation by activating the cells of the dentate gyrus with patterned bursts of high frequency stimulation (Bliss and Lomo, 1973). Subsequent studies have revealed that comparable patterns of bursting activity take place in the brain of freely moving animals, and coincide with memory encoding events and changes in synaptic transmission (Buzsaki et al., 1987). By adjusting experimental stimulation parameters to mimic those occurring under physiologically relevant conditions, experimenters have established that specific networks of the brain exhibit synaptic modification in response to specific patterns of activation (Yun et al., 2002).

Theta-burst stimulation (TBS), characterized by 5 Hz spacing of 100 Hz pulse bursts, is a protocol that is frequently used to induce robust LTP in area CA1 in vitro (Larson et al., 1986; Staubli and Lynch, 1987; Abraham and Huggett, 1997;), and in the dentate gyrus of anesthetized animals (Christie et al., 1995). Bursts of activity at 5 Hz represent an observed activity pattern in hippocampus, and thus TBS protocols are often heralded as physiologically relevant (Larson and Lynch, 1986). Specific to the dentate gyrus, experimental enhancement of synaptic transmission is observed to peak when activating the perforant path with brief bursts of 400 Hz pulses (Douglas and Goddard, 1975;
Winson and Dahl, 1986), even though individual axon fibers of the perforant path do not generally fire at rates exceeding 100 Hz (Rose et al., 1983). LTP and LTD induced in this way can last for many months (Abraham et al., 1994; Abraham et al., 2002). Moreover, recent evidence has demonstrated that high frequency activation of the dentate gyrus could be supported by asynchronous input from both medial and lateral fibers of the perforant path (Abraham et al., 2007). By way of comparison, the efficacy of theta frequency stimulation in the unanaesthetized dentate gyrus is largely unknown (Hargreaves et al., 1998).

The purpose of the present investigation was to parameterize the variables which modulate bidirectional synaptic plasticity within the dentate gyrus of freely behaving rats. The induction and persistence characteristics of bidirectional plasticity were evaluated across three different experimental conditions. The first experimental condition addressed the impact of strain; bidirectional plasticity was compared between Long-Evans and Sprague-Dawley strain rats. The second experimental condition addressed the impact of circadian rhythm; bidirectional plasticity was compared between animals maintained in light cycle and dark cycle circadian environments. The third experimental condition addressed the impact of HFS induction protocol; bidirectional plasticity was induced and compared across three different HFS protocols. Input output (I/O) testing was used to supplement the characterization of experimental differences observed.
1.2 Methods

1.2.1 Surgery

Adult (100-200 days old, 400-700 gm) male Long-Evans and Sprague-Dawley rats were anesthetized (ketamine, 75 mg/kg s.c.; domitor, 0.5 mg/kg s.c.) and chronically implanted with two monopolar stimulating electrodes and one monopolar recording electrode. Electrode implantation was assisted by a Kopf surgical frame, and guided by standard stereotaxic procedures (as described previously, Abraham et al., 1993). Each electrode was custom fabricated from Teflon insulated stainless steel wire (75 μm tip diameter), and possessed a resistance not exceeding 10 Ω. The stimulating electrodes were implanted in the medial (from lambda: 4 mm lateral) and lateral (from lambda: 5 mm lateral) fibers of the perforant path, and adjusted to activate field excitatory postsynaptic potentials (fEPSPs) within spatially discrete layers of the dentate granule cell dendritic tree (Fig. 1.1). Stimulation of medial fibers activated a fEPSP in the middle molecular layer of the dendritic tree; stimulation of the lateral fibers activated a fEPSP in the outer molecular later of the dendritic tree. fEPSPs generated by medial and lateral stimulation were recorded by a single electrode positioned at the base of the dendritic tree, in the dentate hilus (from bregma: 3.8 mm posterior, 2.5 mm lateral). Once positioning was finalized, electrodes were wired into a plastic headplug-socket and secured to the skull with acrylic resin. Upon completion of all surgical procedures, animals were administered antisedan (0.5 mg/kg s.c.) and closely monitored over a 2-week recovery period before being tested for stable electrophysiological recordings. All procedures were approved by the University of Otago Animal Ethics Committee, and complied with all NIH requirements for the humane care and use of laboratory animals.
FIGURE 1.1 Schematic illustration of electrode positions. The middle molecular layer of the granule cell dendritic tree was activated by stimulation of the medial perforant path (black). The outer molecular layer of the dentate granule cell dendritic tree was activated by stimulation of the lateral perforant path (grey). fEPSPs evoked by this activation were recorded by an electrode positioned in the dentate hilus.

1.2.2 Recording procedures

All experimental procedures took place in a recording room which was outfitted with recording and stimulation equipment, and isolated from extraneous distractions. Experimentation was conducted while animals were in a quietly alert state (Abraham et al., 2002), and took place during either the light or dark phase of the circadian rhythm, depending on the experiment. Between procedures, animals were single housed in standard rodent cages, and provided with *ad libitum* access to food and water.
Stimulation was administered in the form of square wave pulses (150 μs half-wave duration). Evoked potentials were amplified, band passed filtered (low: 0.3 Hz, high: 3 kHz), recorded at a sample rate of 10 kHz, and stored on a computer for offline analysis. Stimulation protocols and data acquisition applications were programmed with Labview software (National Instruments, additional coding courtesy of Dr. Michael Eckert).

1.2.3 Electrophysiology

Before formal experimentation began, medial and lateral waveforms were evaluated with a standard set of electrophysiological tests.

The perforant pathway of the dentate gyrus consists of medial and lateral fibers. Paired pulse testing was used to establish that stimulating electrodes were positioned in dissociable fiber pathways (McNaughton and Barnes, 1977). The conditions of separation were satisfied if paired pulse stimulation of either pathway resulted in a fEPSP change greater than when paired pulses were divided between pathways.

Medial and lateral fibers project to adjacent layers within a common dendritic field. Convergence testing was used to verify that medial and lateral electrodes were activating a common postsynaptic target (McNaughton and Barnes, 1977; Abraham and Goddard, 1983). The conditions of convergence were satisfied if simultaneous medial and lateral perforant path stimulation resulted in a PS amplitude at least 2 times larger than either pathway stimulated independently.
Medial and lateral evoked potentials exhibit morphologically dissociable waveforms (Fig. 1.3A; Abraham and Goddard, 1983; Abraham et al., 2001). Medial path waveforms were required to exhibit a fEPSP slope of $\geq 3.5$ mV/ms in association with a population spike amplitude of 2–4 mV, at stimulation currents $\leq 500$ µA. Lateral path waveforms were required to exhibit an amplitude of $\geq 4$ mV at stimulation currents $\leq 500$ µA. For each animal, "test-intensity" was defined as the stimulation current required to evoke a waveform which met criterion specifications. Animals that satisfied the requirements of paired pulse testing, convergence testing, and waveshape criteria were graduated to I/O testing and experimental baseline sessions.

I/O testing was used to evaluate the fEPSP and PS dynamics of medial and lateral waveforms across a range of increasing stimulation intensities. Stimulation pulse intensity began at 20 µA, and increased by incremental steps to a maximum of 200 µA.

During baseline sessions, test-intensity pulses were administered to medial and lateral fibers. Pulses alternated between pathways at a frequency of 0.06 Hz, and continued for a period of 30 minutes. fEPSP slope values were averaged from the last 15 minutes of each session. Baseline sessions were conducted once every two days, and continued until medial and lateral fEPSP slope averages exhibited a variance of $\leq 5\%$ for 4 consecutive sessions.

One day following the establishment of fEPSP baseline stability, one of three high frequency stimulation (HFS) protocols was applied to the medial path: 8 trains of theta-
burst stimulation (8TBS; 5 Hz spacing of 100 Hz pulse trains; Christie & Abraham, 1994), 50 trains of 400 Hz stimulation (50T; Abraham et al., 2002), or 8 trains of a hybrid protocol (8Hyb), which combined elements of both 8TBS and 50T (Fig. 1.2). HFS was delivered immediately after 30-min of baseline stimulation, and followed by an additional hour of stimulation post-HFS. Persistence sessions (identical in format to baseline sessions) were conducted on days 1, 3, 5 & 7 post-HFS, and twice a week thereafter.

Long-term potentiation (LTP) was defined as a ≥15% increase in fEPSP measured 60 min following HFS (Abraham et al., 2002). Long-term depression was defined as ≥15% decrease in fEPSP measured 60 min following HFS.

1.2.4 Statistics

I/O data was expressed in terms of fEPSP slope (mV/ms) and PS amplitudes (mV), and displayed across units of increasing stimulation intensity (µA). I/O data was compared across experimental conditions with a 2-way analysis of variance (ANOVA) with repeated measures. For all analyses, an alpha level of 0.05 was defined as statistically significant. Tukey’s post hoc testing was performed where appropriate.

For fEPSP-spike (E-S) analysis, data from I/O testing was used to plot changes in medial fEPSP slope against changes in medial PS amplitude. The relationship between these variables was best approximated by an exponential function. For statistical analysis, these datasets were log transformed (base 10) to extract linear slopes; slope values were then compared across experimental conditions with analysis of covariance (ANCOVA).
HFS session values were expressed as a percentage change in fEPSP slope. Percentages were calculated by normalizing fEPSP slopes to the average of values taken from the last 4 days of baseline stimulation. All HFS session data was displayed across units of time; induction sessions were displayed across minutes, persistence sessions were displayed across days.

Induction sessions were analyzed by dividing the 60 min period post-HFS into 6, 10-min bins; values were then compared across experimental conditions with two-way ANOVA with repeated measures.

**FIGURE 1.2** Schematic illustration of the HFS protocols. A: Theta burst stimulation (8TBS). B: Hybrid HFS (8Hyb). C: 50 train HFS (50T). 50T HFS is characterized by a rapid 400 Hz pulse frequency, and has been previously shown to induce a persistent expression of bidirectional synaptic plasticity in the dentate gyrus. 8TBS
is characterized by a 5 Hz “theta” spacing of stimulation trains. 8TBS has been shown to induce robust LTP with area CA1 and other regions of cortex. 8Hyb HFS is an unconventional protocol which was designed to incorporate the 400 Hz pulse frequency of 50T stimulation, and the 5 Hz trains frequency of TBS.

1.3 Results

Analysis confirmed that the experimental groups did not differ in average baseline fEPSP slope or PS amplitude before HFS administration (Table 1.1).

TABLE 1.1 Summary of baseline parameters. Strain testing compared Long-Evans and Sprague-Dawley animals, and was conducted in a light-cycle circadian environment (n = 10). Circadian testing compared light and dark-cycle experimental environments, and was conducted with Long-Evans animals (n = 10). Stimulation protocol testing compared the efficacy of three different HFS protocols, and was conducted with Long-Evans animals maintained in a dark-cycle circadian environment (n = 18).

<table>
<thead>
<tr>
<th>Strain</th>
<th>n</th>
<th>Medial fEPSP (mV/ms)</th>
<th>Medial PS (mV)</th>
<th>Lateral fEPSP (mV/ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-Evans</td>
<td>5</td>
<td>5.88 ± 0.68</td>
<td>1.91 ± 0.37</td>
<td>1.44 ± 0.06</td>
</tr>
<tr>
<td>Sprague-Dawley</td>
<td>5</td>
<td>7.30 ± 0.65</td>
<td>2.27 ± 0.52</td>
<td>1.47 ± 0.01</td>
</tr>
<tr>
<td>Circadian</td>
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<tr>
<td>Dark cycle</td>
<td>5</td>
<td>5.65 ± 0.57</td>
<td>3.34 ± 0.61</td>
<td>1.65 ± 0.28</td>
</tr>
<tr>
<td>Light cycle</td>
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<td>5.88 ± 0.68</td>
<td>1.91 ± 0.37</td>
<td>1.44 ± 0.06</td>
</tr>
<tr>
<td>Stimulation protocol</td>
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<tr>
<td>8TBS</td>
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<td>5.89 ± 0.50</td>
<td>2.01 ± 0.21</td>
<td>1.72 ± 0.09</td>
</tr>
<tr>
<td>50T</td>
<td>5</td>
<td>5.65 ± 0.57</td>
<td>3.34 ± 0.61</td>
<td>1.65 ± 0.28</td>
</tr>
<tr>
<td>8Hyb</td>
<td>6</td>
<td>4.41 ± 0.98</td>
<td>2.23 ± 0.57</td>
<td>1.28 ± 0.09</td>
</tr>
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*One-way ANOVA. All group differences found to be non-significant.
1.3.1 Effect of strain on bidirectional synaptic plasticity

Sprague-Dawley (n = 5) and Long-Evans (n = 5) strain rats were maintained in a conventional 12:12 h light/dark circadian cycle. All experimentation took place during the light-cycle of the animal’s circadian rhythm.

I/O testing of medial path responses revealed that Long-Evans and Sprague-Dawley animals exhibited proportional increases in fEPSP slope with increasing stimulation intensity (Fig. 1.3A & B, strain × intensity, \( F_{(9,54)} = 0.734, P = 0.68 \)). Interestingly, the same increases in stimulation intensity evoked significantly higher population spike amplitudes in Long-Evans than Sprague-Dawley animals (Fig. 1.3A & C, strain × intensity, \( F_{(9,54)} = 5.86, P < 0.001 \)). Taken together, these findings indicated that dentate granule cells were more excitable in Long-Evans strain animals. E-S analysis was used to address this question more directly. Curve fitting procedures demonstrated that the relationship between medial fEPSP slope and PS amplitude was best approximated by an exponential function (Fig. 1.3D, Long-Evans \( R^2 = 0.95 \), Sprague-Dawley, \( R^2 = 0.83 \)). Following log transformation, ANCOVA revealed that comparable levels of fEPSP activation were associated with significantly higher amounts cell firing in Long-Evans animals (Fig. 1.3D, strain, \( F_{(1,75)} = 6.2414, P = 0.0146 \)). No strain difference was observed in I/O comparison of lateral fEPSP values (Fig. 1.3A, strain, \( F_{(1,54)} = 0.009, P = 0.926 \)).
FIGURE 1.3 Effect of strain on I/O electrophysiology. A: Waveforms from I/O analysis. Medial (left panel) and lateral (right panel) waveforms were averaged across 2 sweeps, and displayed at stimulation intensities 20%, 40% and 100% of maximum (40 µA, 80 µA and 200 µA stimulation intensity), scale bar: 5 mV, 5 ms. B: Medial fEPSP I/O. Medial fEPSP slopes evoked across increasing stimulation intensities were equivalent between Sprague-Dawley and Long-Evans animals. C: Medial population spike (PS) I/O. PS amplitude increased more rapidly across stimulation intensity for Long-Evans animals than for Sprague-Dawley animals. D: E-S potentiation. Proportionally equivalent fEPSP slopes were associated with higher PS amplitudes in Long-Evans animals than in Sprague-Dawley animals.
For HFS sessions, 50T protocol stimulation was applied to the medial perforant path of Long-Evans and Sprague-Dawley animals. In both strains, increases in medial fEPSPs were sufficient to qualify as LTP (Fig. 1.4A, Long-Evans, $M = 35.55\%$; Sprague-Dawley, $M = 15.72\%$); and decreases in lateral fEPSPs were sufficient to qualify as LTD (Fig. 1.4A, Long-Evans, $M = -28.23\%$; Sprague-Dawley, $M = -39.45\%$).

During the hour following HFS, Long-Evans animals demonstrated significantly greater increases in medial fEPSPs than Sprague-Dawley animals (Fig 1.4A, & B, strain, $F_{(5,40)} = 12.74, P = 0.007$). Persistence testing revealed that this difference in fEPSP slope was sustained for at least one week following HFS (Fig. 1.4C, strain, $F_{(1,39)} = 8.194, P = 0.021$). No strain effect was observed in lateral LTD induction (Fig 1.4A & B, strain, $F_{(1,30)} = 1.63, P = 0.248$) or persistence (Fig 1.4C, strain, $F_{(1,33)} = 1.115, P = 0.326$).
FIGURE 1.4 Effect of strain on bidirectional synaptic plasticity. A: Induction. Following 50T HFS, Long-Evans animals exhibited larger increases in medial fEPSPs than Sprague-Dawley animals. Changes in lateral fEPSPs were proportional across the strains. B: Medial (top) and lateral (bottom) waveforms pre and post 50T HFS. Pre HFS waveforms are displayed as dashed lines, post HFS waveforms are displayed as solid lines. Pre HFS waveforms were averaged from 30 sweeps leading up to 50T administration, post HFS waveforms were averaged from the final 30 sweeps of the induction session. Scale bar: 5 mV, 5 ms C: Persistence. Medial fEPSP changes induced in Long-Evans animals remained higher than Sprague-Dawley for
across days. No strain effect was observed in the persistence of lateral fEPSP changes across time.

1.3.2 Effect of circadian rhythm on bidirectional synaptic plasticity

One group of Long-Evans strain animals (n = 5) was maintained in a conventional 12:12 h light/dark circadian environment, a second group of Long-Evans animals (n = 5) was maintained in a reversed 12:12 h dark/light cycle. Animals in the conventional cycle underwent experimental testing during the "light cycle" of their circadian rhythm. Animals in the reverse cycle group underwent experimental testing during the "dark cycle" of their circadian rhythm.

I/O testing revealed no significant effect of circadian cycle on medial fEPSPs (Fig 1.5A & B, cycle × intensity, $F_{(9,63)} = 1.796, P = 0.087$). Similarly, there was no significant effect of circadian cycle on population spike amplitudes, although there was a tendency for responses to be greater in the dark (1.5A & C, cycle × intensity, $F_{(9,44)} = 0.544, P = 0.834$). Subsequent E-S analysis revealed that the relationship between PS amplitude and fEPSP slope was faithfully approximated by an exponential function (Fig. 1.5D, light $R^2 = 0.95$, dark $R^2 = 0.97$); furthermore, the difference between slope functions describing light and dark cycle animals was highly significant (Fig 1.5D, strain, $F_{(1,66)} = 18.11, P < 0.001$). This suggests that dentate granule cells are more excitable in the dark cycle. No circadian differences were observed in the I/O comparison of lateral fEPSPs (Fig 1.5A, cycle, $F_{(1,54)} = 1.828, P = 0.225$)
FIGURE 1.5 Effect of circadian cycle on I/O electrophysiology. A: Waveforms from I/O analysis. Medial (left panel) and lateral (right panel) waveforms were averaged across 2 sweeps, and displayed at stimulation intensities 20%, 40% and 100% of maximum (40 µA, 80 µA and 200 µA stimulation intensity). Scale bar: 5 mV, 5 ms. B: Medial fEPSP I/O. Medial fEPSP slopes evoked across increasing stimulation intensities were equivalent between light cycle and dark cycle animals. C: Medial PS I/O. Dark cycle and light cycle PS amplitude were not significant different; however, dark cycle PS values tended to be larger. D: E-S potentiation. Equivocal fEPSP slopes were associated with higher population spike amplitudes in dark cycle animals than light cycle animals.
For HFS sessions, 50T stimulation was applied to the medial perforant path of light and dark cycle animals. In both circadian groups, increases in medial fEPSPs were sufficient to qualify as LTP (Fig. 1.6A & B, light cycle, $M = 35.55\%$; dark cycle, $M = 42.53\%$); and decreases in lateral fEPSPs were sufficient to qualify as LTD (Fig. 1.6A & B, light cycle, $M = -28.23\%$; dark cycle, $M = -25.16\%$).

There was no significant main effect of circadian cycle on the induction of LTP (Fig. 1.6A & B, cycle, $F_{(1,40)} = 2.912$, $P = 1.26$). Interestingly, however, analysis revealed a significant cycle by time interaction (Fig. 1.6A, $F_{(5,40)} = 11.634$, $P = 0.001$). Subsequent Tukey’s post hoc analysis indicated that dark cycle medial fEPSPs were significantly higher than light cycle fEPSPs immediately following 50T HFS (Fig. 1.6A, Tukey's, $P = 0.013$). However, this difference was transient; medial light and dark cycle LTP was equal at times >10-min following HFS (Fig. 1.6A Tukey's, $P < 0.05$). Circadian cycle had no significant effect on changes induced in lateral fEPSPs (Fig. 1.6A, cycle, $F_{(1,30)} = 1.038$, $P = 0.348$).

Persistence sessions revealed that circadian cycle did not exert a statistically significant main effect on the maintenance of lateral LTD (Fig. 1.6C, cycle, $F_{(1,40)} = 0.919$, $P = 0.382$). Similar findings were evident in medial fEPSPs. Persistence was marginally higher in dark cycle animals; however, this difference was not statistically significant (Fig. 1.6C, cycle, $F_{(1,40)} = 4.783$, $P = 0.06$). Tukey’s post hoc testing revealed that this difference occasionally reached significance (Fig. 1.6C, Tukey’s, persistence day seven,
$P = 0.042$, day fourteen, $P = 0.035$); however, the main effect was not sufficient to distinguish this persistence from variance.

**FIGURE 1.6** Effect of circadian cycle on bidirectional synaptic plasticity. **A:** Induction. Immediately following 50T HFS, dark cycle animals demonstrated significantly larger increases in medial fEPSPs than light cycle animals; however, this difference did not persist beyond 10-min post-HFS. Changes in lateral fEPSPs were equal between light and dark cycle animals. **B:** Medial (top) and lateral (bottom) waveforms pre and post 50T HFS. Pre HFS waveforms are displayed as
dashed lines, post HFS waveforms are displayed as solid lines. Pre HFS waveforms were averaged from 30 sweeps leading up to 50T administration, post HFS waveforms were averaged from the final 30 sweeps of the induction session. Scale bar: 5 mV, 5 ms C: Across persistence sessions, medial fEPSPs were marginally greater in dark cycle animals than light cycle animals; however, this effect did not reach significance. No circadian difference was observed in the persistence of lateral fEPSPs.

1.3.3 Effect of stimulation protocol on bidirectional synaptic plasticity

Having previously established that circadian rhythm modulates the expression of short-term plasticity (1.3.2), all subsequent experiments were conducted in dark-cycle environment. Three groups of Long-Evans strain animals were maintained in a reverse 12:12 h light/dark schedule. In the first group of animals (n = 5), 50T HFS was applied to the medial perforant path. In the second group of animals (n = 6), 8TBS HFS was applied to the medial perforant path. In the third group of animals (n = 7), 8Hyb HFS was applied to the medial perforant path.

50T and 8Hyb stimulation protocols induced increases in medial fEPSPs sufficient to qualify as LTP (Fig. 1.7A-C, 50T, M = 42.53%; 8Hyb, M = 34.22%); however, changes induced by 8TBS did not reach experimental criterion (Fig. 1.7A-C, 8TBS, M = 9.82%). 50T and 8Hyb stimulations protocols induced decreases in lateral fEPSPs sufficient to qualify as LTD (Fig. 1.7A-C, 50T, M = -25.16%; 8Hyb, M = -20.73%); however,
changes induced by 8TBS did not reach experimental criterion (Fig. 1.7A-C, 8TBS, \( M = -2.42\% \))

Induction data confirmed a highly significant difference in the ability of each HFS protocol to evoke changes in medial (Fig. 1.7A-C, protocol, \( F(2,75) = 16.493, P < 0.001 \)) and lateral fEPSPs (Fig 1.7A-C, protocol, \( F(2,50) = 9.18, P = 0.005 \)). Tukey’s post hoc testing was used to parse out the electrophysiological differences between these protocols across time.

50T HFS induced the largest amount of LTP and LTP out of all protocols tested (Fig. 1.7B). In contrast, 8TBS HFS was largely ineffective, evoking only a small change in medial (Fig. 1.7B, \( M = 9.82\% \)) and lateral fEPSPs (Fig. 1.7B, \( M = -2.42\% \)). Interestingly, modification of 8TBS pulse frequency from 100 Hz to 400 Hz resulted in a dramatic increase in protocol efficacy. This new protocol, dubbed “8Hyb”, demonstrated changes in medial (Fig. 1.7B, \( M = 34.22\% \)) and lateral (Fig. 1.7B, \( M = -20.73\% \)) fEPSPs which far exceeded changes induced by 8TBS (Fig. 1.7B, Tukey’s, 8Hyb vs. 8TBS, \( P = 0.004 \)), and approached an efficacy comparable to 50T (Fig 1.7B, Tukey’s, 8Hyb vs. 50T, \( P = 0.54 \)).
FIGURE 1.7 Effect of stimulation protocol on bidirectional synaptic plasticity. A: Medial (top) and lateral (bottom) waveforms displayed pre and post HFS. Pre HFS waveforms are displayed as dashed lines, post HFS waveforms are displayed as solid lines. Pre HFS waveforms were averaged from 30 sweeps leading up to HFS administration; post HFS waveforms were averaged from the final 30 sweeps of the induction session. Scale bar: 5 mV, 5 ms. B: Summary of induction data. Values are displayed as a percentage change in medial and lateral fEPSPs, and calculated from
the last 10-minute window of the induction session. C: 50T HFS yielded the largest changes in medial and lateral fEPSP slopes, where as TBS HFS was ineffectual. 8Hyb HFS evoked a change in medial fEPSPs which gradually increased to a magnitude comparable to 50T. Lateral fEPSP changes evoked by 8Hyb HFS were equivalent to 50T. D: Persistence. Changes in medial fEPSPs evoked by 50T HFS exhibited greater persistence than changes evoked by 8Hyb. Lateral fEPSP persistence did not differ between 8Hyb and 50T HFS groups.

50T and 8Hyb induced changes in medial fEPSPs demonstrated different trends across time. In the hour following HFS, 50T induced changes were observed to “peak”, and then decline marginally to a plateau (Fig 1.7C). In contrast, 8Hyb induced changes were observed to “develop” following HFS administration, and steadily increase to plateau (Fig 1.7C). Statistics revealed that this trend was significant (Fig. 1.7C, protocol x time, \( F_{(10,107)} = 5.76, P < 0.001 \)). Immediately following HFS, 50T and 8Hyb induced changes were significantly different (Fig 1.7C, Tukey’s, \( P < 0.001 \)); however, values were statistically equivalent by the end of the recording session (Fig. 1.7C, Tukey’s \( P = 0.537 \)). Lateral fEPSP changes induced by 50T and 8Hyb were insignificantly different (Fig. 1.7C, \( P = 0.778 \), Tukey’s).

Persistence data provided additional insight regarding the efficacy of each protocol. 50T and 8Hyb induced equally persistent changes in lateral fEPSPs (Fig. 1.7D, protocol, \( F_{(1,24)} = 1.579, P < 0.254 \)). Interestingly, 50T induced changes in medial fEPSPs persisted significantly longer than 8Hyb induced changes (Fig. 6D, Protocol, \( F_{(1,36)} = 41.092, P < \))
0.001). Changes induced by 8TBS were not deemed sufficient to include in persistence testing.

1.4 Conclusions

LTP was larger and more persistent in Long-Evans strain rats than Sprague-Dawley strain rats. As a possible mechanism of this difference, I/O testing revealed that granule cell excitability was significantly higher in Long-Evans strain rats.

In Long-Evans animals, short-term potentiation was higher when experiments took place during the dark cycle as opposed to light cycle of the circadian rhythm; however, these values were equivalent by the end of the first recording session. Light cycle and dark cycle LTP persistence was not significantly different. Tukey’s post hoc testing revealed that dark cycle persistence was higher on specific days; however, circadian modulation did not affect LTP persistence as a main effect. I/O testing revealed that granule cell excitability was higher in the dark cycle than in the light cycle.

In the final round of experiments, Long-Evans animals were used to evaluate the efficacy of three different HFS protocols. All experiments took place in the dark cycle of the circadian rhythm. 50T HFS is a protocol characterized by 400 Hz pulse frequency, and was found to induce the largest and most persistent forms of LTP and LTD out of the 3 protocols tested. 8TBS is a protocol characterized by 5 Hz spacing of 100 Hz pulse trains, and did not induce changes in medial or lateral fEPSPs that were sufficient to qualify as LTP or LTD. Interestingly, adjustment of 8TBS pulse stimulation from 100 Hz to 400 Hz
induced a magnitude of LTP and LTD comparable to 50T. LTP induced by this new protocol ("8Hyb") was less persistent than 50T; however, LTD persistence was found to be comparable. 400 Hz stimulation appears to be a preferential frequency for induction of bidirectional synaptic plasticity in the dentate gyrus.

1.5 References


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Chapter Two: Allometry Of The Granule Cell Dendritic Tree

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2.1 Introduction

Allometry is a term used by forest ecologists to describe the relationship between tree geometry and the distribution of biomass as branching patterns extend into the canopy (Huxley, 1932; Chave et al., 2005; Calvo-Alvarado et al., 2008). The dentate gyrus is a sub-region of the brain that has been implicated in the storage of memory (Scoville and Milner, 1957; Bliss and Lomo, 1973), and is characterized by a unique pattern of cellular organization. The granule cells of the dentate gyrus are arranged in a laminar fashion, and project their dendritic processes, known as “dendritic trees”, into the upper strata of the molecular layer (Andersen et al., 1969). The dendritic tree is populated by thousands of tiny formations known as “spines”. Dendritic spines are responsible for a majority of excitatory synaptic transmission within the brain, and may be fundamentally responsible for the expression of learning and memory (Harris et al., 2003; Bourne and Harris, 2007; Harms et al., 2008). In the following investigation, we describe how spine distribution scales proportionally with structures of the granule cell dendritic tree. We have termed this proportional distribution, “dendritic allometry”.

Evidence suggests that the shape and branching pattern of a dendritic tree is highly influential in modulating the propagation of synaptically evoked electrical currents (Kim and Connors, 1993; Schaefer et al., 2003). This functional geometry varies significantly across cell types and regions of the brain; thus, it would appear that dendritic structure is intrinsically and/or actively tuned to meet the specifications of the cellular networks in which they reside (Scott and Luo, 2001). Contemporary evidence suggests that the dendritic tree is not a passive participant in modulating electrical conductance (Johnston...
Electrophysiological studies have demonstrated that dendritic branches are interspersed with a myriad of protein ion channels, each capable of exerting a specific influence on signaling conductance (Magee and Johnston, 1995). In summary, evidence suggests that the dendritic tree is rich with computational complexity. This complexity results from the shape of the dendritic tree, and the relative distribution of ion channels and organelles responsible for modulating its activation. An understanding of how these features interrelate is crucial for understanding the function of the dendritic tree.

The dendritic tree is structured to modulate electrical currents resulting from synaptic transmission. Excitatory synaptic transmission may be experimentally induced and recorded with a high degree of fidelity; however, the anatomical structures responsible for synaptic transmission are less easily discerned. Excitatory synapses exist almost exclusively on specialized protrusions known as dendritic spines (Bourne and Harris, 2007). Dendritic spines were first observed by the neuroanatomist Santiago Ramón y Cajal (Cajal, 1888; for review see Garcia-Lopez et al., 2007), and facilitated by the development of a revolutionary new staining procedure known as the Golgi method. With the Golgi method, it became possible to visualize fine structures of the nervous system, and elucidate features of cellular anatomy with an unparalleled level of detail. Interestingly, contemporary application of the Golgi method has demonstrated that spine protrusions are non-uniformly distributed throughout the granule cell dendritic tree, and suggest that the majority of excitatory synaptic contacts are located within the middle third of the dentate molecular layer (Desmond and Levy, 1985). To this day, dendritic
spines remain at the forefront of studies seeking to elucidate the mechanisms of excitatory synaptic transmission; however, several other cellular organelles are necessary to support synaptic function.

Dendritic microtubules are highly dynamic cylindrical filaments that course through the branches of the dendritic tree. The functional significance of microtubules within the dendritic tree has not been sufficiently characterized; however, studies suggest that microtubules play a role in vesicular transport and activity dependent movement of dendritic resources (Hu et al., 2008). Microtubules are not frequently observed within dendritic spines, but the functional interplay of these two structures is strongly implied. The N-methyl-D-aspartic acid (NMDA) receptor is a transmembrane ion channel which is preferentially located within the head of dendritic spines, and is principally responsible for triggering changes in synaptic transmission associated with cellular memory (Bliss and Collingridge, 1993). Biochemical preparations reveal that microtubule related mRNA is present in spines (Chicurel et al., 1993); furthermore, microtubule specific tubulin proteins have been found to bind with subunits of the NMDA receptor (van Rossum et al., 1999). Activation of NMDA receptor currents has been shown to decrease phosphorylation of microtubule associated protein 2 (Quinlan and Halpain, 1996), and recent evidence suggests that microtubules may gravitate towards, and possibly enter, smaller actin-rich dendritic protrusions (Gu et al., 2008). Emerging evidence suggests that microtubules play a critical support role in maintaining the function of the dendritic tree. Owing primarily to their size, (~10 nm) high magnification imaging is required to accurately characterize the role of microtubules.
Modern developments in optical technology have provided substantial increases in magnification since the days of Cajal. Fluorescence imaging is an optical technique that implements properties of stimulated emission to detect structures which have been labeled with a fluorescent dye. The resolving power of fluorescence imaging is sufficient to distinguish individual dendritic spines; however, its maximum usable magnification is limited by the diffraction of light. While sub-diffraction microscopy is possible, quantification of dendritic organelles such as polyribosomes and microtubules remain beyond the practical limitations of optical microscopy (Stevens et al., 1994; Harris et al., 2006).

Electron microscopy is an imaging technique which illuminates specimens with an electron beam, and magnifies through a series of electromagnetic lenses. The wavelength of an electron is substantially smaller than light, thus, the resolving power of electron microscopy is far greater than the theoretical limits of optical techniques. Electron microscopy provides the magnification and resolution required to resolve structures and organelles of the dendritic tree; however, considerable effort is required to prepare specimen samples for imaging.

Transmission electron microscopy (TEM) is a high energy (small wavelength) preparation in which the electron beam is designed to pass through samples, and focus a projected image on a charge-coupled sensor positioned below. TEM requires that tissue samples be thin enough for the electron beam to pass through; as such, samples must
often be trimmed into sub-micron sections (~ 80 nm or thinner). While arduous, sectioning provides an ideal technique to expose-and-image intracellular organelles. The finished product of TEM imaging is a two-dimensional micrograph taken from a section of three-dimensional structure. TEM provides the magnification and resolution required to image the dendritic tree; however, particular care is required when interpreting its results.

Stereology is a method of sample analysis that utilizes statistical inference to estimate measurements of three-dimensional structure from two-dimensional micrographs. Stereology is capable of extracting a high number of samples from a small number of micrographs; thus, stereological methods have a high degree of statistical efficiency. Crucially, however, this statistical efficiency is based on the assumption that inferences in sampling are representative depictions of the structure as a whole. In this way, the validity of a stereological calculation is contingent upon its ability to account for known patterns of variability within the sample space. As these patterns of variability are not always known, stereological calculations must be used with discretion.

Serial section transmission electron microscopy (ssTEM) is a method which uses hundreds of consecutive ultrathin tissue sections to visualize and measure three-dimensional structures (Harris et al., 2006). Samples are first cut into ultrathin sections, and then photographed with a TEM microscope. The resulting micrographs are then aligned in a calibrated stack, and analyzed using specialized reconstruction software. With ssTEM, three-dimensional structures can be measured directly, without the need for
statistical extrapolation or inference. In this sense, ssTEM offers the highest degree of “ground truth” available to any contemporary method of high magnification microscopy.

Physiological studies suggest that the dendritic tree is structured to modulate electrical currents resulting from synaptic transmission (Johnston and Narayanan, 2008). Low magnification studies suggest that structures responsible for excitatory synaptic transmission are non-uniformly distributed throughout the granule cell dendritic tree (Desmond and Levy, 1985). High magnification imaging techniques provide the ability to quantify the distribution and dimensions of dendritic spines with a far greater degree of accuracy. As an additional virtue, high magnification techniques provide the resolution necessary to characterize statistical relationships which exist between spines and various other dendritic organelles.

In the following investigation, we used ssTEM to analyze dendritic segments from IML, MML and OML regions of the rodent dentate granule cell dendritic tree. We found that the spine density within the dendritic tree decreased systematically relative to distance from the cell body layer. Furthermore, we found that this systematic distribution of spine density was accompanied by proportional changes in microtubule number and dendrite caliber. The relationships that ascribe these variables were linear, and remained constant throughout the extent of the dendritic tree. We conclude by discussing how these findings may be applied to improve the sample design of future investigations.
2.2 Method

2.2.1 Animals

Two adult male Long-Evans rats (animal codes "LED 50" and "LED 56"; 623 gm and 448 gm; 179 days and 121 days old at the time of sacrifice, respectively) were used in the present investigation. Animals were single housed in a reverse light cycle (12:12 hr) environment, and provided with \textit{ad libitum} access to food and water. All procedures were approved by the University of Otago Animal Ethics Committee, and complied with NIH requirements for the humane care and use of laboratory animals.

2.2.2 Perfusion

Animals were deeply anesthetized with halothane, and surgically intubated with a respirator tube that supplied vaporized anesthetic in carbogen (4% halothane in 95% O\textsubscript{2}/5% CO\textsubscript{2}; 120 breaths/minute, 1.5 cc tidal volume). Immediately following intubation, animals were transcardially perfused for 10 seconds with oxygenated Krebs-Ringer Carbicarb (KRC), and 1 hour with mixed aldehydes (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer). All solutions were administered at a temperature of 41° C, maintained at a pH of 7.3, and mixed no more than 24 hours before perfusion. Once the perfusion procedure had been completed, brain tissue was removed with bone rongeurs and stored in mixed aldehydes. Perfusion procedures were assisted by Mrs. Sara E. Mason-Parker.
2.2.3 Tissue processing

Sagittal sections (70 µm) were cut through the brain with a VT 1000S Vibroslicer (Leica, Bannockburn, IL.) and placed in 24-well plates filled with a solution of 0.1 M cacodylate buffer (CB). Two sections were selected from symmetrical coordinates of the left and right cerebral hemispheres (3.5 mm lateral from midline), and embedded in 7% agarose; sections were then trimmed to excise a sample region of the hippocampal molecular layer (Fig. 2.1A) and postfixed with 1% OsO₄ and 1.5% NaKeCN in CB, followed by an additional treatment with 1% OsO₄ and 1% uranyl acetate stain. At the completion of staining, tissue sections were dehydrated into embedding treatments with incrementally decreasing ratios of acetone to Epon resin. Final Epon blocks were hardened in an oven for 60 hours.

2.2.4 Ultrasectioning

A Reichert Ultracut S (Leica, Nussloch, Germany) was used to cut a series of ~200 consecutive ultrathin sections (45 nm thickness) from three locations of the dentate granule cell dendritic tree (Fig. 2.1B). IML series were cut ~50 µm dorsal from cell body layer, MML series were cut 125 µm dorsal from cell body layer, and OML series were cut 250 µm dorsal from cell body layer. In animal LED 50, ultrathin series were cut from the left and right hemispheres of OML and MML regions of the dendritic tree (n = 4 series). In animal LED 56, ultrathin series were cut from left and right hemispheres of the OML and IML regions of the dendritic tree (n = 4 series). Each tissue series was collected on Piooloform coated slot grids (Ted Pella, Inc) and stained with treatments of saturated uranyl acetate and 0.2% lead citrate.
The experimenter had intended to obtain match samples of OML and MML tissue from animals LED 50 and LED 56; however, histological analysis revealed that MML tissue from animal LED 56 had been trimmed from an IML coordinate of the granule cell dendritic tree. Henceforth, descriptions of MML region tissue pertain to data derived from animal LED 50, whereas descriptions of IML region tissue pertain to data derived from animal LED 56. OML data were derived from both animal LED 50 and LED 56.

FIGURE 2.1 Serial section transmission electron microscopy (ssTEM) A: Tissue selection, scale bar: 200 µm. 70 µm thick tissue sections were excised from the hippocampal molecular layer (grey box) of the left and right hemispheres (3.5 mm from midline) and processed for ssTEM. B: Serial sectioning. Series of 200 consecutive serial sections (45 nm thickness) were cut from inner molecular layer (red), middle molecular layer (orange), and outer molecular layer (blue) regions. Middle and outer molecular layer samples were sectioned from animal LED 50, whereas inner and outer molecular layer samples were sectioned from animal LED.
Refer to section 2.2.4 for details. C: Conventional region selection. Low magnification region select images (top panel, magnification: 5,000X, scale bar: 18 µm) were used to choose sample frames (bottom panel, magnification: 10,000X, scale bar: 2 µm) that would be imaged for ssTEM analysis.

2.2.5 Serial Section transmission electron microscopy (ssTEM)

Each series was loaded into a rotating cassette and photographed with a Jeol 1200EX (Peabody, MA) transmission electron microscope (80-120 kV). Region select images were photographed at a magnification of 5,000x. Series images were photographed as 2 side-by-side 10,000x magnification images and montaged together with Adobe Photoshop (Adobe, San Jose, CA). All magnification values were calibrated with a diffraction grating replica (Ernest F. Fullam Inc, NY).

2.2.6 Measured variables

Image processing and analysis procedures were conducted with the Reconstruct software application (Fiala, 2005). Series images (~200 per series) were imported into Reconstruct, and manually aligned with a least-squares linear fit procedure (Fiala and Harris, 2001a). Once aligned, each micrograph of the series was calibrated to ensure proper magnification and section thickness measurements. Image magnification was calibrated through use of the diffraction grating replica, section thickness was calibrated with the cylindrical volume method (Fiala and Harris, 2001b).
Following calibration, each dendrite within the series was identified and assigned a unique experimental tag. Experimental tags were used to associate each individual dendrite with its respective measured variables; in addition, experimental tags could act as coordinates with which to locate features within the three-dimensional environment of the series. Dendrites that did not pass through all images of the series, or lost protrusions off image were excluded from analysis. Additionally, dendrites with branch points were excluded from analysis.

Z-length was measured as the length (µm) of a dendrite as it passed through sequential sections of the series (Fig. 2.2B).

Spine density was measured as the total number of spines on a dendrite, divided by the Z-length between the first complete spine, and the last spine (Fig. 2.2B).

Microtubule number was measured from three positions of the dendrite as it passed through sequential sections of the series (Fig. 2.2A); one count was taken at a low section number (within sections 1-50), the second was taken at medium section number (within sections 75-125), the final was taken at a high section number (within sections 150-200). These three values were then averaged to give a final count.

In ssTEM experiments (2.3.1) dendrite caliber was measured as the flat area of dendritic cross-sections averaged from low, medium and high section numbers. In region selection
experiments (2.3.2), dendrite caliber was measured from single image micrographs (Fig. 2.2A).

FIGURE 2.2 Measured variables. A: Quantification of dendrite caliber and microtubule number. (Top panel) single section micrograph of MML dendrite, analysis code d07. (Bottom panel) dendrite d07 superimposed with reconstruct traces; microtubule stamps are shown in blue, dendrite caliber trace is show in green, scale bar: 0.5 µm. B: Quantification of spine density. Reconstruction of
**dendrite d07 showing Z-length calculation (red line) and dendritic spine stamps (red spheres), scale cube: 1 µm³.**

2.2.7 Statistics

Multiple variable datasets were compared with one-way analysis of variance (ANOVA); Holm-Sidak post-hoc testing was used where appropriate. Non-parametric datasets were evaluated with Kruskal-Wallis one-way analysis of variance based on ranks; Dunn’s test post-hoc was used where appropriate.

Dual variable datasets were compared with Student’s t-test; non-parametric datasets were compared with Mann-Whitney U test.

Regression analysis was used to evaluate the strength of linear correlations between measured variables. Slope covariates were compared with analysis of covariance (ANCOVA). For all analyses, an alpha level of 0.05 was defined as significant.

2.3 Results

2.3.1 Allometry of the granule cell dendritic tree

Average dendrite caliber was significantly different between principal layers of the granule cell dendritic tree (Fig. 2.3B, $H = 237.24$, 2 d.f., $P < 0.001$). Caliber was highest within dendrites of the IML ($M = 0.76$), lower in dendrites of the MML ($M = 0.55$), and lowest within dendrites of the OML ($M = 0.47$). Overall, the caliber of the dentate
granule cell dendritic tree was observed to decrease relative to distance from the cell body layer.

A similar pattern of results was observed in the microtubule content of the dendritic tree. Average microtubule number was highest in dendrites of the IML ($M = 47.42$), lower within the MML ($M = 28.80$), and lowest within the OML ($M = 22.29$). Once again, this difference between layers was highly significant (Fig. 2.3C, $H = 57.59$, 2 d.f., $P < 0.001$).

Spine density was significantly different between layers of the dendritic tree (Fig. 2.3D, $H = 16.47$, 2 d.f., $P < 0.001$). Spine density was highest in the IML ($M = 4.29$), lower in the MML ($M = 3.49$), and lowest in the OML ($M = 3.09$). Post-hoc testing revealed that the difference in spine density between MML and OML regions was not significantly different (Fig. 2.3D, Dunn’s test, $Q = 1.38$, 2 d.f., $P < 0.05$).

A significant linear relationship was found to exist between microtubule number and spine density within each layer of the granule cell dendritic tree (Fig. 2.3E-F). Interestingly, the slope coefficient of this linear trend was statistically consistent across layers (Fig. 2.3E-F, ANCOVA, $F_{(2, 97)} = 0.61$, $P = 0.55$), suggesting that microtubule number and spine density were proportionally interrelated throughout the granule cell dendritic tree. To test this hypothesis, regression analysis was performed on a dataset which pooled microtubule number and spine density values from all layers into a single group. The results of this analysis revealed that the relationship between microtubule number and spine density could be represented across all layers of the granule cell.
dendritic tree with a single linear equation\(^1\) (Fig. 2.3E-G, \(R^2 = 0.68\) \(F_{(1, 100)} = 214.59, P < 0.001\)).

FIGURE 2.3 Allometry of the granule cell dendritic tree. A: Schematic diagram illustrating the three principal layers of the dentate granule cell dendritic tree;

\(^1\) Spine density (Y), microtubule number (X); Y = 1.5427 + (0.0632 * X)
OML (blue), MML (orange), IML (red). B-C: Dendrite caliber, microtubule number, and spine density differ systematically between layers the granule cell dendritic tree. E-G: Dendritic spine density correlates positively with microtubule number. (Top panel) $R^2 = 0.614$, $F_{(1,24)} = 36.57$, $P < 0.001$, (middle panel) $R^2 = 0.611$, $F_{(1,33)} = 50.34$, $P < 0.001$, (bottom panel) $R^2 = 0.646$, $F_{(1,41)} = 72.86$, $P < 0.001$. The slope coefficients that defined these trends were statistically consistent across layers of the granule cell dendritic tree, and could be modeled with a single linear function.

2.3.2 A quantitative method for unbiased region selection

Dendritic allometry is a term which has been coined to describe the interrelationship of proportional variables within the dendritic tree. In the following section, we demonstrate how dendritic allometry can be implemented to increase the validity of an informed sampling scheme.

Region select images are low magnification micrographs which provide the experimenter with a “zoomed out” perspective of the sample specimen. In theory, region select images are intended to guide the experimental design, and ensure that high magnification sample frames are focused on appropriately matched locations of the specimen. Conventional methods of electron microscopy use subjective criteria to choose sample frames from region select images; in this way, the quantitative variability present within a region select image is rarely appreciated. Failure to acknowledge patterns of quantitative variability can decrease statistical power, and may result in a type I error. For this reason,
researchers of the present investigation sought to develop a quantitative method for unbiased region selection.

Dendritic spines and microtubules are anatomical features which require serial section analysis for accurate identification; thus, variability of these structures cannot be directly measured from single section region select images. Dendritic allometry has previously been described as a relationship between microtubule number and spine density (2.3.1); however, allometric systems are often multidimensional, and express proportional relationships across a range of different parameters (West et al., 1997). For this reason, the experimenter sought to establish a proxy measure by which dendritic spine density or microtubule number could be predicted; ideally, a measure which could be accurately quantified from a single section.

Analysis from serial section reconstructions revealed the existence of a significant linear relationship between microtubule number and dendrite caliber (Fig. 2.4B). Results indicated that the slope coefficient of this linear trend was statistically consistent across layers (Fig. 2.4B, ANCOVA, \( F_{(2, 129)} = 2.25, P = 0.109 \)). Subsequent regression analysis revealed that the relationship between dendrite caliber and microtubule number could be represented across all layers of the granule cell dendritic tree with a single linear function \(^2\) (Fig. 2.4B, \( F_{(1, 132)} = 267.23, P < 0.001 \)). In summary: dendrite caliber, spine density and microtubule number were found to be proportionally related dimensions of

\[ Y = -0.232 + (57.306 \times X) \]
dendritic allometry. Furthermore, dendrite caliber could be easily resolved from single images. In the next section, dendrite caliber was used to estimate the variability of microtubule number and spine density across different sample frames of single section region select images.

In one animal, region select images were photographed from OML and MML sections of the left and right hemisphere granule cell dendritic tree. In a second animal, region select images were cut from OML and IML sections of the left and right hemisphere granule cell dendritic tree (Fig. 2.4A). All together, this gave a total of 8 region select images. Dendrite caliber was quantified within 4 different sample frames of each region select image (Fig. 2.4C). Approximately 30 measures of dendrite caliber were made within each sample frame, providing a total of 960 overall caliber measures.

Dendrite caliber was found to be significantly different across OML sample frames (Fig. 2.4D, $H = 68.34$, 15 d.f., $P < 0.001$). Dendrite caliber was also significantly different across MML sample frames (Fig. 2.4D, $H = 25.66$, 7 d.f., $P < 0.001$). Differences between IML sample frames were non-significant (Fig. 2.4D, $H = 2.74$, 7 d.f., $P = 0.91$).

Using the linear equations described above $^1$ & $^2$, dendrite caliber measurements were used to calculate extrapolated spine density values. Results indicated that extrapolated spine density values were significantly different within OML and MML sample frames (OML, $P < 0.001$; MML, $P < 0.001$). Differences between IML sample frames were non-significant ($P = 0.91$). Dunn’s post-hoc test did not reveal any regular pattern associated
with sample frame variability, as such, this variability presents a formidable bias for experimenters using subjective methods to match sample frames from within/across region select images. In the next section, we demonstrate how quantitative comparison can be used to reduce sampling bias.

Dunn’s post-hoc test was used to identify region select sample frames which were most similar in dendrite caliber. First order priority was given to matching sample frames across hemispheres (Fig. 2.4D, a vs. b), second order priority was given to matching sample frames between animals (Fig. 2.4D, 1 vs. 2). Results indicated that extrapolated spine density was not significantly different between matched sample frames (OML, $H = 3.89$, 3 d.f., $P = 0.27$; MML, $U = 108$, $n = 20$, $P = 0.10$; IML, $U = 57$, $n = 12$, $P = 0.40$).

To confirm the validity of this extrapolated finding, matched sample frames were prepared for ssTEM and three-dimensional reconstruction. Eight series (~200 sections) were cut from matched sample frames of each region select image. Approximately 10 dendrites per series were traced and analyzed with three-dimensional reconstruction. Results from this analysis confirmed that spine density was not significantly different between matched sample frames (Fig. 2.4E, OML, $F_{(3,24)} = 0.83$, $P = 0.49$; MML, $t_{(34)} = -1.74$, $P = 0.09$; IML, $U = 57$, $n = 12$, $P = 0.40$).

Significant differences in dendrite caliber exist across different sample frames of a single section region select image. These differences in caliber correspond with differences in spine density, and may present considerable bias to experiments attempting to match sample frames for ultrastructural analysis. To account for this variability, the
experimenter has shown that dendrite caliber can be measured from multiple sample frames of the region select image. Thorough use of post hoc testing, the experimenter was able to identify portions of the region select image that were most similar in caliber, and match sample frames based on this quantitative data. Subsequent high magnification ssTEM analysis confirmed that matched regions were not significantly different in spine density.

FIGURE 2.4 A quantitative method for unbiased region selection. A: Schematic diagram illustrating the three principal layers of the dentate granule cell dendritic
tree; OML (blue), MML (orange), IML (red). B: Microtubule number correlates with dendrite caliber. (Top panel) $R^2 = 0.539$, $F_{(1,58)} = 63.91$, $P < 0.001$. (Middle panel) $R^2 = 0.546$, $F_{(1,28)} = 32.482$, $P < 0.001$. (Bottom panel) $R^2 = 0.63$, $F_{(1,44)} = 72.83$, $P < 0.001$. C: Sampling scheme for quantitative unbiased region selection. Sample frames were photographed from different portions of regions select images. D: Dendrite caliber from left (a) and right (b) hemisphere sample frames of animal LED 50 and LED 56 regions select images. Dendrite caliber was significantly different across sample frames of OML ($H = 68.336$, 15 d.f., $P < 0.001$), MML ($H = 25.656$, 7 d.f., $P < 0.001$), and IML ($H = 2.74$, 7 d.f., $P = 0.91$) region select images. Additional calculations indicated that these differences could be associated with significant differences in spine density. Post hoc testing was used to match sample frames that were most similar. E: ssTEM analysis confirmed that spine density was not significantly different between matched sample frames.

2.4 Conclusions

Analysis from high magnification ssTEM reconstructions demonstrated that dendrite caliber, microtubule number and spine density vary significantly between layers of the granule cell dendritic tree. Subsequent analysis revealed that these structures are proportionally distributed, and can be modeled with a series of simple linear equations. Further analysis demonstrated that dendrite caliber can vary significantly across sample frames of a single section region select image. Additional calculations revealed that these differences in caliber can correspond with significant differences in spine density. This variability represents a considerable source of bias for experiments attempting to match
sample frames for ultrastructural analysis. To account for this variability, the experimenter has outlined a quantitative method that can be used to select unbiased sample frames from region select images.

### 2.5 References


Chapter Three: Structural Mechanisms Of Bidirectional Synaptic Plasticity In The Dentate Gyrus Of Freely Behaving Rats

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3.1 Introduction

Bidirectional plasticity is a term which delineates the two principal states of synaptic memory. The first of these states, long-term potentiation (LTP), is characterized by persistent increases in synaptic transmission (Bliss and Lomo, 1973; Douglas and Goddard, 1975). The second of these states, long-term depression (LTD), is characterized by persistent decreases in synaptic transmission (Levy and Steward, 1979; Abraham and Goddard, 1983). In the following investigation we used a chronic electrode implantation procedure to induce bidirectional synaptic plasticity within the dentate gyrus of freely behaving rats. In the experimental hemisphere, high frequency stimulation applied to the medial perforant path induced LTP within afferents projecting to the middle molecular layer (MML) of the dentate granule cell dendritic tree. Simultaneously, this stimulation induced a state of LTD within afferents projecting to the outer molecular layer (OML) of the granule cell dendritic tree. Baseline stimulation was applied to the medial perforant path of the control hemisphere. Thirty min following induction, animals were sacrificed and prepared for serial section transmission electron microscopy (ssTEM). Dendritic segments reconstructed from inner molecular layer (IML), and MML regions of the dendritic tree revealed that the induction of bidirectional synaptic plasticity was associated with systematic changes in spine head size and PSD area. Subsequent analysis revealed that structural changes were balanced between IML and MML regions. These findings reflect a coordinated management of excitatory synaptic resources within the dentate granule cell dendritic tree.
The dentate granule cell dendritic tree innervates three categorically discrete layers as it branches into the outer reaches of the molecular layer (Claiborne et al., 1990). Each of these layers is different in composition, and each exerts a specific influence upon the cellular network of the dentate gyrus. The IML is located in the proximal region of the dendritic tree and is populated by inhibitory interneurons and commissural fibers (Freund and Buzsaki, 1996). The MML resides just above the IML, and receives a monosynaptic input from the fibers of the medial perforant path. The OML is the final stratum of the sequence, and receives a monosynaptic input from the fibers of the lateral perforant path arising from the lateral entorhinal cortex (McNaughton, 1980).

The granule cell dendritic tree exhibits a unique pattern of activation when stimulated. High frequency stimulation (HFS) of the medial perforant path is capable of inducting LTP within the MML (Abraham et al., 2002). HFS of the lateral perforant pathway is capable of inducting LTP within the OML (Abraham et al., 2006). Interestingly, subsequent investigations have demonstrated that induction of LTP in the MML is accompanied by a simultaneous induction LTD of within the OML (Abraham and Goddard, 1983; Abraham et al., 2001). Conversely, induction of LTP within the OML is accompanied by a simultaneous induction of LTD within the MML (Abraham et al., 1985; Doyere et al., 1997). In this way, the constituent states of bidirectional synaptic plasticity appear to be balanced between OML and MML regions of the granule cell dendritic tree.
Spike-timing-dependent plasticity (STDP) is an experimentally observed principle which states that the timing of a synaptic event relative to cell firing is crucial in determining the state of bidirectional plasticity which is induced (Markram et al., 1997). A synaptic event that takes place before a cell fires may result in LTP. A synaptic event that takes place after cell firing may result in LTD. Interestingly, evidence from computational studies suggests that the granule cell dendritic tree is structured to segregate the order in which OML and MML regions experience patterns of cell firing (Benuskova and Abraham, 2007). Dominant activation of one region will set in motion a series of temporal events conducive to LTP; however, regions peripheral to this site of activation will experience these events in a pattern conducive to LTD. The timing of these events is precise; however, STDP provides a mechanism by which bidirectional plasticity could be simultaneously coordinated between layers of the granule cell dendritic tree.

Bidirectional synaptic plasticity is characterized by persistent alterations in synaptic transmission. Excitatory synapses exist almost exclusively on the heads of dendritic spines, thus, dendritic spines represent a logical focus for changes associated with bidirectional synaptic plasticity (Harris, 1999; Fukazawa et al., 2003; Harris et al., 2003; Bourne and Harris, 2007). Many early studies investigating the structural complements of bidirectional synaptic plasticity reported that LTP was accompanied by significant increases in the overall number of dendritic spines (Moser et al., 1994; Trommald et al., 1996; Collin et al., 1997; Andersen and Soleng, 1998; Weeks et al., 1998; Maletic-Savatic et al., 1999). Such findings presented an interesting conceptual solution to the question of potentiation: an increase in the amount of synaptic spines would equate to an
increase in synaptic transmission. Problematically, however, subsequent investigations revealed that spine density could change dramatically according to experimental preparation (Bourne et al., 2007), developmental age (Crain et al., 1973; Seress and Pokorny, 1981), behavioral state (Radley et al., 2006), position within the dendritic tree (2.3.1), estrous cycle (Gould et al., 1990; Woolley et al., 1990; Murphy and Segal, 1996;) and a host of other variables.

As experimental paradigms were gradually refined, a series of studies emerged which suggested that bidirectional synaptic plasticity was expressed through a characteristic remodeling of preexisting spines. Live imaging optical techniques revealed that LTP was associated with substantial increases in spine size (Lang et al., 2004; Matsuzaki et al., 2004; Otmakhov et al., 2004). In addition, LTD was found to be associated with significant decreases in spine size (Okamoto et al., 2004; Zhou et al., 2004). These investigations served to suggest that increases and decreases in spine size were correlated with complementary changes in synaptic transmission.

Computational studies have revealed that alterations in spine dimensions could significantly impact calcium signaling and the compartmentalization of various biochemical processes (Hayashi and Majewska, 2005). Effects such as these could contribute to changes in synaptic function. More dramatically however, Harris and Stevens (1989; Harris et al., 1992) demonstrated that spine head size and postsynaptic density (PSD) area share a positive linear correlation. The PSD is a collection of specialized scaffolding molecules and protein ion channels, and is principally responsible
for synaptic transmission. By way of summary: live imaging studies revealed that bidirectional synaptic plasticity was correlated with systematic changes in spine size. Harris and Stevens (1989, Harris et al., 1992) revealed that spine head size correlated with the mechanisms responsible for synaptic transmission. As a unification of these findings, it would appear that bidirectional synaptic plasticity could be expressed through coordinated changes in spine size and PSD area.

Experimental evidence indicates that individual neurons actively regulate the strength of their excitatory synaptic contacts in an effort to maintain a homeostatic level of cell excitability. This feature of neurophysiology is known as “synaptic scaling” (Turrigiano et al., 1998; Turrigiano, 2008). The dendritic tree is populated by a number of different ion channels that regulate the intrinsic excitability of the cell; the most prominent collection of channels is evident in the PSD. In a landmark study by Bourne & Harris (2010), PSD area was found to consolidate 2-hr following the induction of LTP. Analysis revealed that average values of spine size and PSD area were significantly larger following LTP. Interestingly, however, the total amount of PSD area present on control and LTP spines remained constant. This result indicated that excitatory synaptic resources could be reallocated to regions where they were required; however, the net balance of resources appears to be dynamically maintained. In summary: synaptic scaling is a principle which states that neurons endeavor to maintain a homeostatic level of excitation (Turrigiano, 2008); the findings of Bourne and Harris (2010) suggest that this homeostasis could be achieved through the balancing of excitatory synaptic structures across individual dendritic segments. Owing to the laminar structure of its principal
inputs, the dentate gyrus presents the unique opportunity to determine if excitatory synaptic structures are homeostatically coordinated between adjacent segments of the granule cell dendritic tree.

In the dentate gyrus, bidirectional synaptic plasticity is electrophysiologically coordinated between the medial and lateral fibers of the perforant path. These fibers project to MML and OML regions of the granule cell dendritic tree. In the present investigation, we sought to characterize the structural complements of bidirectional synaptic plasticity induced within the dentate gyrus of freely behaving rats. Furthermore, we were interested to establish how changes in excitatory synaptic structures might be homeostatically regulated across regions of the granule cell dendritic tree.

3.2 Methods

3.2.1 Animals

Two adult male Long Evans rats (animal codes LED 50 and LED 56, as described in 2.2.1) were used in the present investigation. Animals were single housed in a reverse light cycle (12:12 hr) environment, and provided with ad libitum access to food and water. All procedures were approved by the University of Otago Animal Ethics Committee, and complied with NIH requirements for the humane care and use of laboratory animals.
3.2.2 Surgery

The chronic electrode implantation surgery described in 1.2.1 was extended to include both left and right hippocampal formations of the brain.

The left hippocampal formation, to be referred to as the “experimental hemisphere”, was prepared with two stimulating electrodes and one recording electrode. Stimulating electrodes were implanted in medial and lateral portions of the perforant path, and adjusted to activate MML and OML regions of the granule cell dendritic tree. Field excitatory postsynaptic potentials (fEPSPs) evoked at MML and OML locations were recorded by an electrode positioned in the dentate hilus (Fig. 3.1)

The right hippocampal formation, to be referred to as the “control hemisphere”, was prepared with one stimulating electrode and one recording electrode. The stimulating electrode was implanted in the medial portion of the perforant path, and adjusted to activate the MML of the granule cell dendritic tree. fEPSPs evoked at this MML location were recorded by an electrode positioned in the dentate hilus (Fig. 3.1).

Additional methods, coordinates, anesthetic concentrations and electrode characteristics were as described in 1.2.1. Following a 2-week recovery period, animals were taken to a recording room for response criteria testing.
FIGURE 3.1 Schematic illustration of electrode positions. The middle molecular layer of the granule cell dendritic tree was activated by stimulation of the medial perforant path (orange). The outer molecular layer of the dentate granule cell dendritic tree was activated by stimulation of the lateral perforant path (blue). fEPSPs evoked by this activation were recorded by an electrode positioned in the dentate hilus. The experimental hemisphere of each animal was implanted with media, lateral, and recording electrodes; the control hemisphere of each animal was implanted with medial and recording electrodes.

3.2.3 Recording procedures

All experimental procedures took place in a recording room that was outfitted with recording and stimulation equipment, and isolated from extraneous distractions. Instrumentation parameters were prepared as described in 1.2.2. Experimentation was conducted while animals were quietly awake (Abraham et al., 2002), and took place during the dark phase of the circadian rhythm. Between procedures, animals were single housed in standard rodent cages.
3.2.4 Electrophysiology

Before formal experimentation began, medial and lateral waveforms were evaluated with a standard set of electrophysiological tests, as described previously (1.2.3). Animals that satisfied the conditions of these tests were graduated to experimental baseline sessions.

During each baseline session, test-intensity pulses were administered to medial and lateral path of the experimental formation, and the medial path of the control hemisphere; pulses alternated between pathways at a frequency of 0.06 Hz, and continued for a period of 30 minutes. fEPSP slope values were averaged from the last 15 min of each session. Baseline sessions were conducted once every two days, and continued until medial and lateral fEPSP slope averages exhibited a variance of ≤ 5% for 4 consecutive sessions.

One day following the establishment of fEPSP baseline stability, a 50T high frequency stimulation (HFS) protocol (cf. Fig. 1.2C) was applied to the medial perforant path of the experimental hemisphere. HFS was delivered immediately after 30 minutes of baseline test pulse stimulation, and followed by an additional 20 minutes of test pulse stimulation.

The time between the start of 50T HFS administration and the end of the recording session was exactly 30 minutes. At the completion of this session, animals were removed from the recording chamber and perfused.
3.2.5 Perfusion

Animals were deeply anesthetized with halothane before being surgically intubated and transcardially perfused. Once the perfusion procedure had been completed, brain tissue was removed with bone rongeurs and stored in mixed aldehydes (as described in 2.2.2). Perfusion procedures were assisted by Mrs. Sara E. Mason-Parker.

3.2.6 Tissue processing

Sagittal sections (70 µm) were cut through the brain and place in 24-well plates filled with a solution of cacodylate buffer (CB). Two sections were selected from symmetrical coordinates of the left and right cerebral hemispheres of each animal (3.5 mm lateral from midline), trimmed to excise a sample region of the hippocampal molecular layer (cf. Fig. 2.1A) and embedded in Epon resin (as described in 2.2.3). Final Epon blocks were hardened in an oven for 60 hours and assigned unique experimental codes to designate their identity as control or experimental hemisphere tissue.

3.2.7 Ultrasectioning

A Reichert Ultracut S (Leica, Nussloch, Germany) was used to cut a series of ~200 consecutive ultrathin sections (45 nm thickness) from the inner, middle and outer molecular layers of the granule cell dendritic tree (cf. Fig. 2.1B). In animal LED50, ultrathin series were cut from left and right hemisphere MML tissue \((n = 2\) series). In animal LED56, ultrathin series were cut from left and right hemisphere IML and OML tissue \((n = 4\) series). Preliminary OML data from animal LED 56 is presented \((n = 1\) series); however, these data are the subject of an ongoing investigation, and as such,
remain experimentally coded. Each tissue series was collected on Pioloform coated slot grids (Ted Pella, Inc) and post section stained with treatments of saturated uranyl acetate and 0.2% lead citrate (as described in 2.2.4).

3.2.8 *Serial section transmission electron microscopy (ssTEM)*

Each series was photographed with a Jeol 1200EX (Peabody, MA) transmission electron microscope. Region select images were photographed at a magnification of 5,000x, series images were photographed as a montage of two side-by-side 10,000x magnification images (as described in 2.2.5).

Dendritic allometry is a principle which delineates the proportional scaling of resources within a granule cell. To account for this phenomenon, the present investigation utilized a quantitative method of unbiased region selection to ensure that structural analysis was performed on control hemisphere and experimental hemisphere tissue samples that were correctly matched (as described in 2.3.2).

3.2.9 *Measured variables*

Offline analysis of evoked waveforms was achieved with a Labview software application (National Instruments, additional programming courtesy of Dr. Michael Eckert). Waveform values were expressed as a percentage change in fEPSP slope (mV/ms). Percentages were calculated by normalizing fEPSP slopes to the average of values taken from the last 4 days of baseline stimulation.
Each image series was calibrated and analyzed through use of the Reconstruct software application (Fiala, 2005).

Microtubule number was measured from three positions of the dendrite as it passed through sequential sections of each series. These three values were then averaged to give a final count (cf. Fig. 2.2A)

Z-length was measured as the length (μm) of a dendrite as it passed through sequential sections of the series (cf. Fig. 2.2B)

Spine density was measured as the total number of spines on a dendrite, divided by the Z-length between the first complete spine, and the last spine (cf. Fig. 2.2B)

Spine volume (μm³) was measured with a manual function of the Reconstruct software. Spine dimensions were first traced across consecutive serial sections, and then reconstructed within calibrated dimensions of three-dimensional space. Similar methods were used to measure spine head volume (μm³), spine neck volume (μm³), and PSD area (μm²).

3.2.10 Statistics
LTP was defined as a ≥15% increase in fEPSPs averaged from the last 10-min of the recording session. Likewise, LTD was defined as a ≥15% decrease in fEPSPs averaged
from the last 10-min of the recording session. The electrophysiology sample size was not sufficient to qualify valid statistical testing.

Structural differences between control and experimental tissue series were analyzed with Student’s t-test. Comparisons based on more than two groups at a time were conducted with one-way analysis of variance (ANOVA). Holm-Sidak post hoc testing was used where appropriate.

The degrees of freedom used for structural analysis were partitioned according to individual dendrites; as such, statistical reports concerning volume or area measurements reflect multiple observations that were nested within an individual average.

Linear regression was used to evaluate the relationship between spine head volume and PSD area. Slope coefficients calculated from regression analysis were compared with analysis of covariance (ANCOVA). For all analyses, an alpha level of 0.05 was defined as statistically significant.

3.3 Results

3.3.1 Induction of bidirectional synaptic plasticity

Prior to the administration of HFS, baseline fEPSP and population spike values were comparable between animals (Table 3.1).
TABLE 3.1 Summary of electrophysiological baseline parameters.

<table>
<thead>
<tr>
<th>Animal code</th>
<th>Control medial fEPSP (mV/ms)</th>
<th>Control medial PS (mV)</th>
<th>Medial fEPSP (mV/ms)</th>
<th>Medial PS (mV)</th>
<th>Lateral fEPSP (mV/ms)</th>
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<tr>
<td>LED 50</td>
<td>6.2 ± 0.13</td>
<td>2.22 ± 0.14</td>
<td>3.92 ± 0.08</td>
<td>1.75 ± 0.13</td>
<td>1.98 ± 0.10</td>
</tr>
<tr>
<td>LED 56</td>
<td>6.18 ± 0.13</td>
<td>2.08 ± 0.18</td>
<td>3.78 ± 0.08</td>
<td>1.92 ± 0.32</td>
<td>1.81 ± 0.04</td>
</tr>
</tbody>
</table>

50T HFS was applied to the medial perforant path of the experimental hemisphere. Increases in medial fEPSPs were sufficient to qualify as LTP (Fig. 3.2A-C, LED50, $M = 35.42 \pm 1.89\%$; LED56, $M = 41.78 \pm 2.49\%$). Decreases in lateral fEPSPs were sufficient to qualify as LTD (Fig. 3.2A-C, LED50, $M = -34.38 \pm 1.33\%$; LED56, $M = -29.69 \pm 1.91\%$). The magnitude of LTP and LTD induction was similar across animals (Fig. 3.2C). Changes in medial fEPSPs within the control hemisphere were minimal (Fig. 3.2A-C, LED50, $M = 3.30 \pm 1.48\%$; LED56, $M = 3.75 \pm 1.68\%$).
FIGURE 3.2 Induction of bidirectional synaptic plasticity. A: Animal LED50 (top), Animal LED56 (bottom). In the experimental hemisphere, 50T HFS applied to the medial perforant path induced LTP within afferents projecting to the MML (orange), and LTD within afferent projecting to the OML (blue). HFS activation of the experimental hemisphere did not appear to change fEPSPs recorded in the control hemisphere (grey). B: Waveforms pre and post HFS. Pre HFS waveforms
(dashed line) were averaged from 20 sweeps leading up to HFS administration; post
HFS waveforms (solid line) were averaged from the final 20 sweeps of the session.
Scale bar: 5 mV, 5 ms. C: The induction of bidirectional synaptic plasticity was
similar between experimental animals. Values are displayed as a percentage change
in fEPSPs, and calculated from the last 10-minute window of the session.

3.3.2 Systematic changes in spine volume accompany the induction of bidirectional
synaptic plasticity
The granule cell dendritic tree is subdivided into inner, middle and outer molecular
layers. LTP was induced within the afferent projection to the MML. LTD was induced
within the afferent projection to the OML. Collectively, these changes constitute the
properties of bidirectional synaptic plasticity. ssTEM with three-dimensional
reconstruction was used to evaluate the structural complements of bidirectional synaptic
plasticity within IML, MML and OML regions of the dendritic tree.

The induction of bidirectional synaptic plasticity did not alter the number of
microtubules present within the granule cell dendritic tree (Fig. 3.3E); furthermore,
induction did not change the number of spines/μm present along dendritic segments (Fig
3.3F). Interestingly, however, bidirectional synaptic plasticity was accompanied by
systematic changes in spine volume. The induction of LTP was associated with
significant increases in MML spine volume (Fig. 3.3G, \( t_{(11)} = -5.402, P < 0.001 \)).
Increases in MML spine volume were associated with significant decreases in IML spine
volume (Fig. 3.3G \( t_{(10)} = -4.60, P < 0.001 \) ).

82
FIGURE 3.3 Systematic changes in spine volume accompany the induction of bidirectional synaptic plasticity. A-D: Three-dimensional reconstructions of
dendritic shafts (transparent) and spines (solid). Scale cube: 1 µm. A-B: dendritic reconstructions from MML control hemisphere (left) and experimental hemisphere (right) tissue. C-D: dendritic reconstructions from IML control hemispheres (left) and experimental hemisphere (right) tissue. Middle molecular layer samples were sectioned from animal LED 50, whereas inner molecular layer samples were sectioned from animal LED 56 (refer to section 2.2.4 for details). Outer molecular layer samples were sectioned from animal LED 56, and remain experimentally coded. E: The induction of bidirectional synaptic plasticity did not alter dendritic microtubule number within OML (blue, \( t_{(17)} = -3.46, P = 0.41 \)), MML (orange, \( t_{(46)} = -1.298, P = 0.20 \)), or IML (red, \( t_{(33)} = 0.072, P = 0.478 \)) layers of the granule cell dendritic tree. F: The induction of bidirectional synaptic plasticity did not alter dendritic spine density within OML (blue, \( t_{(17)} = -0.645, P = 0.528 \)), MML (orange, \( t_{(32)} = -1.64, P = 0.112 \)), or IML (red, \( t_{(23)} = 0.272, P = 0.788 \)) layers of the granule cell dendritic tree. G: Bidirectional synaptic plasticity was associated with systematic changes in spine volume. HFS induced potentiation of MML fEPSPs was accompanied by significant increases in MML spine volume (middle panel, and significant decreases in IML spine volume (bottom panel).

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3 Data from the OML sample set is the focus of current investigation. As such, investigators are still blinded as to experimental conditions. Preliminary OML data from animal LED 56 is labeled with reference to unique experimental codes.
FIGURE 3.4 Representative micrographs illustrating spine changes associated with bidirectional synaptic plasticity. (Left panel) original micrographs, (right panel)
micrographs with reconstruct traces. Spine trace analysis is shown in yellow; PSD trace analysis is shown in red. A: Spine from control hemisphere MML tissue. B: Spine from experimental hemisphere MML tissue. C: Spine from control hemisphere IML tissue. D: Spine from experimental hemisphere IML tissue. Scale bar: 0.5 µm. Representative micrographs were selected as spines that most closely approximated group means.

3.3.3 Volume changes associated with bidirectional plasticity are most pronounced in the spine head.

Quantitative three-dimensional reconstruction was used to segment and measure specific portions of spine anatomy. Segmentation of control hemisphere tissue revealed that spine necks contributed only a small fraction of total spine volume (Fig. 3.5A-D & 3.5E vs. F). Subsequent analysis indicated that MML spine neck volume did not change in response to LTP (Fig. 3.5F, $t_{(4)} = 1.79, P = 0.148$); however, marginal decreases were observed in IML spine neck volume (Fig. 3.5F, $t_{(4)} = -4.01, P = 0.016$). Proportionally speaking, the discrepancies observed in spine neck volume were not sufficient to explain the magnitude of total volume change associated with bidirectional synaptic plasticity (Fig. 3.5E).

The next target of quantitative three-dimensional reconstruction focused on spine heads. Segmentation of control hemisphere tissue revealed that spine heads occupied a large proportion of total spine volume (Fig. 3.5E vs. G). Spine head volume was found to change dramatically in response to bidirectional synaptic plasticity. Results indicated that LTP was associated with an 81% ($\Delta 0.022 \pm 0.002 \mu m^3$) increase in MML spine head.
volume (Fig. 3.5G, $t_{(4)} = -6.56, P = 0.003$). Interestingly, this change was also associated with a 70% ($\Delta 0.040 \pm 0.004 \mu m^3$) reduction in IML spine head volume (Fig. 3.5G, $t_{(4)} = -5.614 P = 0.005$). These results indicate that changes in spine volume associated with bidirectional synaptic plasticity were localized almost exclusively to spine heads.

**FIGURE 3.5 Volume changes associated with bidirectional plasticity were most pronounced in the spine head. A-D Three-dimensional reconstructions of spine**
necks (transparent) and spine heads (solid). A-B: Spine reconstructions from MML control hemisphere (left) and experimental hemisphere (right) tissue. C-D: Spine reconstructions from IML control hemisphere (left) and experimental hemisphere (right) tissue. Scale cube: 0.1 µm. E: Reiteration of Fig. 3.3G, Bidirectional synaptic plasticity was associated with systematic changes in spine volume. F: Bidirectional synaptic plasticity was associated with negligible changes in spine neck volume. (Top) MML spine neck volume did not change with HFS induced potentiation of MML fEPSPs. (Bottom) Marginal decreases in IML spine neck volume accompanied HFS induced potentiation of the MML. G: Volume changes associated with bidirectional plasticity were most pronounced in the spine head. (Top) HFS potentiation of MML fEPSPs was associated with significant increases MML spine head size, and significant decreases in IML spine head size (bottom).

3.3.4 Spine head changes associated with bidirectional synaptic plasticity are accompanied by changes in PSD area

The postsynaptic density (PSD) is a collection of specialized scaffolding molecules and protein ion channels. Increases in the number of excitatory ion channels present within the PSD could be responsible for increases in synaptic transmission (Malenka and Nicoll, 1999; Kerchner and Nicoll, 2008).

The proteins that comprise the PSD are electron-dense. As such, PSD area could be clearly discerned within transmission electron micrographs, and measured with three-dimensional reconstructions. Experimental findings revealed that LTP was associated
with significant increases in MML PSD area (Fig. 3.6F, $t_{(11)} = -2.385$, $P = 0.036$).

Increases in MML PSD area were associated with significant decreases in IML PSD area (Fig. 3.6F, $t_{(10)} = -3.948$, $P = 0.003$).

**FIGURE 3.6** Spine head changes associated with bidirectional synaptic plasticity were accompanied by changes in PSD area. A-D: Three-dimensional reconstructions of spine heads (transparent) and PSDs (solid). A-B: Spine head and PSD reconstructions from MML control hemisphere (left) and experimental hemisphere.
(right) tissue. C-D: Spine head and PSD reconstructions from IML control hemisphere (left) and experimental hemisphere (right) tissue. Scale cube: 0.1 µm. E: Reiteration of Fig. 3.5G, Volume changes associated with bidirectional plasticity were most pronounced in the spine head. F: (Top) Potentiation of MML fEPSPs was associated with a significant increase in MML PSD area and significant decreases in IML PSD area (bottom).

**TABLE 3.2 Summary of three-dimensional reconstruction data.**

<table>
<thead>
<tr>
<th></th>
<th>Animal code</th>
<th># Spines reconstructed</th>
<th>Average spine volume (µm³)</th>
<th>Average spine head volume (µm³)</th>
<th>Average PSD area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MML</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>LED 50</td>
<td>217</td>
<td>0.039 ± 0.0038</td>
<td>0.0272 ± 0.0022</td>
<td>0.0383 ± 0.0034</td>
</tr>
<tr>
<td>Experimental</td>
<td>LED 50</td>
<td>236</td>
<td>0.0705 ± 0.0043</td>
<td>0.0492 ± 0.0016</td>
<td>0.0508 ± 0.0039</td>
</tr>
<tr>
<td><strong>IML</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>LED 56</td>
<td>203</td>
<td>0.0535 ± 0.0064</td>
<td>0.0534 ± 0.0053</td>
<td>0.0588 ± 0.0047</td>
</tr>
<tr>
<td>Experimental</td>
<td>LED 56</td>
<td>193</td>
<td>0.0219 ± 0.0052</td>
<td>0.0173 ± 0.0036</td>
<td>0.0369 ± 0.0029</td>
</tr>
</tbody>
</table>

* Spine volume and PSD area were measured from all spines sampled. Spine head size was measured from a subset of the sample population; MML control (n = 112), MML experimental (n = 95), IML control (n = 86) IML experimental (n = 104).

**3.3.5 PSD area correlates with spine head volume**

A significant linear relationship was found to exist between the spine head volume and PSD area of each dendritic layer (Fig. 3.7). Interestingly, the slope coefficients which described this trend held constant across all layers of the granule cell dendritic tree, and was unchanged by the induction of bidirectional synaptic plasticity (Fig. 3.7A-D, ANCOVA, $F_{(3,7)} = 3.74, P = 0.68$). This result suggests that spine head volume and PSD area are proportionally interrelated throughout the granule cell dendritic tree. To test this hypothesis, regression analysis was performed on a data set that pooled PSD area and...
spine head volumes across all layers and conditions. The result of this analysis indicated that the relationship between PSD area and spine head volume could be represented across all layers and conditions with a single linear equation \(^4\) (Fig. 3.7A-D, \(R^2 = 0.78, F_{(1,360)} = 1270.87, P < 0.001\)).

**FIGURE 3.7** PSD area correlates with spine head volume. A-D: A significant linear relationship was found to exist between spine volume and PSD area. A: MML, control hemisphere \((R^2 = 0.65, F_{(1,99)} = 180.33, P < 0.001\)). B: MML, experimental hemisphere \((R^2 = 0.83, F_{(1,88)} = 415.876, P < 0.001\)). C: IML, control hemisphere \((R^2 = 0.79, F_{(1,76)} = 288.45, P < 0.001\)). D: IML, experimental hemisphere \((R^2 = 0.92, F_{(1,95)} = 501.12, P < 0.001\)). The slope coefficients that describe these trends were constant across layers of the granule cell dendritic tree, and unchanged by the

\[ Y = 0.0129 + (0.777 \times X) \]

\(^4\) PSD area (Y), spine head volume (X); Y = 0.0129 + (0.777 * X)
induction bidirectional synaptic plasticity. Subsequent analysis revealed that these relationships could be modeled with a single linear function. A-B: HFS induced potentiation of the MML fEPSPs causes an ascending shift (orange arrow) of MML values along the linear function. C-D: HFS potentiation of MML fEPSPs causes a descending shift (red arrow) of IML values along the linear function.

As established earlier, the induction of bidirectional synaptic plasticity was associated with systematic changes in spine head volume and PSD area within MML and IML regions of the granule cell dendritic tree. These effects were significant in opposing directions, but unified by a common linear relationship. As such, the structural consequences of bidirectional synaptic plasticity may be summarized as “ascending” or “descending” shifts along the slope of a linear constant.

LTP was associated with coordinated increases in PSD area and spine head volume within the MML (Fig. 3.8A-D); this effect may be conceptualized as an ascending shift along the linear constant (Fig. 3.8E). Changes in MML structure were associated within coordinated decreases in PSD area and spine head volume within the IML (Fig. 3.8A-D); this effect may be conceptualized as a descending shift along the linear constant (Fig. 3.8E).
3.3.6 Changes in spine head volume and PSD area are balanced across MML and IML layers of the granule cell dendritic tree

Previous results have revealed that granule cell dendritic caliber, microtubule number, and spine density decrease with distance from the cell body layer (cf. Fig. 2.3). Data from the present investigation revealed a significant main effect of spine head volume ($F_{(3, 11)} = 22.86, P < 0.001$) and PSD area ($F_{(3, 24)} = 7.36, P = 0.001$) across layers and experimental conditions. Under control conditions, spine head volume and PSD area were found to be significantly higher in IML regions than MML regions (Holm-Sidak, Spine head volume, $P = 0.013$; PSD area, $P = 0.001$). This result may indicate that average spine head volume and PSD area decrease with distance from the cell body layer.

As mentioned above, spine volume and PSD area were significantly larger in control IML regions than control MML regions. Subsequent analysis revealed that, following LTP, spine head volume and PSD area in MML regions became more like control IML regions (Fig. 3.8E, Holm-Sidak, Spine head volume, $P = 0.44$; PSD area, $P = 0.15$). Furthermore, following LTP, spine head volume and PSD area in IML regions became more like MML control dimensions (Fig. 3.8E, Holm-Sidak, Spine head volume, $P = 0.09$; PSD area, $P = 0.75$). This finding may indicate that excitatory synaptic resources are homeostatically maintained between IML and MML regions of the granule cell dendritic tree.
FIGURE 3.8 Summary of structural changes associated with the induction of bidirectional synaptic plasticity. A-B: Schematic illustration of granule cell dendritic segment from control hemisphere (A) and experimental hemisphere (B) tissue; spine head size is scaled to represent data. HFS potentiation of MML fEPSPs was associated with coordinated increases in MML (orange) spine head volume and PSD area (black); this effect was associated with coordinated decreases in IML (red) spine head volume and PSD area. C-D: Spine head volume (C) and PSD area (D) changes associated with HFS potentiation of MML fEPSPs. Values are expressed relative to control. E: Bidirectional synaptic plasticity can be conceptualized as ascending and descending shifts in the relationship between spine head volume and PSD area. HFS potentiation of MML (orange) fEPSPs was associated with an
ascending shift in spine head volume and PSD area, and a descending shift in IML spine head volume and PSD area. Control hemisphere averages are designated by circles, experimental hemisphere averages are designated by triangles. Following the induction of LTP within the MML, spine head volume and PSD area appeared to balance between MML and IML regions. Prior to LTP, control IML spine head volume and PSD area was significantly larger than MML spine head volume and PSD area. Following LTP, MML spines became more like control spines, and IML spines became more like control MML spines.

3.4 Conclusions

LTP was associated with coordinated increases in spine head volume and PSD area within the MML. These changes were associated with coordinated decreases in spine head volume and PSD area within the IML.

A significant linear relationship was found to exist between spine head volume and PSD area. The slope of this relationship was the same across MML and IML regions of the granule cell dendritic tree, and was unchanged by the induction of bidirectional synaptic plasticity.

Changes in excitatory synaptic structures appear to be balanced between MML and IML regions of the granule cell dendritic tree. In control conditions, IML spine volume and PSD area were significantly larger than MML spine head volume and PSD area.
Following the induction of LTP, MML spines became more like control IML spines, and IML spines became more like control MML spines.

3.5 References


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General Discussion

4.1 Chapter one review

4.1.1 Circadian spines?

Dendritic spines are highly dynamic structures which account for the majority of excitatory synaptic transmission within the brain (Harris et al., 2003; Fukazawa et al., 2003; Bourne et al., 2007a). In chapter three we demonstrated that increases in synaptic transmission were accompanied by systematic changes in spine structure (3.3.2). Furthermore, in chapter one we reported that increases in synaptic transmission were modulated by circadian rhythm (1.3.2). Interestingly, however, little is known regarding the role circadian modulation might play in influencing the structure and function of dendritic spines.

Barnes et al., (1977) demonstrated that fEPSPs evoked by perforant path activation of the granule cell dendritic tree fluctuate in parallel with an animal’s circadian cycle. In diurnal animals, fEPSP slope peaked during the light phase of the circadian rhythm; in nocturnal animals, this peak was observed to take place during the dark phase. In the present investigation, we found that HFS induced increases in fEPSPs were associated with coordinated increases in spine head volume and PSD area (3.3.3). Therefore, if the mechanisms responsible for regulating fEPSP strength are conserved across conditions, it seems logical to suggest that circadian fluctuations in fEPSPs could be associated with comparable fluctuations in spine head volume and PSD area.
Input-output (I/O) testing is used to evaluate the parameters of an evoked waveform across a range of stimulation intensities. The shape of an evoked waveform is indicative of the electrophysiological mechanisms responsible for its expression; in this way, specific waveshape signatures can be measured and interpreted to represent the activity of specific cellular events. fEPSP slope is a measure indicative of synaptic activation. Population spike (PS) amplitude is a measure indicative of synchronized cell firing. The summation of excitatory synaptic activation is required to initiate cell firing, thus, measures of fEPSP slope and PS amplitude are interrelated. Crucially however, a fundamental distinction must be made between the interpretation of fEPSP and PS measures: synaptic transmission can change according to a host of different physiological events, but the amplitude of a single cell firing is always the same. In summary, changes in fEPSP slope may be interpreted as changes in synaptic transmission, but changes in PS amplitude must be interpreted as a change in the total number of cells firing in synchronous activity.

I/O data from chapter one revealed that light and dark cycle animal exhibited equivalent fEPSP values across all stimulation intensities tested. Interestingly, however, the same stimulation intensities evoked significantly higher PS values in dark animals than in light animals (1.3.2). This finding suggests that dark cycle animals fired more cells in response to equivalent amounts of synaptic activation.

Results from the present investigation conflict with the findings of Barnes et al., (1977). A finding that circadian rhythm modulates fEPSP slope could be used to implicate the
involvement of synaptic spines. Instead, results from chapter one suggest that circadian rhythm modulates cell firing. Cell firing contributes to induction of LTP and LTD (Markram et al., 1997), and may be required for the persistence-phase of synaptic plasticity (Raymond, 2008). Therefore, the experimenter hypothesizes that circadian fluctuations in cell excitability could act to modulate the induction and persistence of bidirectional synaptic plasticity.

Contemporary evidence suggest that sleep is a time of consolation, a phase during which the relevant experiences of the day (or night, depending on species) are consolidated to a more persistent form (Walker and Strickgold, 2006; Walker, 2009; Eckel-Mahan and Storm, 2009; for alternative theories see Siegel, 2001). The present investigation was conducted on rats. Rats are nocturnal animals, and thus, are most active during the night. Results from chapter one indicated that cell excitability was higher in the dark phase of the circadian rhythm than it was in the light. Furthermore, this difference was shown to correspond with differences in the induction and persistence of LTP. As a general interpretation: circadian modulation of cell excitability could serve to increase the prevalence of synaptic plasticity when an animal is most active, and decrease the prevalence of synaptic plasticity during times of rest. The specific mechanism of this effect is unknown; however, its expression may be related to the activation of inhibitory synaptic transmission within the dentate gyrus (Austin et al., 1989).
4.1.2 Strain differences: a question of intelligence?

Long-Evans strain rats exhibited larger and more persistent increases in synaptic transmission than their Sprague-Dawley counterparts (1.3.1). Increases in synaptic transmission have been associated with learning ability and memory (Rioul-Pedotti et al., 2000; Pastalkova et al., 2006; Harms et al., 2008) thus, could differences in synaptic plasticity between animal strains be indicative of some underlying difference in intelligence?

To equate plasticity with superior learning ability is not necessarily a valid assumption. For a network to learn, it must be capable of encoding and retaining information through some persistent form of change. A network which is tuned to rapidly encode and retain information will learn quickly; however, providing the mechanisms of retention are not infinite, previously retained memories will be overwritten and lost (Barnes et al., 1994). From the alternative perspective, a network that is tuned to resist storage of new information may learn nothing at all (Davis et al., 1992). In summary: the ideal state of network learning is most likely context dependent (Bienenstock et al., 1982; Abraham and Bear, 1996; Abraham et al., 2001) change is required for memory, however, the magnitude of change does not necessarily reflect a more intelligent system.

Long-Evans and Sprague-Dawley strain rats have the propensity for different patterns of behavior; more specifically, open field testing has shown that Long-Evans animals spend significant more time engaged in walking, rearing, and exploration-related behaviors. Interestingly, these behaviors are associated with categorically different patterns of brain
activation, and in theory, could be associated with different levels of plasticity (van Lier et al., 2003). Stress has been shown to exert a significant effect on the induction of synaptic plasticity; thus, an animal strain that is prone to duress could conceivably exhibit less synaptic plasticity than more amenable strains (Foy et al., 1987; Shors et al., 1997). In addition, studies have shown that exercise can facilitate the expression of synaptic plasticity; thus, an animal strain with sedentary tendencies could exhibit lower amounts of synaptic plasticity in comparison to more active strains (van Praag et al., 1999; O'Callaghan et al., 2007). The present investigation was conducted on freely behaving rats. Experimental measures were taken to ensure that animals were calm at the time of recording, and housed within a consistent environment. Irrespective of these facts, inherent behavioral differences may have influenced the expression of synaptic plasticity between strains.

I/O testing was used to evaluate the fEPSP and PS dynamics of Sprague-Dawley and Long-Evans strain animals. No significant difference was observed between Long-Evans and Sprague-Dawley medial fEPSPs; however, PS amplitude was significantly higher in Long-Evans strain animals than Sprague-Dawley animals. This result suggested that granule cell excitability was higher in Long-Evans animals. Similar to the findings demonstrated in the circadian data set, it would appear that strain differences in synaptic potentiation might be expressed through the influence of a system which acts to modulate cell firing. An interesting interpretation of this finding is available.
Previous studies have shown that exploratory behavior is associated with decreased levels of inhibition with the dentate granule cell body, and significant increases in PS amplitude (Moser, 1996). Additionally, Long-Evans strain rats have been shown to be more active in their explorative tendencies than Sprague-Dawley strain rats (van Lier et al., 2003). In summary: it is plausible to suggest that categorical differences in exploratory behavior and granule cell inhibition could be used to explain the differences in cell excitability and LTP reported between Long-Evans and Sprague-Dawley strains.

Experimental findings from chapter one emphasize the role of cell excitability as a modulator of LTP. The experimenter postulates that inhibitory synaptic transmission could play a key role in the expression of circadian and strain related differences in LTP within the dentate gyrus.

4.1.3 Physiological patterns of memory

Theta burst stimulation (TBS) is an HFS protocol that is characterized by 5 Hz spacing of 100 Hz pulse episodes. 5 Hz activation is a common frequency of cell firing within regions of the hippocampus, thus, TBS protocols are often heralded as physiologically relevant (Larson et al., 1986). TBS has been shown to induce robust forms of synaptic potentiation within the schaffer collateral projection between hippocampal areas CA3 and CA1 (Staubli and Lynch, 1987), and may facilitate the polymerization of actin within dendritic spines (Lin et al., 2005)
Results from chapter one demonstrated that TBS was unable to evoke a criterion level of LTP or LTD within the dentate gyrus of freely moving rats (1.3.3). Instead, results highlighted the role of 400 Hz as a preferential frequency within the circuitry of the dentate. Adjustment of TBS pulse frequency from 100 Hz to 400 Hz yielded a dramatic increase in both LTP and LTD induction. However, persistence testing of this new protocol (“8Hyb”) revealed that LTP decayed more rapidly than effects induced by 50T HFS.

In chapter one, the experimenter sought to evaluate the induction and persistence characteristics of a modified TBS protocol (8Hyb). Previous studies have demonstrated that LTP persistence can depend on both the number and pattern of stimulation episodes administered (Abraham et al., 2002). In particular, more persistent forms of LTP are often observed when stimulation is repeated across a high number of episodes (Abraham et al., 1995; Abraham et al., 2002; Huang and Kandel, 2005). 8Hyb stimulation was based on a conventional TBS pattern, and differed only on the basis of pulse frequency. For comparative purposes, the distinction between 8TBS and 8Hyb protocols was based purely on pattern. Paradoxically, direct comparison of the 8Hyb protocol with 50T is somewhat more problematic. Both 8Hyb and 50T protocols share a 400 Hz pulse frequency; however, 50T HFS was structured as 500 total stimulation pulses, whereas 8TBS totaled only 320. In summary, 50T stimulation induced more persistent changes than 8Hyb; however, it is unclear if this difference is attributable to a difference in protocol structure, or simply a product of pulse number. Future investigations might
evaluate the persistence qualities of the 8Hyb and 50T protocol with a matched number of pulses.

Dentate granule cells do not generally fire at rates above 100 Hz (Rose et al., 1983). The experimental effect of 400 Hz stimulation within the dentate gyrus is profound (1.3.3); however, its physiological relevance is justifiably questionable. Winson and Dahl (1986) suggest that 400 Hz activation could be expressed through the cooperative action of multiple afferents firing in asynchronous activity. Abraham et al., (2007) demonstrated that 400 Hz induction of bidirectional synaptic plasticity within the dentate gyrus is dependent upon activation of both medial and lateral fibers of the perforant pathway. Spike timing dependent plasticity provides a method by which LTP and LTD could be coordinated between MML and OML regions of the granule cell dendritic tree; however, this effect requires that both MML and OML regions be active during periods of cell firing (Markram et al., 1997; Benuskova and Abraham, 2007). Overall, these studies serve to suggest that medial and lateral fibers are cooperative in their activation of the dentate gyrus. Field recording methods clearly implicate 400 Hz stimulation as an influential frequency within the dentate gyrus (Abraham et al., 1995; Abraham et al., 2002; Abraham et al., 2007). Crucially however, 400 Hz stimulation may be activating the dentate in ways that we, as yet, do not fully understand. The present investigation had the virtue of working within the intact neural architecture of the freely behaving rat. Subsequent investigation will be required to elucidate the specific properties of the dentate gyrus that are engaged by 400 Hz stimulation.
4.2 Chapter two review

4.2.1 Dendritic allometry

Data from high magnification ssTEM reconstructions has revealed that dendrite caliber, microtubule number and spine density vary systematically between layers of the granule cell dendritic tree; values were highest at the base of the tree, and decreased proportionally with distance from the cell body layer (2.3). Contemporary studies suggest that intrinsic excitability may serve to actively amplify signals within the dendritic tree (Johnston and Narayanan, 2008). However, physical properties of signal degradation suggest that inputs closest to the cell body are most influential in modifying cell firing (Rall, 1967; Carnevale et al., 1997). Results from chapter two revealed that the most peripheral regions of the granule cell dendritic tree had the lowest number of spines (cf. Fig. 2.3). At first appearance, this finding would seem to discount the influence of synaptic events occurring within peripheral regions of the dendritic tree. Specialized organelles and features of dendritic geometric may play a role in mediating synaptic transmission evoked within OML of the dentate gyrus.

The smooth endoplasmic reticulum (SER) is an interconnected organelle which spans throughout the dendritic tree, and frequently enter dendritic spines (Harris and Stevens, 1989; Spacek and Harris, 1997). Synaptic activity can trigger the release of SER calcium stores, and may serve to amplify excitatory transmission (Sandler and Barbara, 1999). SER can be clearly visualized and measured with ssTEM; however, very little is known regarding the presence of SER within the OML region of the granule cell dendritic tree, or the functional contributions it might make in amplifying synaptic transmission.
The spine apparatus (SA) is a specialized derivative of the SER which is present in approximately 15% of all spines within the MML of the granule cell dendritic tree (Kuwajima et al., 2010). Like SER, the SA has been implicated in calcium signaling, and may contribute to local protein synthesis (Spacek and Harris, 1997; Deller et al., 2007). More research is required to characterize the distribution and function of SA within the granule cell dendritic tree.

Low magnification Golgi studies have estimated the sum length of the granule cell dendritic tree to be ~3,221 µm. At its crown, the tree has spread to a width of 325 X 176 µm, and has branched into a series of proportionally smaller dendritic segments (Desmond and Levy, 1982; Desmond and Levy, 1984; Claiborne et al., 1990). These branch points are crucial important for interpreting the capabilities of the dendritic tree.

Data from chapter two revealed that average dendrite caliber was larger at the base of the dendritic tree than it is at the crown. However, additional consideration is required when making comparisons of this nature. As the granule cell dendritic tree progresses, it branches into subsequent units. This means that, while dendrites at the base of the tree may be larger, dendrites near the crown are more numerous.

The properties of dendritic allometry presented in chapter two suggest that measures of microtubule number, caliber, and spine density are positively correlated throughout the granule cell dendritic tree. In reference to the example provided above: a large caliber
dendrite positioned at the base of the dendrite tree would have a larger amount of microtubules and spines than a small caliber dendrite positioned at the crown. Crucially however, resources within peripheral regions of the dendritic tree may be conserved across multiple branches. Evidence from low magnification Golgi studies suggest that daughter branches are mathematically proportional in size to their parent branch (Desmond and Levy, 1984). This branching is not zero-loss; however, a considerable amount of dendrite caliber appears to be conserved within daughter branches. In summary, branch points represent a crucial dimension in the understanding of dendrite allometry. ssTEM investigation could provide the additional resolution required to characterize the division of dendritic resources at branch points. In contrast to the findings presented in chapter two, such studies might demonstrate that dendritic resources are largely conserved throughout the granule cell dendritic tree.

With these properties of dendrite allometry in mind, the relative significance of the OML requires reassessment. The OML region of the granule cell dendritic tree is populated by small caliber dendrites, each with proportionally low spine densities. When considered in isolation, the activation of any one segment within this region might exert an insignificant influence on cell activation. However, when activated across multiple segments, OML synaptic potentials can be seen as additive. Small segments could gradually merge into larger caliber dendrites, and synaptic potentials would summate as they propagate towards the cell body. In this way, the OML of the granule cell dendritic tree may be conceptualized as a watershed of synaptic activity.
Additional mechanisms of signal propagation may serve to enhance the impact of inputs arising from peripheral regions of the OML. Experimental finding from CA1 pyramidal neurons suggest that fEPSP amplitude increases relative to distance from the cell body (Magee and Cook, 2000). The cause of this distance dependent scaling is likely attributable to intrinsic excitability, and properties of active conductance within the pyramidal cell dendritic tree (Johnston and Narayanan, 2008). Comparable mechanisms of intrinsic excitability within the dentate gyrus could, in theory, act to equalize the ability of MML and OML inputs to evoke granule cell firing. As discussed previously, the electrophysiological expression of bidirectional synaptic plasticity is balanced between MML and OML regions of the dentate gyrus (Abraham and Goddard, 1983; Abraham et al., 2001); furthermore, this effect appears to be driven by temporal mechanisms of cell firing (Markram et al., 1997; Benuskova and Abraham, 2007; see 3.1). Accounting for the watershedding of synaptic inputs, and active properties of dendritic propagation, it is possible that MML and OML regions of the dentate gyrus could act as functionally equivalent "units" across which excitatory input is integrated (Katz et al., 2009). In summary, the granule cell dendritic tree may serve to separate firing events according to their synaptic origin (MML or OML); furthermore, the timing of these firing events may serve to coordinate plasticity between adjacent units. Perhaps most interesting, this coordination of plasticity between dendritic units could be structurally expressed though changes in excitatory synaptic resources. This topic is the subject of discussion in section 4.3.2.
4.2.2 Neurogenesis

Results from chapter three revealed that dendrite caliber could vary significantly across a single section of the granule cell dendritic tree. By implementing properties of dendritic allometry via statistical inference, the experimenter calculated that these differences in caliber could be associated with significant differences in spine density (2.3.2). This variability presents a unique challenge for the electron microscopist attempting to match sample frames for structural comparison.

The source of dendrite variability within single sections is not entirely known. The granule cell dendritic tree has a reasonably systematic branching pattern; however, deviations are not entirely uncommon (Claiborne et al., 1990; Desmond and Levy, 1984). It is possible that single section variability could be explained by the presence of small caliber dendrites which branched prematurely from the tree. As an additional consideration, the cell body layer (CBL) of the granule cell dendritic tree is not single file; some cell bodies are positioned at the top of the CBL, others are positioned at the bottom (Green and Juraska, 1985). In this sense, even if branching pattern was a consistent property of the granule cell dendritic tree, the relative position of cells within the CBL suggests that branch sizes could be different within the same section.

As a more dramatic explanation to this finding: the dentate gyrus is one of only two brain regions where neurogenesis is observed within the adult brain (Gage et al., 1998; Goldman and Luskin, 1998). Newly born cells do not have fully developed dendritic
trees; thus, single section variability could be partially attributable to the presence of new neurons (Kelsch et al., 2008). Interestingly, ssTEM could provide a unique approach to examining these cells within the mature brain.

Conventionally, newly born neurons have been distinguished from their mature counterparts with Bromodeoxyuridine (BrdU) labeling. BrdU is a synthetic thymidine analog which can be incorporated into the DNA of replicating cells, and consequently, used to identify cells which have recently divided (Taupin, 2007). As an alternative approach, doublecortin (DCX) labeling can be used to identify young cells by virtue of their endogenous protein content. DCX is a microtubule protein that is expressed exclusively during the first few weeks of cell maturity (Brown et al., 2003); thus, labeling of DCX protein may be used to distinguish young cells from old cells.

Unfortunately, BrdU and DCX labeling techniques are subject to a shared caveat. Both methods allow for selective labeling of newly born cells; however, to be of any use, the experimenter must be able to visualize these labels in order to identify and study newly born cells. Optical microscopists often use fluorescent tags to identify BrdU or DCX labels. Electron microscopes operate well below the wavelength of light; therefore, an alternative approach to fluorescent tagging is required to identify BrdU and DCX labeled cells.

3,3′-diaminobenzidine tetrahydrochloride (DAB) is an organic compound which may be used to tag the location of BrdU and DCX labels. Once the tag has been applied, DAB
can be photoconverted into an electron opaque precipitate and visualized under an
electron microscope. Unfortunately, the presence of DAB precipitate often obscures the
internal membrane of the cell, making it difficult to discern subcellular organelles.
ssTEM presents a viable solution to this problem.

Serial section transmission electron microscopy (ssTEM) is a method which uses
hundreds of micrographs to visualize and measure three-dimensional structures (Harris et
al., 2006). A structure of interest is first cut into ultrathin sections, and photographed with
a transmission electron microscope. Once micrographs are properly calibrated and
aligned, relevant structures can be followed through consecutive images, traced, and
reconstructed into three-dimensional models. In theory, DCX labeling and DAB
photoconversion could be performed on the first sections of a ssTEM image series. Once
identified, newly born cells could be followed through consecutive, un-labeled images of
the series.

Dendrite caliber has been shown to vary significantly within single sections of the
granule cell dendritic tree, and may contribute to bias when matching regions for
ultrastructural analysis (2.3.2). The source of this variation is unknown; however it may
be attributable to the branching pattern of the dendritic tree, the position of the cell body
within the CBL, and/or the presence of newly born neurons. ssTEM with DCX labeling
presents a novel approach by which newly born neurons could be measured with
unparalleled levels of accuracy, and visualized without compromising analysis of
intracellular organelles.
4.3 Chapter three review

4.3.1 Functional implications of dendritic allometry

Evidence suggests that the granule cell dendritic tree is structured to modulate the propagation of synaptic potentials. This effect is exerted through passive properties of dendrite geometry, and active processes of intrinsic excitability (Kim and Connors, 1993; Scott and Luo, 2001; Schaefer et al., 2003). Synaptic scaling is an experimental principle which suggests that a neuron strives to maintain a consistent level of synaptic excitability (Turrigiano, 2008). Bidirectional synaptic plasticity is a phenomenon whereby synaptic potentials can be persistently increased or decreased (Lee et al., 2000), and spike timing dependent plasticity is a principle which could be responsible for balancing bidirectional synaptic plasticity between regions of the granule cell dendritic tree (Benuskova and Abraham, 2007; Markram et al., 1997). Several studies suggest that bidirectional synaptic plasticity is associated with systematic increases and decreases in spine size (Lang et al., 2004; Okamoto et al., 2004), and Harris & Stevens (1989; Harris et al., 1992) demonstrated that these changes could be correlated with changes in PSD area. In addition to all of this, Bourne & Harris (2010) have recently demonstrated that PSD area can be homeostatically maintained across lengths of dendrite.

The dendritic tree is complex; notably, however, its function is characterized by several key themes. One particularly emergent theme concerns the balance of structures responsible for cell excitability.
Evidence from chapter one demonstrated that the granule cell dendritic tree is capable of expressing dramatic and persistent forms of bidirectional synaptic plasticity. Increases in fEPSPs within the medial projection to the MML were associated with simultaneous decreases in fEPSPs within the lateral projection to the OML.

In chapter two we described a series of proportional interrelationships which were found to exist between dendritic structure and specialized organelles. We reported that these relationships could be modeled with simple linear equations, and seemed to decrease relative to distance from the cell body layer. Furthermore, we acknowledged that subsequent research may demonstrate that these parameters are actually conserve within branches, and expressed equivalently across the extent of the dendritic tree.

In chapter three, we demonstrated that potentiation of the medial path resulted in a coordinated increase in MML spine head volume and PSD area. Interestingly, this change was associated with a coordinated decrease in IML spine head volume and PSD area. Subsequent analysis revealed that changes in spine head volume and PSD area could be related by a single linear function. Lastly, we demonstrated that structural changes appeared to balance between MML and IML regions of the granule cell dendritic tree. As a working hypothesis, the experimenter postulates that structural plasticity could be balanced throughout the granule cell dendritic tree. Additional data from the OML region must be attained to ensure that all principal layers of the dentate gyrus are represented in this analysis.
4.3.2 The “unit” of plasticity

As described previously (c.f. 4.3.1), a synergy of theories and experimental findings suggest that the dendritic tree is active in balancing excitability (Turrigiano et al., 1998; Turrigiano, 2008; Bourne and Harris, 2010). The researchers of the present investigation postulate that this balance could have a structural correlate.

To address this question accurately, the experimenter must define the operational “unit” of plasticity. As an example: If one spine within the dendritic tree became potentiated, it may increase in volume, PSD area, and contribute a greater amount of excitability to the dendritic tree as a whole. In this scenario, do all other spines within the dendritic tree scale to account for this change, or does change occur on more local “units” of scale? Overall excitability appears to be balanced within the dendritic tree; however, it is possible that subsidiary “units” of plasticity are active in modulating excitation on a more local scale. Several independent units could then balance to satisfy the desired state of the cell. Branch points provide an interesting conceptual dimension to this question.

In the present investigation, we excluded dendritic segments with branch points from structural analysis. The rationale for this criterion was simple: from subjective observations, microtubule number and dendrite caliber were reported to divide at branched points. Thus, to compare a branched dendrite to an unbranched dendrite would be invalid. Crucially however, this may be the point. Data from chapter two revealed that dendrite caliber was correlated with microtubule number and spine density. Golgi work from Desmond and Levy (1984) suggests that caliber is consistent across the extent of a
dendritic segment, decreasing only at branch points. As a hypothesis: each of these dendritic segments could be conceptualized as independent units.

According to the equations established in chapter two, dendritic units of equivalent caliber would have equivalent spine densities and microtubule content. Furthermore, results from chapter two suggest that dendrite caliber decreases relative to distance from the cell body; thus, the resource composition of each subsequent unit would decrease incrementally as branches extended to peripheral regions of the dendritic tree. Interestingly, these patterns of resource distribution could also apply to dendritic spine volume and PSD area. Data from chapter three revealed that spine head volume and PSD area were significantly larger in dendritic segments of the IML than segments of the MML. This finding suggests that PSD area and spine head volume might also scale with distance from the cell body layer, and change proportionally according to the relative dimensions of each dendritic segment. In summary, evidence suggests that resource distribution scales proportionally within individual dendritic segments, and varies systematically between dendritic segments. The experimenter has hypothesized that these segments could represent partitioned “units” of plasticity. In theory, it is proposed that activity dependent changes in cell excitability could be expressed through coordinated adjustment of synaptic efficacy within individual dendritic units. Furthermore, it is proposed that net excitation could be homeostatically maintained by balancing synaptic efficacy between active and inactive units of the dendritic tree. Data from chapter three provides interesting insight as to how these dendritic units might function under conditions of activation. It should be emphasized, however, MML and IML samples were
acquired from different animals (MML: animal LED 50; IML: animal LED 56, see 2.2.4). Though both animals were exposed to the same experimental manipulation, subsequent analysis will be required to confirm the operation of homeostatic regulation between layers of the same dendritic tree. This topic is the subject of an ongoing investigation.

Results from chapter three revealed that LTP induced within the medial path projection to the MML was associated with coordinated increases in spine head volume and PSD area. Notably, these changes were also associated with coordinated decreases in spine head volume and PSD area within the IML.

In both MML and IML regions, changes in spine head volume and PSD followed a common linear trend. This finding suggests that synaptic resources were scaled within IML and MML segments in a systematically fashion.

Previously described results revealed that control IML spine dimensions were significantly larger than control MML spine dimensions. Following LTP, MML spine head volume and PSD area became more like control IML measurements. Furthermore, IML spine head volume and PSD area became more like control MML dimensions (cf. Fig. 3.8E) Theoretically, these findings could reflect a homeostatic balance of synaptic efficacy between dendritic units. Increases in synaptic efficacy within MML segments would have increased the overall excitability of the cell. To balance this effect, synaptic
resources within dendritic segments of the IML were scaled down (cf. Fig. 3.8E). This result appears to represent a balancing of synaptic resources between layers.

The homeostatic mechanisms responsible for balancing cell excitability within the dendritic tree are largely unknown. In the previous section, we have outlined a hypothesis which highlights the role of dendritic segments. We propose that activity dependent changes in cell excitability could be expressed through coordinated adjustment of synaptic efficacy within individual dendritic segments, or “units”. Furthermore, we propose that net excitation could be homeostatically maintained by balancing synaptic efficacy between active and inactive units of the dendritic tree. Finally, we hypothesize that the balancing of dendritic units could be expressed as a systematic change in the distribution of synaptic resources.

4.3.3 The persistence of memory

Persistence is a fundamental quality of memory. The retention of learned information reflects the activation of an enduring change. The research detailed in this thesis is part of a collaborative study between the laboratories of Kristen M. Harris and Wickliffe C. Abraham; its purpose was to elucidate the structural mechanisms responsible for the expression of memory within the brain. Paramount to this, the experimenter sought to understand these mechanisms within the intact architecture of a freely behaving animal. In chapter one, we introduced and parameterized a cellular model of memory known as bidirectional synaptic plasticity. In chapter two, we introduced a structure of the brain known as the dentate gyrus, and characterized features of its ultrastructure which have
been implicated in the storage of memory. In chapter three, we demonstrated how memory related structures of the dentate gyrus change in association with bidirectional synaptic plasticity. These structures were analyzed 30 min following the induction of bidirectional plasticity.

The future direction of this study aims to characterize the expression of memory in its persistent form. Psychological investigations suggest that memory is, at very least, a two tiered system (Miller, 1956; Waugh and Norman, 1965). Short-term storage is responsible for retaining small amounts of information across limited periods of time. Long-term storage is responsible for retaining large amounts of information across extended periods of time. The process of consolidation describes the transition of information from short term to long-term stores, and often involves repetition. In many ways, this description of memory may seem like a gross oversimplification; however, it highlights several themes which are exemplified at both cellular and behavioral levels. Repetition is analogous to patterned activation of cellular network, and consolidation is analogous to the structural and functional changes a cell must undergo to maintain a memory.

In chapter one, we demonstrated that bidirectional synaptic plasticity could persist for months within the dentate gyrus. Previous research has revealed that this persistence is characterized by at least two discrete phases. The “early” phase of LTP takes place during the first 30 minute of induction, and is primarily characterize by the activation of protein signaling molecules. The “late” phase of LTP takes place approximately 2-3
hours following induction, and is characterized by its protein synthesis dependence (Sweatt, 1999). Evidence from chapter three suggests that the early phase of LTP is associated with increases in spine head volume and PSD area. If late-LTP is merely a continuation of the state induced during early-LTP, then experimenters might expect to view persistence as a prolonged increase in spine head volume and PSD area. Previous studies have demonstrated that large spines are more prevalent 6 h (Popov et al., 2004) and 24 h (Weeks et al., 1999; Mezey et al., 2004; Medvedev et al., 2010) following the induction of LTP in the MML, and may persist for as long as 5 days (Weeks et al., 2001). Crucially, however, it is unknown how this persistence is managed across adjacent regions of the dendritic tree; and furthermore, what organelles might be required to support the longevity of these changes. Previously mentioned organelles such as SER and SA (4.2) could play a role in maintaining protein synthesis and heightened states of calcium activation. Additionally, polyribosomes have been shown to enter dendritic spines 2-hr following LTP, and may play a role in synthesizing proteins required for persistence (Bourne et al., 2007b; Ostroff et al., 2002).

Several conceptual difficulties face experimenters wishing to characterize the structural mechanisms of late-phase bidirectional synaptic plasticity. The first of these issues concerns signal-to-noise. *In vivo* electrophysiological recording provides an ideal experimental preparation for examining persistence of bidirectional synaptic plasticity; changes may be induced, and then observed across multiple days, weeks, and months thereafter (Abraham et al., 2002). Crucially however, the magnitude of bidirectional synaptic plasticity is often highest at the time immediately after induction, and gradually
fades in the time following (Abraham et al., 1995). This effect may be attributable to the function of the dentate gyrus, it may be attributable to some inherent property of the induction method, or it may just be an accurate representation of memory erasure within an animal that is free to behave and form new associations. Whatever the case, bidirectional synaptic plasticity declines over time, and so too must the structural mechanisms initially responsible for its expression. The mechanisms of persistence may be examined at later time points; however, the experimenter may require the assistance of some new technology to distinguish synaptic mechanisms which have persisted since the initial phase of induction.

### 4.4 Future Directions

#### 4.4.1 ssTEM reconstructions from the OML of the granule cell dendritic tree

The electrophysiological complements of bidirectional synaptic plasticity are expressed as persistent increases (LTP) and decreases (LTD) in synaptic transmission. At present, we have reported that LTP was associated with coordinated increases in synaptic structures within the MML. Reconstruction from the OML will provide data concerning the structural expression of LTD.

In addition, we have shown that increases in MML synaptic structures were associated with complementary decreases in IML synaptic structures. This result suggests that structural changes in excitability may be balanced across regions of the granule cell dendritic tree. Reconstructions from the OML would ensure that all principal layers of the granule cell dendritic tree were represented in the understanding of this dynamic balance.
4.4.2 PSD composition

Results from chapter three revealed that bidirectional synaptic plasticity was associated within systematic changes in PSD area. The PSD contains a multitude of different protein signaling molecules, each exerting a different influence on synaptic function (Malenka and Nicoll, 1999; Kerchner and Nicoll, 2008). Immunogold labeling could be used to characterize the composition of the PSD, and identify molecules that have changed in response to bidirectional synaptic plasticity (Takumi et al., 1999). Of particular relevance, the experimenter is interested to quantify the presence of $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors within dendritic spines, and establish whether the prevalence and/or composition of these signaling molecules are coordinated between layers of the granule cell dendritic tree following the induction of bidirectional synaptic plasticity.

4.4.3 Dendritic allometry

In chapter three, we reported that individual segments of dendrite decreased in caliber, microtubule number, and spine density relative to distance from the cell body layer. As the dendritic tree extends into the molecular layer, it branches into increasingly numerous segments. The experimenter has hypothesized that dendrite resources might, to a certain extent, be conserved across multiple branches of the dendritic tree. To test this theory, researchers would need to characterize the division of dendritic resources at branch points. This could be achieved with ssTEM analysis.
4.4.4 Inhibition

Results from chapter one emphasized the role of inhibition in modulating the expression of strain and circadian differences in LTP. The experimenter suggests that the role of inhibition under these conditions could be evaluated with more extensive paired-pulse facilitation testing (Stuart and Redman, 1991). For circadian testing, fluctuations in inhibition could be examined by conducting test sessions across different phases of each animal’s light/dark cycle.

4.4.5 Dendritic units of plasticity

The experimenter has hypothesized that structural plasticity could be balanced within individual dendritic segments, or “units”. Furthermore, we have proposed that overall cell excitation could be homeostatically maintained by balancing synaptic efficacy between active and inactive units of the dendritic tree. In the present investigation, the experimenter examined a total of 25 dendritic segments, each ~200 section long, and spanning ~10 µm in length. Seress and Pokorny (1981) suggest that each dentate granule cell has 33.5 ± 2.50 dendrite segments, each averaging average 71.47 ± 4.56 µm. This is a prohibitively large sample size for conventional methods of ssTEM. As a potential solution, the Harris lab has recently been involved with the development of a Scanning Transmission Electron Microscope (STEM). The STEM is capable of imaging extremely large fields at high magnification; as an additional benefit, the imaging procedure is largely automated (Mendenhall et al., 2009). The experimenter proposes that consecutive dendritic segments could be analyzed by longitudinally sectioning the dentate granule cell dendritic tree, and imaging with the STEM.
4.4.6 Anesthetic influences on the structural expression of bidirectional synaptic plasticity

In the present investigation, we demonstrated that LTP induced within freely behaving rats was associated with significant increase in spine volume (3.3.2). A recent series of studies has reported that LTP induced changes in dendritic spines were accompanied by increases in spine head curvature (Stewart et al., 2008; Stewart et al., 2010). Notably however, these studies were conducted on anesthetized animals. Anesthetic compounds have been shown to modulate brain activity, and reduce neuropil kinetics (Kaech et al., 1999; Benrath et al., 2007;). The experimenter postulates that curvature could result when spines enlarge within the less-dynamic confines of an anesthetized neuropil. To test this hypothesis, LTP induced spine changes could be compared between anesthetized and freely behaving animals.

4.4.7 Neurogenesis

In 4.2.2, the experimenter outlined a technique using DCX labeling with ssTEM to analyze the structure of newly born neurons in the adult hippocampus. This technique may be implemented within the next sample set.

4.4.8 Additional dependent variables

The present investigation has focused exclusively on postsynaptic dendritic structures. Bidirectional synaptic plasticity is characterized by persistent changes in excitatory post synaptic potentials; however, it is likely that these changes are paralleled by several other
complementary changes within the neuropil. Many of these structures are visible within ssTEM micrographs, and could be measured and analyzed with three-dimensional reconstructions. Future research might focus on the role of presynaptic changes, the distribution and proximity of astrocytes, the location of polyribosomes, the location and balance of inhibitory synaptic contacts, and the presence of SER and SA within spines and across the dendritic tree.

4.4.9 Structures of persistence

The present investigation evaluated the structural correlates of bidirectional synaptic plasticity 30 min following induction. Analysis will soon begin on data sets prepared 2 hrs and 48 hrs following induction.

4.5 References


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