STEREOLOGY AND NEURONAL CONNECTIVITY OF THE RAT HIPPOCAMPUS: FROM 2D IMAGES TO 3D MODELING

by

Deepak Ropireddy
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Committee:

Dr. Giorgio A. Ascoli, Dissertation Director

Dr. James L. Olds, Committee Member

Dr. Ann B. Butler, Committee Member

Dr. Jyh-Ming Lien, Committee Member

Dr. James L. Olds, Department Chairperson

Dr. Timothy Born, Associate Dean for Academic and Student Affairs, College of Science

Dr. Vikas Chandhoke, Dean, College of Science

Date: 7/28/11

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By

Deepak Ropireddy
Master of Science
Texas A&M University-Commerce, 2002
Bachelor of Technology
National Institute of Technology, Warangal, India, 1999

Director: Giorgio A. Ascoli, University Professor
Molecular Neuroscience Department
Krasnow Institute for Advanced Study

Summer Semester 2011
George Mason University
Fairfax, VA
DEDICATION

This dissertation is dedicated to my wonderful wife and our adorable son. I am fortunate to have a loving wife, who in spite of her busy neurology residency curriculum, has been very patient and incredibly supportive of my doctorate education and research over the years. I would also like to dedicate this to my parents and brothers for their love, help and guidance and my mother-in-law who took extra-ordinary care of our son while I was busy with my studies.
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I would like to thank my beloved wife who supported me throughout the duration of my studies, and my parents and friends whom their understanding and endless love gave me strength. My special thanks go to Dr. Ascoli for guiding and encouraging me during my journey through these years. I am also greatly in debt to Dr. Olds for advising me and providing me with intellectual enrichment all along. I am thankful to Dr. Butler whom I benefited the most from her constructive advices and moral support, and to Dr. Jyh-Ming Lien whom I learned a great deal from. I also thank Dr. Bachus for her help with histological preparation and to Dr. Scorcioni for his guidance in the early phase of this project and for his contribution to the CA3 axonal morphometry project. My success in this endeavor would not have been possible without continuing support and feedback from the entire computational neuroanatomy group at Krasnow Institute. I also like to specially thank the NeuroMorpho.Org team for their hard work in making the valuable neuromorphological data available to the community.
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ABSTRACT

STEREOMETRY AND NEURONAL CONNECTIVITY OF THE RAT HIPPOCAMPUS: FROM 2D IMAGES TO 3D MODELING

Deepak Ropireddy, Ph.D.
George Mason University, 2011
Dissertation Director: Dr. Giorgio A. Ascoli

Synaptic micro-circuit properties in neuronal networks are fundamental to information processing in brain systems. These properties, intrinsic to brain structure, are directly related to neural activity and function. Characterization and mapping the micro-circuitry has been a long standing challenge in mammalian hippocampal research. One of the stumbling blocks in this endeavor is the absence of a construct to integrate the available anatomical knowledge. Thus, integrating hippocampal anatomy from neuronal dendrites to whole-system level may help explain its relation to spatial navigation and episodic memory. This dissertation describes a novel approach to map existing morphological data onto an in-silico based template of the rat hippocampus and address important scientific questions on macroscopic stereology and potential connectivity between various cell types in the hippocampus. Towards this aim, we digitally traced the cytoarchitectural boundaries of the dentate gyrus (DG) and areas CA3/CA1 throughout their entire longitudinal extent from high-resolution images of thin cryostatic sections of adult rat
brain. A custom developed computational framework further extends the functionality of this model by transforming the digital trace stack into volumetric representations with arbitrary voxel size. Next, virtually embedding 1.8 million neuronal morphologies stochastically resampled from 244 digital reconstructions, emulated the dense packing of granular and pyramidal layers, and orienting the principal dendritic axes according to local curvature.

Utilizing this unique systems level digital representation, the first part of this research study reports and discusses the macroscopic stereological properties such as volumes, and neuropil occupancy ratios across the various cytoarchitectonic layers of DG & CA regions in the rat hippocampus. The neuropil occupancy reproduced recent electron microscopy data specifically measured in a restricted location. Extension of this analysis across each layer and sub-region throughout the whole longitudinal extent of the hippocampus revealed highly non-homogeneous dendritic density. In CA1, dendritic occupancy was >60% higher temporally than septally (0.46 vs. 0.28). CA3 values varied both across subfields (from 0.35 in CA3b/CA3c to 0.50 in CA3a) and layers (0.48, 0.34, and 0.27 in oriens, radiatum, and lacunosum-moleculare, respectively). Dendritic occupancy was substantially lower in DG, especially in the supra-pyramidal blade (0.18). The computed probability of dendro-dendritic collision significantly correlated with expression of the membrane repulsion signal DSCAM. These heterogeneous stereological properties reflect and complement the non-uniform molecular composition, circuit
connectivity, and computational function of the hippocampus across its hippocampal-
transverse, longitudinal, and laminar organization.

The second part of this dissertation reports and discusses the potential synaptic
connectivity computed by mapping and orienting digital axonal reconstructions of five
principal and two CA3 interneuron classes across the CA pyramidal dendritic network
within this 3D model. In the mammalian cortex, structural plasticity of spines and
boutons makes ‘potential synapses’ functionally relevant to learning capability and
memory capacity. To date, however, potential synapses have only been mapped in the
surrounding of a neuron and relative to its local orientation rather than in a system-level
anatomical reference. Analyzing connectivity in terms of close spatial appositions
between axons and dendrites could thus bridge the opposite scales, from synaptic level to
whole systems. We report the potential connectivity onto pyramidal cell dendrites from
the axons of a dentate granule cell, three CA3 pyramidal cells, one CA2 pyramidal cell,
and 13 CA3b interneurons. The numbers, densities, and distributions of potential
synapses were analyzed in each sub-region (e.g. CA3 vs. CA1), layer (e.g. oriens vs.
radiatum), and septo-temporal location (e.g. dorsal vs. ventral). The overall ratio between
the numbers of actual and potential synapses was ~0.20 for the granule and CA3
pyramidal cells. All potential connectivity patterns are strikingly dependent on the
anatomical location of both pre-synaptic and post-synaptic neurons.
CHAPTER 1: INTRODUCTION

Structural complexity of mammalian brain systems

Interactions occurring among processes in the biological world are highly complex and non-linear in nature. This phenomenon is strikingly evident in brain circuits as in cellular signaling networks, but the architecture of brain circuits stands out in the magnitude of this complexity. The spatial organization and connectivity between cells within these complex circuits play a pivotal role in spatial and temporal processing of the networks within. The formation of synaptic microcircuits and their spatial organization in central nervous system is largely an open question. Specifically, how the complex networks of neurons interact and form canonical circuits both at local and global level is an intriguing and nontrivial question (Shepherd, 2004). This inherent structural complexity is extremely difficult to analyze and thus dissect the complex network organization and their interaction in an experimental preparation at systems level. This gives us a wonderful opportunity and the need to study the complex spatial organization of neuronal networks and the structure-function relationship by reverse engineering brain systems.

Reverse engineering brain systems: The case for rat hippocampus

Reverse engineering brain systems offers great advantages in connecting the cellular to the systems-level architecture. In contemporary neuroscience, there are many databases
that successfully disseminate high-resolution histological brain images to the neuroscience community at large (BrainMaps.org, Mikula et al., 2007). On the other hand, one can effortlessly download neuronal digital reconstructions pertaining to a specific species and brain region from NeuroMorpho.Org database (Ascoli et al., 2007) and perform detailed morphological analysis. Obtaining comprehensive three-dimensional models from the histological images and mapping the morphological data is a viable approach to combine these complementary anatomical data at cellular and systems level. With this novel methodology, one can address the challenging aspect of relating connectivity from synaptic to systems level, in addition to, extracting valuable macroscopic stereological properties evaluated across entire brain regions.

This approach is particularly amenable and important for the rat hippocampus for at least three reasons. The first obvious advantage is that this study results in a better understanding of the structural complexity of the hippocampus and thus potentially contributing towards understanding the neural processes behind memory consolidation and spatial navigation by linking structure and function. Secondly, the neural architecture layout is particularly reproducible in an in-silico model as the geometric orientation and alignment of the principal cell dendrites can be approximated by layer curvatures as revealed in a Golgi stained section of the hippocampus. Thirdly, the connectivity architecture and network topology in the hippocampus is less modular compared to the neocortical columnar structure. An individual pyramidal cell extends thousands of synapses over macroscopic distances (~cm). As such, it is extremely difficult to address
the micro- to macro-scale connectivity between different cell types in the hippocampus using conventional experimental preparations. Thus, this reverse engineering approach can be used to address the fundamental challenge of mapping connectivity at synaptic to whole systems level. In a nutshell, taking the opportunity and need further, this dissertation is a humble effort to reverse engineer the detailed structure of the rat hippocampus and address important scientific questions on macroscopic stereology and potential synaptic connectivity patterns between various cell classes.

**Organization of dissertation**

This dissertation consists of four chapters and two appendices based on three published articles (chapters 2, 4 & 5), and one manuscript finalizing for submission (chapter 3). Except for part of the work described in Chapter Four and part of the experimental procedure, as specified in Chapters Two and Three, the candidate conducted and carried all the research work described in the rest of the three chapters. Following is a synopsis of each chapter and appendix and the specific contributions of the candidate with respect to chapter Four.

Chapter Two introduces to the reader about “Computational Neuroanatomy of the Rat Hippocampus” and how it can be applied to clinical pathologies like epilepsy. The work described in this chapter is a preview to what follows in the next three chapters related to macroscopic stereology and potential synaptic connectivity. The experimental setup for constructing the 3D model are discussed in detail in chapter Three, though briefly
mentioned in this chapter. Additionally, the biophysical importance and influence of layer-specific GABAergic synapse position on a CA1 pyramidal neuronal firing pattern in the context this anatomical framework is presented and discussed.

Chapter Three describes the detailed methodology in constructing the complete three dimensional model of the rat hippocampus from high-resolution histological images of a rat brain and the design of the computational framework for the sub-region and layer based volumetric representation. This chapter also describes the methodology to compute the layer based macroscopic stereological properties such as absolute volumes, neurite occupancy ratios, and dendritic orientation analysis across the DG and CA sub-regions. Results with respect to layer and region specific differences in these macroscopic stereological properties are discussed and presented in this chapter. The heterogeneous stereological properties presented in this chapter reflect and complement the non-uniform molecular composition, circuit connectivity, and computational function of the hippocampus across its hippocampal-transverse, longitudinal, and laminar organization.

In Chapter Four, a new methodology is described to reconstruct fully labeled axonal morphologies by augmenting conventional tracing techniques with computational approaches. This chapter describes the methodology for digitally reconstructing three rat hippocampal pyramidal neurons that were intracellularly filled from different CA3 sub-regions. Quantitative morphometric differences between these three neurons and their length distributions in various sub-regions and layers is discussed and presented. The
CA3 axonal reconstructions were first manually traced using conventional tracing techniques by Bonnie Lasher and later digitized using computational approaches developed by Dr. Scorcioni. The candidate performed the CA3 axonal morphometry research study and incorporated the axons into the 3D hippocampus framework and analyzed their distributions in various sub-regions and layers and drafted the paper.

Chapter Five presents the implementation of existing mathematical framework to compute potential synapses based on axo-dendritic overlaps oriented within this 3D template. Potential synaptic connectivity patterns of the dentate granule mossy fiber, proximal and distal CA3c axons, CA3b and CA2 axons to the CA pyramidal dendritic network are presented and discussed. Specifically, the differences in the number of potential synapses within the various sub-regions and layers are presented for these five principal axons. Additionally, the potential connectivity of 13 CA3b inhibitory interneurons to the CA3 pyramidal network are also presented and discussed. All potential connectivity patterns are strikingly dependent on the anatomical location of both pre-synaptic and post-synaptic neurons.

The two appendices at the end of the dissertation contain details on the 3D hippocampus voxel database that is available to download and the computational framework to compute the potential synapses for use as a manual for users as well as programmers for future use. All the code, scripts and run time executables are included in the digital version of this dissertation.
Conclusion

In conclusion, the scientific significance and implications for this research are substantial and has the potential to advance our knowledge and understanding of the organization of the micro-circuitry within the hippocampus. In most of the previous computational modeling studies, there has been a missing link from cellular to systems level connectivity. Incorporating these anatomically realistic connectivity patterns into these models would give another dimension to these models and thus allow the study of how spatial information processing in the hippocampus leads to unique cognitive functions such as spatial navigation, short term memory and memory consolidation.
ABSTRACT

The hippocampus subserves important cognitive functions of the mammalian brain, including the consolidation of episodic and declarative memories in humans, and spatial navigation in rodents. Much is known about hippocampal anatomy, electrophysiology, and behavioral involvement. Yet, the relationship between structure, activity, and function in the hippocampus is far from obvious. To aid data integration between the cellular and system levels, we completely reconstructed and segmented the fine cytoarchitectural structure of the rat hippocampus from thin cryostatic slices. A custom developed computational framework transforms the digital trace stack into volumetric representations with arbitrary voxel size. This enables the 3D embedding of morphological reconstructions of individual neurons, oriented according to the hippocampal-transverse and longitudinal hippocampal curvatures. Virtually packing the neuropil volume of all layers and subregions with available cellular arborizations, allows the estimation of macroscopic stereological properties such as space occupancy and
neuritic overlap. We present a preliminary volumetric analysis of the individual
cytoarchitectural layers within the whole hippocampus and illustrate the stereological
analysis of spatial occupancy and overlap for a portion of the dentate region. Moreover,
we describe the suitability of this approach to compute potential synaptic connectivity
patterns for specific cell classes as a function of local spatial position. Finally, we
exemplify the use of a single cell computational model to examine the regulation of
neuronal spiking by synaptic inhibition, a phenomenon particularly relevant to the
pathophysiology of epilepsy. Our novel anatomical construct could lead to a dramatic
improvement in the realism, predictive power, and experimental relevance of these
numerical simulations.

INTRODUCTION

The cognitive role of the mammalian hippocampus in learning and memory gained
recognition after the seminal clinical case of the epileptic patient HM, whose bilateral
surgical resection of the medial temporal lobes resulted in a severe impairment in
forming new memories (Scoville and Milner, 1957). Countless studies over past decades
have corroborated the involvement of the hippocampus in the storage and retrieval of
episodic memory in humans (Eichenbaum and Cohen, 2001; O’Keefe and Nadel, 1978;
Squire and Zola-Morgan, 1991) and in the representation and encoding of spatial memory
in infrahumans (Eichenbaum and Cohen, 2001; Morris et al., 1982; O’Keefe and Nadel,
1978). The neural substrates behind these important cognitive processes have been under
rigorous investigation over the last couple of decades. In particular, the rodent hippocampus has become and remains one of the most popular and active research areas in neuroscience.

This endeavor has greatly enhanced our understanding of hippocampal structure and physiology. Anatomical studies have generated a wealth of detailed knowledge on the organization of the intrinsic hippocampal circuitry (Witter and Amaral, 2004), revealing greater complexity than imagined with the “lamellar hypothesis” embraced by earlier physiologists (Andersen et al., 1971). At the cellular level, modern day experimental and computational techniques (Ascoli et al., 2001; Van Pelt et al. 2001) have highlighted tremendous morphological variation between and within neuronal classes in the hippocampus (Cannon and Wheal, 1999; Claiborne et al., 1990; Ishizuka et al., 1995; Pyapali et al., 1998; Rihn and Claiborne, 1990; Scorcioni et al., 2004). With respect to biophysical properties, hippocampal neurons have been shown to possess a rich array of active conductances (Migliore and Shepherd, 2002; Reyes, 2001), which play a decisive role in shaping electrophysiological behavior. At the network level, population oscillations such as theta, gamma and ripples (Buzsáki, 1989, 2002; Buzsáki and Draguhn, 2004; Traub et al., 2000) are theorized to play a key role in the memory consolidation processes.

Several computational studies attempted to investigate directly the relationship between structure and activity over the past decade, bridging the two fields of anatomy and
physiology at the level of single cells (Krichmar et al., 2002; Mainen and Sejnowski, 1996; Migliore et al., 1999; Migliore, 2003; Migliore et al. 2004; Poirazi et al., 2003a; Poirazi et al., 2003b). In particular, the compartmental simulation approach incorporates complete morphologies and appropriate ion channel distributions, providing fundamental insights into the link between neuronal geometry and function and complementing experimental evidence (Schaefer et al., 2003; Vetter et al., 2001). In spite of these advances, the functioning of the hippocampus is far from understood at the system level.

In particular, the difficulty to characterize the details of anatomical structure comprehensively and quantitatively continues to constitute a formidable challenge in systems neuroscience, which is particularly non-amenable to direct experimental investigation. Two specific characteristics of cortical architecture stand out as major impediments in this quest. Firstly, the principal cells have diffuse projections spread over all spatial directions (e.g. in the hippocampus proper, both septo-temporally and in the hippocampal-transverse direction, as observed by e.g. Li et al., 1994). How this intrinsic architecture of the circuitry contributes to cognitive function remains an open question. Secondly, cortical principal cells are heavily modulated by GABAergic inhibition across the dendritic trees as well as peri-somatically. At least 16 distinct types of interneurons have been described in the CA1 region of the hippocampus alone (Freund and Buzsáki, 1996; Somogyi and Klausberger, 2005), identified through their electrophysiological characteristics and specific calcium binding markers, providing both feed forward and feedback inhibition to the pyramidal cells. These complex interneuronal networks play a
prominent but as of yet not fully determined role in the generation of population oscillations.

The local “canonical” circuits within the broader organization of the hippocampus are determined by the position of synapses and further modulated by the biophysical properties underlying synaptic integration, such as calcium spike propagation (Shepherd, 2004). Synaptic circuitry constitutes an important component in capturing network dynamics, which is interdependent with and complementary to the connectivity within and between the various neuronal classes. Activity dependent changes in synaptic efficiency through long-term potentiation (Bliss and Collingridge, 1993) validated Hebb’s original hypothesis (Hebb, 1945). These network changes, possibly accompanied by the continuing evolution of cell assemblies (Harris et al., 2003; Maurer et al., 2006), could underlie a fundamental functional mechanism in the hippocampus. The importance of structural studies in this endeavor cannot be overemphasized.

In order to integrate the current architectural and functional knowledge, and to attempt linking the structure and activity, a framework is needed to relate information regarding the characteristic of intrinsic connectivity patterns, like small-world networks (Watts and Strogatz, 1998), all-to-all (Kalisman et al., 2005) or random graphs, to the simulation of anatomically realistic neural networks (Ascoli and Atkeson, 2005; Bernard and Wheal, 1994; Patton and McNaughton, 1995). In this regard, we view a complete, 3D digital model of the hippocampus with a cellular level resolution as a necessary condition. In
addition to mapping out potential connectivity patterns, such a construct would enable a layer-by-layer stereological analysis across the entire hippocampus, which is completely impractical with current experimental techniques (Fiala and Harris, 2001; Schmitz and Hof et al., 2005).

In keeping with these goals, we have completed a full digital reconstruction of the adult rat hippocampus from thin cryostatic sections. Moreover, we are developing a robust computational framework to embed reconstructed cells and transform the digital trace stack into arbitrary voxel size. Previous efforts in this direction were initiated by Scorcioni et al. (2001) in the dentate gyrus, but were not extended to a volumetric representation. Preliminary results of this work were presented in conference formats (Ropireddy et al., 2005). In this chapter, we describe the methodology and potential applications of our approach.

We start by reporting a detailed volumetric analysis of individual cyto-architectonic layers and of the total volume of the hippocampus. We then continue with an illustration of the potential for stereological analysis when a section of neuropil volume is packed with principal cells. This enables us to estimate the distribution of space occupancy fractions and overlaps of dendritic trees and their variations upon the position within the cyto-architectonic layers and the septo-temporal position. Moreover, we discuss future extensions to estimate the intrinsic potential connectivity patterns in the hippocampus using available digital reconstructions of axonal morphologies. Finally, we offer a
compartmental model-based example of the importance of synaptic position (perisomatic vs. distal) in the inhibition of principal cells. Thus, the construct described in this chapter could be qualitatively and quantitatively utilized towards investigating the biophysics of excitatory and inhibitory dynamics, and their relevance to seizure generation and spread in epilepsy. More generally, this approach could potentially contribute to a better understanding of the structure, function and activity of the hippocampus as well as of their inter-relations, bringing us closer to the formulation of a unified theory of the resulting cognitive processes.

Experimental Design

A. Image and Data Acquisition

Brains of three male 45 day old Long-Evans rats (226 - 237 g) were cryostatically sectioned at 16 µm in dorso-ventral, ventro-dorsal and lateral-medial orientations, respectively, and mounted on gelatin-subbed slides as described in Chapter Three. The dorso-ventral approach resulted in the least distortion in the hippocampal region and was thus selected for further analysis (presented here). In order to evaluate the shrinkage factor in the two dimensional plane of the section, we acquired whole mount pictures of the block face prior to cutting for 10 slices in the medial portions of the hippocampus, using a Kodak digital camera at 300 dpi resolution. Slides were Nissl stained with standard protocol (Simmons and Swanson, 1993) and cover-slipped. Images were
acquired using an EPSON 3200 dpi scanner and contrast enhanced using the Matlab routine *imcontrast*. By overlaying these whole mount pictures over the corresponding histological scanned images, we computed a shrinkage factor of $7(\pm 0.78)\%$, assumed isotropic in the XY plane.

After loading the image stack into the Reconstruct tool (Fiala, 2005) with appropriate pixel/µm conversion (see Figure 2.1), a mid-line guided image alignment was manually performed (see Figure 2.1B). For every section, we traced inner and outer boundaries of the cyto-architectonic layers of the hippocampus based on Swanson’s and Paxinos’ standard rat atlases as validation references. Each resulting digital contour consisted of a set of pixels representing these boundaries as closed polygons (see Figure 2.1A). In particular, the following layers were identified and traced: stratum granulosum (GC), stratum moleculare (ML) and hilus proper (HI) of the dentate gyrus; and stratum lacunosum moleculare (LM), stratum radiatum (RA), stratum pyramidale (PC) and stratum oriens (OR) of Ammon’s horn. Missed slices (42 out of 290) were interpolated based on their neighboring sections (see Figure 2.1C).

**B. Triangulation Algorithm and Surface Representation**

Each individual pixel-based closed polygon representing a cyto-architectonic layer boundary in a given section was ‘triangulated’ to identify or ‘fill’ all pixels inside the
contour, representing an inner position of the respective anatomical subregion. The novel algorithm, written in C++ and illustrated in Figure 2.2, uses circular linked lists

![Figure 2.1: Image and data acquisition: (a) An example of a rat brain Nissl stained section captured with EPSON 3200 dpi scanner. Inset shows the dorsal hippocampus at a magnified resolution. Clearly, the natural cyto-architectonic layers in the hippocampus are distinguishable leading to a reliable manual segmentation. (b) Preliminary manual mid-line guided registration (c) Digital representation of a contour as a “closed pixel based polygon” (d) Manual segmentation of the internal and external boundaries of the hippocampus. Three different rostro-caudal sections are shown depicting the dorsal, ventral and medial/caudal hippocampus. A surface view of the hippocampus in the medio-lateral view is also shown.](image-url)
(Kernighan and Ritchie, 1998) and is applicable to both convex and concave polygons. This procedure is analogous to the Marching Cube approach (Lorensen and Cline, 1987), but extends it beyond iso-surface rendering of volumetric data. Additionally, this process also estimates the centroid of the polygon. The locations of the outer boundary centroids of each individual section were used to refine further the manual registration. In particular, ten zones were visually identified based on the geometrical discontinuity of these boundaries along the rostro-caudal axis. For example, the ventral hippocampus emerges approximately at one-third of the rostro-caudal extent (slice 105) and the dorsal and ventral regions merge together at one half of the same span (slice 146). The segregation into these ten zones accounted for these changes and avoided the drastic shift in the centroid position between adjacent boundaries. A three-point average was then applied on the centroid location until convexity or concavity was reached in each separate zone. Finally, every section was translated based on the new centroid coordinates. This approach yielded a smooth and satisfactory post-alignment registration.

The resulting data can be displayed based on surface or volume. Surface rendering (Figure 2.3A–G) is particularly amenable to visualization in Virtual Reality Modeling Language (VRML, Ames et al., 1997). Voxel-based volume representation (Figure 2.3H,I), on the other hand, enables the direct implementation of stereological analyses. The triangulation/filling algorithm described above can yield an arbitrary pixel size, or resolution of the digital reconstruction. In order to maintain 3D isotropy (cubic voxels), a
shrinkage-corrected pixel size of 16 μm was selected, corresponding to the nominal cryostatic section thickness.

![Flowchart of the steps to triangulate the 'pixel-based closed polygon' and pixilate the individual triangles.](Image)

**Figure 2.2**: Triangulation and filling algorithm. Top: schematic representation of the process. Bottom: flowchart of the steps to triangulate the ‘pixel-based closed polygon’ and pixilate the individual triangles. The program initially reads the contour and structures the vertices of the polygon in a circular linked list with the left most vertex/node (‘least X value’) as its head. The recursive procedure starts with forming a speculative triangle with the head and its adjacent nodes and checks for any vertices/nodes inside this triangle. If none are present, the triangle is pixilated by changing the corresponding 2D array null values into ‘1’s. The first node is then removed, the list re-sorted and the recursive triangle check repeated. If a vertex/node is present inside the triangle, a new triangle is formed with the head node, inside vertex/node and the left most other vertex/node. This triangle is pixilated in the 2D array.
and, if this operation splits the lists into two, the iteration is applied to both. The recursive triangulation ends when there are no additional vertex/nodes and the final triangle is pixilated.

Figure 2.3: Surface (A–G) and volumetric (H,I) views of the outer and inner boundaries. ‘S’ and ‘T’ mark septal and temporal poles respectively. (A) Stratum granulosum, (B) stratum moleculare, (C) hilus proper, (D) stratum lacunosum moleculare, (E) stratum radiatum, (F) stratum pyramidale, (G) stratum oriens, (H) middle portion of stratum pyramidale, (I) middle portion of stratum granulosum.

C. Volumetric Analysis

The computational framework described above decomposes the 3D volume of the hippocampus into 4096 m³ (16 µm*16 µm*16 µm) voxels. The centers of the voxels
within the granule and pyramidal cell layers are assigned to correspond to a soma location, approximating the tight neuronal packing of principal cells in the real tissue (Claiborne et al., 1990; Ishizuka et al., 1995). The resulting number of soma positions in the granule cell layer covering the whole septo-temporal extent was $\sim 0.9 \times 10^6$. For comparison, the stereological estimates are around $10^6$, varying with the age and strain of the animal (Gaarskjaer, 1978; Boss et al., 1987; Amaral et al., 1990). Throughout the hippocampal volume, voxels are named according to their cytoarchitectonic layers in order to facilitate information retrieval within the computational framework. The volume of each layer is computed by voxel counting or by summing the total areas of individual triangles multiplied by the section thickness (16 $\mu$m). The total hippocampal volume, given by the sum of the seven individual layers (including both dentate gyrus and Ammon’s horn) across the rostro-caudal extent amounted to 34.89 mm$^3$.

The reconstructed volume was analyzed based upon hippocampal sub regions, layers and stereotaxic-transverse slicing order (Figure 2.4). Volume distributions are not uniform within the hippocampus, with the medial region contributing more (16.25 mm$^3$) than the dorsal (12.31 mm$^3$) and ventral (6.33 mm$^3$) regions. Percentage wise, the rostro-dorsal volume accounted for 35% of the hippocampus proper, with the rostro-ventral region amounting to 18% and the remaining portion (over 46%) taken up by the caudal hippocampus. In the highly curved posterior (or caudal) regions (Figure 2.4B), Ammon’s horn (CA) regions were altogether nearly three times larger than dentate gyrus (DG)
volumes, but they had similar sizes in the rostral portions (ventral plus dorsal volumes: DG $\sim 7$ mm$^3$, CA $\sim 11$ mm$^3$). The CA layers in the posterior region exceeded those in the dorsal or ventral regions (Figure 2.4A), but this relation was reversed for DG layers, where the dorsal portion is predominant (Figure 2.4C). Interestingly, LM is least in the ventral regions compared to the dorsal and medial regions (Figure 2.4F), while the RA layer is predominantly in the medial region (Figure 2.4 A,C,D). The change in volume fraction in the stereotaxic-transverse slicing order is more incremental for CA (except for LM) than for DG layers (Figure 2.4E). These volumetric measures are consistent with (but substantially extend) earlier reports (Boss et al., 1987; Tinsley et al., 2001).
Figure 2.4: Volumetric measures of the reconstructed hippocampus. Absolute (A, B, D) and relative (C, E, F) analyses refer to individual layers (A, C, D, E, F), regions (B), and sections (A, B, C, F). Dorsal, ventral and medial sections, demarcated by landmarks as shown in Figure 2.1C, encompass slices 1–145, 105–145 and 146–290, respectively.

D. Cellular Embedding and Spatial Occupancy

The volumetric representation of the hippocampal reconstruction lends itself to further stereological analysis. In particular, the inclusion of cellular morphology (e.g. 3D dendritic arborizations) in this construct enables the estimation of spatial occupancy and
overlaps, as well as of the potential connectivity across various neuronal classes in the hippocampus. An important prerequisite for this kind of analysis is the ability to embed reconstructed cells within the proper volume based upon their location and alignment in the hippocampus. In our framework, digital morphological reconstructions of pyramidal and granule cells, available at NeuroMorpho.Org (Ascoli, 2006), are algorithmically embedded based upon the following criteria: (a) the first principal axis of the dendritic arborization passes through the soma and is perpendicular to the cellular layer (Scorcioni et al., 2002); (b) the second (orthogonal) axis lies on the septo-temporal plane (Claiborne et al., 1990; Ishizuka et al., 1995); (c) the third axis is orthogonal to the above two. The 3D coordinates of the dendritic and axonal arborizations are read from the digital reconstruction files (Ascoli et al., 2001) and rotated along the above principal axes using standard numerical recipes (Press et al., 1992).

VRML files can be generated on the fly for visualization purposes with the freeware vrmlview viewer (www.sim.no). Figure 2.5 illustrates renderings of such embedding along with septo-temporal and coronal views. This process accurately reflects the organization of the neuronal arbors observed in vitro: the granule cells dendritic tree spread in the septo-temporal and hippocampal-transverse directions in our construct averaged 351.2 µm and 170.59 µm, respectively, compared to the reported experimental values of 347 µm and 182 µm, respectively (Claiborne et al., 1990).
Figure 2.5: A macroscopic view of granule dendritic tree (blue) embedded within the appropriate location and orientation shown in the septo-temporal view. Further examples of reconstructed pyramidal cells embedded in various (algorithmically identified) CA subregions including CA3c, CA3b, CA2, CA1 proximal, CA1 medial, CA1 distal shown in coronal view. Also shown are inner cellular and outer boundaries with oriens in pink, pyramidal layer in yellow, molecular layer in green, granule layer in red.

We used these data and approach to estimate the dendritic occupancy and overlap as a function of the 3D position. Spatial occupancy is the volume fraction occupied by
embedded dendrites and averaged across all the voxels of a given sub region and cyto-
architectonic layer. Spatial overlap is the number of occurrences in which one and the
same 3D location is occupied by distinct dendritic branches. Here, we present the results
of the initial analysis carried out on a portion of the dentate gyrus. In particular, we
embedded replicas of 39 reconstructed granule cells (Rihn and Claiborne, 1990) in their
corresponding supra-pyramidal blade locations within the middle third of the septo-
temporal extent.

The septo-temporal length of the whole hippocampus calculated along the centroid
positions is 6.50 mm, which leaves 2.17 mm for the middle third portion as the extent
available for cell packing. However, voxels at the edges of this extent are not suitable for
estimation of space occupancy and overlaps, because they are only invaded by the
arborizations of the cells from one direction and not the other. To account for this edge
factor, the volume that encompasses half of the maximum arborization span in the
corresponding orientation (the tree ‘depth’) at both of the edges is discarded from the
computation of spatial occupancy and overlap. In turn, the region of interest corresponds
to the septo-temporal range from 2.29 mm to 4.19 mm. An analogous edge factor is also
applied in the hippocampal-transverse direction, thus preventing the computation of
space occupancy and overlap in the volume corresponding to half of the maximum tree
width at the dentate crest and tip (the edges of the suprapyramidal blade). Even though
these volumes are excluded from the computation of space occupancy, they are still
sampled for the embedding process.
In order to emulate the high packing density of principal cells in vivo, all granule layer voxels within the range of potential overlap with a given position are embedded with a granule cell soma and the corresponding arborization is oriented accordingly in the surrounding space. The region of interest is sequentially traversed in unit boxes crossing the entire granular and molecular layers and sized as the average hippocampal-transverse and septo-temporal spreads of the 39 available granule cells. The spatial range of potential overlap whose granular layer needs to be packed with embedded cells to estimate occupancy in these unit boxes extends half the maximum width and depth in both directions. The resulting embedding space around each unit box is ~ 825 µm wide and ~ 430 µm deep.

From the pool of reconstructed cells, whose reported position (Rihn and Claiborne, 1990) is consistent with the appropriate hippocampal-transverse location are sampled for each voxel. To approximate the natural alignment variability of the principal dendritic component with the direction normal to the local surface, the primary axis of each cell is stochastically tilted between 0 and 5 degrees around a random (0–360 degree) rotation angle. The resultant direction falls within a 5 degree cone centrally aligned with the original normal axis. The other two orthogonal axes are correspondingly adjusted for both the tilt and rotation factor.
The distance from the granule cell soma (or initial embedding voxel) to the outer molecular layer boundary (hippocampal fissure) is also measured along the unit normal. The vertical span of the dendritic tree (height) along the normal vector is then calculated and used to scale the geometry of the tree appropriately. In particular, the three coordinates and diameters of all branches in each embedded neuron are multiplied by the ratio between the soma-fissure distance and the original dendritic height, ensuring that the farthest tips of the dendrites terminate at the end of the molecular layer.

To evaluate efficiently the spatial occupancy of dendritic trees, we sequentially probe the voxels that fall within the three-dimensional span of each given tree. This process is repeated through all the soma-points. Digital reconstructions of neuronal morphology consist of sets of cylinders. Three conditions are checked to determine whether a cylinder intersects a voxel. The first tests for the presence of the two cylinder end points in (or on the planes of) the voxel. If this condition is satisfied, the entire volume of the cylinder is added into the occupied fraction for the voxel. If only one end point lies inside the voxel, the intersection between the cylinder and one of the face planes of the voxel is calculated and the corresponding (partial) volume added to the occupied fraction. If neither of the above conditions is met, the cylinder end points lie outside the voxel. In this case, the two intersecting points and resulting volume, if any, are calculated and treated as above.

For both spatial occupancy and overlaps, results are averaged over one embedding unit box (single neuron span) based on the cyto-architectonic position, namely granule cell
layer (GC) and proximal, medial and distal thirds of the molecular layer (ML-1/3, ML-2/3, and ML-3/3, respectively). The region of interest of this analysis spanned 20 embedding units in the longitudinal axis (Figure 2.6) and two in the hippocampal-transverse axis (adjacent to the blade crest and tip, respectively).

![Figure 2.6: Spatial occupancy (top) and overlaps (bottom) of granule cell dendrites in the crest (left) and tip (right) halves of the supra-pyramidal blade of the dentate gyrus. Four sub-layers are distinguished and analyzed across their relevant longitudinal extent: granular (GC), inner molecular (ML-1/3), middle molecular (ML-2/3), and outer molecular (ML-3/3).](image)

Spatial occupancy has a moderate dependence on the septo-temporal position (Figure 2.6A,B) and varies according to layer and blade position (crest or tip). The ML-2/3 voxels have the maximum occupancy compared to other regions, with a range of 0.4–0.7.
Interestingly, the ordering of the rest of the voxels varies over the septo-temporal position. These results suggest that the dendritic volume changes non-uniformly with the distance along the extent of the molecular layer. Recent studies based on electron microscopy have shown that the wiring fraction (dendritic and axonal volumes) constitutes 55% of the neuropil in the CA1 radiatum area (Chklovskii et al., 2002). These authors observe that this fraction might not hold for the dentate gyrus. Thus, our report has interesting implications with respect to the volume available to axons, spines, boutons and glia within the dentate. The outer two-thirds of the ML layer are highly innervated by the entorhinal perforant path projections emanating from layer II (Witter and Amaral, 2004). The difference in space occupancy between ML-2/3 and ML-3/3 could correspond to a change in the density of spines along the granule cell dendritic tree.

The average number of spatial overlaps (Figure 2.6C,D) also changes with the position in the DG. The ML-2/3 and GC voxels have respectively the maximum and minimum number of overlaps. Noticeably, the number of overlaps in the tip exceeded that in the crest. The high incidence of overlaps could reflect an intrinsic signaling mechanism to form multineurite sites (Cove et al., 2006) in order to economize the wiring pattern (Chklovskii et al., 2002; Buzsáki et al., 2004). The difference in the number of overlaps in the crest and tip regions could be due to a differential dendritic recognition mechanism to avoid or promote contacts between adjacent cells (Sestan et al., 1999).

**E. INTRINSIC POTENTIAL CONNECTIVITY PATTERNS**
Electron microscopy showed that synaptic specialization may exist without a physical contact between an axon and a dendrite (Palay, 1958; Gray, 1959a, 1959b). Experiments have demonstrated the plastic nature of spines in establishing and remodeling a synaptic contact with the presynaptic machinery (Lendvai et al., 2000; Matsuzaki et al., 2004). In this context, a potential synapse can be defined as the condition of an axonal segment passing within a specified distance (depending on the type of synapse) from a dendritic segment. For excitatory synapses, the typical spine length (~ 2 μm) is taken as the distance parameter (Spacek and Hartmann, 1983; Harris and Stevens, 1989; Harris, 1999; Kalisman et al., 2003; Stepanyants and Chklovskii, 2005). For (presumably inhibitory) synapses on shafts and gap junctions, the critical distance can be obtained as the sum of the radii of the dendrite and axonal segments, typically 0.4–1.0 μm (Stepanyants and Chklovskii, 2005).

Several studies over the past years have estimated potential synaptic connectivity in the neocortex from detailed morphological data (Kalisman et al., 2003; Stepanyants et al., 2004) and simplified arbor shapes (Liley and Wright, 1994) through analytical and computational means. In addition, the functional connectivity between CA3 and CA1 has been investigated experimentally (Brivanlou et al., 2004). However, most approaches based on in vitro (slice) conditions are typically limited to a narrow anatomical region and cannot be assumed to extend representatively to surrounding areas. Our framework is in principle suitable to map out probabilistic connectivity patterns across the septo-
temporal extent quantitatively. Digital reconstructions of dendritic arborizations are especially abundant in the morphology database (NeuroMorpho.Org) for the rat hippocampus. Complete projection axons were traced algorithmically from available in vivo data (Tamamaki et al., 1988; Tamamaki and Nojyo, 1990, 1991; Scorcioni and Ascoli, 2005). For example, Figure 2.7A shows the axodendritic spread of a CA2 pyramidal cell with a CA1 cell co-embedded in the framework.

To account for variability in the arbor shapes due to pooling of data from different rat brains and imprecision in measurements, the arbor skeleton can be convolved with a Gaussian filter (Stepanyants and Chklovskii, 2005). After embedding the neuronal tree in the appropriate hippocampal volume, the voxels appropriate to the axonal density can be probed for the occurrence of potential synapses (see Figure 2.7B,C). This protocol is suitable to map out the intrinsic connectivity patterns among principal cells and between principal cells and interneurons within the whole hippocampus. This strategy will thus establish a quantitative relation between the spatial positions of the pre- and postsynaptic cell and their synaptic connectivity in probabilistic terms. Such a result would constitute a monumental step in the full characterization of the hippocampal circuitry.
Figure 2.7: Estimating potential connectivity: - (a) CA2 cell with (blue dendritic tree) having axonal arborization (in white) invades the large space in the CA radiatum region can have potential synaptic contacts with CA1 cell (in orange). (b) In the inset the spread of the axonal collaterals and the possible overlaps with the CA1 dendritic tree (in orange) is shown. For estimation of a potential contact, a putative synapse exists if the axonal segment passes within 1 μm of the dendritic segment. (c) A magnified view of the Figure 2.7b showing potential synapses (circles).

E. INFLUENCE OF INHIBITORY SYNAPSE POSITION ON CA1 PYRAMIDAL CELL FIRING
At least 16 distinct types of inhibitory interneurons synapse on CA1 pyramidal cells (Gulyas et al., 1999; Somogyi and Klausberger, 2005). These highly layer specific contacts play a pivotal role in the generation of population oscillations by sculpting the firing pattern of pyramidal cells by both feedforward and feedback inhibition. The laminar organization of this architecture raises the question of the biophysical importance of GABAergic synapse position. A single CA1 pyramidal cell synapse can make an interneuron fire (Gulyas et al., 1993; Marshall et al. 2002). If this interneuron has feedforward inhibitory connection onto another CA1 pyramidal cell, how does the efficacy of this latter synapse depend on its somato-dendritic placement? Can a single peri-somatic or axo-dendritic inhibitory synapse prevent pyramidal cell firing? To explore this problem, we adapted an existing CA1 pyramidal cell model (Migliore et al., 1999; http://senselab.med.yale.edu) available for the NEURON simulation environment (Hines and Carnevale, 2001). The model had a total of 200 compartments (soma: 13, apical: 127, basal: 60). We distributed the inhibitory synapses on the dendritic tree following the available experimental characterization (Megias et al., 2001). Accordingly, an inhibitory synapse was associated with one of the compartments in somatic, proximal radiatum, medial radiatum, distal radiatum and lacunosum-moleculare compartments (Figure 2.8).

The simulated firing frequency of the CA1 pyramidal cell is 40 Hz (Figure 2.8A) in response to a 0.15 nA somatic current injection (Migliore et al., 1999; Graham, 2001).
Figure 2.8: Effect of the inhibitory synapse location on the dendritic tree of a CA1 pyramidal cell. Side panel shows a real CA1pc morphology image with synapse positions. All the inhibitory synapses are activated at t=50 msec at 10 Hz frequency (a) 0.15 nA soma current injection leading to 40 Hz Oscillations. inhibitory synapses on (b) soma compartment leading to inhibition in CA1pc (c) radiatum proximal layer leading to inhibition in CA1pc (d) radiatum medial layer leading to decreased firing rate of CA1pc (e) radiatum distal layer has no effect on the firing pattern of CA1pc (f) LM layer has no effect on the firing pattern.

Inhibitory synapses were activated at a regular, constant rate of 10 Hz, corresponding to the spontaneous firing of stratum lacunosum-moleculare interneurons (Lacaille and Schwartzkroin, 1988). The rise and decay times for the inhibitory synapses, corresponding to tau1 and tau2 in the NEURON Exp2Syn object, were 0.75 and 37 ms, respectively (Kapur et al., 1997), with a reversal potential of −80 mV and a synaptic
current of 0.001 nA. The effect of activating individual inhibitory synapses after 50 ms varies dramatically with synaptic location (see Figure 2.8). A single GABAergic input in the peri-somatic and proximal dendritic region can inhibit the neuron from firing (see Figure 2.8B,C). Inhibition in the intermediate apical tree can slow down, but not fully block, spiking (see Figure 2.8D). In the most distal (outer radiatum and lacunosum-moleculare) layers, four synchronous inhibitory synapses are still insufficient to affect firing (Figure 2.8E,F). These simulations illustrate the importance of synaptic placement on CA1 pyramidal cell firing pattern. A thorough modeling study implemented with realistic synaptic connectivity could help investigate hippocampal dynamics.

F. IMPLICATIONS AND APPLICATIONS TO EPILEPSY

Homeostasis in brain circuits is essential for normal functioning and its disruption can lead to pathological states such as temporal lobe epilepsy (TLE). A critical way to maintain homeostasis is a delicate balance between excitatory and inhibitory circuits. This balance requires a careful assembly of highly complex topological and structural elements. Seemingly small changes in this configuration may lead to a hyperexcitable state, tipping the system into recurrent seizures.

Almost every pathological case of epilepsy seems to be associated with an anatomical abnormality, such as in the lamination of the hippocampus (Forster et al., 2006). Animal models attribute the genesis of seizure activity in the dentate gyrus to mossy fiber
sprouting (‘synaptogenesis’), or alternatively to hilar mossy cell loss (Sutula, 2002; Avoli et al., 2005). In contrast, the surviving CA1 pyramidal cells in human epileptic tissue receive intact peri-somatic inhibitory input (Wittner et al., 2005). However, mesial TLE patients have sclerosis of tissue and cell loss in the dentate hilus, as well as in the CA region (Mathern et al., 1994). As a result, there are significant volume and morphological changes in the cellular and non-cellular layers in these pathological cases. These changes in turn can affect the intrinsic circuitry and the stereological properties, such as space occupancy and overlaps, which have a direct effect on the biophysical state of the system. These factors could be collectively dubbed as ‘anatomical correlates of epilepsy’. In order to understand the dynamics of seizure initiation and spread, the anatomical complexity and organization should be taken into consideration in computational models of epilepsy. In recent years, there has been considerable effort to embrace a data-driven and connectivity based approach to simulate and investigate epileptiform activity (Netoff et al., 2004; Santhakumar et al., 2005; Dyhrfjeld-Johnsen et al., 2007). The framework described in this chapter is well positioned to strengthen and expand these kinds of studies by providing in vivo-like spatial connectivity patterns within various cell groups depending on their spatial position. A modeling approach as illustrated in the previous section, and implemented using a realistic spatial organization and morphology of principal and inhibitory cells in normal and pathological cases, could yield insights into the mechanism of TLE. These models could also become useful in the detection of pre-seizure states (Wong et al., 2007) and to provide a feedback signal to seizure detection and prevention devices.
CHAPTER 3: NON-HOMOGENEOUS STEREEOLOGICAL PROPERTIES OF THE RAT HIPPOCAMPUS FROM HIGH-RESOLUTION 3D SERIAL RECONSTRUCTION OF THIN HISTOLOGICAL SECTIONS

ABSTRACT

Integrating hippocampal anatomy from neuronal dendrites to whole-system level may help explain its relation to spatial navigation and episodic memory. Towards this aim, we digitally traced the cytoarchitectural boundaries of the dentate gyrus (DG) and areas CA3/CA1 throughout their entire longitudinal extent from high-resolution images of thin cryostatic sections of adult rat brain. The 3D computational reconstruction identified all isotropic 16 μm voxels with appropriate sub-regions and layers http://krasnow.gmu.edu/cn3/hippocampus3d). Overall, DG, CA3, and CA1 occupied comparable volumes (15.3, 12.2, and 18.8 mm$^3$, respectively), but displayed substantial rostro-caudal volumetric gradients, whereas CA1 made up more than half of the posterior hippocampus while CA3 and DG were more prominent in the anterior regions. The CA3/CA1 ratio increased from ~0.4 to ~1 septo-temporally, due to a specific change in stratum radiatum volume. Next we virtually embedded 1.8 million neuronal morphologies stochastically resampled from 244 digital reconstructions, emulating the dense packing of granular and pyramidal layers, and orienting the principal dendritic axes according to local curvature. The resulting neuropil occupancy reproduced recent electron microscopy data specifically measured in a restricted location. Extension of this
analysis across each layer and sub-region throughout the whole longitudinal extent of the hippocampus revealed highly non-homogeneous dendritic density. In CA1, dendritic occupancy was >60% higher temporally than septally (0.46 vs. 0.28, s.e.m. ~0.05). CA3 values varied both across subfields (from 0.35 in CA3b/CA3c to 0.50 in CA3a) and layers (0.48, 0.34, and 0.27 in oriens, radiatum, and lacunosum-moleculare, respectively). Dendritic occupancy was substantially lower in DG, especially in the supra-pyramidal blade (0.18). The computed probability of dendro-dendritic collision significantly correlated with expression of the membrane repulsion signal DSCAM. These heterogeneous stereological properties reflect and complement the non-uniform molecular composition, circuit connectivity, and computational function of the hippocampus across its hippocampal-transverse, longitudinal, and laminar organization.

INTRODUCTION

The hippocampus, a brain system in the medial temporal lobe, is theorized to play a prominent role in spatial and episodic memory processes in mammals including rodents, primates and humans alike (Scoville and Milner, 1957; O'Keefe and Nadel, 1978; Morris et al., 1982; Squire and Zola-Morgan, 1991; Eichenbaum and Cohen, 2001). The hippocampus consists of two major anatomical domains namely, Dentate Gyrus (DG) and Cornu Ammonis (CA), which sub-serve different functional aspects of these memory processes (Vazdarjanova and Guzowski, 2004; Leutgeb et al., 2007). CA is further divided anatomically into CA3, CA2 and CA1 regions accounting to their different neural
architecture layout (Lorente de No, 1934; Amaral et al., 1990; Witter and Amaral, 2004) and functional significance (Treves, 2004; Vazdarjanova and Guzowski, 2004; Kunec et al., 2005). Owing to these anatomically and functionally distinct domains within the hippocampus, there exist differences in their genetic, molecular, morphological, physiological and behavioral makeup (Ishizuka et al., 1995; Granger et al., 1996; Cannon et al., 1999; Vazdarjanova and Guzowski, 2004; Newrzella et al., 2007; Datson et al., 2009). Recently, there has been a growing body of evidence suggesting non-homogeneity of genetic to behavioral makeup within these distinct domains (Moser and Moser, 1998; Fanselow and Dong, 2010). Specifically, recent literature report dorso-ventral differentiation in molecular and genetic (Leonardo et al., 2006; Newrzella et al., 2007; Thompson et al., 2008; Datson et al., 2009; Dong et al., 2009; Greene et al., 2009), physiological (Royer et al., 2010) and behavioral properties (Bannerman et al., 1999; Kesner, 2007; Hunsaker and Kesner, 2008; Hunsaker et al., 2008) within each region of the hippocampus. Studies delving into morphometric analysis of individual digital reconstructions report dorso-ventral and sub-regional differences among the CA and DG principal neurons (Claiborne et al., 1990; Rihn and Claiborne, 1990; Turner et al., 1995; Pyapali et al., 1998). Additionally, there are also reports of dorso-ventral differences in the structural properties like cell densities in the principal layers of the rodent hippocampus (Ahmad et al., 2002; Daisu et al., 2009; Jinno and Kosaka, 2009).

In light of the non-homogeneous distribution of genetic to behavioral properties within the various regions of the hippocampus, one might hypothesize that the macroscopic
stereological properties like volume distributions and neurite occupancy ratios follow similar pattern of non-homogeneity. In deciphering function and activity, these stereological properties which are directly dependent on the structural organization and neural architecture layout of the hippocampus play a fundamental role. Volumetric measurements are conventionally carried out on randomly sampled histological sections using statistical based stereological methods (Schmitz and Hof, 2005) on particular regions of interest, like individual layers (Boss et al., 1987; West, 1990; Tinsley et al., 2001; Ahmad et al., 2002; Geinisman et al., 2004; Schmitt et al., 2004; Akdogan et al., 2008) or the complete hippocampus (Ahmad et al., 2002; Schmitt et al., 2004). Only on rare instances (West et al., 1978) have attempts been made previously to estimate the volumes of regions (DG, CA3 and CA1) by segmenting the cyto-architectonic layers over the entire extent of the rat hippocampus. However, these studies lacked the technological know-how to reconstruct a three dimensional (3D) model. In this context, a comprehensive in-silico based 3D model of the rat hippocampus facilitates our study of the volume distributions of individual layers and regions across the canonical anatomical and hippocampal axes that otherwise are not possible at systems level. The partial volumetric measurements based on conventional stereological protocols have significant limitations in our quest to understand the regional and divisional differences in volume distributions across the entire hippocampus. Additionally, a robust computational framework for digitally packing neuronal arbors within this 3D template facilitates the quantitative estimation of the neurite occupancy (or wiring) ratios (Chklovskii et al., 2002) across the various layers and regions of the hippocampus.
As the hippocampus is laminated (Forster et al., 2006) and divided into cyto-architectonic layers attributed to different axonal projections (Witter and Amaral, 2004), there could be differential volume distributions and wiring ratios in effect within these layers. The dense packing of cell densities places an important constraint on cell growth within these stipulated boundaries and volumes such as “tiling effect” (Cook and Chalupa, 2000), wherein different signaling mechanisms drive the neurite’s growth (Sestan et al., 1999; Cove et al., 2006). Because of the dense packing of dendrites within the hippocampal non-principal layers, neurons could employ inherent signaling mechanisms, like Down syndrome cell-adhesion molecule (DSCAM), in order to avoid dendro-dendritic (homotypic or heterotypic) contacts (Matthews et al., 2007; Schmucker, 2007; Fuerst et al., 2008; Millard and Zipursky, 2008). The expression of these molecules could be high in the highly curved regions because of probable high dendritic densities. Additionally, in a densely packed neuropil the anisotropic or isotropic nature of the orientation of the neurites would directly affect the field potential and current source distribution (Nicholson, 1973).

In order to address these questions with respect to systems level analysis of the stereological properties, we created a detailed 3D model of the rat hippocampus from high resolution thin histological sections. We developed a robust in-house computational framework to embed reconstructed neurons based on the tissue curvature. We further extended this framework by digitally packing the cellular layers with reconstructed arbors
and estimated the dendritic occupancy ratios (DORs) by mapping the available hippocampal morphological data onto this 3D template based on the reported positional information in the literature. Previous efforts in this direction were initiated by (Scorcioni et al., 2002) in the dentate gyrus, but were not extended to a volumetric representation and thus the detailed stereological analysis was not carried out. Here, we present the volumetric distributions and also elaborate on the occupancy ratio distributions in these layers and regions with respect to the canonical anatomical and hippocampal axes. We also evaluated the anisotropic nature of the orientation of dendritic segments in the various layers derived from the nematic order parameter with the principal axis taken as the reference axis (Gennes and Prost, 1993). These macroscopic stereological properties are a key for us to understand the true organization of the hippocampus and enable the integration of structure and function.

MATERIALS AND METHODS

Experimental procedure and raw image data acquisition

Four male Long-Evans hooded rats (226-237 g) were obtained at postnatal day (P) 22 (Harlan, Indianapolis, IN). Rats were housed individually in metal wire-hanging cages and maintained at ambient temperature (22-24 °C), on a 12 h L/D cycle (lights on at 0700 h) with access to ad libitum food and water. Animal care was in accordance with George
Mason University and National Institute of Health Guide for the Care and Use of Laboratory Animals guidelines.

Rats were sacrificed by decapitation with the aid of a guillotine at P45, and the brains quickly removed and frozen in powdered dry ice and kept in air-tight ziplock plastic bags at –80 °C until cryostat-sectioning. The decapitation and brain extraction was carried by Dr. Susan Bachus. Each brain is then mounted in a cryostat kept at -18 °C and coronally sectioned at 16 µm in three different orientations. Two of these brains were sectioned in dorso-ventral and one each in ventro-dorsal and lateral-medial orientations respectively. For each brain, the series of stereotaxic-transverse sections covering the entire extent of the hippocampus are mounted on gelatin-subbed glass slides and Nissl stained with standard protocol (Wouterlood, 1993) and cover-slipped. Of the four Nissl datasets, two in the dorso-ventral direction resulted in the least distortion of the tissue in the hippocampal region and thus were selected for the stereological analysis in this study. For one of the dorso-ventral dataset, the complete three dimensional extent of the hippocampal formation was segmented with the cyto-architectonic boundaries (Figure 3.1) and for the other dataset the medial portions of the hippocampal layers were segmented.

In order to evaluate the planar shrinkage and distortion in the XY plane, we acquired whole mount pictures of the block face prior to sectioning for 10 slices in the medial
Figure 3.1: Hippocampus whole brain segmentation in the rostro-caudal extent
Segmentation of the cytoarchitectonic boundaries of the hippocampus at different rostro-caudal levels (dorsal (A); medial (B); posterior(C)) and blow up of the segmented dorsal hippocampus is shown in panel a1 with CA and DG hippocampal-transverse regions labeled. The sub-regions labeled are CA3c, CA3b, CA3a, CA1c, CA1b, CA1a for CA and Infra Tip and Crest, Crest, Supra Crest and Tip for DG. In the segmentation process, contours of DG Hilar (HILUS), granular (GC), molecular (ML), CA pyramidal (PC), radiatum (RAD), lacunosum moleculare (LM) and oriens (OR) layers are validated using standard rat atlases (Swanson’s and Paxinos’). The insets in panel a1 show further division of the CA and DG neuropil to sub-layers. Within the CA neuropil, Oriens (OR) is divided into distal and proximal OR, radiatum (RAD) into proximal, medial and distal RAD. Within the DG neuropil, molecular (ML) is divided into proximal, medial and distal ML. Scale bars shown in panel A, a1 and B are 4.0, 0.50 and 3.0 mm respectively.

portions (slide#140-150) of the hippocampus using a Kodak digital camera at 300 dpi resolution (see Figure 3.2A). We did not estimate the shrinkage in the z-axis because we took the physical interslice distance (16 μm) in our analysis of the stereological properties. The image stacks were acquired using an EPSON 3200 dpi scanner and
contrast enhanced using the Matlab routine ‘imcontrast’. By overlaying these whole mount pictures over the corresponding histological scanned images, we estimated the shrinkage factor by taking the ratio of the difference in the areas between the block face (\(A_B\)) and the Nissl scanned image (\(A_N\)) to the area of the block face image (see Figure 3.2A). For distortion analysis, the block face image and the scaled Nissl scanned images are overlaid so as to estimate their overlap area (\(A_O\)). The distortion factor is estimated by taking the ratio of the difference in the areas of the block face image and the overlay image (\(A_O\)) to the area of the block face image (see Figure 3.2A). By following this process we obtained a planar shrinkage factor of 7 ± 0.78 % (N = 10) and distortion factor of 1.49 ± 0.06 % (N=10), assuming isotropic shrinkage in the XY plane.

Figure 3.2: Experimental set-up and digital representation
A) Shrinkage and distortion analysis from block face (\(A_B\)), Nissl scanned image (\(A_N\)) and overlap of the block face and scaled Nissl scan image (\(A_O\)) with the formulas shown for shrinkage, scaling and distortion. B) Granule cell layer traced showing boundary values of the contour as “pixel based closed polygon” with the triangulation and filling process shown in the bottom row.
In addition to the experimental acquisition of histological data sets in our lab, we obtained MR microscopy (or μMRI) of a rat brain dataset from David Lester (Lester et al., 2001). This μMRI imaging dataset was captured by in vivo microscopy of excised rat brain at a voxel resolution of 47 μm in the stereotaxic-transverse plane.

**Data Digitization: 3D Hippocampus Model Reconstruction**

The image stacks of the two dorso-ventral datasets are loaded into the Reconstruct tool (Fiala, 2005; http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm) with appropriate pixel/μm conversion factor (4.28). In order to tackle registration of images, initially a mid-line guided image alignment was performed and then an algorithmic approach is applied after the data digitization step. In order to segment the histological images, we identified seven different cyto-architectonic layers within Cornu Ammonis (CA) and Dentate Gyrus (DG) based on Swanson and Paxinos standard rat atlases as validation references. For one data set, we traced inner and outer boundaries of the cyto-architectonic layers of the hippocampus based on these references in the entire rostro-caudal extent. In particular, the following layers were identified and traced. For DG, molecular layer (ML), granule cell layer (GC) and hilus proper (HILUS) were identified in the Nissl stained sections and segmented. For CA, oriens (OR), pyramidal cell (PC), radiatum (RAD), and lacunosum moleculare (LM) layers were identified and traced on these sections (Figure 3.1). The CA and DG layers segmented and shown in Figure 3.1 are in blue and red shades respectively. Missed slices (numbering 42) were interpolated
based on their neighboring sections. A total of 290 digital sections (after interpolation for the missing slices) represented the entire rostro-caudal extent of the hippocampus of this data set. In another data set, we traced only the inner and outer layer boundaries within the middle third of the hippocampus in the rostro-caudal axis because of the sheer amount of manual work in the segmentation process. In CA, the lucidum was not delineated as a separate layer but included as part of radiatum and the alveus (or fimbria) was also not segmented. Though the Subiculum layers are segmented, this hippocampal region is not included in our study. For the μMRI dataset, because of lower contrast compared to Nissl only the cellular (PC, GC) and outer (OR, ML) layers of CA and DG in the dorsal hippocampus were segmented.

The digital contours resulting in each section consist of a set of pixels representing these boundaries as closed polygons (see Figure 3.2B). Each individual pixel based closed polygon representing a cytoarchitectonic layer boundary in a given section was “triangulated” to identify or “fill” all pixels inside the contour, representing an inner position of the respective anatomical sub-region. The novel algorithm, written in C++, uses circular linked lists (Kernighan and Ritchie, 1998) and is applicable to both convex and concave polygons (see Figure 3.2B; Ropireddy et al., 2008). This algorithm is extended further to obtain 3D voxels from neighboring slices of a predefined width analogous to the marching cube approach (Lorensen and Cline, 1987). The centroid of the outer boundary of the hippocampus in each sectional plane was used to further refine the manual registration. In particular, several zones were manually identified based on the
geometry of these boundaries. The geometry of these boundaries changes as the shape of the hippocampus evolves along the rostro-caudal axis (see Figure 3.1). For example, approximately at one-third rostro-caudal extent (anterior-posterior position - ~4.16mm) ventral hippocampus emerges and at half of the rostro-caudal extent (anterior-posterior position - ~4.80mm) the dorsal and ventral hippocampus regions merge (Paxinos & Watson, 1986). In order to account for these changes and to avoid the drastic shift in the centroid position between adjacent boundaries, the segregation of the entire region into distinct zones was a necessary step. A three-point average was then applied on the centroid location within each zone until convexity or concavity was reached in each separate zone. Finally, every section was translated based on the new centroid coordinates. This approach yielded a smoother and satisfactory post-alignment registration. The septo-temporal axis is based on these series of centroids of the outer boundary calculated from the above described triangulation algorithm. 3D curve fitting analysis of the septo-temporal axis revealed that it fits (using least sum of squared absolute error) with the following sigmoidal function (with offset) computed using Matlab:

\[
Z = \left[ \frac{a}{\left( (1.0 + e^{(b - cx)}) \times (1.0 + e^{(d - ey)}) \right)} + f \right] \quad \text{Eq. 1.}
\]

where the values of the coefficients are: \(a = 2.09e^{03}\), \(b = 3.16\), \(c = 2.03\ e^{-03}\), \(d = -4.56\), \(e = -4.91e^{-04}\) and \(f = 7.53\).
Surface and Volumetric Representation

This highly complex 3D architecture of the hippocampus can be represented in either surface or volumetric approaches. Surface representation (or rendering) is particularly amenable to visualization of this 3D model in Virtual Reality Modeling Language (Figure 3.3). Voxel-based volume representation, on the other hand, enables the direct implementation of stereological analyses. The triangulation/filling algorithm (see Figure 3.2B) described above can yield an arbitrary pixel size, or resolution of the digital reconstruction. In order to maintain 3D isotropy (cubic voxels), a voxel size of 16 µm was selected, corresponding to the nominal cryostatic section thickness. Volumetric representation is convenient to carry out a detailed macroscopic stereological analysis within the hippocampus whose technical details are discussed in the following sections.
Figure 3.3: Hippocampus 3D renderings. 3D Hippocampus renderings of CA and DG with external and cellular boundaries are shown in two views, rostral (left) and ventral (right). For CA, the pyramidal layer is shown in dark blue and the outer boundary is shown in light blue. For DG, the granule layer is shown in dark red and the outer boundary is shown in light red.

The volumetric representation results in voxels corresponding to all seven cytoarchitectonic layers of this 3D template (Ropireddy et al., 2008). We catalogued all the voxels with respect to two reference axes (hippocampal and stereotactic) resulting in a six-dimensional reference system for each voxel. The cataloging process in the hippocampal reference system results in three coordinates namely ‘septo-temporal’, ‘hippocampal-transverse’ and ‘depth’ positions for all the voxels in each layer. The stereotactic reference system results in the bregma/lamda and lateral position using the standard atlases (Paxinos’ and Swanson’s) as references. Contrastingly, defining the coordinates for the natural hippocampal axes is quite complex. In order to solve this problem, we implemented the following approach. We took 60 reference equidistant hippocampal-transverse virtual planes along the septo-temporal axis and generated the classical double ‘C’ shape of the hippocampal hippocampal-transverse cross-section. For each voxel on this plane, we defined the hippocampal-transverse and septo-temporal positions. Hippocampal-transverse position is the relative distance with respect to the ‘C’ for granule and pyramidal cell layers. As we traverse along the DG hippocampal-transverse axis, the infra changes to the supra position thus resulting in two types of DG voxels, infra (‘I’) and supra (‘S’). For CA, as we traverse along its hippocampal-transverse axis, the voxel type changes from CA3c (proximal to hilus) to CA1a (proximal
to subiculum) thus defining seven types of CA voxels (CA3c, CA3b, CA3a, CA2, CA1c, CA1b and CA1a). The septo-temporal position was estimated by translating each voxel parallel to the longitudinal axis defined and the hippocampal-transverse position was estimated by translating to the nearest of these 60 planes along the septo-temporal axis. Each voxel, as a result of the cataloging process, has a septo-temporal, hippocampal-transverse, depth and bregma/lamda coordinates tagged and stored in a custom developed database.

**Stereological Cell Density Estimates**

The volumetric representation as described above results in a very high number of voxels for the principal cell layers alone with CA3, CA1 PC and GC layers having 540K, 730K and 890K voxel numbers respectively. In order to create a true in-vivo representation of the dense packing in the principal cell layers, we need to identify the sampling factors for the number of principal cells in the hippocampal cell layers as estimated from stereological methods. Based on literature, there is a great variation in the number of principal cells depending on the strain and age of the animal. For our study here, we used numbers reported by Rapp and Gallagher (1996) because these stereological estimations are based on the same strain and age group as the animals used in our study. They report that there are 1.2 million granule cells, 225K CA3/2 and 390K CA1 pyramidal neurons in Long-Evans strain of the rat hippocampus. To get a sense of the variation in the number of neurons between different strains of rodents, for example, two-month old Wistar rats
contain on an average 1.08 granule cells, 188K CA3/2 and 324K CA1 pyramidal cells (Hosseini-Sharifabad and Nyengaard, 2007). Similar studies report differences in other rat strains like F344, Sprague-Dawley and various strains of mice (Boss et al., 1987; West, 1990; West et al., 1991).

In addition to strain-based variation in absolute number of neurons, there are also reports of numerical density (ND) differences along the dorso-ventral axis of the mouse hippocampus in the cellular layers of DG and CA (Jinno and Kosaka, 2009). In the mouse hippocampus NDs of CA1 principal neurons in the dorsal hippocampus is thrice that of the ventral hippocampus and in DG the NDs of granule cells is significantly higher in dorsal compared to the ventral level. Previous research suggests that corresponding density differences in the rat hippocampus are inconclusive and report conflicting results. In particular, West et al. (1991) report that there are no cell densities differences in the dorsal and ventral divisions of CA1, CA2 or DG of the rat hippocampus. On the other hand, Daisu et al. (2009) report significant dorso-ventral differences in NDs for CA1 and non-significant differences for CA3 regions.

In light of these inconsistencies in cell densities in the dorso-ventral axis, we carried out stereological analysis of the cell count and in turn derived the NDs of the principal neurons in CA and DG in dorsal and ventral sections using the optical fractionator probe (Schmitz and Hof, 2005) with the aid of MicroBrightField StereoInvestigator software (www.mbfbioscience.com). The optical fractionator probe is a two-stage unbiased
sampling method used to estimate number of objects in a specified region of interest (ROI). It combines the optical dissector method with the fractionator systematic random sampling (West et al., 1991) in obtaining the number of cells by applying counting frames (Schmitz and Hof, 2005). In carrying out these experiments we delineated the CA3, CA1 and GC layers as our ROI in dorsal and ventral sections and applied the

![Comparison of cell densities for CA3, CA1 and DG in rat and mouse hippocampus.](image)

*Figure 3.4: Comparison of cell densities for CA3, CA1 and DG in rat and mouse hippocampus.*
fractionator probe with the aid of an achromatic 100x immersion oil lens with a numerical aperture of 1.25 on an Olympus BX51 microscope. In contrast to the differences found in Jinno and Kosaka (2009), we found that there are significant differences between the CA1 dorsal and ventral divisions but non-significant differences in CA3 and DG regions (see Figure 3.4). Specifically, the CA1 dorsal region has 1.27 times the ND of that in the ventral region and in the CA3 region the ventral portion has higher ND (by 1.14 times) than dorsal portion. In order to represent the true packing nature of the principal neurons in-vivo, we identified the sampling factor (one soma position corresponding to a certain number of voxels) for embedding reconstructions in DG, CA3 and CA1 principal cell layers. For DG, the number of granule cells is higher than the number of voxels in our volumetric representation, so we have a granule cell embedded with a voxel sampling ratio of 0.75 and for CA3 this ratio is 2.7 with no gradient. For the CA1 region, because of the dorso-ventral gradient, voxel sampling factor increases from 1.4 in the dorsal division to 2.2 in the ventral region which is implemented in a linearly increasing fashion. These sampling factors were measured based on the above reported differences in the dorso-ventral differences in NDs and assuming that there is a linear decrease of the NDs in CA1 and no change for DG and CA3 across the septo-temporal axis.

Neuronal Arbor Reconstructions (Archives)
The rat hippocampal neuronal reconstructions were downloaded from NeuroMorpho.Org (Ascoli et al., 2007) from different archives within DG, CA3/2 and CA1 regions. When reporting the positional information for morphological reconstructions, researchers report either the stereotactic (bregma and lambda) or the natural hippocampal coordinate systems, that is along the septo-temporal (or longitudinal), hippocampal-transverse and across the depth (layers). This positional information is acquired from the literature and used to map the morphological data availability to the 3D template model of the hippocampus. An error adjustment is made to the reported coordinates based on whether they are reported in absolute or relative coordinates as is explained below for each archive.

For DG, 81 granule cells (Claiborne and Turner archives) are mapped onto the 3D template based on the reported positional information obtained from Carnevale et al. (1997) and Rihn and Claiborne (1990). The granule cells from Claiborne archives were located within the middle third of the septo-temporal axis and 39 of these cells were located in the supra-pyramidal blade. According to Rihn and Claiborne (1990), the supra-pyramidal blade and the depth of the GC layer were divided into six divisions and each of these 39 granule cells belonged to one of these divisions. For mapping these cell locations in our framework, all the GC voxels within the middle third of the septo-temporal axis and those that matched one of the blade and layer divisions were tagged with these cell positions.
For CA3, 54 CA3 pyramidal neuronal reconstructions were mapped based on the reported positional information from five neuronal archives (Amaral (20): Ishizuka et al., 1995; Barrionuevo (8): Henze et al., 1996; Claiborne (4): Carnevale et al., 1997; Jaffe (6): Jaffe and Carnevale, 1999; Turner (16): Turner et al., 1995). All five neuronal archives except Jaffe’s had the CA3 type (CA3c or CA3b or CA3a) specific information and other positional information. Amaral’s archive has comprehensive information for each of the CA3 neuronal reconstructions, with each cell belonging to either CA3c or CA3b or CA3a type within the middle third of the septo-temporal axis. The hippocampal-transverse axis and depth positions were given as percentage along the ‘C’ axis and the PC layer respectively (Ishizuka et al., 1995). For mapping these CA3 neurons, all the PC voxels within the middle-third of the septo-temporal axis and those matching the hippocampal-transverse and depth relative positions with a ±5% error were tagged with these neurons. The neurons in the Barrionuevo archive are of the CA3b type and have no septo-temporal or hippocampal-transverse position specified. Claiborne CA3 neurons are of the CA3c type situated within the middle third of the septo-temporal axis with no hippocampal-transverse or depth information reported, so all the CA3c,b PC voxels in the middle third of the septo-temporal axis are tagged with these CA3c,b neurons for the Claiborne and Barrionuevo archives respectively. Finally, the Turner archive consisted of all the three CA3 sub-regions and the septo-temporal and hippocampal-transverse positions reported in their paper (Turner et al., 1995) in relative coordinates. For the septo-temporal position, we obtained the relative position for each neuron from Fig. 1 of Turner et al. (1995). After scaling these positions with the total septo-temporal axis
(‘ST’) length, we introduce a 5% error in this axis with a correction of ±0.16mm (5% of 6.5mm total length of ‘ST’ axis) for the estimated position. The hippocampal-transverse position is given as percent of location across the ‘C’, so we correct this with a ±5% adjustment in this relative position.

For CA1, 109 CA1 pyramidal neuronal reconstructions were mapped based on the reported positional information from eight neuronal archives (Amaral (23): Ishizuka et al., 1995; Ascoli (2): Brown et al., 2005; Claiborne (7): Carnevale et al., 1997; Gulyas (18): Megias et al., 2001; Spruston (3): Golding et al., 2005; Turner (56): Pyapali and Turner, 1994; Pyapali and Turner, 1996; Pyapali et al., 1998). Out of these eight archives, only Amaral and Claiborne archives had the cell type information. Similar to the CA3 Amaral archive, the CA1 pyramidal neuronal reconstructions of this archive had comprehensive information on the 23 CA1 pyramidal neurons with each cell categorized as CA1c, CA1b or CA1a type within the middle-third of the hippocampus. Additionally, the hippocampal-transverse and depth positions were given as percentage along the ‘C’ axis and the PC layer respectively (Ishizuka et al., 1995). For mapping these CA1 neurons, all the PC voxels within the middle-third of the septo-temporal axis and those matching the hippocampal-transverse and depth relative positions with a ±5% error were tagged with these neurons. The 7 CA1 pyramidal neurons from Claiborne archive were situated within the middle third of the septo-temporal axis with no hippocampal-transverse or depth information reported, so all the CA1 PC voxels in the middle third of the septo-temporal axis are tagged with these neurons.
For the CA1 pyramidal neurons from Gulyas and Turner archives, the stereotactic coordinates were provided as the cell labeling was carried in vivo (Pyapali and Turner, 1994; Pyapali and Turner, 1996; Pyapali et al., 1998; Megias et al., 2001). For these neurons, bregma/lambda and lateral positions were corrected by ±0.05 mm of the reported values. So, all the CA1 PC voxels corresponding to these stereotactic coordinates are tagged with these cells. For the archives where no positional information is specified an assumption was made that these cells could belong to any position which include the DG Turner and CA1 Spruston (Golding et al., 2005) archives.

**Computational Framework**

All the algorithms (including the triangulation algorithm in Figure 3.2B) are designed and developed in-house in C/C++ programming language on an Intel based fedora core 8 Linux environment. The simulations are run on eight node dual-cpu Intel Pentium IV 3.0 GHz microprocessor cluster machines.

*a) Volume Estimation and Analysis*

Volumes of all seven cyto-archetectonic layers are estimated in five different axes of which three are anatomical (stereotaxic-transverse, sagittal and horizontal) and two are hippocampus centric (septo-temporal and hippocampal-transverse). The volumes for all
the seven layers are obtained by integrating the voxel volume in any of these five axes. For the anatomical axis, the volumes of the seven layers are obtained by integrating in the stereotaxic-transverse, horizontal, and sagittal axes respectively. For the hippocampal axes (septo-temporal and hippocampal-transverse) estimating these volumes in these two axes is based on the hippocampal coordinate system. The cornu ammonis (CA) volume is obtained by integrating the individual layer volumes of OR, PC, RAD and LM and that of dentate gyrus (DG) is obtained by integrating the individual layer volumes of ML, GC and HILUS over the reference axes. The total hippocampal volume is the sum of the volumes of CA and DG.

b) Hippocampal Cell Mapping Process

Cell location is important for categorizing the type of neuron and thus aiding in the process of linking structure and function. In our framework, the hippocampal cell mapping process is accomplished through a probabilistic approach. The morphological hippocampal data downloaded from NeuroMorpho.Org (Ascoli et al., 2007) with the given location information are probabilistically mapped to the voxel database catalogued with the hippocamapal and canonical brain axes reference system. For this we defined two indices called, Available Neuronal Density Index (ANDI) and Available Neuronal Count (ANC) for every voxel in the database. ANDI is the cumulative probabilities of all the cells belonging to each voxel averaged across depth. In other words, this index shows the propensity of data availability for a particular voxel with a given septo-temporal and
hippocampal-transverse position. ANC, on the other hand is the sum of the number of cells belonging to a given voxel in this probabilistic matching method over. In addition to estimating these two indices for each voxel, we also calculated the conditional probability of a particular cell belonging to a voxel, given that a certain number of cells match that voxel’s criteria, defined as $P_{\text{AverageWeight}} (P_{AW})$. The ANDI, ANC and $P_{AW}$ equations are shown below:

$$ANDI = \left( \sum_{i=1}^{n} \left( \frac{1}{\sum_{j=1}^{c} (1/N_j)} \right) \right)$$  \hspace{1cm} \text{--- Eq. 2}$$

$$ANC = \sum_{i=1}^{n} (c)$$  \hspace{1cm} \text{--- Eq. 3}$$

$$P_{AW} = \left( \frac{1/N}{\sum_{j=1}^{c} (1/N_j)} \right)$$  \hspace{1cm} \text{--- Eq. 4}$$

where, $n$ is the number of voxels with unique septo-temporal and hippocampal-transverse but different depth positions.

c is the number of cells matched for a voxel

N is the number of voxels matched for a cell

c) Neuronal Embedding Process
The digital morphological reconstructions of pyramidal and granule cells are algorithmically embedded based on the following criteria: (a) the first principal axis of the dendritic arborization passes through the soma and is perpendicular to the cellular layer; (b) the second (orthogonal) axis lies on the septo-temporal plane; (c) the third axis is orthogonal to the above two axes (Ropireddy et al., 2008; Ropireddy et al., 2010). The 3D coordinates of the dendritic and axonal arborizations are read from the digital reconstruction files and transformed and rotated along the above principal axes using standard numerical recipes (Press, 1988). The heights of the arbors are scaled according to the depth of the layer until the border of ML/LM (fissure) and OR. In particular, the three coordinates of all branches in each embedded neuron are multiplied by the ratio between the soma-OR and soma-fissure distance and the original dendritic height, ensuring that the farthest tips of the dendrites terminate at the end of the outermost layer. Virtual Reality Modeling Language (VRML) files (Ames et al., 1997) can be generated on the fly for visualization purposes with the freeware VRML viewer: view3dscene (http://vrmlengine.sourceforge.net/view3dscene.php).

\( d) \) \textit{Estimation of Dendritic Occupancy Ratios}

Dendritic occupancy ratio (DOR) is the fraction of neuropil volume occupied by dendrites. The general approach we adopted in order to estimate DORs across the entire septo-temporal extent of the hippocampus was to digitally pack the cellular layers of CA and DG with appropriate principal cell density and voxel sampling ratios (Rapp and
Gallagher, 1996; Jinno and Kosaka, 2009) and estimated the dendritic volume occupancy in different layers. For this we designed a unique protocol by parceling the entire 3D space of the hippocampus into uniform neuropil volumes, the dimensions of which were estimated by the average span of dendritic arbors of CA and DG principal neurons in the septo-temporal and hippocampal-transverse axes. We define these uniform neuropil volumes as cell-boxes. Based on the span of the dendritic extent in the hippocampal-transverse axis for CA, coincidentally we were able to fit seven cell-boxes corresponding to CA3c, CA3b, CA3a, CA2, CA1c, CA1b and CA1a sub-regions (see Figure 3.1a1). As a result the entire extent of CA was parceled into 12*7(84) cell-boxes. For DG, the hippocampal-transverse extent was compartmentalized into five cell-boxes corresponding to Infra Tip, Infra Crest, Crest, Supra Crest and Supra Tip sub-regions (Figura 3.1a1). Similar to CA, the entire extent of DG was parceled into 25*5(125) cell-boxes.

While digitally packing the cellular layers in this 3D construct with the voxel sampling ratios for DG, CA3 and CA1 regions, all the voxels falling under an extended cell-box (called ‘embed-box’) are sampled. The dimensions of the ‘embed-box’ were defined as the average dendritic span in the septo-temporal and hippocampal-transverse axes (‘cell-box’ dimensions) plus the maximum span of the entire CA and DG principal neurons in these two axes. While compartmentalizing the entire 3D space, necessary steps were taken to negate the edge-effect by discarding the edges in both the hippocampal-transverse and septo-temporal directions because of the risk that the cell-boxes are
invaded by arborizations on only one direction. However, the edge voxels were sampled for embedding principal arbors.

The cell-boxes covering the depth of the layers of CA and DG are further sub-divided into sub-layers. Specifically, the Oriens (OR) neuropil is divided into two sub-layers: Distal and Medial OR (see Figure 3.1a1). The Radiatum (RAD) neuropil is divided into three sub-layers: Proximal, Medial and Distal RAD. The neuropil volume of Lacunosum Moleculare (LM) is considered as a whole. For DG, the molecular layer (ML) is divided into three sub-layers: Proximal, Medial and Distal ML corresponding to molecular layer inner, middle and outer thirds respectively. As a result, DORs are computed in individual sub-layers of CA (Distal and Proximal OR, Proximal, Medial and Distal RAD and LM) and DG (Proximal, Medial and Distal ML) as depicted in Figure 3.1a1.

In order to compute the DOR in each layer at a particular septo-temporal position and for each sub-region, the computational process consists of series of steps. First, the entire set of principal layer voxels falling inside the embed-box are sampled according to the voxel sampling ratio. Then, a neuron is randomly sampled by generating a random weight between 0 and 1 and the cell whose $P_{AW}$ falls within this range is picked and embedded. While embedding, the normal is stochastically tilted between 0 and 5 degrees around a random 0-360 degree rotation angle in order to approximate the natural variability in the alignment of principal dendritic component (Claiborne et al., 1990; Rihn and Claiborne, 1990; Ishizuka et al., 1995). The other two orthogonal axes are correspondingly adjusted.
for both the tilt and rotation factor. Finally, the DORs are obtained by taking the ratio of the dendritic to the total volume available in each sub-layer within each sub-region. The raw DORs are weight-averaged with the volume of each sub-layer across the entire extent of the septo-temporal and the hippocampal-transverse (i.e. sub-regions) axes.

e)  Mean DSCAM Expression for CA and DG derived from Allen Brain Atlas

The Down syndrome cell adhesion molecule (DSCAM) in-situ hybridization expression images in the sagittal sectional plane are downloaded from Allen Brain Atlas (ABA) at mouse.brain-map.org (Ng et al., 2009). The ABA images have a blue-red (low to high) color scale associated with the DSCAM expression and seven intermediate levels in the color spectrum. A relative scale is created with seven distinct divisions based on this blue-red color scale. In order to quantify the DSCAM expression in each of the seven CA and five DG sub-regions, the hippocampus is segmented into these 12 distinct sub-regions. Then, their xy coordinates with the pixel RGB values are extracted using the ImageJ (rsb.info.nih.gov/ij) image processing plug-in (‘Analyze/Tools/save xy coordinates’). The DSCAM expression levels in these sub-regions are quantified based on the relative difference in their RGB values with the created color scale. These expression levels are averaged across all the pixels in each section and the overall average obtained for each sub-region. The relative DSCAM expression is obtained by normalizing with the maximum expression level and compared with the mean squared occupancy levels for each CA and DG sub-region.
f) **Estimation of Nematic Order Parameters (S)**

Nematic order parameter (NOP) is mainly used in the field of material science to analyze the orientations of fibers/nanotubes with respect to a reference axis and characterize their nematic phases (Gennes and Prost, 1993). We applied this concept to analyze the orientation of dendritic segments within the various cyto-architectonic layers of CA and DG through our computational framework. The reference axis in our framework taken was the primary axis of the dendritic arbor (normal to the tissue curvature). The NOP (S) is directly dependent on the square of the cosine of the angle \( \theta \) the dendritic segments make with the reference axis which, in other words, is given by the average of Legendre second order polynomial and is measured by Eq. 5 (Gennes and Prost, 1993). The ‘S’ value obtained is a spatial average of all the segments within each sub-layer and are evaluated for each segment instead of averaging the raw \( \theta \) values because of the three dimensional nature of the dendritic arbor geometry.

\[
S = \left[0.5 \times \left(3 \times \cos^2(\theta) - 1\right)\right] \quad \text{---- Eq. 5}
\]

The ‘S’ value obtained from the above equation is weight-averaged with the length of the segments in each sub-layer and their overall average is derived within each layer of CA and DG. We can deduce from the above equation that if S=1, then the segments are parallel to the reference axis or the principal axis of the neuron. On the other hand, if S=-
0.5, then the segments run parallel to the reference axis, which in our case is the cell body layer. Finally, if S=0 then the segments are oriented in isotropic nature. From the above equation, the angle ($\theta$) corresponding to S=0 is 55° because of solid angles (Gennes and Prost, 1993) and not 45°. We extended our computational framework designed to estimate occupancy ratios and applied the above equation in order to calculate the weight-averaged values of ‘S’ in CA (‘OR’, ‘RAD’, ‘LM’) and DG (ML) layers. We obtained the average angles of these ‘S’ values from multiple seeds across the septo-temporal axis. Finally, we derived the angular deviation of the dendritic segments from the isotropic angle of 55° and characterized the isotropic or anisotropic nature of dendritic neurite orientations in each of these layers.

g) Availability of data pertaining to the 3D model

The high resolution 3D hippocampus data are uploaded to a cloud storage space, the details and links of which are provided here (http://krasnow.gmu.edu/cn3/hippocampus3ddata/3DHippocampus-Data.html). The data files that can be downloaded include the raw hippocampus stereotaxic-transverse Nissl images in the entire rostro-caudal extent, the hippocampus segmentation/tracing XML files that can be imported and visualized in the Reconstruct tool (http://synapses.clm.utexas.edu/tools/reconstruct/reconstruct.stm) and the voxel database coordinate files for CA and DG layers. Finally, the surface rendering (VRML) files of the CA and DG layers can also be downloaded and visualized in a VRML viewer.
RESULTS

3D Model of Rat Hippocampus

The three dimensional geometry of the rat hippocampus is very different in shape and conformity from the primate and human hippocampus, albeit the name of the hippocampus suggests a ‘sea horse’ shaped structure (Witter and Amaral, 2004). The rat hippocampus is a banana shaped structure with a bulging appearance in the middle with its septo-temporal (or longitudinal) axis represented as a sigmoidal equation expressed in three dimensional Cartesian coordinates (see Equation 1 in methods). As observed from Figure 3.1, the hippocampus is not a homogenous structure as one proceeds along the rostro-caudal axis. This is evident from the appearance of the histological based stereotaxic-transverse cross-sections of the hippocampus at dorsal (Figure 3.1A), medial (Figure 3.1B) and posterior (Figure 3.1C) levels with the ventral hippocampus appearing in the medial portion and merging with dorsal division at posterior levels. As described in methods, the segmentation of the histological sections into cyto-architectonic layers are validated using standard rat atlases and the parcellation of CA3, CA1 and DG regions into various sub-regions is shown in Figure 3.1a1 and is based on the terminology defined originally by Lorente de No (Lorente de No, 1934).
The extent of the hippocampus in the canonical brain and natural hippocampal axes is not symmetrically oriented along these axes. Specifically, the extension of the hippocampus in stereotaxic-transverse and hippocampal-transverse axes is around 5 mm and that in the longitudinal, horizontal and sagittal axes is around 7 mm. The 3D renderings of the complete structure of the hippocampus in multiple views are shown in Figure 3.3 with CA in blue and DG in red, where the internal layers are shown in darker and external layers in lighter colors. Typically, histological experiments are carried on cryostatic sections that are obtained by sectioning the rat brain in one of the three canonical brain (stereotaxic-transverse, horizontal or sagittal) axes. In standard atlases, the hippocampal histological cross-sections have different shape and contour geometry (Witter and Amaral, 2004; Amaral and Lavenex, 2007) but the classical ‘C’ shaped conformity corresponding to CA and DG is conserved across the septo-temporal axis. In fact, this property of the hippocampus is utilized to identify and parcel the distinct CA and DG sub-regions and carry out the detailed stereological analysis described in subsequent sections.

**Divisional and Regional Volume Differences in CA and DG**

As one would expect, the total volume of CA and DG was different (Figure 3.5A), where the DG volume was one-third (33%, 15.31 mm$^3$) of the total hippocampus volume (46.33 mm$^3$). One striking difference between the volume distributions of CA and DG divisions is that, the anterior and posterior divisions in CA had almost equal volumes (15.13 and
Figure 3.5: Divisional and region based volume differences in CA and DG A) Stacked bar plots of overall volume distributions for CA and DG characterized based on divisions (Anterior Vs Posterior) and the dorsal, ventral and posterior volumes. B) Stacked bar plot of CA3 and CA1 volumes in anterior and posterior divisions with (OR+PC) and
(RAD+LM) layer volumes. Inset shows the ratio of (RAD+LM) and (OR+PC) volumes in the six CA3 and CA1 hippocampal-transverse regions. C) Stacked bar plot of DG molecular and non-molecular layer (GC+HILUS) volumes in infra and supra regions.

15.89 mm$^3$ respectively), but within DG, the anterior volume (9.4 mm$^3$) was significantly greater than the posterior volume (5.92 mm$^3$). Additionally, the posterior volume in DG was almost equal to its dorsal volume (see Figure 3.5A). Incidentally, the volume in anterior CA closely matches that of the total volume of DG.

Within CA, the regional volume analysis revealed a non-uniform volume distribution of CA1 and CA3 among anterior and posterior divisions (see Figure 3.5B). For example in the anterior division, CA1 volume is marginally higher than CA3. But within the posterior division, CA1 volume is three times higher than that of CA3 (Figure 3.5B). Additionally, when analyzing the layer volume differences between the six CA3 and CA1 sub-regions, the volume ratio of (RAD+LM)/(OR+PC) increases from CA3c to CA1a (inset of Figure 3.5B). When comparing these ratios for the entire CA3 and CA1 regions, the (RAD+LM) combined volume is twice that of (OR+PC) volume in CA1 but just above one for CA3. In complete contrast to the CA divisional distribution, DG had equal ratio of supra to infra (1.6) volume distributions for both anterior and posterior divisions. This same ratio (1.6) was obtained for the ratios of total supra to infra volumes (see Figure 3.5C). Moreover, the total volume in infra (5.9 mm$^3$) is similar to the ML volume in the supra region (9.35 mm$^3$). The absolute volumes of each individual layer of CA and DG regions in the dorsal, posterior and ventral portions are tabulated in Table 3.2. In addition to the divisions in the rostro-caudal axis, we also divided it into septal, medial
and temporal divisions along the septo-temporal axis. The ratio of septal/dorsal, medial/posterior and temporal/ventral volumes was around one for all three ratios (results not shown here).

**Validation of volumes**

Comparison of CA and DG layer volumes between the complete and partial (Nissl#1 Vs Nissl#2) Nissl dataset, the µMRI dataset (Nissl Vs µMRI) and volume estimations from West et al. (1978) (Long-Evans Vs Wistar) are tabulated in Table 3.1. For (Nissl#1 Vs Nissl#2) comparison, all the seven layer volumes within the middle-third of the hippocampus are computed for both the complete and partial Nissl datasets and tabulated in column 2. For (Nissl#1 Vs µMRI) comparison, the cellular and outer layer volumes within CA and DG in the dorsal hippocampus are computed and tabulated in column 3. For (Long-Evans Vs Wistar) comparison, the combined volumes of (OR+PC), (RAD+LM) for CA and ML, (GC+HILUS) for DG are computed for the complete Nissl dataset and Wistar rats (West et al., 1978) and tabulated in column 4.

Table 3.1: Validation of volumes Individual CA (OR, PC, RAD, LM) and DG (ML, GC, HILUS) layer volume estimates of the complete Nissl (Nissl#1, Long-Evans) are compared with the partial Nissl (Nissl#2), µMRI and Wistar datasets and are tabulated in columns 2, 3 & 4.
The Nissl volume estimates from the two datasets reveals that they are close and within an error of 10%. The comparison between the μMRI dataset and the Nissl reveals that they closely match and are within an error of 5%. For comparison with (West et al., 1978), the combined (OR+PC) layer volumes is higher by 7.75% than Wistar and the combined (RAD+LM) layer volumes is lower by -1.77%. For DG, the ML layer volumes is closely related (only 0.34% difference) between these two strains. The biggest difference comes in the combined volumes of (GC+HILUS) where the Long-Evans is higher by 23.6% than the Wistar rats which might be due to difference in their strains.

Table 3.2: Absolute volumes of CA and DG layers characterized based on dorsal, posterior and ventral divisions.
Despite this huge difference in (GC+HILUS) layers, the total volumes of the hippocampus (CA+DG) differ only by 4.51%, with Long-Evans rats having a volume of 46.33 mm$^3$ and Wistar rats occupying a volume of 44.24 mm$^3$. Additionally, a comparison of the total volume of the hippocampus in our complete reconstruction closely matched the volumetric measurements from multiple structural MRI studies of
the rat hippocampus (Wolf et al., 2002a; Wolf et al., 2002b; Kalisch et al., 2006; Lee et al., 2009) within an error of 5%. Interestingly, we observed that the volumetric measurements of the total hippocampus obtained from the statistical based stereological assessments from histological data are under estimated by at least 15% (Ahmad et al., 2002; Schmitt et al., 2004) though the individual volumetric measurements of pyramidal cell layers are in close agreement (Ahmad et al., 2002; Akdogan et al., 2008). The importance and implications behind these volume differences is further elaborated in the discussion section.

**Uniform Non-Principal to Principal Layer Ratios in CA and DG**

The hippocampus is laminated into cyto-architectonic layers with quite distinct structural and histo-chemical identity associated with each layer (Witter and Amaral, 2004; Forster et al., 2006). Quantifying the volumes and their ratios has important implications because principal and non-principal layer cell densities and the occupancy ratios of neuritic and non-neuritic processes depend directly on the volume availability in these individual layers. Even though we obtained the sections by slicing the brain in rostro-caudal orientation, we took advantage of the high resolution 3D model and analyzed the volumetric data of each layer in four other reference axes. Specifically, we analyzed the volumetric distributions of each layer and their relationships in CA and DG in the three standard canonical brain axes, stereotaxic-transverse, horizontal and sagittal. Additionally, the volumetric data was analyzed in the natural hippocampal axes, septo-
temporal and hippocampal-transverse orientations (see legend in Figure 3.6D for cartoon of the five brain orientations).

**Figure 3.6: Uniform layer ratios for CA and DG**

A) Absolute CA layer (OR, PC, RAD, LM) volume distributions with 95% confidence interval as shaded colors are plotted in the stereotaxic-transverse axis with the dorsal, ventral, and posterior divisions marked at the bottom of the x-axis. The rostral and caudal poles are also shown at the beginning and end of the scale of the x-axis. The scatter plot with the linear fit shows the scaled ratio of OR and PC (OR/PC) layers in this axis. C) Absolute DG layer (ML, GC, HILUS) volume distributions with 95% confidence intervals as shaded colors are plotted in the hippocampal-transverse axis with infra and supra regions marked at the bottom of the x-axis. The scatter plot shows the scaled ratio of ML and GC (ML/GC) layers with the linear fit (black line). B and D) Bar plots of the average non-principal to principal layer
ratios in five different (stereotaxic-transverse, horizontal, sagittal, septo-temporal and hippocampal-transverse) axes for CA (panel C) and DG (panel D) ratios are shown. The overall mean (μ) and the Coefficient of Variation <CV> is shown on the top of the bar plots in each category (N ~ 40, mean ± SD). Analysis of Variance (ANOVA) statistical comparison with F and p values for OR/PC\(^1\), RAD/PC\(^2\), LM/PC\(^3\), ML/GC\(^4\) and HILUS/GC\(^5\) ratios are shown below.

* - The ratio of OR/PC scatter points is scaled by a factor of 10 to fit in the plot in panel A where the actual ratio is 0.96. ** - The ratio of ML/GC scatter points is scaled by a factor of 100 to fit in the plot in panel B where the actual ratio is 2.3. \(^1\) - F=1.1, p>0.3; \(^2\) - F=2.3, p>0.06; \(^3\) - F=0.6, p>0.6; \(^4\) - F=0.07, p>0.99; \(^5\) - F=0.62, p>0.6.

The CA layer volumes are spread across a stereotaxic-transverse axis span of over 4.5 mm except for the LM layer (red) which does not appear until 1 mm into the rostral pole (see Figure 3.6A). The majority of the volume for all the CA layers is distributed in the medial portions of the hippocampus as indicated by the dorsal, ventral, posterior extensions in the stereotaxic-transverse axis (bottom labels in Figure 3.6A). In particular, the dorsal part extends until 2.4 mm, ventral starts at 1.5 mm and ends at 2.4 mm and posterior occupies the rest of the stereotaxic-transverse axis extent. Incidentally, the absolute volume of all the layers (except LM) peak at or around the anterior/posterior cusp position with RAD layer (green) peaking over 0.35 mm\(^3\) and occupying most of the volume in this axis. Similar to the CA layer volume distributions, the DG layers are spread across 4 mm in the hippocampal-transverse axis with ML layer (yellow) peaking over 0.08 mm\(^3\) at the intersection of infra and supra regions (see Figure 3.6C).

Given these absolute volume distributions for CA and DG layers, we estimated the ratio of OR and ML to their respective cellular layers (PC for CA and GC for DG) in the stereotaxic-transverse and hippocampal-transverse axes and shown as scatter plots.
(dotted points in Figure 3.6A&C) respectively. The OR/PC ratio was uniform across the stereotaxic-transverse axis as evident from the linear fit with low $r^2$ value (~0.08) with the mean ratio being 0.96. Similar to the OR/PC ratio, the ML/GC ratio was also uniform across the hippocampal-transverse axis as depicted by the linear regression fit (solid black line in Figure 3.6C) with low $r^2$ value (~0.13) and having a mean value of 2.3.

Are the uniform layer ratios (OR/PC and ML/GC) demonstrated in panel A and C of Figure 3.6 for CA and DG similar for other ratios in all orientations? We found that this is indeed true for all the five non-principal to principal ratios of CA (OR/PC, RAD/PC, LM/PC) and DG (ML/GC, HILUS/GC) in all the five orientations (Figure 3.6B&D). The overall mean ($\mu$) ratios for the three CA ratios are 0.99, 2.76 and 0.66 for OR/PC, RAD/PC and LM/PC respectively (Figure 3.6B). There is also no significant variation for each of the three CA ratio’s average values in the five orientations as shown from the low coefficient of variation $<CV>$ estimates (see Figure 3.6B). Additionally, there is no significant difference in the averages (N ~ 40, mean ± SD) of these ratios in each orientation as evident from the ANOVA analysis (see Figure 3.6B). For the DG ratios, the overall mean ($\mu$) ratios for the two DG ratios are 2.38 and 0.60 for ML/GC and HILUS/GC respectively (see Figure 3.6D). As in the CA ratios, the average ratios in the five orientations have less variation as evident from the low $<CV>$ values (see Figure 3.6D). Additionally as in the case of CA ratios, there is no significant difference in the averages (N ~ 40, mean ± SD) of these ratios in each orientation as evident from the ANOVA analysis (see Figure 3.6D). These results suggest that intrinsically in the
structural development of the hippocampus, the non-principal layers are geared towards maintaining a balance in space and volume with respect to the principal cell layer in the canonical and hippocampal orientations.

**Non-Uniform Area Ratios for CA in Stereotaxic-transverse and Septo-temporal Axes**

In light of the functionality differences between CA3 and CA1 regions (Vazdarjanova and Guzowski, 2004; Leutgeb et al., 2007), we analyzed the distribution and ratios of CA3 to CA1 cumulative and individual layer volumes in all the five orientations. Following the non-uniformity in total CA3 and CA1 volumes in the anterior-posterior direction and uniform layer ratios in all the orientations as demonstrated earlier (see Figures 3.5&3.6); we addressed the question of how the CA3/CA1 ratio varies across all the orientations. In order to address this question, we took advantage of our volumetric representation where we categorized all the CA voxels into either CA3 or CA2 or CA1 based on their position along the hippocampal-transverse axis (see methods). For simplicity, the CA2 voxel volumes are added to the CA3 regional volumes. The ratios of the cumulative and individual layer regional (CA3 Vs CA1) volumes for CA are analyzed and plotted (see Figures 3.7&3.8). Similarly, the DG voxels were categorized as either infra or supra based on their hippocampal-transverse location (see methods) and their ratios plotted (see Figure 3.9).
Figure 3.7: Non-uniform area ratios for CA in stereotaxic-transverse and septo-temporal axes A and B) Cumulative CA3 (light grey) and CA1 (dark grey) volume distribution plots are shown with the solid black line as the mean and color shades as 95% confidence interval for stereotaxic-transverse (panel A) and septo-temporal (panel B) axis. In both these plots, the scatter points show the CA3/CA1 volume ratio with the linear regression fit (black line). C and D) Scatter plots of the CA3/CA1 volumes ratios for each of the four CA layers (OR: black; PC: red; RAD: green; LM: blue) with the corresponding colored line showing the linear regression fit in stereotaxic-transverse (panel C) and septo-temporal (panel D) axes.

There is a non-uniform distribution of the cumulative CA3 (light grey) and CA1 (dark grey) volumes in both stereotaxic-transverse and septo-temporal axes (see Figures 3.7A & B). In the stereotaxic-transverse axis, the CA3 and CA1 volumes have a normal
Figure 3.8: CA3 and CA1 cumulative volume distributions and CA3/CA1 ratios in horizontal and sagittal axes.

Distribution where the CA3 volume dominates in the rostral and CA1 dominates in the caudal pole, as is evidenced by linear fit of the ratio of CA3 to CA1 volumes (see Figure 3.7A). Linear regression analysis results in a high r² value (~0.93) which provides further evidence of the decreasing CA3/CA1 ratio from the rostral to caudal pole. In contrast to the stereotaxic-transverse axis, the CA3 and CA1 volume distributions follow an opposite trend (Figure 3.7B) in the septo-temporal axis. That is, at the septal pole, the CA1 volume dominates and at the temporal pole the CA3 volume either is higher or comparable to that of CA1 volume (Figure 3.7B). The increasing trend of CA3/CA1 ratio is evident from the linear regression analysis which produces moderate to high r² value (~0.54). The CA3 and CA1 volumes in two other axes (horizontal and sagittal) did not show similar patterns.
of distribution as in the stereotaxic-transverse or septo-temporal axes (Figure 3.8). The linear regression of

![Graphs showing volume distributions in different axes](image)

**Figure 3.9:** DG Infra and Supra cumulative volume distributions and Infra/Supra ratios in stereotaxic-transverse, septo-temporal, sagittal and horizontal axes. CA3/CA1 ratios resulted in low $r^2$ values for both horizontal (~0.0004) and sagittal (~0.2)
orientations (see Figure 3.8). For DG, none of the four orientations had any significant relationship between the infra to supra ratio as is evident from the low regression $r^2$ values (see Figure 3.9). However, the supra volumes were consistently higher than the infra volumes in all the four orientations (see Figure 3.9) which could be predicted from the cumulative supra/infra volume ratio of 1.6 as shown in Figure 3.5C.

An interesting question arises from the decreasing and increasing CA3/CA1 ratios in the stereotaxic-transverse and septo-temporal axes (Figure 3.7A&B). Which of the CA layers contribute to this unequal distribution of the CA3 and CA1 volumes in these two orientations? In order to answer this question we also analyzed the individual layer CA3/CA1 ratios in both stereotaxic-transverse (Figure 3.7C) and septo-temporal (Figure 3.7D) axes. In the stereotaxic-transverse axis, all four CA layers except LM had a decreasing CA3/CA1 ratio with high linear regression $r^2$ (~0.8) values. The low $r^2$ (~0.08) value for LM layer is depicted by a flat line (see Figure 3.7C). In contrast, only the RAD layer in the septo-temporal axis had increasing CA3/CA1 ratio with moderate to high linear regression $r^2$ values (~0.60) and the other three layers had low $r^2$ (~0.02) values as depicted with flat lines (see Figure 3.7D). The inhomogeneous distribution of the cumulative CA3 and CA1 volumes affecting occupancy ratios and its functional implications is discussed further.

**Hippocampal Cell Mapping: Availability of Morphological Data**
Three dimensional morphometric analysis are regularly employed to study the complex geometry and shape of neuronal arbors digitally reconstructed from stained brain tissue (Ascoli et al., 2001; Van Pelt et al., 2001; Ascoli, 2002). In pathological conditions, the arbor geometry and branching structure is compared to that in the healthy conditions and the cellular and molecular factors affecting the differential geometry are studied.
Figure 3.10: Hippocampal morphological reconstructions data availability. Hippocampal morphological data availability grey scale maps are shown for the two indices, Available Neuronal Count (ANC, panel A) and Available Neuronal Density Index (ANDI, panel B). The two ANC maps shown in panel A correspond to CA (left) and DG (right) principal neuronal availability mapped onto the 3D model with respect to the septo-temporal and hippocampal-transverse axes. Similarly, the two ANDI maps shown in panel B correspond to CA (left) and DG (right) principal neuronal availability in these two axes. For the CA maps, the sub-regions of CA3 (CA3cba), CA2 and CA1 divisions are shown. For the DG maps, the infra and supra divisions are shown. The color scale in each grey scale map represents the range of values for ANC and ANDI.

(Kaufmann and Moser, 2000). Additionally, 3D geometry of the neuronal arbors is incorporated in detailed biophysical models in order to study the dendritic structure affecting signal propagation and integration at single cell level (Mainen and Sejnowski, 1996; Poirazi et al., 2003a; Komendantov and Ascoli, 2009). In all these studies, cell type and location is important for linking structure-function relationship.

In order to integrate all the hippocampal morphological data acquired till date, we performed a probabilistic mapping onto this 3D template based on the reported positional information in the literature (see methods). All together a total of ~250 (CA: 163, DG: 81) hippocampal cells were mapped onto this template acquired from sixteen different archives downloaded from Neuromorpho.org (Ascoli et al., 2007). As described in methods we created two indices, ANC and ANDI for the mapping process. In summary, ANC shows the number of reconstructed cell data available and ANDI, on the other hand, shows the localization of neuronal data at a given hippocampal-transverse and septo-temporal position. The results of the mapping for excitatory principal cells are plotted using the R package ‘ggplot2’ (http://had.co.nz/ggplot2/) and shown in grey scale for
ANC and ANDI maps (Figure 3.10A&B) for both CA and DG (left and right column of Figure 3.10).

For CA principal neurons, the middle third of CA1 region has the highest pyramidal cell data availability as evident from the dark pixels in the left graph of Figure 3.10A with neuronal count reaching ~60 for these positions. The rest of the CA1 region, that is the septal and temporal thirds, also have a high availability of CA1 principal cells (40-50). Compared to CA1, the CA3 region has lesser data availability with a range of 10-40 cells for each position. Similar to CA1, the middle CA3 region except the CA3a sub-region has the most data availability. In contrast to the ANC maps for CA principal neurons, the pixels in the middle third CA1 region do not correspond to the maximum ANDI values (left graph of Figure 3.10B). This is because ANDI and ANC show different but complementary cell data availability information. In other words, more precise the septo-temporal and hippocampal-transverse positions are known for a particular cell, higher the ANDI value for that particular location.

We can easily distinguish this difference in the CA principal cell layer. The highest values of ANDI (~0.31) correspond to the hippocampal voxel positions in the dorsal CA1 region. But from the ANC index map (left graph in Figure 3.10A), the same voxel positions do not have the highest ANC where the voxels in the middle third of CA1 have the highest ANC index. The voxel positions with the highest ANDI values are where the cell data whose stereotactic coordinates are known in the dorsal CA1 region which corresponds to the CA1 neuronal reconstructions from Gulyas archive (see methods). It is
worth noting that some portion of CA2 region is devoid of any data as we can see from the white pixels within the CA2 region in these graphs (Figure 3.10A&B). For DG, the middle third GC layer voxels have higher ANC (right graph of Figure 3.10A) values. Within the middle third, the supra pyramidal GC voxels have the highest ANC values in the range of 50-65 and the highest ANDI values (right graph in Figure 3.10B).

**Visualization of Neuronal Embeddings**

![Digital neuronal embedding illustrations](image)

**Figure 3.11:** Digital neuronal embedding illustrations A) Illustrations of CA3/CA1 dendritic arbors embedded within the 3D Template and two views are shown here, septo-temporal and stereotaxic-transverse views. Layers shown here are PC (yellow), OR (pink), GC (red) and ML (green) with dendritic arbors shown in blue. (B) Illustration of CA2 axonal arbor (green) embedded within this 3D framework. The two views shown here are septo-temporal and rostro-caudal views. Layers shown here are PC (blue) and OR (red) with CA2 axonal arbor shown in green.
As mentioned previously, the cell mapping process (Figure 3.10) results in establishing a database where each voxel is populated with a list of cell-ids and their associated probabilistic weight for random sampling. The novel computational framework, as described in methods, facilitates the embedding of neuronal arbors within this 3D construct by approximating the principal components along the septo-temporal, normal to the tissue curvature and the hippocampal-transverse axes (Ropireddy et al., 2008; Ropireddy et al., 2010). Since neuronal position is important for functional significance, this process not only allows embedding of arbors in a specific sub-region but also at a particular location. For example, with this robust framework we can randomly sample a cell belonging to a voxel at mid-septotemporal levels and at 50% into the CA hippocampal-transverse axis in the CA3b sub-region. With such a robust and powerful computational ability to embed arbors, this construct enables the digital packing of neuronal arbors at NDs that are similar to in vivo conditions and compute key stereological parameters like the dendritic occupancy ratios and layer based dendritic orientation angles across the entire hippocampus which are discussed in the following sections.

The neuronal embeddings within this 3D template are rendered and visualized using VRML viewer view3dscene (view3dscene.sourceforge.net) as illustrated in Figure 3.11. The dendritic arbors embedded and illustrated here (Figure 3.11A) are obtained from Amaral archive, whose position is mapped as explained in earlier section. In this panel, examples of CA3 and CA1 embeddings are shown in two views (septo-temporal and
coronal). The dendritic tree is scaled in depth to fit within the available volume from the alveus to the fissure. The neuronal data are pooled together from different animals of varied ages, rat strains, and incorporated into our 3D template. In order to allay our concerns of the variability in the data and their organization within the hippocampus, we validated the spread of the dendritic arbor in the septo-temporal and hippocampal-transverse axes for CA and DG principal neurons. The average spread of granule cells (taken from Claiborne archive) in these two orientations computed was $351 \pm 39 \, \mu m$ and $171 \pm 16 \, \mu m$, which are within an error of 10% as measured experimentally by Claiborne et al. (1990). In the CA3 region, the CA3b dendritic arbors were symmetrically oriented along these two axes with their spread of $310 \pm 102 \, \mu m$ and $333 \pm 59 \, \mu m$ respectively which is within a 13% error of that reported in literature (Henze et al., 1996). This reflects that the organization of neuronal arbors observed in vitro is captured in our 3D framework which is comforting as we demonstrate the application of this embedding process to estimate occupancy ratios and layer based dendritic orientation angles in further sections. Apart from dendritic arbor embedding, we also oriented axonal arbors into this 3D space. Figure 3.11B illustrates the embedding of CA2 axonal arbors oriented in this 3D space based on the dendritic orientation. The CA2 axonal arbor (orange) clearly occupies half of the hippocampus in the septo-temporal axis and the two views shown here are septo-temporal and rostro-caudal views.

**Regional Differences in Dendritic Occupancy Ratios for CA and DG**
The hippocampal neuropil contains one the highest neurite and cell packing ratios in the cortical circuits (Stepanyants et al., 2002; Escobar et al., 2008). It is intriguing to know the relative percent volume occupied by wire (axons and dendrites) and non-wire (boutons, spines, glia, extra-cellular matrix and vasculature) in a given neuropil volume. Previous work in addressing this question was carried out by Chklovskii et al. (2002) in cortical circuits. The optimal value of wiring ratio in cortical and CA1 hippocampal circuits was shown to be close to 0.6 which does not deviate much from the biophysically predicted value (Chklovskii et al., 2002). These optimal values were demonstrated to be applicable in the CA1 radiatum layer. As shown and discussed in previous sections, there

Figure 3.12: Validation of occupancy ratios (A) and nematic order parameters (B) with EM based approach in the mid-radiatum of CA1.
is a non-uniform and inhomogeneous distributions of volumes in individual layers and within regions across the entire extent of the hippocampus (see Figures 3.5, 3.6, 3.7). Additionally, in the scenario of differential NDs in the dorsal (or septal) and ventral (or temporal) divisions in CA1 (see Figure 3.4; Jinno and Kosaka, 2009), it is highly questionable that this optimal wiring ratio holds across the septo-temporal axis of the rodent hippocampus. In order to address these questions, we designed a robust computational framework to digitally pack the cellular layers with reconstructed principal neurons and computed the dendritic occupancy ratios whose methodology is explained in the methods section.

In order to validate our framework with electron microscopy (EM) based measurements, we computed DORs for the mid-radiatum layer in the three CA1 sub-regions at mid-septo-temporal level (Figure 3.12). The EM based measurements of dendritic occupancy ratios were carried out in automatically reconstructed minute neuropil volumes in CA1 mid-radiatum layer at mid-septotemporal levels and the dendritic occupancy ratio estimated within these minute neuropil volumes was 0.4 (Mischenko et al., 2010). The weight-averaged DOR at this location estimated in our framework was 0.38 ± 0.01 (N=10 simulations, mean ± SD) which is close to that estimated by EM approach (Figure 3.12A). Finally, the DORs estimated over the entire extent of the hippocampus in our framework are weight-averaged across the depth (layers) and sub-regions with their volumes and their distribution plotted in the septo-temporal axis for CA and DG (Figure 3.13).
Figure 3.13: Dendritic occupancy in CA3, CA1, and DG. (A) Distribution along the longitudinal axis of CA1 and CA3 dendritic occupancy weight-averaged by volume across layers and hippocampal-transverse sub-regions. The CA1, but not CA3, values (and corresponding linear fits) show separate septal and temporal components. Inset: average CA1 and CA3 dendritic occupancies (N=7, mean±SD) in the septal and temporal halves. B) Distribution along the longitudinal axis of DG dendritic occupancy in the infra- and supra-pyramidal blades. Inset: average DG dendritic occupancies (N=13, mean±SD) in the septal and temporal divisions.
The CA3 and CA1 regions have different patterns of DORs in the septo-temporal axis (Figure 3.13A). The CA1 region has two distinct patterns in the septal and temporal divisions and in contrast the CA3 region is uniform across the length of the hippocampus. Within the individual septal and temporal divisions, the DORs in the CA1 region are more or less uniform as depicted by the light and dark grey linear fitted lines of the correspondingly colored scattered points having low-medium $r^2$ values (CA1-Septal: ~0.53; CA1-Temporal: ~0.32). Compared to the CA1 DOR values, the CA3 region has no such distinction between septal and temporal divisions, but is uniform across the septo-temporal axis. The linear fit for the CA3 DOR values result in a low $r^2$ value (~0.16). The average DORs in the septal and temporal divisions for CA1 region are 0.29 and 0.46 respectively (inset in Figure 3.13A).

For CA3, these average values are 0.44 and 0.37 for septal and temporal divisions respectively (inset in Figure 13A). The ratio of CA3/CA1 for the DORs in the septal and temporal division is 1.56 and 0.8 respectively. This trend in the DOR values for CA3 and CA1 is reflected in the CA3/CA1 cumulative volume ratio plot in Figure 3.7B where high CA1 volumes in septal division leads to low DOR values and vice versa in the temporal division. The pattern for the CA3 region is reverse to that followed by the CA1 region. That is, lower CA3 volume availability in septal division leads to higher DOR values and vice versa for the temporal division though marginally.
The pattern of DOR distribution for CA1 and CA3 is also reflected in their individual sub-regions across the septo-temporal axis (see Figure 3.14A&B). Clearly we can see that all the three CA1 sub-divisions have two distinct patterns corresponding to the septal and temporal divisions. In the septal and temporal divisions, all three divisions have no change with respect to the septo-temporal axis which is reflected in low $r^2$ values (see Figure 3.14A). The average CA1c,b,a DORs in the septal divisions are 0.26, 0.27 and 0.31 respectively. In the temporal division these values are 0.48, 0.42, & 0.48. Similarly for CA3, the distribution of DORs for individual CA3 sub-regions show no dependence on their position across the septo-temporal axis as depicted by their low $r^2$ values (see Figure 3.14B). When comparing the average DORs for these three sub-regions, the CA3a DOR is higher by at least 35% compared to CA3c or CA3b sub-regions (see Figure 3.14B).

The DOR distributions are weight-averaged across the septo-temporal axis and the overall averages are compared for the individual layers and sub-regions. In Table 3.2, the differences in the OR, RAD and LM layer in the individual as well as the grand weight-averaged of CA3 and CA1 sub-regions are shown. In between the overall CA3 and CA1 average DOR, there is a pronounced difference in OR and RAD layers. Specifically, the OR layer has significantly higher mean occupancy ratio (0.48) compared to that of RAD layer (0.34) in CA3 by over 40%. In contrast, the RAD layer (0.40) has higher mean occupancy ratio than the OR layer (0.33) in CA1 by 20%. This difference in the OR and RAD mean DORs between CA3 and CA1 regions could be attributed to the difference in average principal dendritic length and volume differences between OR and RAD layers.
Figure 3.14: Occupancy ratio distributions in CA and DG sub-regions across the septo-temporal extent.
in CA3 and CA1. It is interesting to note that the average dendritic length within the OR and RAD layers for CA3 and CA1 pyramidal neurons are complementary (Ishizuka et al., 1995). The average CA3 and CA1 pyramidal neuronal dendritic length within the OR layer is 5645.6 μm and 4585.8 μm respectively. Conversely, the dendritic length in RAD layer for CA3 and CA1 principal cells is 4382.4 μm and 6306.5 μm. Intuitively, we can deduce that the occupancy ratios follow the same pattern. But, since the volumes are non-uniformly distributed for RAD and not for OR in the septo-temporal axis (see Figure 3.5), this results in the adjustment of the occupancy ratios in the OR and RAD layers in CA3 and CA1 regions. Within the individual sub-regions of CA3 and CA1, the DORs in the individual sub-layers also showed a similar pattern across the septo-temporal axis. The three sub-regions in CA1 have septal and temporal distinctive divisions and CA3 sub-regions have a uniform distribution across the septo-temporal axis (see Figure 3.14 C&D) which is consistent with the observations reported in the main figure (Figure 3.13A). In the temporal division of CA1, the RAD layer has higher occupancy ratio by at least 20% compared to the OR layer (see Figure 3.14C), but no significant differences exist in the septal CA1 division. For the CA3 layers, there is no such distinction of septal and temporal divisions as shown by the low r² values for OR, RAD and LM (see Figure 3.14D).

The DOR distributions in the DG regions, in contrast to CA, were uniform throughout the septo-temporal axis for both infra and supra regions (Figure 3.13B). This is evident from
the low $r^2$ values of the linear fits for both infra (~0.035) and supra (~0.006) regions (see Figure 3.13B). Apart from the uniform distribution of the DORs for DG regions, the infra has higher occupancy ratios than the supra region by at least 20% in both the septal and temporal divisions (see inset in Figure 3.13B). Additionally, in both these two divisions

**Table 3.3: Summary of dendritic occupancy ratios for each layer within CA3, CA1 and DG sub-regions obtained by weight-averaging across the septo-temporal extent.**

<table>
<thead>
<tr>
<th>OR</th>
<th>RAD</th>
<th>LM</th>
<th>All Layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA3c</td>
<td>0.44 (± 0.09)</td>
<td>0.31 (± 0.07)</td>
<td>-</td>
</tr>
<tr>
<td>CA3b</td>
<td>0.48 (± 0.10)</td>
<td>0.33 (± 0.08)</td>
<td>0.22 (± 0.05)</td>
</tr>
<tr>
<td>CA3a</td>
<td>0.66 (± 0.14)</td>
<td>0.41 (± 0.10)</td>
<td>0.39 (± 0.06)</td>
</tr>
<tr>
<td>CA3</td>
<td>0.48 (± 0.12)</td>
<td>0.34 (± 0.09)</td>
<td>0.27 (± 0.07)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OR</th>
<th>RAD</th>
<th>LM</th>
<th>All Layers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA1c</td>
<td>0.35 (± 0.08)</td>
<td>0.43 (± 0.10)</td>
<td>0.34 (± 0.06)</td>
</tr>
<tr>
<td>CA1b</td>
<td>0.32 (± 0.07)</td>
<td>0.35 (± 0.11)</td>
<td>0.39 (± 0.08)</td>
</tr>
<tr>
<td>CA1a</td>
<td>0.36 (± 0.08)</td>
<td>0.42 (± 0.09)</td>
<td>0.38 (± 0.11)</td>
</tr>
<tr>
<td>CA1</td>
<td>0.33 (± 0.07)</td>
<td>0.40 (± 0.11)</td>
<td>0.35 (± 0.09)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Proximal ML</th>
<th>Medial ML</th>
<th>Distal ML</th>
<th>ML</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infra Tip</td>
<td>0.20 (± 0.07)</td>
<td>0.22 (± 0.05)</td>
<td>0.39 (± 0.12)</td>
</tr>
<tr>
<td>Infra Crest</td>
<td>0.17 (± 0.03)</td>
<td>0.21 (± 0.05)</td>
<td>0.35 (± 0.12)</td>
</tr>
<tr>
<td>Supra Crest</td>
<td>0.16 (± 0.03)</td>
<td>0.21 (± 0.06)</td>
<td>0.23 (± 0.06)</td>
</tr>
<tr>
<td>Supra Tip</td>
<td>0.14 (± 0.03)</td>
<td>0.19 (± 0.03)</td>
<td>0.24 (± 0.08)</td>
</tr>
<tr>
<td>DG</td>
<td>0.17 (± 0.03)</td>
<td>0.21 (± 0.02)</td>
<td>0.30 (± 0.08)</td>
</tr>
</tbody>
</table>
the infra/supra DOR ratio is similar reflecting the uniform volumetric infra/supra ratios (see Figure 3.5C). The differences between the individual sub-layers and within the sub-regions of DG are obtained by weight-averaging across the septo-temporal axis (see Table 3.3). For all the sub-regions of DG, the distal ML has higher occupancy ratios compared to the proximal and medial ML by at least 30% (see Table 3.3). The difference in the occupancy ratios between infra and supra regions as demonstrated in Figure 3.13B arise from the major difference between the DORs of distal ML values between the infra and supra sub-regions (see Table 3.3). Except within the Infra Tip sub-region, these uniform pattern of DORs across the septo-temporal axis (Figure 3.13B) are conserved across all the sub-regions of DG and for all three molecular sub-layers (see panels Figure 3.14E-H). These results are in direct agreement with the empirical observations on the differential dendritic density of the granule cell arbors in the proximal, medial and distal ML and in the infra and supra regions (Claiborne et al., 1990; Rihn and Claiborne, 1990).

**Occupancy² Correlation to DSCAM expression**

The density and space occupancy of dendritic segments within the cortical neuropil volumes places a huge constraint on the growth of neurites within stipulated cyto-archetectonic boundaries (Parrish et al., 2007) which is otherwise known as the ‘tiling effect’. This phenomenon is evident in hippocampal neuropil where the principal cells of DG and CA (granule and pyramidal) neurons extend their dendritic fields until only the fissure border (Witter & Amaral, 2004). In addition to the tiling effect, through
development, neurons are theorized to express intrinsic molecular guidance mechanisms to avoid homotypic and heterotypic dendritic overlaps (Sestan et al., 1999; Parrish et al., 2007). In recent years, a number of studies in drosophila and mouse models have shown that DSCAM molecule is a likely candidate for avoidance between these dendro-dendritic contacts (Matthews et al., 2007; Schmucker, 2007; Fuerst et al., 2008; Millard and Zipursky, 2008).
Figure 3.15: Correlation of occupancy\(^2\) values with DSCAM expression across hippocampal-transverse regions of CA and D. A) Regression analysis of occupancy\(^2\) values for the six CA sub-regions compared to the normalized DSCAM expression. B) Regression analysis of occupancy\(^2\) values for DG sub-regions compared to the normalized DSCAM expression. The means represented here are averaged over dorsal sections of ABA and the occupancy\(^2\) values (N=5, mean ± SE).
In previous sections, we showed that there is non-uniformity in the distribution of volumes within various layers, sub-regions and divisions of CA and DG. Accordingly, the occupancy ratios across the layers and regions are non-homogeneous in nature. Specifically, the highly curved regions (CA3a/CA2) have higher occupancy ratios due to the higher neurite density in these sub-regions. As a result, we hypothesized that the DSCAM gene expression is correlated with the squared occupancy ratios (occupancy$^2$) within the individual CA and DG sub-regions. As explained in methods, the relative DSCAM expression is quantified from sagittal DSCAM in situ hybridization images of the ABA (mouse.brain-map.org, Ng et al., 2009) of the mouse hippocampus from raw color coded images. We correlated the relative DSCAM expression to the occupancy$^2$ values for CA and DG sub-regions which are obtained by weight-averaging across the septo-temporal axis in the dorsal extent (see Figure 3.15). The reason for correlating these two quantities in the dorsal extent is that the ISH images from ABA are sagittally obtained and the individual CA and DG sub-regions could only be identified in the dorsal division in these images.

In agreement with our hypothesis that the curved regions in CA have higher expression of DSCAM, the CA3a/CA2 regions have the highest correlation between occupancy$^2$ and relative CA DSCAM expression (see Figure 3.15A). Qualitatively, the relative degree of DSCAM expression in the CA sub-regions can be seen in the inset of Figure 3.15A with curved regions (CA3a/CA2) having more red pixels compared to other sub-regions. Overall, there is a high correlation between the occupancy$^2$ values and the DSCAM
expression as evident from the high $r^2$ of the linear fit (~0.94). Incidentally, CA1 sub-regions have the lowest DSCAM expression in parallel with their low occupancy$^2$ values in the dorsal (or septal) division as discussed previously (see Figure 3.15A). The significance of this effect is that, in highly curved regions like CA3a/CA2, there is a higher need for expression of DSCAM like molecules which has been implicated in assisting the phenomenon of avoidance of homotypic and heterotypic dendro-dendritic contacts. In contrast to the CA hippocampal-transverse regions, the DG sub-regions do not display the same trend of the relation of curvedness to DSCAM expression (Figure 3.15B). But, the infra sub-regions have comparatively higher correlation than the supra regions which can be attributed to the higher degree of DORs in the infra compared to the supra regions (see Figures. 3.13B and Table 3.2). Overall, DG sub-regions display high correlation between the occupancy$^2$ and DSCAM expression as evident from the linear regression with moderate to high $r^2$ values (~0.77). Though, there is not much difference of the relative DSCAM expression between the five DG sub-regions where the supra and infra tip sub-regions differ by only 5%. This can also be intuitively observed from the inset of DSCAM image in the hippocampus where the color intensity (redness) of the pixels in the GC layer does not vary much (see inset of Figure 3.15B). In contrast to CA, the quasi-uniform and high levels of DSCAM expression reflects the dense packing of granule cells and the high density of its dendritic segments across the DG hippocampal-transverse axis from infra to supra tip in order to avoid the dendro-dendritic contacts.

**Anisotropicity in Dendritic Orientation Within CA and DG Layers**
Using the computational framework for estimating occupancy ratios, we also analyzed the dendritic micro-architecture orientation within the cyto-architectonic layers of the hippocampus from the nematic order parameter (‘S’) values. The angular deviation ($\Delta \theta$) of the dendritic segments from the isotropic angle ($\theta = 55^\circ$, $\Delta \theta = 0$) was derived by weight-averaging over the entire extent of the hippocampus (see methods) and tabulated in Table 3.3 for the three CA layers (OR, RAD and LM) in CA3, CA1, and for DG-ML.

In this table, the cartoon representation shows two cases of anisotropy where $\Delta \theta \neq 0$ corresponding to $\theta = 0^\circ$ or $\theta = 90^\circ$ and the isotropic case where $\Delta \theta = 0$ for $\theta = 55^\circ$. In both the anisotropic cases, the dendritic segments are either parallel or perpendicular to the director (or principal) axis (see Table 3.3). Similar to the DORs validation with EM based measurements; here also we validated our estimates of angular deviation of CA1 dendrites in the mid-radiatum neuropil volume (see Figure 3.12B). The weight-averaged angular deviation of CA1 dendrites in the mid-radiatum at mid-septotemporal levels obtained from the three CA1 sub-regions is $-10.4^\circ \pm 1.4^\circ$ (N=10 simulations, mean ± SD), while that obtained from the EM based neuropil is $-11.7^\circ$ corresponding to $S=0.3$ (personal communication, Chklovskii, 2009).

In both the CA3 and CA1 regions, the terminal dendrites in the LM layer are organized in a random (or isotropic) orientation which is evident from the low angular deviation of $-0.9^\circ \pm 3.20^\circ$ for CA3 and $1.7^\circ \pm 4.64^\circ$ for CA1 from the isotropic angle of $55^\circ$ (see last column in Table 3.4). In contrast to the terminal dendrites in LM, the basal and apical dendrites in OR and RAD deviate more from the isotropic nature with a range of
Table 3.4: Angular deviation of the dendritic segment orientation from the isotropic angle (55°) in CA and DG layers. The angular deviation (Δθ) for the three CA layers (‘OR’, ‘RAD’ and ‘LM’) are shown in columns 2, 3, 4 for CA3 and CA1 regions. For DG, the angular deviation (Δθ) for the ML layer is shown in the last row. For all the Δθ values the mean with SD is reported based on N=10 simulations. Asterisks denote statistical significance of the one-way ANOVA analysis with Bonferroni’s multiple comparison tests with the control being that of the angular deviations of CA3-LM and CA1-LM for OR, RAD and ML layers. The p-values corresponding to *, **, *** are p<0.05, p<0.03 and p<0.001 respectively. Cartoon representation in this table shows examples of isotropicity and anisotropicity in terms of angular deviation (Δθ) and the angle of incidence of the dendritic segments with the reference axis (θ).

<table>
<thead>
<tr>
<th></th>
<th>OR (Δθ ± SD, N=10)</th>
<th>RAD (Δθ ± SD, N=10)</th>
<th>LM (Δθ ± SD, N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA3</td>
<td>-10.2° ± 4.90° **</td>
<td>-10.2° ± 5.50° **</td>
<td>-0.9° ± 3.20°</td>
</tr>
<tr>
<td>CA1</td>
<td>-8.1° ± 3.98° **</td>
<td>-12.8° ± 4.42° ***</td>
<td>1.7° ± 4.64*</td>
</tr>
<tr>
<td>DG-ML</td>
<td>-15.9 ± 3.98° ***</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

deviation of 8° to 13° to the isotropic angle (see column#2&3 in Table 3.4). In CA3, the angular deviation for OR and RAD is similar in the order of 10°, but in CA1, the apical dendrites in RAD layer deviate more than that in the OR layer by 5°. In the case of DG, the granule cell dendrite orientation displays the most anisotropic pattern with at least a 15° angular deviation from the isotropic angle in the ML layer. The statistical comparison of the angular deviation between LM and other layers (OR, RAD in CA3 and CA1, DG-ML) reveals statistically significant deviation from the isotropicity of LM (as denoted by asterisks in Table 3.4). The statistical analysis was performed by doing a one-way
ANOVA analysis with Bonferroni’s multiple comparison tests with the control being that of the angular deviations of CA3-LM and CA1-LM. Both the comparisons to the CA3-LM and CA1-LM had statistically significant differences (p<0.05). Additionally, the differences between OR and RAD layer dendritic orientations in CA3 and CA1 were not significant. In terms of the maximum anisotropicity the ML layer has the most angular deviation from the isotropic angle which is denoted by lowest p-value (p<0.001). These observations are in agreement with the anisotropic tissue architecture in hippocampal layers studied from high-resolution diffusion tensor imaging and tractography of isolated rat hippocampus (Shepherd et al., 2006).

DISCUSSION
Three dimensional morphometric analyses of dendritic arbors are commonly employed to study the nature of the complexity of the branching structure and shape of neurons (Ascoli et al., 2001; van Pelt et al., 2001). In almost all of these studies, the three dimensional geometry of the neuronal arbors is studied outside the entity of the brain system they represent. In creating a three dimensional representation of the hippocampus, we facilitated a qualitative and quantitative integration between single neuronal morphology and systems neuroanatomy of the hippocampus. To the best of our knowledge this is the first and foremost study to embed digital neuronal arbors and compute the macroscopic stereological properties (volumes and occupancy ratios) within a high-resolution 3D construct of the rat hippocampus. Previous efforts in estimating individual and regional volumes across the entire extent of the hippocampus based on
histological sections was carried by West et al. (1978) by segmenting the cyto-
architectonic layers across the canonical brain axes and whose work comes close to our
approach with respect to total volume estimations. But, they lacked the technological and
computational power to analyze the layer and regional volumes in a way we present here
which is only possible with a comprehensive 3D model.

Volume estimation within cyto-architectonic layers or whole anatomical regions is
fundamental to our understanding of the structural organization in any brain system. In
the case of the hippocampus, it is even more important because of the distinction between
principal and non-principal layer architecture and the region (DG Vs CA3 Vs CA1) based
functional differentiation (Vazdarjanova and Guzowski, 2004; Leutgeb et al., 2007). The
volume occupancy in principal layers directly affects the principal cell absolute numbers
and their densities which in-turn influences the wiring ratios in the non-principal layers
(Chklovskii et al., 2002). Additionally, in many pathological conditions and animal
models there are concomitant changes in brain volumes at regional or at whole systems
level compared to their controls (Wolf et al., 2002a; Lee et al., 2009).

As a result, a number of studies have been carried out for estimating the volumes of
individual layers within the hippocampus by applying statistical based stereological
methods (Schmitz and Hof, 2005) which employ random unbiased sampling carried out
on histological sections (Boss et al., 1987; Tinsley et al., 2001; Ahmad et al., 2002;
Akdogan et al., 2008). As noted in results, except for the CA PC layer volume estimate
by Ahmad et al. (2002), all other estimates of layer and total hippocampal volumes are underestimated by at least 15-30%. One might attribute these differences to the different age and strain of the animals used. A comparison of volumes, however, between our model and the ones estimated by West et al. (1978, see Table 1) or the μMRI based studies (Wolf et al., 2002a; Wolf et al., 2002b; Kalisch et al., 2006; Lee et al., 2009) reveals that the total hippocampus volume is within ±5% of our estimate in which different age and strain of the rats are used. So, in the case of design-based stereological approach it is very important to carry these experiments in a methodical way because it relies heavily on estimating the volumes through error correction methods based on random sampling (West et al., 1991; Schmitz and Hof, 2005; Slomianka and West, 2005). In light of these observations in volume differences, it may be necessary to refine the statistical based error estimation protocols in brain volume quantification based on histological sections by taking the reconstructed tissue as controls as demonstrated here.

A major disadvantage of our study though is that we are presenting the volume analysis based on one rat hippocampus model because manual reconstruction is laborious. But, without a complete 3D model of the hippocampus, it is not possible to analyze the volume distributions of the layers in three canonical (stereotaxic-transverse, horizontal and sagittal) and the natural hippocampal (septo-temporal and hippocampal-transverse) axes. With this robust and invaluable construct, we found out that there is a uniform non-principal to principal layer volumetric ratios in both CA and DG in all these five axes (see Figure 3.6). It wouldn’t have been possible to observe the reverse relationship
between the CA3/CA1 volume ratio in the stereotaxic-transverse and septo-temporal axis (see Figure 3.7). By digitally packing the cellular layers with reconstructed neurons of the respective type, we computationally extracted and quantified the distributions of DORs within the different regions and layers of the hippocampus. The non-homogeneous volume distributions affected the distribution of occupancy ratios as discussed before (Figure 3.13, 3.14 and Table 3.2). Specifically, we found that there are regional differences in the DOR distributions both in CA and DG. In the CA3 region, the DOR distribution was uniform while in CA1, the septal DORs were less than the temporal divisions by 60%. Within DG, even though the DORs were uniform across the septo-temporal axis, the infra ratios were 20% higher than the supra region. These occupancy ratios reflect the non-uniform volume distributions in these regions as shown and discussed earlier (see Figs. 4&5&6 & Table 1). As shown in Table 2, there are also layer based differences in all these three regions which could be attributed to the different cyto-architectonic and layer-specific intrinsic and extrinsic projections to the hippocampus (Witter & Amaral, 2004; Amaral & Lavenex, 2007).

In extracting the occupancy ratios, we pulled morphological data from different animals of varied strain and ages and incorporated them into our model. Previous research on hippocampal morphological variability showed non-significant differences among different strains and reconstructed under different preparations and laboratory settings within the rodent hippocampus (Scorcioni et al., 2004). Keeping in mind this limitation, it is virtually impossible to extract occupancy ratios and study their distributions at systems
level, that is, across the setpo-temporal axis using other methodologies like EM based approaches (Chklovskii et al., 2002, Mishchenko et al., 2010). A huge limitation of estimating the wiring ratios from EM based approaches is that we can only assess tiny portion of neuropil volumes (Mishchenko, 2009; Mishchenko, 2010; Mischenko et al., 2010). In spite of these practical challenges, we compared the DORs within the CA1 mid-radiatum layer at mid-septotemporal level with that of data obtained from EM reconstructed neuropil volume in the rat hippocampus (Mischenko et al., 2010). As described in results, the comparison resulted in ~5% difference between our measurements and the EM neuropil analysis.

One of the hallmarks of the brain’s architectural layout is its capacity to economically pack spatial volumes with unprecedented neuronal densities (Boss et al., 1987; Amaral et al., 1990; Braitenberg and Schultz, 1998; Stepanyants et al., 2002). This organizational property places a tremendous importance on the optimal use of the spatial as well as the energy resources (Levy and Baxter, 1996; Laughlin et al., 1998; Ames, 2000; Chklovskii et al., 2002; Levy and Baxter, 2002; Karbowski, 2007). There is, in fact, a greater need for efficiently utilizing brain volumes in high cell density brain regions such as the hippocampus where the neuronal and neurite densities (Stepanyants et al., 2002; Escobar et al., 2008) are comparable or even higher than other brain regions. One way to efficiently utilize the spatial resources is through wiring optimization (Chklovskii et al., 2002; Buzsaki et al., 2004) where the cyto-archetectonic layers are optimally packed with neurites (dendrites and axons) and non-neuritic components such as glia, extracellular
space and blood vessels. Previous work on wiring optimization resulted in an optimal estimation of 2/5 for dendrites, including spine volumes (Chklovskii et al., 2002). Based on our results presented here, there is variation in the volume occupancy of dendrites in different layers and is different from 2/5. Except for the CA1-RAD layer in which the weigh-averaged DOR was 2/5, there were differences in the other five CA3 and CA1 layers (see Table 2). Specifically, CA3-OR was particularly higher in its average dendritic occupancy ratio which was about 1/2. These differences in individual layer occupancy ratios signify the distinct structural characteristics like dendritic density, spine morphology, and volume availability in different layers and regions in CA3 and CA1.

The most striking observation was the deviation of the occupancy ratios in the DG layers from the optimal value of 2/5. Whereas the layers in CA were closer in their optimal occupancy ratios of 0.4, the DG layers (Proximal, Medial and Distal) ML ranged from only 0.17 to 0.30 which is close to the estimates of other experimental studies (Amaral et al., 1990; Claiborne et al., 1990; Rihn and Claiborne, 1990; Rahimi and Claiborne, 2007) in spite of the granule cell layer having very high numerical cell densities (Jinno and Kosaka, 2009). These studies have shown that the Distal ML sub-layer (upper third ML) contains 30% of the granule cell dendrites. These results have practical implications because of the fact that in DG “neurogenesis” takes place across the entire lifespan of rodent and mammals alike (Kaplan and Hinds, 1977; Kuhn et al., 1996; Kempermann et al., 1998; Gould et al., 1999). The most plausible reason for the low occupancy ratios in the ML layer is that it might facilitate the growth of new granule cells and their
incorporation into the dentate neuronal network and participate in the memory processes (Gould et al., 1999). Other than the neurogenesis factor in DG, the differential DORs in both CA and DG have implications with respect to axonal neurite and non-neurite ratios.

We also demonstrated that the occupancy values correlate with the mouse DSCAM relative expression levels in both CA and DG (see Figure 3.15). Although DSCAM’s role as a recognition molecule for dendritic self-avoidance (homotypic and heterotypic) contacts and in maintaining proper dendritic field organization is extensively documented in the drosophila (Matthews et al., 2007; Schmucker, 2007; Millard and Zipursky, 2008), there are a few instances where studies showed that DSCAM is required for neurite arborization and mosaic spacing in the mammals like in the mouse retina (Fuerst et al., 2008). In the drosophila system, DSCAM is alternative spliced to give rise to thousands of protein isoforms which contributes to isoneuronal and heteroneuronal self-avoidance (Schmucker, 2007). In the mammalian system, though DSCAM genes do not undergo extensive splicing, they undergo homophilic and paralogue-specific binding (Agarwala et al., 2000; Agarwala et al., 2001; Yamagata and Sanes, 2008). Additionally, the vertebrate systems have other DSCAM gene family members, such as DSCAML1 (DSCAMLike1), which could contribute to the complexity of self-recognition (Agarwala et al., 2001; Barlow et al., 2001; Barlow et al., 2002; Fuerst et al., 2008).

One would suggest that in creating a reference 3D template, it is advisable to warp different brains into one brain using multiple landmarks which is ideal in its approach
(Toga and Mazziotta, 2002). This process has been adopted and has been successfully carried out in many invertebrate studies including the fly brain (Rein et al., 2002; Brandt et al., 2005; Kurylas et al., 2008; Rybak et al., 2010). Comparatively speaking, the invertebrate brains are smaller than vertebrates and it is relatively easy to carry out the whole brain reconstruction on multiple brains on high-resolution confocal images and create a single reference 3D brain system for these invertebrates (Rybak et al., 2010). In order to successfully carry out such kinds of sophisticated image analysis and create a reference 3D brain system from multiple rodent brains, the only possible way to accomplish this task is by solving the problem of automated segmentation. Even if a novel methodology is designed to create a reference 3D rodent brain system, we need the unique computational framework to map and digitally pack dendritic arbors in order to relate the single neuronal to systems neuroanatomy as presented here. In this endeavor, Rybak and co. (Rybak et al., 2010) have successfully created a reference template of the honey brain from multiple datasets and embedded reconstructed arbors into the 3D template but did extend to a detailed stereological analysis as presented here.

Other than the stereological analysis described here, this high-resolution and multi-dimensional 3D system can be applied to neuroanatomical ontology (Bota and Swanson, 2008), where the detailed segmentation of layers and sub-regions could come in aid in the design and implementation of ontologies (Rybak et al., 2010). Most importantly, this unique computational framework and the 3D template can be extended to extract potential synaptic connectivity patterns at systems level based on spatial overlap of
dendritic and axonal arbors (Stepanyants and Chklovskii, 2005; Jefferis et al., 2007; Stepanyants et al., 2008). In this context, we are currently computing the potential synaptic patterns between the various principal and inhibitory classes of neurons within this 3D template by following the spatial overlap concept. The detailed results and analysis of this study are forthcoming and preliminary analysis was presented at a previous conference (Ropireddy et al., 2010).

In conclusion, the study of brain structure is quintessential in our quest for understanding the neural basis for brain function and this unique approach in our assessment would link the two together. The importance and influence of neuronal structure on its function has clearly been elucidated in a number of studies over the past decades (Krichmar et al., 2002; Poirazi et al., 2003b; Komendantov and Ascoli, 2009). Extending this hypothesis beyond the individual neuronal level and applying it onto how structure affects function and activity is not clear at systems level. A probable reason for this difficulty is our poor understanding of structural organization of the hippocampus and can be attributed to our inability to link structure, function and activity. Previous research on genetic to behavioral studies have elucidated differences within various regions (DG Vs CA3 Vs CA1) and especially the dorso-ventral differentiation in the hippocampus (Moser and Moser, 1998; Bannerman et al., 1999; Leonardo et al., 2006; Kesner, 2007; Hunsaker and Kesner, 2008; Thompson et al., 2008; Dong et al., 2009; Fanselow and Dong, 2010; Royer et al., 2010). In elucidating the neural processes behind “learning and memory”
within the hippocampus, the anatomical properties described here play an important role in exploring the structure, function and activity linkage.
CHAPTER 4: AXONAL MORPHOMETRY OF HIPPOCAMPAL PYRAMIDAL NEURONS SEMI-AUTOMATICALLY RECONSTRUCTED AFTER IN-VIVO LABELING IN DIFFERENT CA3 LOCATIONS

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ABSTRACT

Axonal arbors of principal neurons form the backbone of neuronal networks in the mammalian cortex. Three-dimensional reconstructions of complete axonal trees are invaluable for quantitative analysis and modeling. However, digital data are still sparse due to labor intensity of reconstructing these complex structures. We augmented conventional tracing techniques with computational approaches to reconstruct fully labeled axonal morphologies. We digitized the axons of three rat hippocampal pyramidal cells intracellularly filled in-vivo from different CA3 sub-regions: two from areas CA3b and CA3c, respectively, toward the septal pole, and one from the posterior/ventral area (CA3pv) near the temporal pole. The reconstruction system was validated by comparing the morphology of the CA3c neuron with that traced from the same cell by a different operator on a standard commercial setup. Morphometric analysis revealed substantial differences among neurons. Total length ranged from 200mm (CA3b) to 500mm (CA3c), and axonal branching complexity peaked between 1mm (CA3b and CA3pv) and 2mm.
(CA3c) of Euclidean distance from the soma. Length distribution was analyzed among sub-regions (CA3a,b,c and CA1a,b,c), cytoarchitectonic layers, and longitudinal extent within a three-dimensional template of the rat hippocampus. The CA3b axon extended thrice more collaterals within CA3 than into CA1. On the contrary, the CA3c projection was double into CA1 than within CA3. Moreover, the CA3b axon extension was equal between strata oriens and radiatum, while the CA3c axon displayed an oriens/radiatum ratio of 1:6. The axonal distribution of the CA3pv neuron was intermediate between those of the CA3b and CA3c neurons both relative to sub-regions and layers, with uniform collateral presence across CA3/CA1 and moderate preponderance of radiatum over oriens. In contrast with the dramatic sub-region and layer differences, the axon longitudinal spread around the soma was similar for the three neurons. To fully characterize the axonal diversity of CA3 principal neurons will require higher-throughput reconstruction systems beyond the three-fold speed-up of the method adopted here.

**INTRODUCTION**

The pioneering work of Ramón y Cajal more than a century ago revealed the intricate structure of dendritic and axonal processes in the central nervous system (Ramón y Cajal 1911). Since then, a great deal of information has been gathered about the complex three-dimensional morphology of principal neurons in the mammalian hippocampus (e.g. Ishizuka et al. 1995; Turner et al. 1995; Pyapali et al. 1998). In particular, axonal arbors of pyramidal cells in area CA3 are much more extensive than their dendritic counterparts,
reaching out to hundreds of thousands of potential post-synaptic targets (Ishizuka et al. 1990; Li et al. 1994; Wittner et al. 2007). The CA3 region emanates the richest network of axonal projections in the rodent hippocampus, with collaterals and commissurals projecting bilaterally to CA3 and CA1 principal cells as well as interneurons (Li et al. 1994; Witter and Amaral 2004).

On the one hand, the dense and far-reaching arborization of CA3 pyramidal cell axons provides the backbone for the circuitry underlying autoassociative computation, a putatively fundamental function of the hippocampus (Rolls 2007). The importance of region CA3 in the hippocampus is well documented from memory encoding and retrieval (Treves and Rolls 1994; Treves 2004; Kunec et al. 2005) through generation of hippocampal rhythms (Buzsáki 1986; Csicsvari et al. 2003). The structure and connectivity of CA3 pyramidal neurons vary substantially with their hippocampal-transverse and longitudinal locations (Witter 2007). Thus, a comprehensive understanding of the dynamic mechanisms of hippocampal learning will likely require a quantitative map of the entire axonal arbors originating from different network sub-regions.

On the other hand, this same structural complexity renders the 3D digital reconstruction of these axons exceedingly labor-intensive, time-consuming, and error-prone with conventional methods (such as Microbrightfield Neurolucida). Only one complete axon has been digitally reconstructed from CA3 pyramidal cells (Wittner et al. 2007), while
other reports were based on serial two-dimensional tracings that, while more practical, only allow limited analysis (Sik et al. 1993; Li et al. 1994). More generally, among the morphological reconstructions publicly available at NeuroMorpho.Org (Ascoli et al. 2007), the proportion of dendrites far outweighs that of axons (even if incomplete). To alleviate this problem, we recently devised a reconstruction technique combining 2D tracing with an algorithmic approach. Similar hybrid methodologies had been only previously applied to dendritic trees (Wolf et al. 1995). We employed our original design (Scorcioni and Ascoli 2005) to reconstruct entire 3D axonal arbors from two-dimensional tablet tracings of several principal neurons from various regions of the hippocampal complex (e.g. Tamamaki et al. 1988; Tamamaki and Nojyo 1995).

Here, we further developed our method to extend its applicability to the more general Camera Lucida (pencil-on-paper) tracings. Such refinement enables the digital retrieval of full 3D reconstructions of neuronal arbors manually traced from serial section staining. Using this technique, we reconstructed the axonal arbors of hippocampal pyramidal cells intracellularly labeled in-vivo from three sub-regions of area CA3 (Li et al. 1994). Two neurons were from areas CA3b and CA3c, respectively, of the dorsal hippocampus (towards the septal pole). The third neuron was from the posterior/ventral area (CA3pv) in the temporal pole. The reconstruction system was validated by comparing the morphology of the CA3c neuron with that traced from the same cell by a different operator on a standard commercial setup (Wittner et al. 2007).
In addition to quantifying the intrinsic morphology of these axons, we evaluated their spatial extension within various hippocampal regions and layers by digitally embedding the arbors within a 3D template of the rat hippocampus based on high-resolution imaging of thin histological sections (Ropireddy et al. 2008). This analysis reveals similarities between the two reconstructions of the same CA3c cell, and dramatic differences from the other two neurons (CA3b and CA3pv). We discuss the implications of these findings for systems level connectivity and the potential functional consequences for existing theories of hippocampal cognitive processing.

**MATERIALS AND METHODS**

In the present work, a new axonal reconstruction pipeline is developed for and applied to *Sprague-Dawley* rat CA3 pyramidal neurons intracellularly filled in vivo with biocytin (Li et al. 1994; Wittner et al. 2007). The experimental details of the histological preparation are described in those previous reports and are only summarized here. Briefly, neurons were located stereotactically and identified electrophysiologically, and animals were perfused 2 hours after injection. After brain removal and fixation, 70 µm coronal sections were incubated in avidin-biotin horseradish peroxidase (HRP) complex and stained by 3,3’-diaminobenzidine-4HCl (DAB) intensified with Ni(NH₄)SO₄. Slices were mounted on gelatin coated slides and covered with DePeX. The reconstruction and analysis procedures described below extend our previous development (Scorcioni and Ascoli 2005) and are expected to be also suitable for rapid Golgi preparations.
Nomenclature

In this study, we follow the hippocampal anatomical terminology originally introduced by Lorente de Nó (1934) and subsequently adopted in numerous reports (e.g. Ishizuka et al. 1990; 1995; Li et al. 1994; Witter and Amaral 2004). The long axis of the hippocampus curves from the septal pole located dorsally in the most anterior end, to the temporal pole, located ventrally after passing the most posterior end. Transverse to this longitudinal curvature, Cornu Ammonis (CA) is divided into seven adjacent sub-regions, namely CA1a, CA1b, CA1c, CA2, CA3a, CA1b, and CA3c. CA1a lies near the subiculum, while CA3c is located between the supra- and infra-pyramidal blades of the dentate gyrus (Figure 4.1a). CA3a is the region of maximum curvature at the origin of the fornix, while CA1b may be recognized as the straightest CA1 sub-region. Because of its small size, CA2 is not always identifiable in all slices, and here we consider it lumped together with CA3a. Along the depth of the hippocampal-transverse plane, four cytoarchitectonic layers are identifiable throughout CA, named (from outermost to innermost) stratum oriens (bordering the alveus), stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare (bordering the fissure). Since stratum lucidum, the mossy fiber layer, is present in CA3 but not CA1, here we consider it lumped together with stratum radiatum.
In this work, three CA3 pyramidal cells are characterized. The somata of two of them are located in the dorsal hippocampus, in sub-regions CA3c and CA3b, respectively. The soma of the third neuron is in the posterior/ventral (CA3pv) region. For the sake of interpretability, we refer to these cells in the text by the names of these sub-regions, but this notation should not be confused to indicate the axonal distribution of these cells, which is spread across all sub-regions. In order to maximize clarity of cross-referencing, here and throughout the captions within the figures we report the correspondence between these cells and their original identifiers used in previous papers. In particular, our reconstructions of neurons CA3b and CA3pv correspond to cells 51 and 60a, respectively (Turner et al. 1995). Our reconstructions of neuron CA3c correspond to cell D256 (Wittner et al. 2007). This CA3c cell was also previously traced with the standard commercial system Microbrightfield NeuroLucida. This earlier digital reconstruction is referred here by the use of the superscript NL (CA3c\textsuperscript{NL}) to distinguish it from the morphology reconstructed with the system described below.

**Slide inspection, sequential alignment, and tracing**

Microscope slides are initially evaluated in order to identify and number the tissue slices containing portions of the stained neuron arbors (Figure 4.1a). The amount of rotational difference between adjacent slices is calculated based on matching blood vessels and other landmarks so as to allow later alignment of traced neuronal data. Tracing is performed on an Olympus BX51 microscope equipped with Camera Lucida and an
achromatic 40× dry objective with numerical aperture of 0.65. All visible dendritic and
axonal segments through the slice depth are manually traced on regular paper pre-printed
with a black box demarking 1 inch margins. The fields of view within these boxes
represent unitary regions of interest (Figure 4.1b) whose relative spatial position is
labeled on the sheet with a univocal alphanumeric designation. For example, the adjacent
boxes on the top, right, bottom, and left of the box in sheet M3 are found on sheets L3,
M4, N3, and M2, respectively. Neurites and landmarks falling within an inch from the
box borders are re-traced outside of the box borders of the appropriate adjacent sheets.
These duplicate tracings allow later alignment of corresponding fields of views.

Distinguishing between bifurcation points and axonal crossings constitutes one of the
toughest challenges in automated tracing (see e.g. http://diademchallenge.org). In most
cases, however, this determination is relatively easy for the human eye. In the
reconstructions described here, such distinction always occurs at the time of pencil-on-
paper tracing, and not as an unsupervised algorithmic step. Moreover, multiple colors are
used to indicate variable thickness estimates: axons are assigned approximate diameters
of 1, 0.5, and 0.1 µm, and dendrites of 3 and 0.3 µm, respectively. When axons and
dendrites are both present in the same field of view, they are drawn on separate sheets of
paper with identical alphanumerical identity. This produces a separate reconstruction of
dendrites that is later merged to the axonal reconstruction during the digital
reconstruction process (described below). The choice to only discriminate among a
limited set of neurite thickness values was the fruit of a compromise between manual
labor and retained biological information. Soma and landmarks were also distinctly color-coded. Subsequent image processing was found to be sensitive to ink quality, and produced optimal results with Pigma Micron (http://sakuraofamerica.com/Pen-Archival).

Figure 4.1: Axonal reconstruction process: from micrographs to digital trees. 

a) Representative micrographs of dorsal hippocampus captured by lenses with different magnification power: 4× (top) with captioned sub-regions (Sub, Supra, and Infra indicate subiculum and the two granular blades of the dentate gyrus, respectively) and asterisk marking the somatic position within CA3c; 8× (bottom) with visible dendritic tree further enlarged at 20× and 40× (boxed insets indicated by black arrows) to highlight branches and spines (white ‘s’ arrows), as well as two axonal stretches enlarged at 40×. b) Illustration of a single labeled slice tracing, assembled from dozens of US letter-sized sheets of paper tiled together, each with manually pencil-traced neurites. One sheet is enlarged on the left, showing representative line traces. c) After high-resolution serial scanning, tracing images (here represented for one sheet only) are digitized into pixel format and vectorized. Dashed lines represent untraced segments joined by nearest-neighborhood. d) In the 3D arbor-stitching step, all planar vector sets, each corresponding to a histological section, are first aligned by translation and rotation. Then, they are joined together in 3D using information of the apparent terminations marked to be in focus at the top or bottom of the slice.
Apparent neurite terminations observed within the top and bottom 10% of the slice depth, assumed to constitute slicing truncations, are marked with small (~2 mm diameter) and large (~5 mm diameter) pink circles, respectively. These circle tracings are utilized in the digital reconstruction process (described below) in order to both identify inter-slice continuations and assign their Z coordinate. In the absence of a circle, neurite tips are treated as real terminations. The axons reconstructed in the present work traversed between 50 and 60 tissue slices. Every slice required up to 75 sheets to trace. Each neuron required approximately three weeks of full-time work to trace.

**Image acquisition and pre-processing**

Tracing papers are digitized with a MicroTeK ScanMaker 5950 automated scanner (http://microtek.com), with 1200 dpi resolution and a color depth of 24 bits. Up to 30 sheets of paper are fed to the machine at a time. To minimize deformation due to lamp warm-up, a batch of 30 scans are rescanned if the scanner was not in use for more than 2 hours. The resulting digital picture is manually renamed with the sheet alphanumerical designation within that particular slice (for example, if the paper sheet is labeled N20, its output scan is saved as N20.jpg). All images belonging to a particular slice are stored in a directory whose name reflects the slice number itself.

The color intensity of the scanner output must be digitally equalized to control for the effects of ambient temperature, vibration, light bulb lifetime, and use hours. During
equalization all papers are digitally processed to identify the average white background level (by averaging all pixels in the picture) and the darkest black present (by selecting the pixel with the lowest intensity). Once these two values are identified, all colors are equalized between these two extremes. All digitized tracings are processed on a Windows-based PC (dual Xeon 3 GHz, 2 GB RAM, 120 GB disk) with a set of ad-hoc Java and Perl scripts. At this stage, the optimal translation offset is also manually estimated between all pairs of adjacent images based on corresponding landmarks and on the overlap of the neurites double-traced outside of the black box boundaries.

All scanned papers are processed to crop the margins outside of the boxed area. While reducing acquisition time, automated scanning introduces two types of distortions in the final scanned image: mechanical deformations and color quality reproduction. The latter is particularly apparent by the non-uniform range of gray shades corresponding to the pixels of black frame. Both of these issues render the cropping phase problematic, and are solved with an additional post-processing step, also implemented in Java. In particular, the most likely box position is identified with a pixel-by-pixel search algorithm based on color intensity and an extended spatial range of 10 pixels from the maximum values. To simplify the correct color identification of dendritic and axonal thickness in the subsequent reconstruction, landmarks are also removed at this point. This step uses the Adobe Photoshop color removal tool with a similarity threshold of 60% to recursively identify and erase all relevant shades of pink. After human-validation for quality, all cropped images are algorithmically tiled into a digital canvas based on their
alphanumerical identifiers and the pair-wise offset information obtained from the duplicate tracing overlaps (Figure 4.1b).

**Digital Reconstruction of Arbor Morphology**

The algorithmic reconstruction of the scanned images into digital arbors requires converting raster images (pixel coordinates) to vectors (segments). This step is performed on each of the 2D digital canvases individually with the freeware version of the Wintopo commercial package (Softsoft.net, Bedfordshire, UK). In particular, optimal vector extraction is obtained by selecting the “Stentiford thinning” method and setting the tolerance of the “Smooth Polyline Vectorization” to 30 (the units of this parameter are tenths of pixel widths, so this setting corresponds to 3 pixels). The value of the option “Reduce Polylines During Vectorization” is also set to 30. Wintopo output is saved in ASCII format (Figure 4.1c).

The Z coordinate of each point is first determined on the basis of the sequential order of the specific section. Points that lie either at the top or bottom of the slice are identified respectively by small and large circles as described above. Circles are algorithmically recognized as poly-lines with starting and ending point characterized by the same 2D coordinates. The circle position and radius are then computed as the mean and standard deviation of the X and Y coordinates of all the points belonging to the poly-line. The Z coordinates of the neurite closest to the circle position is then corrected by ±33% of the
inter-slice distance depending on the circle diameter. Sequential digital canvases in the Z stack were manually aligned by translation and rotation (Figure 4.1d).

Next, diameter values are added to the 3D vector data based on color. Each individual point color is assigned to its corresponding diameter and neurite type, as described above. Unfortunately, however, color varies by the specific batch of paper, as well as by the angle, speed and pressure of the pen used for the tracing. Since these properties can only be controlled in a limited way, custom software is used for color identification. A sample of each color pen trait is first scanned and assigned to its color, averaged across all its neighborhood pixels that are not part of the background (typically ~10 pixels). This average is compared to the mean color components, and the component closest to the measured value is assigned to the pixel.

Finally, 3D arbors are connected with the algorithm previously developed to reconstruct axonal trees from tablet data (Scorcioni and Ascoli 2005). Since this procedure was described previously in detail, and employed without modification, it is only summarized here. All vector data with their assigned type are loaded into memory. Each point is then compared to all other points that do not belong to the same line. At each step the set of points that is closest to each other are joined in the same line. This process is repeated until only one line is left. The soma position, identified by its color type, is selected as the starting point. The structure is then recursively parsed to create the corresponding digital output in SWC format (Ascoli et al. 2007).
Conversions among unit distances in the slice, tracing paper, scanned images, and digital reconstructions were calculated by first tracing the grid from a micron-scale calibration slide. The resulting length was then measured on the sheet with a standard ruler. Next, the number of pixels in the unit lines was counted on the corresponding image. Finally, the resulting numerical coordinates in the vectorized digital reconstruction were converted back to the original micron values. In particular, 1 cm on paper corresponded to 12.3 µm on the slide and in the digital reconstruction file, and to 78.35 image pixels (200 dots-per-inch or dpi), corresponding to 6.37 pixels/µm.

**Hippocampus 3D Embedding and Morphometric Analysis**

In order to evaluate the morphological organization of the axonal arbors within the hippocampal cytoarchitecture, we embedded the digital reconstructions into a 3D template of the rat hippocampus previously created from thin histological sections. The details of this 3D template were extensively described (Ropireddy et al. 2008) and are thus only briefly reported here. Serial stereotaxic-transverse images were obtained with an EPSON 3200 dpi scanner from Nissl-stained 16 µm cryostatic slices. Four cytoarchitectonic layers of Cornu Ammonis were segmented in each image, namely strata oriens, pyramidale, radiatum, and lacunosum-moleculare. The images and 2D segmentations were sequentially registered over the whole rostro-caudal extent. Digitized 3D volumes were extracted with a voxelizing algorithm (custom-developed in C/C++) at 16 µm isotropic resolution. Each voxel in the template is tagged with two sets of
coordinates. The first set corresponds to the main hippocampal axes: longitudinal, along the septo-temporal curvature; hippocampal-transverse, along the DG and CA “C”-like shapes; and depth, perpendicular to the first two. The second set of coordinates follows the canonical brain orientations, namely rostro-caudal, medio-lateral, and dorso-ventral. Moreover, voxels are also marked in the template with their sub-region and layer identity (e.g. CA3c stratum oriens, DG supra-pyramidal blade stratum moleculare, etc.).

After the voxelization step, the digitally reconstructed neuronal arbors are embedded within the 3D volume of the hippocampus such as to map their somatic position to the location reported for the intracellular electrode (Li et al. 1994; Turner et al. 1995 and Wittner et al. 2007). In particular, the CA3b (cell 51 in Li et al. 1994) and CA3pv (cell 60 in Li et al. 1994, also called 60a in Turner et al. 1995) neurons had the same pair of stereotactic coordinates AP=2.4 mm and ML=2.5 mm from bregma. These original papers also describe the locations of the first two neurons as septal/dorsal (CA3b cell) and posterior/ventral (CA3pv cell), which allows the unique identification of the depth position corresponding to the pyramidal layer Figure 4.2A). The CA3c neuron (cell D256, Wittner et al. 2007) had stereotactic coordinates AP=3.5 mm and ML=2.5 mm from bregma. The somatic location in the 3D template was determined for each neuron as the center of the sets of voxels within stratum pyramidale corresponding to the reported stereotactic coordinates. The digital arbors are initially oriented so that their primary dendritic axis is perpendicular to the longitudinal curvature of the principal layer, and their secondary dendritic axis is parallel to the hippocampal-transverse plane (Ropireddy
et al. 2008). This initial orientation is then manually fine-tuned so as to maximize the portion of the axonal tree contained within the volume boundaries of the hippocampus. This additional step of manual optimization was limited in all cases to an angular value of ± 30°.

Intrinsic morphometric parameters, such as axonal path length and bifurcation numbers, are extracted using L-Measure (http://krasnow.gmu.edu/cn3), a freeware tool for morphological analyses of neuronal arbors (Scorcioni et al. 2008). The axonal arbors embedded in the 3D template are amenable to a detailed analysis of the distribution of length along the hippocampal and brain coordinates, and within individual cytoarchitectonic layers and sub-regions. For accomplishing this task, an algorithm is designed to compute the coordinates of intersection between an axonal segment and the boundaries of the voxel. This process enables the measurement of the axonal length enclosed within a voxel. After all voxels are probed, axonal length is summed according to one of the above described hippocampal or brain coordinates, as well as to specific sub-regions and layers. All graphs are plotted with OriginPro 8.0 software (http://originlab.com) unless otherwise noted.

RESULTS

Practical Assessment of the Reconstruction Technique
The devised semi-automated reconstruction technique combines conventional tracing methodology (pencil-on-paper) with algorithmic processing to digitize the drawn morphology into a vector format (Figure 4.1). Intracellularly filled CA3 pyramidal cells display sufficient contrast for reliable tracing at high magnification (Figure 4.1a). However, the sheer size of the axonal trees of these neurons and the spatial extent of the tissue region they invade pose a formidable challenge for the complete reconstructions of these arbors. Conventional tracing is much faster and more practical than the direct digital reconstruction of neuronal arbors enabled by modern computer-interfaced microscopes and commercial software (e.g. Microbrightfield NeuroLucida). For example, the CA3c pyramidal cell described both here and previously (Wittner et al. 2007) required more than six months of full-time labor to reconstruct the entire axonal arbor with NeuroLucida (CA3cNL), compared to only three weeks of pencil-on-paper tracing. By itself, however, this process only yields a very large number of letter sized sheets for each of the serial sections (Figure 4.1b), which sum up to several thousand for the whole axonal tree (e.g. ~3200 for neuron CA3c).

This amount of raw data still constitutes a substantial limiting factor in the implementation of the algorithmic pipeline described in the Materials and Methods. The number of peculiarities and exceptions in the data is too large to fully automate every processing step. Therefore, although the actual computing time is relatively negligible, the sheer execution of the procedure leading from the manual tracings to the digital reconstruction requires almost as much time as the drawing itself. Moreover, the product
of this procedure still requires considerable human intervention to ensure rigorous quality control. In particular, partial 2D projections of the digital reconstruction must be checked against the corresponding tiled images. Once again, although this operation is not unique or even uncommon, the exquisite number of sections and size of each makes this necessary step notably time consuming. More specifically, quality checks and related corrections and adjustments cost in our hands approximately the same amount of time as each of the previous two aspects (manual tracing and execution of the algorithm pipeline).

As a result, the full reconstruction of complete CA3 pyramidal cell axons from mounted slides to finalized digital files is estimated to take approximately nine weeks of full-time skilled labor per neuron with this technique. This is a definitive improvement compared to the six months required by existing state-of-the-art solutions, and enabled the first quantitative comparative analysis of three axonal morphologies, which is described below.

**3D Appearance and Intrinsic Morphometry of CA3 Pyramidal Cell Axons**

The digital reconstructions of the traced neurons were embedded in the hippocampal 3D template (Figure 4.2) as described in the Materials and Methods. The three somata were located in area CA3c near the septal pole (cell D256 in Wittner et al. 2007), area CA3b towards the middle of the longitudinal axis (cell 51 in Li et al. 1994), and area posterior/ventral or CA3pv near the temporal pole (cell 60 in Figures 1, 2, and 12 of Li et
al. 1994; cell 60a in Turner et al. 1995), respectively (Figure 4.2a). Each of the three neurons displayed a uniquely distinct arbor shape. The digital reconstruction previously acquired with the standard NeuroLucida system by a different operator (CA3c\textsuperscript{NL}) was recognizably similar to the morphology traced from the same slides with the present system (Figure 4.2b). In each of the three neurons, the axonal extent spanned a sizeable proportion of the whole hippocampus (Figure 4.2a), far exceeding the reach of the same neuron’s dendrites. The ratios between total dendritic and axonal lengths were 1:13 (CA3b), 1:22 (CA3pv), and 1:27 (CA3c). The CA3c neuron had the longest axonal branching (nearly half-a-meter), and this value differed less than 0.5% from that of CA3c\textsuperscript{NL}.  


Figure 4.2: Digital rendering of reconstructed CA3 pyramidal cell axons. a) Axonal arbors of neurons with somatic locations (black dots) in different CA3 sub-regions (CA3b, red; CA3pv, blue; and CA3c, green) 3D-embedded within the rat hippocampal template, with arrow captions indicating dorso-ventral (D/V), medio-lateral (M/L) and rostro-caudal (R/C) orientations. b) Individual renderings of the four CA3 axonal reconstructions (including CA3c^{NL}), with fiduciary boundaries showing strata oriens (OR) and radiatum (RAD) and the pyramidal layer marked in light blue color.

The large axonal extent of CA3 pyramidal neurons prompts the question of how this length is distributed in space. A simple characterization ("Sholl" plot) can be obtained
from conventional tracings by drawing several concentric circles around the soma location and plotting the number of branch intersections against the circle radius. Digital reconstructions allow a more direct representation of the axonal amount by providing a path length measure instead of relying on intersections. Moreover, 3D information can be extracted by using Euclidean distance from the soma instead of circular projections. The CA3 pyramidal cell reconstructions were compared with this approach (Figure 4.3a). In the face of the large differences in total axonal lengths (the CA3c neuron being twice as long as the CA3b one), the contrast among the reconstructed neurons in other aspects appeared more modest. For example, the maximum Euclidean distance from the soma reached by the axonal trees only varied from above 3 mm for the CA3b neuron to below 4 mm for the CA3c one. Similarly, the “median” distance corresponding to the radius of a sphere encompassing 50% of the whole axonal extension was 1.3 mm and 1.7 mm, respectively, for the same two neurons. Both maximum and median distances, but not total length, were also very similar between the CA3b and CA3pv neurons.

An alternative variation of Sholl plots analyses the proportion of bifurcation points as a function of the 3D distance from the soma (Figure 4.3b), providing an indication of tree complexity. These measures fall at similar distances from the soma for the CA3b and
Figure 4.3: Intrinsic morphometry of CA3 pyramidal cell axons. **a)** Cumulative axonal length as a function of Euclidean distance from the soma for each of the four neurons (CA3b, red; CA3pv, blue; CA3c, light green; and CA3c\(^{NL}\): dark green). **b)** Distribution of the proportion of arbor bifurcations with respect to Euclidean distance from the soma, overlaid with Gaussian curve fits.

CA3pv neurons (1.1 mm and 1.2 mm, respectively), but farther for the CA3c cell (1.7 mm). The spread of these distributions (computed as standard deviation) is essentially identical for all cells, tightly ranging from 1 to 1.1 mm. Similarly, the peak values of these distributions are also constrained within a narrow span between 13.4% (CA3b), and
Moreover, the measures of all of these parameters for the CA3c neuron differ by less than 5% from the corresponding values of CA3c\textsuperscript{NL}, suggesting that these summary metrics of digital reconstructions of the same neuron are reproducible across tracing techniques and operators.

**Distinct 3D Axonal Projections of CA3 Pyramidal Cells Across the Hippocampus**

The embedding of the digital reconstruction in the 3D hippocampus template allows the analysis of axonal length within anatomically identifiable dimensions (Figure 4.4). We first measured the axonal distributions across the septo-temporal direction, which represents the main longitudinal axis of the hippocampus. The somatic locations of the reconstructed neurons are uniformly distanced along this axis (Figure 4.4a). With minimal overlap, the three axonal arbors collectively cover almost the entirety of the longitudinal span. The septo-temporal extent (the “width” of the curves in Figure 4.4a) is similar for all neurons, with a half-height span of \(\sim 1.5\) mm. While the length distribution is approximately symmetric around the soma for neuron CA3c, it is skewed septally in neuron CA3b and temporally in neuron CA3pv. Interestingly, however, the peak of the distribution is septal relative to the soma for all the three neuronal types.

Next, we examined the axonal projections against the canonical brain axes most univocally relevant to the hippocampal orientation, namely dorso-ventral and medio-lateral, corresponding to horizontal and sagittal planes, respectively (Figure 4.4b). In the dorso-ventral direction, the axonal span differs substantially among the CA3 pyramidal
Figure 4.4: Axonal length distribution along main hippocampus axes and canonical brain orientations. **a)** Differential distribution of axonal projections in the septo-temporal axis for the reconstructed neurons (CA3b, red; CA3pv, blue; CA3c, light green; and CA3c\textsuperscript{NL}: dark green). The captions ‘S’ and ‘T’ on the abscissa indicate septal and temporal directions, respectively and arrows point to corresponding soma positions. The **inset** illustrates the position of the hippocampus (light blue) within an outline of the rat brain, relating the canonical hippocampus and brain axes. **b)** Cumulative axonal length distributions in the medio-lateral (left panel) and dorso-ventral (right panel) orientations. Solid circles, stars, and triangles represent the 5\textsuperscript{th}, 50\textsuperscript{th}, and 95\textsuperscript{th} percentiles of the axonal length for every neuron. The captions ‘M’, ‘L’, ‘V’, and ‘D’ on the abscissa indicate medial, lateral, ventral, and dorsal directions, respectively.

cells. This is evident from the position at which each of these axonal arbors crosses the 5\textsuperscript{th}, 50\textsuperscript{th}, and 95\textsuperscript{th} length percentiles (Figure 4.4b, left panel). For the CA3c neuron, 90%
of the total axonal length falls within less than a millimeter. For the CA3b neuron the
same relative span is nearly three times broader (>2.5 mm). A similar trend is observed in
the medio-lateral direction (Figure 4.4b, right panel). In all cases, the median position
falls close to the soma.

Similar to the intrinsic morphometrics, the two digital morphologies traced from the same
neuron by independent operators and with different reconstruction systems (CA3c and
CA3cNL) have comparable length distributions relative both to the hippocampus (Figure
4.4a) and brain (Figure 4.4b) axes.

**CA3 Pyramidal Cell Axons Differ in Their Sub-Region and Layer Patterns**

A distinguishing aspect of CA3 pyramidal neurons within the hippocampal circuit is that
their axons overlap with both basal and apical dendrites of principal cells throughout
CA3 (recurrent collaterals) and CA1 (Schaffer collaterals). The digital embedding of
axonal reconstructions in a 3D cytoarchitectonic template provides the exquisite
opportunity to comparatively quantify among individual morphologies the axonal
proportions in different layers and sub-regions throughout their longitudinal extents
(Figure 4.5). The three reconstructed neurons vary strikingly in the axonal arbor length
invading CA3 vs. CA1. The definite majority (three fourths) of the axonal arbor from the
CA3b cell is confined in CA3. On the contrary, only a minority (two fifth) of the axon of
the CA3c neuron remains in CA3. Interestingly, the CA3pv cell presents an intermediate
pattern, with a nearly identical split between CA3 and CA1 axonal projections.
Figure 4.5: Axonal length distributions across hippocampal sub-regions and layers. a) Axonal collaterals for the four reconstructed neurons within each of six CA sub-regions (CA3c, CA3b, CA3a, CA1c, CA1b and CA1a). Bar lengths are proportional to absolute measures, while the numerical labels in the bars indicate percentages within every neuron. b) Axonal collaterals for the four reconstructed neurons within each of four individual layers (oriens, OR; pyramidal cell, PC; radiatum, RAD; and lacunosum-moleculare, LM). Bar heights are proportional to absolute measures, while the numerical labels in the bars indicate percentages within every neuron.

These relative fractions are in qualitative agreement with previous observations (Ishizuka et al. 1990; Li et al. 1994), yet they follow non-trivially from the absolute lengths (Figure 4.5a). In light of their considerable difference in total extent, the amount of axon cable invading CA3 is remarkably similar among the three neurons (e.g. 160 mm for the CA3b
cell vs. 175 mm for the CA3c cell). In contrast, absolute length differences are exacerbated in the projections to CA1, with the CA3c cell (315 mm) overwhelming the CA3b (95 mm) more than three-fold.

The axonal patterns of the three CA3 pyramidal cell morphologies are also highly non-uniform across the individual sub-regions of CA3 and CA1. For both the CA3b and CA3c cells, more than half of the CA3 axonal collaterals recur within the somatic sub-region (Figure 4.5a). In contrast, the CA3pv axons are more evenly spread in CA3, with only a modest preference for CA3a. Contrasting patterns are observed in the CA1 sub-regions, where two thirds of the axon from the CA3b neuron are located in CA1c, while two thirds of the axon from the CA3c neuron are located in CA1a. Once again the axon of the CA3pv neuron has features in common with both of the other cells, with a more balanced presence in CA1a and CA1c, but almost disregarding CA1b.

Consistent differences also emerge in the axonal distributions of the three pyramidal cells across layers. The CA3b neuron axons extend evenly between the superficial layers (strata oriens and pyramidale) and the deeper layers (strata radiatum and lacunosum-moleculare). In stark contrast, the axonal projection of the CA3c neuron is four times more prominent in deep than superficial layers (Figure 4.5b). As a reoccurring theme, the deep/superficial ratio is intermediate for the CA3pv neuron. Similar to the observation regarding the sub-regional patterns, these relative proportions reflect opposing absolute distributions. The total axonal length in the superficial layers is nearly the same for the three CA3 pyramidal cells (just above 100 mm), whilst the axonal presence of the CA3c
neuron in the deep CA layers is more than 3 times that of the CA3b cell. Also as in the other analyses, both absolute and relative axonal values appear to be robust between the CA3c and CA3\textsuperscript{NL} reconstructions both across layers and sub-regions (Figure 4.5).

The approximately 10% of axonal length found in the pyramidal layer may appear surprising since these fibers do not form synapses on somata. A possible explanation for this finding is that digital reconstructions do not differentiate explicitly main axons from axon collaterals with boutons. We measured the mean axonal diameter in the pyramidal vs. non-pyramidal layer, and we found no differences in any of the cells. Thus, the portions of the axons in the pyramidal cell layer are not thicker than the average branches. Another possibility is that axons in the pyramidal layer contact dendrites on the basal arbor of superficial neurons and apical arbors of deeper neurons.

**Unique Absolute and Relative Axonal Distributions in specific CA3/CA1 Circuit Parcels**

A more detailed view of the spatial distribution of the axonal arborization from each individual neuron is provided by a comparative examination of the length invading every layer and sub-region. This analysis effectively yields a map of the potential output of these CA3 pyramidal cells on the hippocampal-transverse “lamella” that captures much of Cornu Ammonis’ poly-synaptic circuit. The three pyramidal cells are largely non-overlapping along the longitudinal axis of the hippocampus, rendering moot a direct comparison of their axonal distributions as a function of septo-temporal location. For
each of the neurons, we first examined the relative sub-regional and layer composition of
axonal length from the two portions of the arbor located septally and temporally relative
to the soma. Having found only negligible differences (Figures 4.2 and 4.4), we report
axonal length data summed across the longitudinal direction.

The absolute and relative distributions of axonal length by sub-region and layer are
quantitatively represented by a pie chart for each of the neurons (Figure 4.6). The total
surface area of each chart is scaled relative to the total length of the corresponding
neuronal arborizations, namely CA3b (top), CA3pv (middle), and CA3c (bottom). Within
each pie chart, every slice has a surface area proportional to the axonal length in each of
the three CA3 (white background) and three CA1 (gray background) sub-regions. All
slices are further divided in four sectors with surface areas sized according to the
represented cytoarchitectonic layers (from outermost to innermost: strata oriens,
pyramidale, radiatum, and lacunosum-moleculare).
Figure 4.6: Absolute and relative axonal length distributions within individual sub-regional layers. For each of the three neurons (CA3b, top; CA3pv, middle; CA3c, bottom), the size of the pie charts is scaled according to total axonal length. The surface area of each pie slice is proportional to the axonal distributions in the respective three CA3 (white) and three CA1 (grey) sub-regions, and further divided based on the arbor length in each cytoarchitectonic layer (OR, PC, RAD and LM). These pie charts were generated by measuring sector surface areas with ImageJ (v.1.38, http://rsb.info.nih.gov/ij), and marking the appropriate slice boundary positions with Paint Shop Pro (v.8.0, http://corel.com).
Therefore, the ensemble view of the pie charts enables a relative assessment of the axonal composition among neurons, sub-regions, and layers. At the same time, the surface area of each of the slice sectors (and any of their combinations) constitutes a measure of the actual axonal length of the given cell in the corresponding hippocampal sub-region and layer, thus allowing absolute comparisons.

The axonal distributions among sub-regional layers follow the same general trends reported separately for the sub-regional compositions summed across layers and the layer compositions summed across sub-regions reported in Figure 4.5. However, this more detailed analysis additionally reveals specific differences, which are most extreme between the CA3b and the CA3c cells. In the CA3b neuron, the oriens layer in CA3b alone accounts for nearly one fifth (18%) of the entire axonal extent, compared e.g. with a meager 2% in the radiatum layer of CA1a. In contrast, almost one third of the whole axon of the CA3c neuron (32%) is found in the CA1a radiatum layer, as opposed to a negligible presence (<2%) in the CA3b oriens layer. Interestingly, the CA3b cell extends more oriens than radiatum axonal length in CA3, but more radiatum than oriens in CA1.

The differences between the CA3c and CA3b cells are somewhat blended in the CA3pv neuron, where stratum radiatum has a uniform axonal majority in all six CA sub-regions. Interesting trends are also shared among all three reconstructed neurons. In all cases, for example, the axonal length ratio between oriens and radiatum is greater in CA3 than in CA1 and, within CA1, increases moving transversally from the subicular end toward
CA3 (CA1a to CA1c). In other words, there is more axon in radiatum than in oriens in both CA1 and CA3, but the proportion of the total that is in oriens is greater in CA1 and increases from the subiculum end of CA1 to the CA2 border.

The substantial differences in total axonal length among the three neurons yield a different perspective when comparing axonal distributions throughout the sub-regional layers in absolute, rather than relative, terms. For instance, the absolute axonal length of the CA3b neuron invading the oriens layer of area CA3b (39.3 mm) is very similar to the amount of CA3c axon invading the radiatum layer of area CA1b (41.3 mm), even if they constitute very different fractions of their respective arbors (18% vs. 8.4%, respectively). In contrast, the same 8.4% fraction of the CA3b neuron occupying the oriens layer of area CA3a only amounts to a length of 18.4 mm. Likewise, the axonal length of the CA3b neuron found in the radiatum layer of area CA3b (26.2 mm) closely matches the value for the CA3c neuron in the radiatum layer of area CA1c (25.5 mm), while their relative proportions are more than 2-fold off (12% vs. 5.2%). A similar 5.1% proportion of the CA3b neuron invading the radiatum layer of area CA3c corresponds to an absolute length of just 11 mm.

In some cases, the absolute size differences among neurons accentuate, rather than compensate, the non-uniform relative distributions across sub-regional layers. For example, in terms of absolute length, stratum radiatum of area CA1a receives 36 times more axon from the CA3c neuron than from the CA3b neuron (156 mm vs. 4.3 mm).
These observations were qualitatively confirmed post-hoc by direct microscopic inspection of the slices. For example, inspection of the CA3b neuron slide revealed very sparse axonal presence in the radiatum layer of area CA1a, while the same region of interest displayed dense axonal labeling in the CA3c neuron slide.

**DISCUSSION**

Axonal morphology sculpts network connectivity (Stepanyants and Chklovskii 2005; Stepanyants et al. 2008) and intrinsic neuronal processing (Manor et al. 1991; Debanne 2004). Thus, its quantitative characterization is crucial to understand structure-function relationship in the brain. Modern computer-interfaced microscopes and commercial software (e.g. NeuroLucida) allow the direct reconstruction of imaged neuronal arbors into digital format (Ascoli 2006). In practice, however, the ergonomics of the human-equipment interaction typically restrict this possibility to neurites of limited extent, such as dendritic trees or local axons. Larger projections, like axons of principal cells in the mammalian cortex, are more commonly only traced “pencil-on-paper” by simple Camera Lucida technique. Unfortunately, the resulting analog drawings are less conducive to quantitative analysis.

In this work, we extended a semi-automated approach for digitally reconstructing neuronal arbors all the way back to the initial step of basic Camera Lucida, allowing the first quantitative analysis of multiple CA3 pyramidal cell axons intracelluarly labeled in-vivo. Evaluating intrinsic cell-to-cell variability beyond single neuron samples from each
of distinct area (CA3c, CA3b, and CA3pv) will require even greater automation (Ascoli 2008). At the same time, full axonal arbors are dozens of times larger than typical dendritic trees, making these reconstructions remarkably data-rich. Previous studies proved even individual axon reconstructions to be deeply informative (e.g. Sik et al. 1993; Tamamaki et al. 1988). Here, we used the only one of these previously acquired data files that is independently available in digital format (Wittner et al. 2007) to validate our novel semi-automated digitization system by reconstructing the same cell from the same slides. All morphometric measures employed in this report showed excellent consistency between these two data files.

To appreciate the massiveness of these axonal tracings it is useful to compare these morphologies with other digital reconstructions available from NeuroMorpho.Org, the largest available collection of neuronal tracings (Ascoli et al. 2007), and relevant hippocampal literature. A dentate gyrus granule cell filled in vivo has total axonal lengths of approximately 9 mm (Scorcioni and Ascoli 2005), which is only ~3 times that of the dendritic arborization for the same cell type (e.g. Claiborne et al. 1990). Hippocampal interneurons from CA3b strata radiatum and lacunosum-moleculare have typical axonal and dendritic lengths of ~20 mm and ~3 mm, respectively (Ascoli et al. 2009), but their completeness cannot be established conclusively since these were injected in slices. One of the interneuron described from the dentate gyrus had very extensive axonal arbor, potentially comparable to that of pyramidal cells (Sik et al. 1997). Although not fully reconstructed, long-range interneurons also have very dense axons (Sik et al. 1994).
Because of their substantially larger axon caliber (Jinno et al. 2007) the total axonal volume of these long range interneurons can be several times larger than that of pyramidal cells.

At the same time, other principal neurons from the mammalian cerebral cortex in NeuroMorpho.Org have axonal length of the same order of magnitude as the values reported here, such as a pyramidal cell from the visual cortex of the cat at ~110 mm even if incomplete (Hirsch et al. 2002), a spiny stellate cell from the entorhinal cortex of the rat at ~160 mm without including full local collaterals (Tamamaki and Nojyo 1995), and a CA2 pyramidal cell from the rat hippocampus at ~430 mm (Tamamaki et al. 1988). Except for the cat visual cortex example, all these other principal cell axonal reconstructions were obtained with the previous version of the semi-automated reconstruction technique employed here (Scorcioni et al. 2005). In sharp contrast, axons traced from cells intracellularly filled in the slice have much more contained axonal length most probably due to axotomy. For example, pyramidal cells from monkey prefrontal cortex have total axonal length of ~35 mm (Gonzalez-Burgos et al. 2004). Therefore, in terms of data density, the three CA3 pyramidal cell axonal reconstructions described here and publicly distributed at NeuroMorpho.Org contribute great potential for subsequent analysis and modeling.

The ipsilateral axonal projections of CA3 pyramidal neurons generate two crucial associational pathways in the neurobiology of learning and memory: the recurrent
collaterals within area CA3 and the Schaffer collaterals into area CA1. Previous studies (Ishizuka et al. 1990; Li et al. 1994) indicated that the relative balance of these two systems depends on the longitudinal and hippocampal-transverse location of the presynaptic cell, as well as the targeted sub-regions (CA3/CA1a,b,c) and layers (oriens/radiatum). By embedding three digitally reconstructed axons within a 3D template of the rat hippocampus, our approach quantified specific patterns of axonal collaterals throughout every cytoarchitectonic location along the septo-temporal axis.

In dorsal hippocampus, dramatic quantitative differences were measured between CA3c and CA3b pyramidal cells. The CA3c neuron projected twice as much axon to CA1 as to CA3, and, within CA1, twice as much in CA1a (distally) than in CA1b and CA1c combined. Throughout these regions, this axonal arbor overwhelmingly preferred stratum radiatum to oriens by a 6:1 ratio. In contrast, the CA3b neuron projected thrice as much axon to CA3 than to CA1, and, within CA3, 50% more to CA3b (locally) than to CA3a and CA3c combined. Within CA1, this neuron extended 50% more length in CA1c (proximally) than in CA1a and CA1b combined. While within CA3b the recurrent collateral preferred stratum oriens to radiatum by 50% margin, the trend was reversed in the Schaffer projections to CA1, where radiatum trumped oriens 3:1.

Such diametrical opposition suggests a possible sub-regional and cytoarchitectonic specialization for the dual organization of CA3 axons into recurrent and Schaffer collaterals, as also reflected by physiological studies (Csicsvari et al. 2000; 2003). Distal
projections to CA1 predominate in CA3c and preferentially target the radiatum layer. Local arbors predominate in CA3b and preferentially target the oriens layer. This putative division could in turn hint to an alteration of the classic view of the hippocampal synaptic circuit. Rather than region uniform receiving entorhinal input (directly and through the dentate gyrus) and transmitting output to CA1, area CA3 might be serially composed of a first station (CA3b) suitable to receive and locally distribute input within CA3, followed by a second station (CA3c) mainly dedicated to propagate the output downstream to CA1. This alternative view is also supported by prominent dendritic branching (>15% of total dendritic length) of CA3b pyramidal cells in stratum lacunosum-moleculare (the layer invaded by the entorhinal perforant pathway) compared to almost negligible dendritic presence (<3% of total dendritic length) of CA3c neurons in this layer (Ishizuka et al. 1995).

The opposite trends observed in CA3c and CA3b neurons from the dorsal hippocampus are somewhat blended in the pyramidal cells reconstructed from the posterior/ventral area, with axonal collaterals more uniformly distributed throughout all sub-regions. The substantial differences in total arbor length among the reconstructed neurons combine with their relative differences in sub-region and layer distributions, resulting in relative constant absolute values of axonal extent e.g. in the combined oriens and pyramidal layers (very close to 100 mm for all 3 cells) or within sub-regions CA3a,b,c together (~170 mm for all 3 cells). Thus, pyramidal cell output might be specifically differentiated for selective targets (CA1 and radiatum), yet relatively stable for others.
Recent studies reported CA3 back-projections to the dentate gyrus (Scharfman 2007), especially towards the temporal pole (Witter 2007). Also at least one neuron described in the ventral hippocampus (cell r32 in Li et al. 1994) extensively collateralized in the dentate molecular layer. For each of our three neurons, the axonal length within the hilus fell within the margin of measurement error (2-2.5%) and was neglected. Another open question not tackled by the present study regards the relative extent of the commissural projection to the contralateral hippocampus.

Functionally, the CA3 region is recognized to play important roles in memory encoding and retrieval (Treves and Rolls 1994; Treves 2004; Kunec et al. 2005). In most physiological, behavioral, and computational studies, CA3 is considered a single functional region. Recently, however, lesion studies in the rat showed dissociation between CA3 sub-regions (Hunsaker et al. 2008). Specifically, CA3c lesions yielded greater spatial deficits than lesions in sub-regions CA3a and CA3b. These sub-regions are also differentially involved in initiating and transferring population patterns (Csicsvari et al. 2000). Such functional specialization of individual CA3 sub-regions (Kesner 2007) is consistent with the differential axonal collateral patterns of CA3c and CA3b pyramidal cells demonstrated here.

Lesion studies also revealed dorso-ventral functional differentiation (Bannerman et al. 1999; Hock and Bunsey 1998; Hunsaker and Kesner 2008), whereas dorsal, but not
ventral, rat hippocampus appears involved in spatial behavior, as also supported by physiological observations (Royer et al. 2010). Differing circuit organizations might underlie the functional dissociation of dorsal and ventral hippocampus (Moser and Moser 1998). Among the neurons we reconstructed, the posterior/ventral pyramidal cell displayed an axonal distribution pattern intermediate between those of CA3c and CA3b neurons in dorsal hippocampus. This might indicate a less distinct sub-regional differentiation in ventral than in dorsal hippocampus, consistent as well with the relatively more uniform axonal distribution of the CA3pv cell among individual cytoarchitectonic layers throughout the six CA3/CA1 areas.

Axonal reconstructions can be utilized to estimate potential synaptic connectivity patterns based on geometrical overlaps with dendritic arbors (Stepanyants et al. 2002; Kalisman et al. 2003). While thousands of digital dendritic reconstructions are readily available in the public domain (Ascoli et al. 2007), no axons have been fully traced for most known neuron classes. The free distribution of the digital morphologies described here through the NeuroMorpho.Org database should thus constitute a useful addition to shared neuroscience resources. Axonal territory provides a map of necessary, but not sufficient conditions to characterize synaptic connectivity, as individual synapses might abide specific, localized, and compartmentalized criteria (Shepherd and Harris 1998). In the hippocampus, however, axo-dendritic overlap predicts actual contacts in at least some neuron types (Stepanyants et al. 2004). In the heydays of connectomics (Kennedy 2010), the key information about networks is quantitative neuronal connectivity in vivo.
Complete axonal arbors of single neurons are still unsolvable with any of the ongoing connectomics approaches due to limits of resolution (non-invasive imaging), sampling (fluorescent genetic labeling) or field-of-view (electron microscopy). Although time-consuming, intracellular labeling is critical to elucidate connections between neuron types.

Axonal signal propagation also depends on the geometry of its arbor. The intrinsic axonal length properties of CA3 pyramidal cells could affect action potential conduction velocity and even branch point failure in these complex 3D arbors. These effects might influence spike timing and delays, therefore imposing noteworthy computational constraints on temporal coding (Carr and Konishi 1990; McAlpine and Grothe 2003). Although we are still at the beginning of the path towards understanding how the functions of the nervous system emerge from the structure and activity of its circuit, this study represents a step forward in elucidating the morphological organization of one of the most plastic networks in the adult mammalian brain.
CHAPTER 5: POTENTIAL SYNAPTIC CONNECTIVITY OF DIFFERENT NEURONS ONTO PYRAMIDAL CELLS IN A 3D RECONSTRUCTION OF THE RAT HIPPOCAMPUS

ABSTRACT

Most existing connectomic data and ongoing efforts focus either on individual synapses (e.g. with electron microscopy) or on regional connectivity (tract tracing). An individual pyramidal cell extends thousands of synapses over macroscopic distances (~cm). The contrasting requirements of high resolution and large field of view make it too challenging to acquire the entire synaptic connectivity for even a single typical cortical neuron. Light microscopy can image whole neuronal arbors and resolve dendritic branches. Analyzing connectivity in terms of close spatial appositions between axons and dendrites could thus bridge the opposite scales, from synaptic level to whole systems. In the mammalian cortex, structural plasticity of spines and boutons makes these ‘potential synapses’ functionally relevant to learning capability and memory capacity. To date, however, potential synapses have only been mapped in the surrounding of a neuron and relative to its local orientation rather than in a system-level anatomical reference. Here we overcome this limitation by estimating the potential connectivity of different neurons embedded into a detailed 3D reconstruction of the rat hippocampus. Axonal and dendritic trees were oriented with respect to hippocampal cytoarchitecture according to longitudinal and hippocampal-transverse curvatures. We report the potential connectivity
onto pyramidal cell dendrites from the axons of a dentate granule cell, three CA3 pyramidal cells, one CA2 pyramidal cell, and 13 CA3b interneurons. The numbers, densities, and distributions of potential synapses were analyzed in each sub-region (e.g. CA3 vs. CA1), layer (e.g. oriens vs. radiatum), and septo-temporal location (e.g. dorsal vs. ventral). The overall ratio between the numbers of actual and potential synapses was ~0.20 for the granule and CA3 pyramidal cells. All potential connectivity patterns are strikingly dependent on the anatomical location of both pre-synaptic and post-synaptic neurons.

INTRODUCTION

Mammalian brains have complex network architectures (Sporns, 2010), with each neuron connecting to thousands of others. Connectivity must be characterized at both synaptic and regional levels to advance our knowledge of cognitive and computational functions of nervous systems (Buzsaki, 2007; Sporns et al., 2005). Numerous studies recently explored structural and functional connectivity with different experimental modalities, including non-invasive imaging (Bressler and Menon, 2010; Bullmore and Sporns, 2009; Honey et al., 2009), electrophysiology (Kalisman et al., 2005), light microscopy (Wittner et al., 2007; Ishizuka et al., 1990; Li et al., 1994; Sik et al., 1993), and electron microscopy (Mishchenko et al., 2010). Non-invasive imaging such as DTI allows investigation of the whole human brain, but is only amenable to analyzing regional connectivity. Electron microscopy has complementary strengths and limitations, as it can
unambiguously identify all synapses, but only in a narrow region of interest. Unfortunately, neither technique is suitable to acquire the whole synaptic connectivity of a typical cortical neuron. Light microscopy provides an optimal balance of resolution and field of view for this neuronal connectomic level bridging the micro- and macro-scale.

In particular, the connectivity maps of entire neurons can be investigated computationally based on light-level digital reconstructions of axonal and dendritic morphologies in simplified (Amirikian, 2005) or detailed (Escobar et al., 2008; Stepanyants and Chklovskii, 2005; Stepanyants et al., 2008) geometrical representations. In most such studies, putative connectivity is established on the basis of the close proximity between a pair of pre-synaptic (axonal) and post-synaptic (dendritic) segments (Kalisman et al., 2003; Stepanyants et al., 2004). These spatial overlaps have been defined as ‘potential synapses’ because of strong evidence of spine and bouton motility and their essential role for synapse formation and memory consolidation (Chklovskii et al., 2004; Knott et al., 2006; Lendvai et al., 2000). This conceptual framework is appropriate for the mammalian hippocampus, as this region displays structural plasticity throughout adulthood and is clearly involved in learning (Squire and Zola-Morgan, 1991; Eichenbaum and Cohen 2001). To date, however, potential synapses have only been mapped in the surrounding of a neuron and relative to its local orientation rather than in a system-level anatomical reference. This limitation is particularly evident in the hippocampus, because of its peculiarly curved and laminar organization.
The methodology presented here applies an existing mathematical framework to estimate potential synaptic connectivity (Stepanyants and Chklovskii, 2005) to detailed arbor geometries embedded into a 3D reconstruction of the rat hippocampus. The hippocampal model was built by assembling the cytoarchitectonic layers segmented from high-resolution histological images of thin cryostatic brain sections, and transforming the resulting space into a volumetric representation with uniformly-sized voxels (Ropireddy et al., 2008). A custom-designed computational framework was developed to embed digital neuromorphological reconstructions with respect to the natural hippocampal axes (Ropireddy et al., 2011). The key advantage of this methodological extension is the ability to analyze potential synaptic maps of different neurons within the proper anatomical frame of reference. As the principal neurons of the hippocampus have long-range projections (Li et al., 1994; Tamamaki et al., 1988) forming synapses across different sub-regions and layers, this framework provides a unique opportunity to quantify potential synaptic patterns across the entire hippocampal extent.

The present analysis is based on all publicly available digital reconstructions of hippocampal pyramidal cell dendrites (Ascoli et al., 2007) and a representative sample of complete 3D axonal tracings. In particular, the pre-synaptic neuronal selection consists of a dentate granule cell, 4 pyramidal cells (with somata in proximal CA3c, distal CA3c, CA3b, and CA2, respectively), and 13 CA3b radiatum and lacunosum-moleculare interneurons. For each case, we analyzed the numbers, densities, and distributions of
potential synapses in every sub-region (e.g. CA3 vs. CA1), layer (e.g. oriens vs. radiatum), and septo-temporal location (e.g. dorsal vs. ventral).

MATERIALS AND METHODS

Previous work described in detail our high-resolution 3D reconstruction of the rat hippocampus from thin histological sections (Ropireddy et al., 2008) and the embedding of digitally traced neuronal morphologies (Ropireddy et al., 2011). Here, we only present a brief overview emphasizing just the procedures that specifically pertain to the present analysis.

Digital 3D reconstruction of the rat hippocampus

A 45 day old, male, Long-Evans hooded rat (226-237 g; Harlan, Indianapolis, IN) was sacrificed by guillotine and its brain quickly removed and stored air-tight at -80 °C. The brain was sectioned coronally at 16 µm in the dorso-ventral direction with a cryostat at -18 °C. The 290 sections encompassing the entire hippocampus were mounted on slides and cover-slipped after Nissl staining. Slices were imaged at 3200 dpi with an EPSON scanner and contrast enhanced by the Matlab routine 'imcontrast'. The image stack was loaded into ‘Reconstruct’ (Fiala, 2005) with a pixel/µm conversion factor (4.28) accounting for the measured planar shrinkage. Images were initially registered by mid-line guided manual alignment. Seven clearly identifiable cytoarchitectonic layers were
Figure 5.1: Hippocampus 3D template and potential synapse illustration. (A) Nissl-stained stereotaxic-transverse section with CA (blue) and DG (red) cyto-architectonic layers segmented in the dorsal hippocampus. The inset shows an enlarged view of the
segmentation labeling the CA (CA1 and CA3a,b,c) and DG (infra and supra) sub-regions and layers. Layer abbreviations: GC, granule cell; ML, molecular; PC, pyramidal cell; RAD, radiatum; LM, lacunosum-moleculare; OR, oriens. (B) The septo-temporal and hippocampal-transverse axes of the hippocampus (‘S’ and ‘T’ mark the septal and temporal poles). The rat brain depiction in lateral-medial orientation is adapted from Amaral and Witter (1989) with permission from Elsevier. (C) Maps marking the CA and DG sub-region borders with respect to the septo-temporal and hippocampal-transverse axes. (D) Representation of the DG (red) and CA (blue) principal layers of the hippocampus 3D reconstruction, with an embedded CA3b$^{SR}$ interneuron and a CA3b pyramidal cell dendritic tree. (E) Enlarged view of the CA3b$^{SR}$ interneuron (axon: yellow; dendrite: green) and the CA3b pyramidal cell (red), illustrating three potential synapses (light blue). Inset shows a further zoom-in on one of the potential synapses, identifying a location where the interneuron axon is within 1 µm of the CA3b pyramidal cell dendrite.

manually segmented (Figure 5.1A): hilus, granule cell (GC), and molecular layer (ML) in the dentate gyrus (DG); and oriens (OR), pyramidal cell (PC), radiatum (RAD), and lacunosum-moleculare (LM) layers in CA3 and CA1. Serial tracing produced sets of pixels representing layer contours as closed polygons. All locations inside the boundaries of a given layer were identified by triangulation, extending a previous algorithm to yield 3D voxels from neighboring slices. Cubic voxels were thus defined with side corresponding to the inter-slice distance (16 µm). The initial registration was refined by iterative three-point average of the centroids through the longest hippocampal axis until reaching geometric convexity.

The 3D location of each voxel was mapped both onto canonical brain planes (stereotaxic-transverse, sagittal, and horizontal), and in a natural hippocampus reference frame denoting longitudinal and hippocampal-transverse positions. Longitudinal coordinates were calculated as path distances from the septal to the temporal poles (Figure 5.1B).
Hippocampal-transverse coordinates were computed by first virtually generating 60 cross-sectional planes, which displayed the classic double ‘C’ shape corresponding to the principal cell layers. The medial axes of these granular and pyramidal contours defined the DG and CA hippocampal-transverse positions as the distances from the infra-pyramidal tip and from the CA3c end, respectively (Figure 5.1B). Additionally, each voxel was assigned appropriate stereotactic coordinates and tagged by its sub-region, namely DG infra- (‘I’) and supra-pyramidal (‘S’) blades, and CA3c, CA3b, CA3a, CA2, and CA1 (Figure 5.1C).

**Embedding of digital neuronal morphologies**

A selection of 18 fully 3D traced axons from different sub-regions of the hippocampus was obtained from NeuroMorpho.Org (Ascoli et al., 2007): one granule cell, 4 pyramidal neurons, and 13 interneurons. The somata of these pre-synaptic neurons were positioned according to the stereotactic coordinates reported in the original reports. The dentate granule cell and the distal CA3c pyramidal neuron were at AP = 2.5-3 mm and ML = 2.5 mm from bregma (Tamamaki and Nojyo, 1991). The proximal CA3c pyramidal neuron was at AP = 3.5 mm and ML = 2.5 mm (Wittner et al., 2007). The CA3b pyramidal neuron was at AP = 2.4 mm and ML = 3.5 mm (Li et al., 1994). The CA2 pyramidal neuron was at AP = 2-2.5 mm and ML = 2.5 mm (Tamamaki et al., 1988). All interneurons had somata in CA3b, six in the radiatum layer, and seven in lacunosum-moleculare. The range of their stereotactic coordinates were AP = 1.95-3.85 mm and ML.
= 2.4-2.6 mm (Ascoli et al., 2009). Since all these somatic locations for the pre-synaptic neurons are reported with a precision of 0.1 mm, they identify a range of six locations in the hippocampus reconstruction, each corresponding to voxels with 16 μm side (16 μm × 6 = 0.1 mm). Cells were positioned in the location closest to the center of this range. In one case (granule cell) the entire range of 5×5 adjacent locations was tested (see Results).

The dendritic reconstructions of pyramidal cells were embedded as potential post-synaptic targets. For CA3, 54 pyramidal neurons were pooled from 5 different studies (Ishizuka et al., 1995; Henze et al., 1996; Carnevale et al., 1997; Jaffe and Carnevale, 1999; Turner et al., 1995). For CA1, 103 pyramidal neurons were pooled from 2 of the above studies and 5 additional ones (Ishizuka et al., 1995; Carnevale et al., 1997; Brown et al., 2005; Megias et al., 2001; Pyapali et al., 1998; Pyapali and Turner, 1996; Pyapali and Turner, 1994). In all cases, the somata were positioned based on the location ranges reported in the respective papers. As for the pre-synaptic neurons just discussed, the range of suitable voxels in the hippocampus 3D reconstruction was extended to account for the limited precision of the published positional information. Altogether, the location spans of these 10 datasets covered the entire hippocampal space invaded by the 18 selected axons. The pyramidal layers were densely packed by stochastically re-sampling the above 157 post-synaptic neurons, each within its appropriate voxel range. The pyramidal cell layer was packed with CA3 and CA1 neurons with appropriate somatic densities so as to reflect the known total number of cells in these regions (Rapp and Gallagher, 1996).
Pyramidal and granule cells were oriented in the 3D hippocampal reconstruction (Figure 5.1D) such that the principal axis of their dendritic arborization was perpendicular to the cellular layer and the secondary axis lay on the hippocampal-transverse plane (Scorcioni et al., 2002). To account for the natural variability observed in this general alignment (Claiborne et al., 1990; Ishizuka et al., 1995), the initial orientations were stochastically tilted between 0° and 5° around a random 0-360° rotation. Principal and secondary axes were computed relative to the soma by single value decomposition of the digital reconstructions (Scorcioni et al., 2002) using standard numerical recipes (Press, 1988). Dendritic trees were scaled so as to reach the appropriate cytoarchitectonic boundaries (ML for granule, LM for pyramidal apical, and OR for basal trees). To ensure that the entire axonal arbors of the pre-synaptic cells fell within the 3D boundaries of the hippocampus, the orientations of these neurons were further manually fine-tuned within ±30° relative to their automatic embedding using quaternions (Hanson, 2006)

**Potential synaptic connectivity measurement**

A potential synapse occurs when a pre-synaptic segment comes in close proximity of a post-synaptic segment (Figure 5.1E). For excitatory synapses, the interaction distance is generally considered as a spine length or ~2 µm (Harris and Stevens, 1989; Harris, 1999; Kalisman et al., 2003; Spacek and Hartmann, 1983). For inhibitory synapses, the interaction distance can be approximated as the sum of the radii of the dendritic and
axonal segments, typically 1 µm (Wierenga et al., 2008). The number of potential synapses can be derived from the geometry of pre- and post-synaptic neurons based on a published mathematical formula (Stepanyants and Chklovskii, 2005):

\[
N_p(\tilde{R}_a, \tilde{R}_d) = 2s \sum_{i,j} l'_a l'_d |\sin(\hat{n}'_a, \hat{n}'_d)| \exp \left[ - \left( \tilde{r}'_a - \tilde{r}'_d \right)^2 / 4\sigma^2 \right] \left( 4\pi \sigma^2 \right)^{3/2}
\]

This equation defines the number of potential synapses between the axons of one neuron and the dendrites of a second neuron, \(N_p(\tilde{R}_a, \tilde{R}_d)\) as a function of their position in space, whereas the somata of the two cells are placed at coordinates \(\tilde{R}_a\) and \(\tilde{R}_d\), respectively. In the right hand side, \(s\) is the axo-dendritic interaction distance (in our case, 2 µm and 1 µm for principal cells and interneurons, respectively), \(l'_a\) and \(l'_d\) represent the unit-volume lengths of the axonal and dendritic segments along the unit vectors \(\hat{n}'_a\) and \(\hat{n}'_d\), and |\(\sin(\hat{n}'_a, \hat{n}'_d)\)| is the absolute value of the sine of the angle between unit vectors \(\hat{n}'_a\) and \(\hat{n}'_d\).

These neuronal reconstructions are taken to represent the morphology of a class of similar, but non-identical, neurons. Thus, a Gaussian filter with standard deviation \(\sigma\) is applied around the centers of the axonal and dendritic segments with vectors \(\tilde{r}'_a\) and \(\tilde{r}'_d\). The parameter \(\sigma\) was fixed at 10 µm, corresponding to the average length of all segments. This equation is iteratively applied to all of the axonal and dendritic segments (indicated with \(i\) and \(j\), respectively). Thus, the diameter measurements of the axon and dendrite segments are not used in computing the number of potential synapses.

**Computational details and data analysis**
The core computational framework was written in C/C++ and compiled with the GNU compiler under UNIX. Potential connectivity computations were executed on an SGI cluster of 80 Altix 8200 nodes, each containing two quad core Intel Xeon E5440 2.83 GHz processors with 16 GB of RAM. Instead of parallelizing the C/C++ code, we maximized the number of available nodes by submitting to the server a number of PBS (Portable Batch System) scripts that split the job into independent tasks (http://www.unix-info.org). At the same time, the code was optimized using pthreads (Silberschatz et al., 2009) to capitalize on the multi-processors of each node. As expected, the computing time decreased sub-linearly on the cluster machine compared to a single CPU. For instance, computing the potential connectivity for the proximal CA3c axon took ~24 hrs on a single computer vs. ~2 hrs on the cluster.

The program returns the number of potential contacts between a pre-synaptic neuron and all post-synaptic targets. Moreover, it records the anatomical locations of every potential synapse both within the hippocampus (in each of the coordinate systems described above) and in terms of distances from the somata of the pre- and post-synaptic neurons along the axonal and dendritic paths, respectively. Two-dimensional color maps (e.g. Figure 5.2) were generated using the R package ‘ggplot2’ (http://had.co.nz/ggplot2). Linear dependencies were computed from these potential connectivity maps as regression fits of the septo-temporal positions weight-averaged by the corresponding numbers of potential synapses at a given hippocampal-transverse position. Virtual Reality Modeling Language
files were generated with the freeware viewer ‘view3dscene’

RESULTS

The results presented here are intended as a proof-of-concept of the 3D framework in computing the full potential connectivity of single neurons throughout system-level regional maps. As such, the research design was data- rather than hypothesis-driven.

We named the various neurons with a region_cell-type nomenclature convention, using subscripts AX and DE for pre- and post-synaptic cells, respectively and superscripts to specify sub-regions as needed. Thus, the dentate granule cell is labeled DG_GCA\text{X}. The pre-synaptic pyramidal cells in various sub-regions are referred to as CA3c\text{prox}_{PC}\text{AX}, CA3c\text{dist}_{PC}\text{AX}, CA3b_{PC}\text{AX}, and CA2_{PC}\text{AX}. The interneurons with somata in the radiatum and lacunosum-moleculare layers are distinguished as CA3b_{R}\text{IN}_{AX} and CA3b_{LM}\text{IN}_{AX}, respectively. The post-synaptic pyramidal cell targets are referred to as CA3_{PC}\text{DE} and CA1_{PC}\text{DE}.

Potential synaptic framework illustration

The dentate mossy fibers exemplify a well-known axonal arbor system within the hippocampus, with projections to all three sub-regions of the CA3 pyramidal network.
Figure 5.2: Potential connectivity analysis for DG mossy fiber onto CA3 pyramidal cells. (A) Relative orientation of a dentate granule cell (axon: yellow, dendrite: green) and of the dendritic trees of two pyramidal cells from CA3b and CA3a. Inset shows the arbor embeddings within the 3D hippocampus reconstruction. (B) Potential synapse map: The average number (Np) of potential contacts (gray scale) is based on simulations of 25 pre-synaptic positions around the central granule cell location, which is marked by a star in the top (DG) right panel. The bottom (CA3/CA1) right panel demarcates the sub-regions invaded by the axon (square box), corresponding to the main panel. The fitting line is a cubic spline function ($r^2=0.85$) of septo-temporal vs. hippocampal-transverse positions weight-averaged by the potential synapses numbers. (C) Average number of potential contacts in each of the CA3 sub-regions, fitted with two Gaussian curves. Inset shows the density of potential synapses. (D) Number (left axis, red) and cumulative count (right axis, green) of potential synapses along axonal path distance. The solid and dashed colored lines correspond to averages and 95% confidence interval around the mean.

(Figure 5.2A). The axons of dentate granule cells contact both pyramidal cells and interneurons in CA3 stratum lucidum (Acsady et al., 1998). Here we only considered pyramidal cells as the potential post-synaptic targets. Previous research on the
organization of the dentate mossy fiber pathway revealed that the main axonal path initially traverses the CA3 region in a septal direction. Then at the CA3b/CA3a border it abruptly changes course to a temporal/caudal orientation relative to the soma position of the granule cell (Acsady et al., 1998). This peculiar shape of the mossy fibers is evident from the visualization of the axonal embedding within the 3D hippocampal reconstruction (Figure 5.2A) and the potential synaptic map in CA3 (Figure 5.2B). The map is built from 25 granule cell somatic positions around the center of the spatial range in the supra-pyramidal region corresponding to the reported stereotactic coordinates (star in the DG schematic of Figure 5.2B).

The total number of potential synapses made by the granule cell on pyramidal dendrites is $103.8 \pm 4.1$ (mean$\pm$SD, $N=25$), with an almost equal distribution among the three sub-regions of CA3: $32.2 \pm 2.3$ in CA3c, $36.6 \pm 2.1$ in CA3b, and $35.0 \pm 1.1$ in CA3a. However, both the number of potential contacts and the proportions among the sub-fields vary non-uniformly along the longitudinal axis. In particular, the distribution along the septo-temporal axis follows a double Gaussian (Figure 5.2C). The first peak is centered around the somatic septo-temporal position of the granule cell, and largely consists of potential synapses in areas CA3c and CA3b. The second peak (displaced ~0.6 mm temporally) is twice as wide but of smaller (~1:5) amplitude, and almost entirely corresponds to potential contacts in CA3a. In contrast to the absolute numbers of potential contacts, the potential synaptic density is significantly higher in CA3c compared to CA3b and CA3a (Figure 5.2C inset, $p<0.0001$, unpaired two-tailed t-test). The uneven density can be
explained by analyzing the number of potential synapses with respect to the axonal path
distance (Figure 5.2D). The potential synaptic count rises sharply and peaks at \( \sim 1 \) mm
within CA3c, but then decreases farther along the path in correspondence to the CA3b
and CA3a sub-regions. These results parallel the empirical observations on mossy bouton
and actual synapse counts within each CA3 sub-region (Acsady et al., 1998; Henze et al.,
2000).

**Distinct potential synaptic connectivity patterns in the pyramidal cell network**

The axonal trees of the four examined pyramidal neurons share common general
characteristics, at the same time exhibiting exquisitely distinct potential synaptic maps
onto the CA3/CA1 principal neuronal network (Figure 5.3). All four arbors project to
large portions of the CA3 and CA1 sub-regions, with \( \sim 3 \) mm longitudinal coverage. The
numbers of potential synapses, however, vary widely from \( \sim 176K \) for CA3c\(_{\text{prox}}\)\_PC\(_{AX}\)
and \( \sim 160K \) for CA2\(_{PC}\_AX\), to \( \sim 100K \) for CA3c\(_{\text{dist}}\)\_PC\(_{AX}\) and \( \sim 80K \) for CA3b\(_{PC}\_AX\).
These differences, considerable even within sub-region, largely reflect variation in axonal
length (Ropireddy et al., 2011).

Except for CA3b\(_{PC}\_AX\), all other three neurons have potential synapses across every CA
sub-field, with relative differences in their count of potential contacts between CA3 and
CA1. A prominent difference among the maps concerns their orientations with respect to
the two natural axes of the hippocampus, reflected by the linear regressions of the
Figure 5.3: Potential connectivity maps of four pre-synaptic CA3/2 principal neurons onto pyramidal cells. (A) CA3c$^{\text{prox}}$ _PC$_{AX}$ (gray), (B) CA3c$^{\text{dist}}$ _PC$_{AX}$ (blue), (C) CA3b$_{PCAX}$ (green), and (D) CA2$_{PCAX}$ (red). In all maps, the white star denotes the hippocampal-transverse and septo-temporal coordinates of the pre-synaptic soma. Insets show the linear regression and 95% confidence interval of the septo-temporal vs. hippocampal-transverse positions weight-averaged by the potential synapse numbers.

Weighted averages (Figure 5.3 insets). Specifically, no particular trend is apparent for CA3c$^{\text{prox}}$ _PC$_{AX}$ (Figure 5.3A). In contrast, CA3c$^{\text{dist}}$ _PC$_{AX}$ (Figure 5.3B) and CA2$_{PCAX}$ (Figure 5.3D) clearly favor septal locations in the proximal hippocampal-transverse regions (CA3 towards the DG), and temporal locations in the distal hippocampal-transverse regions (CA1 towards the subiculum), with a significantly positive correlation
($r=0.87$ and $r=0.98$, respectively). Conversely, CA3b_PCA displays the opposite
tendency (Figure 5.3C) due to a fairly uniform spread of CA3 contacts around the
somatic position and a ~1 mm septal shift in CA1 ($r=-0.81$).

![Figure 5.4: Sub-region and layer specificity of pyramidal cell potential connectivity.](image)

(A and B) Potential synapse distributions along the hippocampal-transverse axis for
CA3c$_{prox}$ _PCAX (A, gray), CA3c$_{dist}$ _PCAX (A, blue), CA3b_PCA (B, green) and CA2_PCA
(B, red). The solid colored arrows indicate the soma hippocampal-transverse positions.
For each of the four axonal arbors, bar plots summarize the total counts in each CA3
sub-region and in CA1. (C) Proportion of potential contacts in CA3 (light) and CA1.
(dark) layers. The layers not primarily targeted by these axons (PC and LM) are striped. 

(D) Density of potential synapses in the layers of each CA3 sub-region (light) and CA1 (dark).

The distinct patterns of potential connectivity are particularly evident along the hippocampal-transverse axis (Figure 5.4A,B). The CA3c<sup>prox</sup>PC<sub>AX</sub> bimodal distribution peaks in CA3c and in mid-CA1. The multi-modal shape of CA3c<sup>dist</sup>PC<sub>AX</sub> reveals a preference for the CA3c and CA3a sub-regions, but tails off within proximal CA1. These contrasting tendencies are summarized in the counts of potential contacts within CA3 (and its sub-fields) and CA1 (bar plots of Figure 5.4A). To quantify the opposite differences statistically, we repeated the embedding of all post-synaptic pyramidal cells 10 times with different random seeds for the exact positions and orientations. For the CA3c<sup>prox</sup>PC<sub>AX</sub>, the potential synaptic count is 1.5 times larger in CA1 than in CA3, whereas for CA3c<sup>dist</sup>PC<sub>AX</sub> this ratio is 0.45 (both p<0.0001, unpaired two-tailed t-test). Moreover, CA3c<sup>prox</sup>PC<sub>AX</sub> makes three-fourth of its potential synapses in CA3c, while half of the CA3c<sup>dist</sup>PC<sub>AX</sub> potential contacts are in CA3a. The CA3b_PC<sub>AX</sub> neuron exhibits the most localized potential connectivity, peaking around the somatic position in CA3b and extending nearly an order of magnitude fewer potential synapses in CA1 than in CA3. The CA2_PC<sub>AX</sub> hippocampal-transverse distribution is bimodal with peaks in CA3a and mid-CA1. The total number of CA1 potential contacts is twice that in CA3 (p<0.0001).
The four pyramidal cells also display general similarities and distinct differences in potential connectivity across layers (Figure 5.4C). As expected, the vast majority of potential synapses are always found in radiatum (RAD) and oriens (OR), with only small fractions in the pyramidal (PC) and lacunosum-moleculare (LM) layers. The CA3 and CA1 radiatum proportions notably differentiate the four pre-synaptic neurons. For CA3c\textsubscript{prox}\_PC\textsubscript{AX}, the RAD potential contacts are 1.7 times more abundant in CA1 than in CA3, while the inverse ratio (0.4) applied to CA3c\textsubscript{dist}\_PC\textsubscript{AX}. In both neurons, only less than or close to one tenth of potential synapses are contributed by OR, as opposed to more than two thirds by RAD. For CA3b\_PC\textsubscript{AX}, radiatum is still dominant in the minor CA1 component, but for the major CA3 contribution, oriens and radiatum provide an approximately even number of potential synapses. A mirror situation occurs in CA2\_PC\textsubscript{AX}, where radiatum has greater representation in the minor CA3 component, but oriens claims an equal split in the synaptically richer CA1 field. For both CA3b\_PC\textsubscript{AX} and CA2\_PC\textsubscript{AX}, less than 50% of potential synapses are found in RAD, and more than a third in OR.

In addition to the count of potential contacts, the potential synapse densities also vary across sub-regions and layers among the four axons (Figure 5.4D). For CA3c\textsubscript{prox}\_PC\textsubscript{AX}, the highest density of potential synapses occurs in CA3c even though the total count is greater in CA1. In contrast, CA3c\textsubscript{dist}\_PC\textsubscript{AX} shows similar potential synapse densities in all CA3 and CA1 sub-regions. For CA3b\_PC\textsubscript{AX}, the potential synapse density is highest in CA3b and CA1, again in spite of the lower CA1 total count, indicating a focal
concentration of potential connectivity within a smaller area (cf. Figure 5.4B). The CA2_PCAX potential synapse density is maximal in CA3a followed by CA1, where more than two thirds of the potential contacts are made. Thus, each of the four neurons displays unique patterns of preferred density domains. Conversely, in all cases and for each sub-field, the potential synaptic density is substantially greater in radiatum than in oriens with only three exceptions in which the two values are similar: CA3c and CA3a for CA3b_PCAX, and CA1 for CA2_PCAX.

**Potential synapse distributions along the axonal and dendritic paths**

The distance from the soma of a pre-synaptic terminal along the axonal path may affect spike propagation delay and reliability. Thus, we investigated how the dependence on axonal distance of potential connectivity varies among neurons (Figure 5.5A). The four pyramidal cells exhibit linear increases with similar slopes in the cumulative count of potential synapses up to ~1.5 mm, indicating a uniform distribution of potential pre-synaptic contacts in the first part of the axonal path. Beyond this distance, the neuron with the longest axon (CA3c_{prox_PCA}) has a shallow accumulation of potential contacts (mostly in CA3) within the more proximal one-third of path (the initial ~7 mm), and makes progressively more CA1 potential synapses in the next ~7 mm. The neuron with the fewest potential contacts (CA3b_PCA) is also essentially confined within CA3 and in fact ends shortly after the initial 7 mm of path, though it displays a faster potential synapse increase reaching a plateau in the first ~5 mm. Within this same path, the other
two neurons have the steepest (and very similar) accumulation of potential contacts. The neuron with the shortest path (CA3c\textsuperscript{dist}_PC\textsubscript{AX}) synapses mostly in CA3 for the first half of its path and only in CA1 for the second half. CA2\_PC\textsubscript{AX} also forms potential contacts exclusively in CA3 for the first \~2.5 mm, but then continues for \~10 mm after switching to CA1.

![Figure 5.5: Distribution of potential synapses along axonal and dendritic paths. (A) Cumulative count of potential synapses made by the four CA3/2 pyramidal cells as a function of their axonal path distances from the soma. The dotted colored lines correspond to the potential contacts onto CA3 pyramidal cells. The difference between the solid and dotted lines represent the cumulative potential synapse numbers onto the CA1 network. (B) Distribution of the number of potential synapses received by the CA3 (light) and CA1 (dark) pyramidal cells contacted by any of the four axonal arbors. Inset shows the cumulative proportion of pyramidal cells contacted with up to a given number of potential synapses. (C and D) Potential synapse distributions for the four CA3/2](image-url)
axonal arbors along the dendrites of CA3 (C) and CA1 (D) pyramidal cells. Negative and positive path distances correspond to basal and apical dendrites, respectively. The background neuron images are representative dendritic arbors of CA3 and CA1 pyramidal cells from NeuroMorpho.Org.

The four axonal arbors also differ in terms of the proportion of post-synaptic targets they contact, either out of all (225,000 CA3 and 390,000 CA1) pyramidal cells or only considering those with dendrites within the spatial reach of each axon (Table 5.1). These numbers vary widely among neurons and between CA3 and CA1. In contrast, the spatial coverage is similar among the four axons in CA3 (~40%) and CA1 (~25%). The average

Table 5.1: Percentage of pyramidal cells (PCs) contacted by the four axonal CA3/CA2pc arbors and number of potential synapses they receive. The “overall % PCs contacted” is computed based on a total of 225,000 and 390,000 CA3 and CA1 pyramidal neurons respectively. The “Np per PC contacted” only counts the target PCs receiving potential synapses. “Spatial coverage” represents the proportion of PCs with dendrites within the spatial reach of each pre-synaptic axon.

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<tr>
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<th>CA3cPCax</th>
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<td>CA3</td>
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<td>overall % PCs contacted</td>
<td>5.9%</td>
<td>12.0%</td>
<td>8.9%</td>
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<tr>
<td>Np per PC contacted (μ ± σ)</td>
<td>5.6 ± 0.20</td>
<td>2.5 ± 0.18</td>
<td>3.4 ± 0.30</td>
<td>5.1 ± 0.12</td>
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<td>39%</td>
<td>45%</td>
<td>42%</td>
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<tr>
<td>% PCs contacted in spatial coverage</td>
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<td>30.8%</td>
<td>19.8%</td>
<td>11.7%</td>
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<tr>
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<td>0.8%</td>
<td>7.3%</td>
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<td>2.2 ± 0.40</td>
<td>3.8 ± 0.15</td>
<td>4.0 ± 0.39</td>
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<td>23%</td>
<td>26%</td>
<td>24%</td>
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<td>37.5%</td>
<td>16.1%</td>
<td>3.1%</td>
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number of potential contacts per post-synaptic neuron ranged from ~2.2 to 5.6, with small coefficients of variation (typically below 0.1). Thus, most neurons receiving potential
synapses tended to be contacted multiple times. In fact, individual connections only accounted for one third of the cases. However, the distributions of the number of potential synapses per contacted pyramidal cell varied dramatically depending on the pre-synaptic axon (Figure 5.5B). Interestingly, the grand average of potential synapses per pyramidal cell over the whole post-synaptic pool (the product of overall % PCs contacted by their averaged received contacts) is similar for all axonal arbors on CA3 pyramidal dendrites (~0.3) but not on CA1 pyramidal dendrites, where it spans an order of magnitude from 0.29 for CA2_PC\textsubscript{AX} to 0.03 for CA3b_PC\textsubscript{AX}. These divergence ratios are consistent with previous findings (Li et al., 1994).

The distance of a synapse from the soma along the dendritic path can affect signal integration both by cable filtering and active membrane properties. The distributions of potential synapses in the apical and basal dendrites of pyramidal cells reflect the patterns observed in the oriens and radiatum layers, respectively, in CA3 (Figure 5.5C) and CA1 (Figure 5.5D). All four neurons have a peak of potential synapses on the basal dendrites at a similar distance along the path (~200 µm). However, CA3b_PC\textsubscript{AX} and CA3c\textsubscript{prox}\_PC\textsubscript{AX} have the largest number of basal potential contacts in CA3 and the smallest in CA1. In contrast, CA2_PC\textsubscript{AX} is dominant in CA1, and CA3c\textsubscript{dist}\_PC\textsubscript{AX} has a similar number in both regions. The four neurons display more similar distributions of potential contacts along the CA3 apical paths, except for the more proximal peak of CA3c\textsubscript{prox}\_PC\textsubscript{AX}. The patterns along the CA1 apical paths, in contrast, are completely distinct in the four cases. CA3c\textsubscript{prox}\_PC\textsubscript{AX} and CA2_PC\textsubscript{AX} have the largest and second
largest peaks, but are located at opposite extremes (distal and proximal, respectively) of the apical dendrites, almost 750 µm apart. The remaining two CA3 neurons have smaller peaks (especially CA3b_{PCAX}) at intermediate path distances.

**Potential synapse distributions of Radiatum and Lacunosum-Moleculare CA3b interneurons**

Interneurons with somata in CA3b radiatum and lacunosum-moleculare layers have distinct electrophysiology and synaptic plasticity, yet similar axonal morphology (Ascoli et al., 2009). The collective potential synaptic map for the 13 interneurons (6 CA3b^{R}_{INAX} and 7 CA3b^{LM}_{INAX}) depicts localized CA3 distributions spanning approximately the same spatial extent in the septo-temporal and longitudinal dimensions (Figure 5.6A). CA3b^{R}_{INAX} and CA3b^{LM}_{INAX} make approximately 50,000 and 40,000 potential contacts, respectively, with CA3 pyramidal cell dendrites. The difference between these mean values was not statistically significantly (p>0.5, unpaired two-tailed t-test). The spatial extent in the septo-temporal and hippocampal-transverse axes were 0.60 ± 0.09 mm and 2.91 ± 0.55 mm (mean ± SD, N=13), respectively. The potential synaptic map area was 1.38 ± 0.23 mm². The coefficient of variation for these three parameters is thus under 0.2. We also computed the mean square deviation (MSD) for the same three parameters by comparing the connectivity map of each neuron with the convolved map (shown in Figure 5.6A). The corresponding measurements were 0.02 mm, 0.31 mm, and 0.09 mm², demonstrating a modest variation among these 13 CA3b interneurons.
Interestingly, however, the two groups of interneurons can be clearly differentiated based on their potential connectivity patterns along the hippocampal-transverse axis (Figure 5.6B). The number of potential synapses from CA3b\textsuperscript{R}_IN\textsubscript{AX} gradually increases from CA3c, peaking just past the CA3b/CA3a border, and dropping sharply within CA3a. In contrast, CA3b\textsuperscript{LM}_IN\textsubscript{AX} displays a bimodal distribution, with a prominent potential synapse peak at the CA3c/CA3b border, a dip in the middle of CA3b, and a secondary peak in CA3a. In contrast, the potential connectivity patterns along the septo-temporal position are symmetric around the somata and indistinguishable between the two interneuron types (Figure 5.6C).
Figure 5.6: Potential connectivity analysis for CA3b interneurons onto CA3 pyramidal cells. (A) Map of the convolved common area of potential synapses formed by the axons of 13 CA3b interneurons with somata in the radiatum (R) or lacunosum-moleculare (LM) layers onto CA3 pyramidal cell dendrites. The inner closed line represents the area in which all pre-synaptic cells establish potential synapses. The outer close line delineates the region in which any of these interneurons makes potential contacts. The star marks the average somatic location of all 13 cells. The size of the star approximately corresponds to the location range. There were no statistically significant differences in the septo-temporal and hippocampal-transverse positions between the R and LM neurons. (B) Mean potential synapse distributions and 95% confidence intervals for 6 CA3b\_IN\_AX (purple) and 7 CA3b\_LM\_IN\_AX (gold) along the hippocampal-transverse axis. The solid colored arrows indicate the average hippocampal-transverse positions of the somata. The vertical dashed lines demarcate the boundaries between the three CA3 sub-regions. (C) Gaussian fits of the means and 95% confidence intervals for the septo-temporal distributions of potential synapses. (D) Hippocampal-transverse patterns (‘towards CA3c’ vs. ‘towards CA3a’) and layer specificity in potential synapse densities for the two CA3b interneuron types. (E) Potential synapse distribution along the dendritic path distance of CA3 pyramidal cells.

The differences along the hippocampal-transverse axis suggest opposite propensities of CA3b\_IN\_AX and CA3b\_LM\_IN\_AX to make potential contacts towards CA3c and CA3a, respectively, as evidenced by potential synaptic density analysis (Figure 5.6D). Further inspection of layer specificity shows in the density bar graphs that the minor proportion of potential contacts in the oriens layer is unique of CA3b\_IN\_AX cells, and entirely confined to the CA3c region. In contrast, in both groups the concentration of potential contacts in lacunosum-moleculare grows towards CA3a. The lack of CA3b\_LM\_IN\_AX potential synapses in oriens is reflected in the distribution along the dendritic path distance of the target pyramidal cells (Figure 5.6E), which is similar for the two interneuron types on the apical, but not basal arbors. Lastly, interneurons with higher and lower dendritic branch numbers (called HiDe and LoDe in Ascoli et al., 2009) cannot be distinguished by their potential connectivity (not shown).
DISCUSSION

The approach introduced here enables the analysis of potential connectivity patterns from individual axo-dendritic overlaps across the entire hippocampus. The spatial scale defined by the span of whole axonal arborizations bridges the conceptual levels of potential synapses and regional anatomy. The hippocampus is especially suitable for studying systems-level connectivity, because of its unique structural organization and relative wealth of cellular morphological data. At the same time, the highly diverse hippocampal principal cells and interneurons form complex microcircuits whose computational function is only beginning to be understood. Functionally, the hippocampus is theorized to play a central role in spatial navigation and memory processing. Thus, our results might facilitate incorporation of realistic connectivity patterns into models investigating structure-function relationship.

We observe unique distributions of potential synapses across the various sub-regions and cyto-architectonic layers of the hippocampus, based on entire axonal reconstructions from seven neuron types and the dendritic arbors representing the whole CA pyramidal cell network. Although axonal morphology constitutes the most prominent determinant of synaptic connectivity, the present study demonstrates the importance of integration both across data sources and data types. The 3D whole-hippocampus reconstruction enables the embedding of complete axonal arbors traced from disparate histological preparations
into the same framework (e.g. Scorcioni and Ascoli, 2005; Ascoli et al., 2009; Ropireddy et al., 2011). Moreover, the dense dendritic embedding allows actual computation of potential synaptic maps, as well as quantitative analysis such as the dendritic and axonal distributions of potential contacts and the ratio between the numbers of actual and potential synapses.

In this analysis, the pre-synaptic neuron, the post-synaptic neuron, and the embedding tissue are reconstructed from different animals. Thus, this framework cannot capture the specific correlations among pairs of cells within their surroundings. At the same time, potential connectivity patterns can reveal general statistical principles of the specific interaction probabilities among particular cell classes that might apply across individuals. In particular, if validated with a larger sample, the CA3 axonal potential connectivity might indicate that proximal CA3c neurons (towards hilus) communicate primarily with CA1, while distal CA3 neurons (e.g. in CA3b) mostly form recurrent axo-dendritic overlaps within CA3. Moreover, the strikingly different potential connectivity patterns of the two CA3c neurons (proximal and distal) suggest that even principal cells from the same sub-region can have contrasting potential synaptic distributions throughout the hippocampus. While this finding may have interesting implications on the information processing in CA3 and CA1, the empirical observation must be corroborated on a representative sample of neurons before generalizing to the whole population of proximal and distal CA3 pyramidal cells.
We adopted the concept of potential synapses to characterize circuitry based on light microscopy data. Functionally, potential connectivity relates to the capability to create actual synapses leveraging the structural plasticity of dendritic spines and axonal boutons (Holtmaat and Svoboda, 2009). Evidence for this phenomenon is particularly compelling in the hippocampus, where it might bear direct functional relevance to information storage (Chklovskii et al., 2004). From this viewpoint, it is interesting to evaluate the ratio of actual to potential synapses, called connectivity fraction (Escobar et al., 2008) or filling fraction (Stepanyants et al., 2002). The only available dense electron microscopy reconstruction of a small hippocampal volume from the rat CA1 radiatum layer (Mishchenko et al., 2010) confirmed earlier estimates of connectivity fraction in the 0.20-0.25 range. Considering, as in these recent and earlier reports (Bannister and Larkman, 1995; Megias et al., 2001), bouton and/or spine counts as proxies for actual synapse numbers, we obtain values fully consistent with the existing data for that region. At the same time, our results can be further used to derive the connectivity fractions for other components of the hippocampal circuit.

Dentate mossy fibers are estimated to form ~18 mossy terminals each (Acsady et al., 1998; Henze et al., 2000), leading to connectivity fractions of 0.17-0.18 in all three sub-fields CA3a,b,c. Similarly, the bouton numbers reported for the proximal CA3c pyramidal axon (Wittner et al. 2007) combined with our potential synapse count yield an overall connectivity fraction of ~0.23. However, this value changes drastically throughout the hippocampus, from 0.06 in CA1 OR to ~0.17 in CA3 OR and RAD, to
0.32 in CA1 RAD. This differentiation by sub-region and layer emphasizes the importance of analyzing connectivity through the entire anatomical region invaded by an axonal arborization as opposed to the narrow region of interest typically covered by electron microscopy.

Summing together the potential synapses made by the four analyzed pyramidal axons on all pyramidal cell dendrites, ~26% of the contacts are made in the oriens layer, and ~59% in radiatum. These fractions, virtually identical in CA3 and CA1, can be compared to the relative densities of dendritic spines observed in the same layers. The values in CA3 (Drakew et al., 1996) constitute an almost perfect match, with 28% of spines in oriens and 59% in radiatum. Such correspondence suggests equal overall connectivity fraction between basal and apical dendrites. Interestingly, the proportion of CA1 spines found in radiatum (~55%) is also very similar to the potential synapse fraction, while the value in oriens (~39%) is higher (Bannister and Larkman, 1995; Megias et al., 2001), consistent with the addition of recurrent collaterals from CA1 pyramidal cells on the basal dendrites.

A drawback of this study is that the potential connectivity patterns are based on only one axonal reconstruction for each principal neuron. This is due to the difficulty of obtaining complete digital reconstructions of projecting axons from extremely laborious in vivo preparations. With the more contained interneurons, axons can be reconstructed from slices, enabling analysis of larger samples. In the case of CA3b Radiatum and Lacunosum-Moleculare cells, we found different potential synaptic patterns across the
hippocampal-transverse axis (i.e. among the CA3 sub-fields). Our previous intrinsic morphometric analysis could not detect these differences between the two groups (Ascoli et al., 2009). This observation stresses the importance of studying connectivity patterns within the context of a 3D system-level anatomical framework.

Based on the axonal and dendritic reconstruction data available in NeuroMorpho.Org, our computational framework can allow the estimation of potential interconnectivity of additional neuron classes in the rodent hippocampus. In particular, complete axonal reconstructions of principal neurons from posterior-ventral region of CA3, CA1a, CA1b, CA1c, subiculum, and entorhinal cortex layer 2 (e.g. Scorcioni and Ascoli, 2005) can be embedded to map their hippocampal potential connectivity. Similarly, 18 axonal reconstructions of interneurons from DG hilus, CA3 stratum lucidum and CA1 stratum oriens can also be included in future studies. Additionally, 223 dendritic reconstructions of interneurons throughout all DG, CA3, and CA1 sub-fields and layers can be embedded to extend all potential connectivity maps beyond the pyramidal cell targets examined here. It is also expected that the number of available reconstructions will continue to grow, enabling further refinements of these results.

Comparing the potential connectivity obtained for the same pre-synaptic cells, but using the dendritic trees of different neuronal classes (e.g. interneurons vs. pyramidal cells), can also be useful as a control to examine the role of dendritic specificity in establishing potential synapses. This could be achieved, for example, by contrasting the connectivity
patterns of CA3 pyramidal axons to CA1 basket cell dendrites with those to CA1 pyramidal cell dendrites. An alternative possibility is to use random cable for the dendrite as a control. These extensions will be explored in future investigations.
APPENDIX I: 3D HIPPOCAMPUS VOXEL DATABASE

The data files corresponding to the high resolution three dimensional (3D) structure of the rat hippocampus that we reconstructed from histological sections are being shared on the Windows Live™ cloud space provided by Microsoft®. Downloadable data files include the Nissl histological images, the hippocampus layer tracings that can be visualized alone or superimposed to the corresponding Nissl images, the voxel database coordinates, and the surface rendering VRML files. All the files are uploaded into a common folder and the downloadable links are provided at http://krasnow.gmu.edu/cn3/hippocampus3d. Within this parent folder there are four subfolders (Hippocampus-NisslImages, Hippocampus-Tracings, Hippocampus-VoxelDB, and 3D Surface Rendering).

**Hippocampus Nissl Images**

The high resolution histological Nissl images obtained at 16 μm inter-slice distance for the Long-Evans rat hippocampus can be downloaded from the cloud. This dataset consists of 230 jpeg images that cover the hippocampus from rostral to caudal poles. This image dataset is uploaded in seven parts as rar files part1-7.rar).
As an alternative to downloading, the images can also be directly viewed in a browser whose link is also provided on the wrapper html web page. Use thumbnails to navigate and click on the larger images to obtain the higher resolution data.

**Hippocampus Layer Tracings**

The seven hippocampus layers ‘ML’, ‘GC’, ‘HILUS’ in DG and ‘LM’, ‘RAD’, ‘PC’, ‘OR’ in CA were segmented (traced) using the Reconstruct tool which can be downloaded from Synapse web. This tool outputs all the tracings for each image in XML format. The XML tracing files for all these seven layers for each of the above Nissl images are zipped into one file and can also be downloaded. In order to open and view the XML tracing files, decompress all the Nissl rar archives and the zipped tracings in one directory and open the master Reconstruct file (Series_10.ser) in Reconstruct. The instructions for using this tool are also provided at Synapse web.

**Hippocampus VoxelDB**

The 3D hippocampus reconstructed is volumetrically transformed into 16 μm sized voxels for all the seven layers. Each voxel is reported according to multiple coordinate systems, namely in Cartesian, along the natural hippocampal dimensions, and in reference to the canonical brain planes. The voxel database file is created in ascii format. The single voxel database file was split into three rar archive files (Hippocampus-VoxelDB.part01-03.rar). Please note that the three rar archive files should be downloaded and decompressed in a single directory in order to obtain the single voxel data file.
(Hippocampus-VoxelDB.txt). The details of the organization of this voxel database file are discussed in the README file described below.

3D Surface Renderings
Surface rendering (or triangulation) VRML files of the DG and CA layers can also be downloaded from the cloud space. This is a rar archive file with a single VRML file containing the surface rendering of DG and CA layers. This VRML file can be opened and visualized in any VRML viewer, e.g. the open source software view3dscene (http://vrmlengine.sourceforge.net/view3dscene.php).

README File Description
# README file for Hippocampus-VoxelDB rar archive files.
# The 3D hippocampus voxel coordinate database file consists of three rar archive files (Hippocampus-VoxelDB.part01.rar, .part02.rar, .part03.rar). These archive files contain Dentate Gyrus (DG) and Cornu Ammonis (CA) voxel coordinates. After downloading and decompressing the three rar files in the same folder, the single voxel coordinates database file (hippocampus-voxeldb.txt) can be obtained. The total number of voxels for the entire hippocampus is 10,107,899.

# In this voxel database file, the DG region voxels are listed first, followed by CA region voxels. Within each region, voxels are listed in order from superficial to deep layers. The DG voxels are listed in Molecular Layer (‘ML’), Granule Layer (‘GC’), and 'Hilus' layer
order. The CA layer voxels are listed in Lacunosum-Moleculare ('LM'), Radiatum ('RAD'), Pyramidal Cell ('PC'), and Oriens ('OR') layer order. Within a layer, voxels are ordered according to sub-regions (infra- to supra-blade in DG and CA3c to CA1a for CA). Finally, within sub-regions, voxels are listed according to their dorso-posterior-ventral positions.

# Each row contains 9 columns with the following specifics.

# Column 1: Center of voxel, X coordinate (μm).
# Column 2: Center of voxel, Y coordinate (μm).
# Column 3: Center of voxel, Z coordinate (μm).
# Column 4: Septo-Temporal (or Longitudinal) Position (μm).
# Column 5: Hippocampal-transverse Position (μm), which is the position along the 'C'-shape of DG or CA.
# Column 6: Depth (in the layer direction) position (μm).
# Column 7: Bregma position (μm).
# Column 8: Lambda or Inter-Aural Distance (μm).
# Column 9: Type of voxel. This is a number from 1 to 102, depending on the region (CA or DG), layer (ML, GC,Hilus, OR, PC, RAD, LM), sub-region (Infra, Supra, CA3c,b,a, CA2, CA1c,b,a) and division (Dorsal, Posterior, Ventral) of the voxel. The specifics of each voxel type are denoted below along with the corresponding numbers of voxels.

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APPENDIX II: 3D HIPPOCAMPUS-POTENTIAL SYNAPTIC FRAMEWORK
MANUAL

**Hippocampal Dendritic and Axonal Data**

This manual details the computational framework for potential connectivity results described and discussed in chapter 5. One must understand the ‘swc’ file architecture for representation of neuronal morphology and the basics of morphometrics in order to use this framework and compute the potential connectivity. These topics can be learnt from other lab resources like L-Measure and NeuroMorpho.Org tools and databases. It would be very useful to read and follow this manual with the Doxygen generated documentation for the potential synaptic C/C++ code and framework that can be viewed on the web browser (/3DHippocampus-PotentialConnectivityFramework/Computational_Framework/Doxygen-Documentation/PotentialConnectivity/html/index.html). Additionally, please go through the 3D Hippocampus database described at the wrapper page (krasnow.gmu.edu/cn3/hippocampus3d/) which has links to the voxel database uploaded to the Microsoft cloud server. Specifically, please read the file description that explains the voxel database format.
Table A2.1 contains the number of axonal reconstructions available for each type displayed in the first column. Specifically, there are 1 GC mossy fiber, 13 hilus interneurons, 1 proximal CA3c, 1 distal CA3c, 1 CA3b, 1 CA3pv (posterior-ventral), 1 CA2, 1 CA1c, 1 CA1b and 1 CA1a pyramidal neuronal reconstructions. In addition, there are 6 CA3bSR (stratum radiatum) interneuron, 7 CA3bSLM (stratum lacunosum moleculare) interneuron, 2 CA3SL (stratum lucidum), 3 CA1SO (stratum oriens) interneuron axonal reconstructions. There are also 1 Subiculum and 1 Entorhinal stellate layer-II axonal reconstruction.

The headers in the first row show the nine types of dendritic (post-synaptic) neurons which are GC, HILUS, CA3pc (pc-pyramidal cell), CA2pc, CA1pc, CA3int (interneuron), CA1int (interneuron), Subiculum (Sub) and EC-II (entorhinal cortex layer-II). From second row, columns# 2-10 in this table show the number of pre/post-synaptic arbors for the probable connectivity scenario.

For ex: The second row lists 1/13, 1/62, 1/78 for HILUS, CA3pc and CA3int, which means, for the single mossy fiber reconstruction one can estimate the potential connectivity by sampling the 13 HILUS, 62 CA3pc and 78 CA3int dendritic reconstructions and so forth for the rest of the neurons. Also in this table, blue is excitatory and red is inhibitory type.
**Table A2.1: Axonal (Pre)/Dendritic(Post) Data**

Total Axons=42

<table>
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<th>GC</th>
<th>HILUS</th>
<th>CA3pc</th>
<th>CA2pc</th>
<th>CA1pc</th>
<th>CA3int</th>
<th>CA1int</th>
<th>Sub</th>
<th>EC-II</th>
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<td>1/62</td>
<td>1/2</td>
<td>1/117</td>
<td>1/78</td>
<td>1/132</td>
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Table A2.2 displays archive (# 13) based number of reconstructions (axonal+dendritic) available from the NeuroMorpho.Org database for each type of neuron classified in Table A1.1. In this table, blue denotes principal and red denotes inhibitory reconstructions. The
Axonal data is denoted by a superscript (axon/s) in bold after the number of reconstructions available.

### Table A2.2: Archive Based Number of Cells for Hippocampus: blue – excitatory red - inhibitory, Total=489(excluding Danzer and Garcia archives)

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<th>Claiborne</th>
<th>Danzer</th>
<th>Garcia-Cairasco</th>
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The following potential connectivity results are analyzed and reported in chapter 5 of this dissertation and also in Ropireddy and Ascoli (2011).

1) $\text{DG}_G \text{C}_{\text{AX}} \rightarrow \text{CA3}_{\text{PC}_{\text{DE}}}$

2) $\text{CA3}_{\text{C}^{\text{prox}}}_{\text{PC}_{\text{AX}}} \rightarrow \text{CA}_{\text{PC}_{\text{DE}}}$

3) $\text{CA3}_{\text{C}^{\text{dist}}}_{\text{PC}_{\text{AX}}} \rightarrow \text{CA}_{\text{PC}_{\text{DE}}}$

4) $\text{CA3b}_{\text{PC}_{\text{AX}}} \rightarrow \text{CA}_{\text{PC}_{\text{DE}}}$

5) $\text{CA2}_{\text{PC}_{\text{AX}}} \rightarrow \text{CA}_{\text{PC}_{\text{DE}}}$

6) $\text{CA3b}_{\text{R}^{\text{IN}}}_{\text{AX}} \rightarrow \text{CA3}_{\text{PC}_{\text{DE}}}$
Computational Framework

The core computational framework was written in C/C++ and compiled with the GNU compiler under UNIX. Potential connectivity computations were executed on the gmice cluster machines (SGI cluster of 80 Altix 8200) accessible through gmice.gmu.edu, each containing two quad core Intel Xeon E5440 2.83 GHz processors with 16 GB of RAM. Instead of parallelizing the C/C++ code, the numbers of available nodes were maximized by submitting to the server a number of PBS (Portable Batch System) scripts that split the job into independent tasks (http://www.unix-info.org). At the same time, the code was optimized using pthreads to capitalize on the multi-processors of each node. As part of this manual, all the code, BASH and PBS scripts, runtime executables and data are being provided and detailed in the following sections.

Overall Process Workflow

The overall process workflow is shown in Figure A2.1 for the estimation of potential connectivity. In the first step, the executable reads the command line parameters which includes parameters such as, the pre-synaptic soma voxel position, the pre-synaptic cell index, sigma and ‘s’ values for the equation (Eq. 1) for estimating potential synapses between the pre- and post-synaptic arbors. These parameters are read into the arguments array and passed onto the threadcalling function. In the threadcalling function, all the calls for data structure initiations are made. Firstly, the function call to read the voxel
coordinates for the pre-synaptic soma position is made through readaxonalf file process. For a given axon, it reads the voxels corresponding to that axon and returns two parameters called “pcfileindex” and “pcindex”.

The embed function includes calls to other functions specific to an axon to transform and orient the axonal cell at the selected voxel position defined by “pcfileindex” and “pcindex”. As a first step in the embed function call for each axon, the axes information are obtained from getaxis function call. The normal unit vectors are read from the readpcvoxelnorms function call. Later the septo-temporal axis information is loaded into the data structures. The third axis is calculated as a perpendicular to the above two axes. After obtaining the axes information, the axonal geometry is read from the getswwccell and through the swccall function calls. In the swccall function, the axonal tree is read from swcread function call and processed for refining the axonal arbor.

*Optimizing Axonal Orientations*

After reading the axonal arbor geometry into the relevant data structures, the axonal arbor coordinates are transformed with respect to the three references axes using the algorithms explained in the numerical recipes book. The algorithms used in order to transform the arbor coordinates are explained in chapter 2 (Solution of Linear Algebra Equations) of this book. The specific algorithms used are Gaussian Elimination with Back Substitution (pages 41-43) and Single Value Decomposition (pages 59-70). The calculated axes reflect the orientations for the dendrites since they are radially oriented and their orientation can

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be approximated with the three axes defined above. As a result we have to refine the orientation of the axons so that the maximum extent of the arbor lies within the 3D space of the hippocampus. This optimization is implemented using Quaternions (please see following paragraph on quaternions, rotation using Euler angles and gimbal lock) and the algorithms are implemented from the following sources:


//http://www.cprogramming.com/tutorial/3d/quaternions.html


Rotations of an object around a particular reference axis can be performed using either Euler angles and Quaternions. Rotation using the Euler angles can be implemented using rotation matrices which poses a major disadvantage. Sometimes, the rotations need to be performed in multiple reference axes and using the Euler angle rotation matrices, there is a possibility that the rotation in one axis will be mapped onto another rotation axis, which is defined as gimbal lock. The only alternative to this is the implementation of rotation and orientation using Quaternions. Quaternions are a four coordinate system defined using the four floating point values | w x y z | where x, y, & z are the three coordinates for the rotation axis and w is the rotation angle. If you like to know more on quaternions, please look at Hanson (2006).

The embedding process of the CA principal dendrites, in comparison to that of the axons, follows a simple rule, such that they can be aligned according to their tissue curvature.
The orientations of all of the principal and interneuron axons, after following the
dendritic curvature approximation, are optimized so that the entire arbor falls within the
3D space of the hippocampus. This optimization is done manually and, in most cases,
was within a range of ± 30°, taking the dendritic orientation axes as the reference system.
In some cases multiple orientation steps were performed to optimize their orientation. In
order to avoid gimbal lock when performing a rotation using Euler angles, we
implemented a quaternion defined as \( q = w + xi + yj + zk \) where \( w, x, y \) and \( z \) are real
numbers. If we consider \( \theta \) as our angle of rotation around a reference axis with a unit
vector \( \hat{n} \) \( (x_0, y_0, z_0) \) then the quaternion is represented as \( \cos(\theta/2), x_0 \cdot \sin(\theta/2), y_0 \cdot \sin(\theta/2) \)
and \( z_0 \cdot \sin(\theta/2) \). A matrix is created using this quaternion and applied to the entire arbor so
that the desired rotation is performed.

**Potential Synapse Calculation**

After the axonal tree is oriented and embedded in this 3D space, the call for estimating
the connectivity function (estimateconnectivity) is called from the threadcalling function.
In the estimateconnectivity function, the calls to the embeddend function and
calcpotentialsynapses are made to read and embed the dendritic trees and calculate the
potential synapses according to the formula from Stepanyants and Chklovskii, 2005
based on the pre- and post-synaptic soma positions, arbor geometry, the interaction
distance and the sigma (see below for its description) values are passed. The number of
potential synapses can be derived from the geometry of pre- and post-synaptic neurons
based on a published mathematical formula (Stepanyants and Chklovskii, 2005):
\[ Np(\vec{R}_a, \vec{R}_d) = 2s \sum_{i,j} l'_a |l'_d| \sin(\hat{n}'_a, \hat{n}'_d) \exp \left[ -\left( \vec{r}'_a - \vec{r}'_d \right)^2 / 4\sigma^2 \right] (4\Pi\sigma^2)^{3/2} \]

This equation defines the number of potential synapses between the axons of one neuron and the dendrites of a second neuron, \( Np(\vec{R}_a, \vec{R}_d) \) as a function of their position in Cartesian space, whereas the somata of the two cells are placed at Cartesian coordinates \( \vec{R}_a \) and \( \vec{R}_d \), respectively. In the right hand side, \( s \) is the axo-dendritic interaction distance (in our case, 2 \( \mu \text{m} \) and 1 \( \mu \text{m} \) for principal cells and interneurons, respectively), \( l'_a \) and \( l'_d \) represent the unit-volume lengths of the axonal and dendritic segments along the unit vectors \( \hat{n}'_a \) and \( \hat{n}'_d \), and \( |\sin(\hat{n}'_a, \hat{n}'_d)| \) is the absolute value of the sine of the angle between unit vectors \( \hat{n}'_a \) and \( \hat{n}'_d \). These neuronal reconstructions are taken to represent the morphology of a class of similar, but non-identical, neurons. Thus, a Gaussian filter with standard deviation \( \sigma \) (sigma) is applied around the centers of the axonal and dendritic segments (in the swc file) with vectors \( \vec{r}'_a \) and \( \vec{r}'_d \). The parameter \( \sigma \) was fixed at 10 \( \mu \text{m} \), corresponding to the average length of all segments. This equation is iteratively applied to all of the axonal and dendritic segments (indicated with \( i \) and \( j \), respectively).

In addition, function calls to readpcvoxelnorms and readpcvoxelcelldb are also made to extract each voxel’s coordinates, unit normals and the post-synaptic neurons corresponding to each voxel. In the embeddend function, the calls to getaxisdend and getdendswccell are made to sample the cell database for reading a dendritic arbor and extract the axes information for the voxel sampled. The main output files from the
estimate connectivity function call are synapseresults.txt and synapselocations.txt. Please note the exact naming of these files changes with respect to the axon type and the parameters passed.

**Contents in the main folder**

*a. files and folders*

The main folder (3DHippocampus-PotentialConnectivityFramework) consists of the following files and folders (names in italics):

1. This manual file in doc format (*manual.doc*).
2. PreSyn-Postsyn tables (Table A1.1 and Table A1.2) details the number of pre- and post-synaptic neurons within each archive.
3. The main framework containing the code, executables and script files for running the simulations, and for analyzing the raw files (*Computational_Framework*).
4. Doxygen generated documentation folders in html and rtf formats (*Doxygen-Documentation*).
5. R graphing scripts for generating the 2D maps (*R_Scripts*).
2.1: Overall process workflow

b. Directory Tree Structure

This is the directory tree structure of the main folder (3DHippocampus-PotentialConnectivityFramework) showing the immediate folders and also its subdirectories.

./3DHippocampus-PotentialConnectivityFramework

|-- Computational_Framework
Analysis-Results

|-- CA2axon
  |-- 2DMaps
  |-- Percentcellscontacted
  |-- SubRegionsLayers-NpSumDensities
  |-- Summation
  |-- CA3bSLMInterneuronaxons

|-- 2DMaps
  |-- Percentcellscontacted
  |-- SubRegionsLayers-NpSumDensities
  |-- Summation
  |-- CA3bSRInterneuronaxons

|-- 2DMaps
  |-- Percentcellscontacted
  |-- SubRegionsLayers-NpSumDensities
  |-- Summation
  |-- CA3baxon

|-- 2DMaps
  |-- Percentcellscontacted
  |-- SubRegionsLayers-NpSumDensities
  |-- Summation
  |-- CA3cdistalaxon
| | | -- 2DMaps
| | | -- Percentcellscontacted
| | | -- SubRegionsLayers-NpSumDensities
| | | -- Summation
| | -- CA3cproximalaxon
| | | -- 2DMaps
| | | -- Percentcellscontacted
| | | -- SubRegionsLayers-NpSumDensities
| | | -- Summation
| | -- gcmossyfiberaxon
| -- Code
| | -- Analysis
| | | -- Maps
| | | -- PercentCellsContacted
| | | -- Refine-CPosition
| | | -- SubRegions-AvgDensity
| | | -- Summation
| | -- PotentialConnectivity
| -- DataFolder
| | -- AxonalVoxels-List  (Contains the mapped pre-synaptic voxel positions for each axon)
|   |   |-- CellDB            (Contains the list of files with cell names and cell ids for all layers)
|   |   |-- Cells
|   |   |   |-- CA           (Contains the swc files for CA)
|   |   |   |-- DG           (Contains the swc files for DG)
|   |   |-- NewCPosition
|   |   |   |-- PC            (Contains the refined transversal position for the voxels)
|   |   |-- NewDepth
|   |   |   |-- PC            (Contains the refined depth for the voxels)
|   |   |-- Normals_Sorted_STPlaneSlices
|   |   |   |-- OR_NormDist   (Contains the oriens layer voxel files with coordinates & depth info)
|   |   |   |-- PC_NormDist   (Contains the pyramidal layer voxel files with coordinates, depth & normals info)
|   |   |   |-- RAD_NormDist  (Contains the radiatum layer voxel files with coordinates & depth info)
|   |   |   |-- LM_NormDist   (Contains the lacunosum layer voxel files with coordinates & depth info)
|   |   |   |-- ML_NormDist   (Contains the molecular layer voxel files with coordinates & depth info)
|   |   |   |-- GC_NormDist   (Contains the granular layer voxel files with coordinates, depth & normal info)

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|-- HILUS_NormDist  (Contains the hilus layer voxel files with coordinates &
depth info)
|-- Sorted_VoxelCellDBSTSlices
  |-- OR  (Contains the oriens layer voxels with list of cell ids mapped
          for each voxel)
  |-- PC  (Contains the pyramidal layer voxels with list of cell ids
          mapped for each voxel)
  |-- RAD (Contains the radiatum layer voxels with list of cell ids
          mapped for each voxel)
  |-- LM  (Contains the lacunosum moleculare voxels with list of cell
          ids mapped for each voxel)
  |-- ML  (Contains the molecular layer voxels with list of cell ids
          mapped for each voxel)
  |-- GC  (Contains the granule cell layer voxels with list of cell ids
          mapped for each voxel)
  |-- HILUS (Contains the hilus layer voxels with list of cell ids mapped
            for each voxel)
  |-- Executables
  |-- Scripts
    |-- Analysis-Scripts
      |-- CA2axon
      |-- CA3b-SLMInterneuronaxons
PotentialSynapses-ComputeScripts

Simulation-Results

CA2-Axon

CA3b-Axon

CA3b-SLMInterneuronAxons

CellID31

CellID32

CellID33

CellID34

CellID35
b. Computational_Framework Folder

The “Computational_Framework” folder contains the code, scripts and executables for running the simulations, and analyzing the raw files to be run on a linux machine. There are also PBS scripts to run the simulations on the gmice cluster machine. This folder contains sub-folders: Analysis-Results, Code, DataFolder, Executables, Scripts, Simulation-Results.

The ./Computational_Framework/Analysis-Results/ folder is where the output files of the analysis-scripts are stored in individual sub-folders named under each type of axon. Within each axon, there are four folders which store the output files in the folders, 2DMaps, Percentcellscontacted, SubRegionsLayers-NpSumDensities & Summation.
The */Computational_Framework/Code* folder contains the core framework for running simulations in the */Computationalramework/Code/PotentialConnectivity* folder and also the code for analyzing the raw output files in */Computational_Framework/Code/Analysis* folder.

In the */Computational_Framework/Code/Analysis* folder, there are individual folders for outputting the results from the raw files such as 2D maps for the potential synaptic maps (in *Maps* folder), summation of the potential synapses in the septo-temporal and hippocampal-transverse axes (in *Summation* folder), the number and density of potential synapses in different sub-regions and layers (in *SubRegions-AvgDensity* folder) and percent cells contacted and number of cells with >=1 potential synapses (in *PercentCellsContacted* folder). The *Refine-CPosition* folder contains the code for refining the hippocampal-transverse position of the voxels.

The */DataFolder* contains the input files with respect to reading cell database files (in */DataFolder/CellDB* folder), swc files for the CA & DG axons and dendrites (in */DataFolder/Cells/* folder), the unit normals for each voxel (in */DataFolder/Normals_Sorted_STPlaneSlices/*), the voxel database files containing the voxels with cells mapped according to voxel position (in */DataFolder/Sorted_VoxelCellDBSTSlice* folder) and the list of voxels mapped for each axon (in */DataFolder/AxonalVoxels-List*). Please see the above Directory Tree Structure which shows the contents of each subfolder in the *DataFolder*. 

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The ./Executables/ folder contains the run time executables for computing potential synapses (ConnectivityExecutable) and the analysis of the raw output files. The lists of other executables are refineposition, potentialsynapticmaps, summation, subregiondensity and percentcontacts. These executables are called in the scripts which are detailed in the ./Scripts folder.

The ./Scripts/ folder contains the PBS and bash scripts to run the simulations (in PotentialSynapses-ComputeScripts folder) and analyze the raw output files (in Analysis-Scripts folder). In the ./Scripts/ PotentialSynapses-ComputeScripts/ folder, the PBS scripts for running the simulations for computing the potential synapses for each axon listed in each folder named under the axonal type. The ./Scripts/Analysis-Scripts/ folder contains the bash scripting files for analyzing the raw input files detailed in each folder named under the axonal type.

The ./Simulation-Results/ folder is where the raw output files from the simulations for potential synapses are stored in separate sub-folders named under CA3c-N5ProximalAxon, CA3c-TamamakiDistalAxon, CA3b-Axon, CA2-Axon, GC-MossyFiberAxon, CA3b-SRIterneuronAxons & CA3b-SLMInterneuronAxons.

c. Doxygen-Documentation Folder
The Doxygen (doxygen.org) is a documentation tool for C/C++ and other programming languages with an ability to generate on-line documentation and also output the ‘caller’ and ‘call by’ graphs for each class or function. It outputs both in ‘rtf” and ‘html’ format.

The main index html page can be viewed in the ./Doxygen-Documentation/html/ folder (/./Doxygen-Documentation/html/index.html). This doxygen documentation is generated for only the main potential synaptic connectivity code in the ./Code/PotentialConnectivity folder, but not for the analysis code for analyzing the raw output files in ./Code/Analysis folder. For the PotentialConnectivity code, the class list, header file, the function list and the cpp files can be seen by clicking on tabs of “Classes” and “Files” listed on this index page.

**List of CPP files for the PotentialConnectivity code with a summary statement**

These files can be found in ./Doxygen-Documentation/PotentialConnectivity/html folder (or rtf).

1) header.h – header for declaring structs, arrays, constants and functions.

2) makefile – makefile for compiling, linking and making the executable – ConnectivityExecutable

3) main.cpp – main function for reading the command line arguments, and calling the thread function.

4) threadcallingfunction.cpp – all the function calls are made in this cpp file....from loading the data structures to estimating connectivity function

5) readca3cn5axonalfilecpp – reads the axonalvoxels-list file for proximal ca3c axon from ca3c-n5axonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/ca3c-
n5axonalvoxels.txt corresponding to the ca3cn5axonfileindex parameter passed in the function call

6) readca3ctamakiaxonalfile.cpp – reads the axonalvoxels-list file for distal ca3c axon from ca3c-tamakiaxonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/ca3c-tamakiaxonalvoxels.txt corresponding to the ca3tamakiaxonalfileindex parameter passed in the function call

7) readca3bn2axonalfile.cpp – reads the axonalvoxels-list file for ca3b axon from ca3b-n2axonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/ca3b-n2axonalvoxels.txt corresponding to the ca3bn2axonalfileindex parameter passed in the function call

8) readca2axonalfile.cpp – reads the axonalvoxels-list file for ca2 axon from ca2axonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/ca2axonalvoxels.txt corresponding to the ca2axonfileindex parameter passed in the function call

9) readgcaxonalfile.cpp – reads the axonalvoxels-list file for gc axon from gcaxonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/gcaxonalvoxels-depth.txt corresponding to the gcaxonfileindex parameter passed in the function call

10) readradaxonalfile.cpp – reads the axonalvoxels-list file for ca3b rad interneuron axon from ca3bsrint-cellidaxonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/ corresponding to the radaxonfileindex parameter passed in the function call

11) readslmaxonalfile.cpp – reads the axonalvoxels-list file for ca3b slm interneuron axon from ca3bslmint-cellidaxonalvoxels.txt stored in ./DataFolder/AxonalVoxels-List/ corresponding to the slmaxonfileindex parameter passed in the function call

12) loadgccelldb.cpp – reads the gccelldb.txt file that contains the names of the neurons for the gc layer (dendrites+axons) contained in ./DataFolder/CellDB/gccelldb.txt and loads them into the gccelldb[] struct array

13) loadpccelldb.cpp – reads the pccelldb.txt file that contains the names of the neurons for the pc layer (dendrites+axons) contained in ./DataFolder/CellDB/pccelldb.txt and loads them into the pccelldb[] struct array
14) loadradcelldb.cpp – reads the radcelldb file that contains the names of the neurons for the rad layer (dendrites+axons) contained in /DataFolder/CellDB/radcelldb.txt and loads them into the radcelldb[] struct array.

15) loadslmcelldb.cpp – reads the slmcelldb file that contains the names of the neurons for the slm layer (dendrites+axons) contained in /DataFolder/CellDB/slmcelldb.txt and loads them into the slmcelldb[] struct array.

16) loadpccellrefaxisdb.cpp – reads the pccelldb_refaxis.txt in ./DataFolder/CellDB/ and load to the refaxisdb[] struct array.

17) initstaxis.cpp – reads the staxis.txt file that contains the coordinates of the staxis and loads in the staxis array.

18) initstplanes.cpp – reads the plane equations for the 60 hippocampal-transverse planes along the septo-temporal axis and loads in the struct array – transplane

19) embed.cpp – from this file, the function calls for embedding and refining the orientations of the axonal arbors are made for the selected axon-- the pcfileindex and pcindex are parameters that are retrieved from from the readaxonal files for the file and pc voxel indices also this is the place to add function calls to embed more axon types in future.

20) embedca3cproximalaxon.cpp – This function call embeds and orients the proximal (Buzsaki-N5) ca3c axon....the call realign_axonalarbor realigns the axonal arbor by using quaternions.

21) embedca3cdistalaxon.cpp – This function call embeds and orients the distal (tamamaki) ca3c axon....the call realign_axonalarbor realigns the axonal arbor by using quaternions.

22) embedca3baxon.cpp – This function call embeds and orients the ca3b axon....the call realign_axonalarbor realigns the axonal arbor by using quaternions.

23) embedca2axon.cpp – This function call embeds and orients the ca2 axon....the call realign_axonalarbor realigns the axonal arbor by using quaternions.
24) embedgcmossyfiberaxon.cpp – This function call embeds and orients the gc mossy fiber axon....the call realign_axonalarbor realigns the axonal arbor by using quaternions.

25) embedca3bsrinterneuronaxons.cpp – This function call embeds and orients the 6 ca3b SR interneuron axons....the call realign_axonalarbor realigns the axonal arbor by using quaternions.

26) embedca3bslminterneuronaxons.cpp – This function call embeds and orients the 6 ca3b SLM interneuron axons....the call realign_axonalarbor realigns the axonal arbor by using quaternions.

27) getaxis.cpp – This function reads the axes unit vectors....of septo-temporal and normal vectors.....and estimates the third axes ...the axes are stored in the 2D float array array_coeff[][].

28) readpcvvoxelnorms.cpp – This function reads the voxel properties line septo-position, hippocampal-transverse position (cposition) and the depth info....also it reads the normal unit vectors and the height to the oriens/alveus and lm/ml fissure boundary height.....this all loaded into the struct pcvvoxelnormdb

29) readpcvvoxelcelldb.cpp – reads the voxel cell db file in ./DataFolder/Sorted_VoxelCellDBSTSlice/ text files and loads into the struct pcvvoxelcelldb. This text files contain the list of cells and their associated mapping probabilities for sampling the dendrites for embedding and estimation of number of potential synapses.

30) randomnoise.cpp – function call for randomizing the orientation of the normal by tilting the normal by 0 to 5 degrees and rotation by 0 to 360 degree

31) getswccell.cpp – Function call for reading the axonal arbors....from the swc_call function

32) swccall.cpp – function call for loading the axonal arbor tree into the data structure....the arbor tree is read from the swc file from SWC_Read function.....the swc file data structure is swcworknode_ptr[] struct

33) swcread.cpp – function call for reading the axonal arbors from swc file in the folder Cells
34) `transform_swc.cpp` – function call for transforming coordinates of the axonal morphological tree according to the axes loaded from the `get_axis` function call. This algorithm is implemented from numerical recipies book.

35) `estimateaxonalpathdistance.cpp` – function call for estimating the axonal path distance for each branch segment in the axonal morphology file. The pathdistance is stored in the variable of the struct array `swcworknode_ptr[].pathdistance`.

36) `estimateconnectivity.cpp` – function call for reading the voxel coordinates in each fileindex and embed the post-synaptic dendritic arbor. Also the call for calculating potential synapses between the axon and dendritic segments is also made in this cpp file.

37) `embed_dend.cpp` – function call for getting the axis information, sampling the dendritic cells and embedding for computing number of potential synapses.

38) `getaxisdend.cpp` – function call for reading and calculating the axes for embedding the post-synaptic pyramidal neurons.

39) `getdendswccell.cpp` – function call for random sampling the cell database and calling the function to read the swc file.

40) `swcdendcall.cpp` – function call for loading the swc file into the struct array `swcworknode_dendptr[]`.

41) `transform_dendswc.cpp` – function call for transforming the coordinates of the post-synaptic dendrite and embed and orient according to the axes.

42) `mlprojectiondistance.cpp` – this function estimates the apical dendrites to the fissure height.

43) `orprojectiondistance.cpp` – this function estimates the basal dendrites to the oriens/alveus border.

44) `morphmlprojection.cpp` – this function scales the apical dendrites to the fissure height.
45) morphorprojection.cpp – this function scales the basal dendrites to the oriens/alveus border

46) estimatedendpathdistance.cpp – function call for estimating the dendritic path distance for each branch segment in the dendritic morphology file.....the pathdistance is stored in the variable of the struct array swcworknode_dendptr[].pathdistance

47) calcpotentialsynapses.cpp – function for calculating the potential synapses between the axonal arbor and dendritic arbor ...... this is the function where the equation in Stepanyants & Chklovskii, 2005 is implemented

48) output.cpp – function call for vrml output of axonal arbor tree

49) outputdend.cpp – function call for vrml output of dendritic arbor tree

50) layersoutput.cpp – function call for vrml output of layers

51) writeoutline1.cpp – function call for vrml output of layers

d. R graphing scripts (R-Scripts) folder

Two-dimensional color maps (for ex. Figure 5.3) are generated using the R package ‘ggplot2’ (http://had.co.nz/ggplot2). This folder contains the ‘R’ scripts for generating the 2D color maps of the potential synapses for each of the axons for CA3cproximal, CA3cdistal, CA3b, CA2, DG mossy fiber, CA3b SR and SLM interneurons. Each folder for the five principal axon types contain two ‘.r’ and ‘.txt’ files for the actual and logarithm of the number of potential synapses. The results are included in here to show as examples on how to generate potential synaptic maps.

User’s Guide

a. Setting up the framework and running the potential synaptic framework
First, copy the “Computational_Framework” folder onto a linux machine or gmice cluster in the user’s home folder (or any folder). The simulations can be run on any unix machine, but to take advantage of the cluster of 80 nodes on the gmice.gmu.edu cluster, the scripts for running the simulations for computing potential synapses are PBS bash scripts. If you run the scripts on a non-gmice machine, please use the scripts that have “nongmice” name in them.

The scripts for running the simulations for computing potential synapses are in the ./Computational_Framework/Scripts/PotentialSynapses-ComputeScripts/ folder for all the seven axon types. There are four scripts in each folder of the axon type. For running the simulations, copy the scripts to the folder (Computational_Framework). For example, for computing the potential synapses between the proximal ca3c axon and the ca pyramidal network, copy the ca3cprox-outsidescript.sh, & computepotentialsynapses-ca3cproximalaxon.sh scripting files to the base folder. The first script (ca3cprox-outsidescript.sh) uses the ‘qsub’ PBS command to submit a job which is detailed in the second script (computepotentialsynapses-ca3cproximalaxon.sh).

From the first script the arguments initialized are the voxelindex and the starting and ending file index numbers for packing the principal cell layer with pyramidal dendrites within the range of these file indices. By running the first script, 10 PBS jobs are submitted that covers the entire spatial coverage of the ca3c proximal axon. Then to check the status of the submitted jobs, use the pbs command ‘qstat –a’. The voxelindex argument corresponds to the sampled voxel in the ca3c proximal axonal voxel list file.
In order to see how the number of potential synapses changes with respect to the change in the pre-synaptic soma position, sample any of the mapped ~1000 voxel positions for the proximal ca3c axon. Copy the ./Computational_Framework/Executables/ConnectivityExecutable to the main folder (./Computational_Framework) before running the above two scripts. The main executable takes the following five arguments which are passed from the scripts:

argument 1 (voxelindex): line number corresponding to the voxel number picked from the axonal voxel list files for the seven types of axons listed in the folder ./Computational_Framework/DataFolder/AxonalVoxels-List.

argument 2: neuron type number
  ca3c proximal (buzsaki) axon: 1
  ca3c distal (tamamaki) axon: 2
  ca3b (buzsaki) axon: 3
  ca2 (tamamaki) axon: 4
  gc mossy fiber (tamamaki) axon: 5
  ca3b radiatum axons (ascoli): 6-11
  ca3b lacunosum moleculare axons (ascoli): 12-18

argument 3: sigma value in the equation

argument 4: s value in the equation

argument 5: starting file index for sampling post-synaptic positions

argument 6: ending file index for sampling post-synaptic positions
The raw output files are written to ./Simulation-Results/CA3c-N5ProximalAxon/voxelindex*-simulations/ folder in the form of synapseresults*_10_2.000000.txt & synapselocations*_10_2.000000.txt. The ‘*’ refers to the number passed as the first argument in the shell script. This number refers to the line number corresponding to the voxel number picked from the axonal voxel list files. This is a way to identify the results based on the axonal voxel selected when the program is run. The synapseresults file contains the number of potential synapses for each post-synaptic voxel position. The synapselocations contains the potential synapse location details i.e., the layer information, sub-region & the axonal and dendritic path distances. Similarly, the simulation results for the other six axon types can be computed and the raw output files are written to the respective folders in ./Computational_Framework/Simulation-Results/.

**Steps to run the simulations on nongmice machines:**

1. Copy the two nongmice scripts for each axonal type in the ./Scripts/PotentialSynapses-ComputeScripts/ folder

2. Copy the “ConnectivityExecutable” to the base folder

3. Run the script with –outside- in its name with voxelindex as an argument passed to this script. For example, for running the proximal ca3c axon, use the following command.

   ‘./ca3cprox-outsidescript-nongmice.sh’ voxelindex
Here voxelindex corresponds to which voxel is sampled for embedding the axon from the list of 988 voxels listed in ./DataFolder/AxonalVoxels-List/ca3c-n5axonalvoxels.txt text file.

4. The simulation results are written to the respective axonal folder in the ./Simulation-Results/ folder. The program creates a separate folder for each pre-synaptic voxel position chosen. For example, if you want to compute the potential synapses for the first and last of the pre-synaptic voxel list displayed in the ./DataFolder/AxonalVoxels-List/ca3c-n5axonalvoxels.txt file for the ca3c proximal axon, the program creates two folders for writing the results: ./Simulation-Results/ CA3c-N5ProximalAxon/voxelindex1-simulations/ and ./Simulation-Results/ CA3c-N5ProximalAxon/voxelindex988-simulations/

5. There are three types of files written to the above described output folders for each axonal type for each starting and ending file index numbers inputted through the shell script. One is the “synapseresults_*_10_2_startfileindex_endfileindex.txt”, “synapselflocations_*_10_2_startfileindex_endfileindex.txt”, & “potentialsynapses-cellindex-cellname-sigma-startfileindex-endfileindex.txt”. The first file lists all the potential synapses for each post-synaptic position for the list of PC voxels within the starting and ending file indices. The second file lists all the potential synapse locations for each potential synapse within the starting and ending file indices.
6. Since the results are outputted in a list of files from the starting to ending file indices, we need to concatenate all the files together. The concat.sh script file in each of the axonal type sub-folders can be used to solve this purpose. Just run the script with the voxelindex as the argument.

b. Analysis of the raw output files

The above two raw output files are analyzed by the scripts in the
./Computational_Framework/Scripts/Analysis-Scripts/ folder. The four run time executables in the ./Executables folder “summation”, “subregiondensity”, “2dpotentialsynapsemaps”, & “percentcontacts” output summation of the potential synapses in the septo-temporal & hippocampal-transverse axes, sum and densities of Np in layers and sub-regions, 2d color maps for the potential synapse maps and statistics of number of cells having >= potential synapses. In order to output the files in the ./Analysis-Results/ folder, copy the scripts to the base folder and run the scripts. The output files from these scripts can be used to plot in any plotting tool.

**Step by step instructions for generating the analysis output files:**

1) First, copy all the analysis script files located in the ./Scripts/Analysis-Scripts/[axonaltypesubfolder] folder to the base folder. Here, [axonaltypesubfolder] refers to the folder corresponding to the axonal type that being chosen to generate the analysis output files. There are six bash scripts in each of the [axonaltypesubfolder] subfolder: 2dpotentialsynapsemaps.sh, execute_analysisisscripts.sh,
percentcontacts.sh, refinecposition.sh, subregiondensity.sh, & summation.sh.

The “2dpotentialsynapsemaps.sh” script generates the input files for

generating the 2D color maps of potential synapses used in the ‘R’ scripts.

The “execute_analysisscripts.sh” script is included for convenience to the user

so that all the other five scripts can be run & executed using this script. The

“percentcontacts.sh” script generates the input files that contain the statistics

on number of CA3 and CA1 pyramidal cells having 0 or >=1 potential

synapses. The “refinecposition.sh” script refines the transverse position in the

raw output files. The “subregiondensity.sh” generates the output file that

contains the statistics on the number of potential synapses for each layer in all

CA sub-region. Finally, the “summation.sh” script generates output files that

contain the summation of the potential synapses in the septo-temporal and

transverse axes.

2) In the second step, execute the “execute_analysisscripts.sh” script by passing

the [voxelindex#] as the only parameter. The [voxelindex#] is the same

argument that is passed when running the potential connectivity framework

which corresponds to the voxel sampled for the axonal type.

3) The output files after running these script files are written in the ./Analys-

Results/[axonalttype]/ subfolders. Please go through the

“READMEFILEFORANALYSISOUTPUTFILES.txt” file in ./Analys-

Results/ folder to get a grasp of the format of the different output files.
4) Except for the output files generated by “2dpotentialsynapsemaps.sh”, all the analysis files can be plotted using any tool. For the output files with “2dpotentialsynapsemaps.sh”, the ‘R’ scripts provided in the ./R-Scripts/ folder can be used to generate the 2D potential synaptic maps (see below).

c. 2D Potential synaptic maps

The ‘R’ scripts and the example input files are listed in the ./R-Scripts folder. Please download and install ‘R’ from http://cran.r-project.org/. Additionally, please install the package ‘ggplot2’ for running these R scripts. These ‘R’ scripts generate the potential synaptic color maps corresponding to the hippocampal-transverse & septo-temporal positions.

Programmer’s Guide

a. Embedding additional axons & computing potential synapses

The potential synaptic framework can be extended easily to include additional axons described in the first section and any additional axonal reconstructions added to the database in the future. This framework is carried out for the five principal axons and the 13 CA3b interneuron axons to the CA pyramidal network. In order to add more axons to this framework, create another function in the form of embed[axonaltype]axon.cpp (for ex: embedca3cproximalaxon.cpp) and call that function from embed.cpp (please refer to ./DoxygenDocumentation/PotentialConnectivity/html/embed.cpp). The orientation of the new axons can be refined by using the quaternion system. In order to compute the
potential synapses from any axon to the CA pyramidal cell network, this code can be used without any changes except for the addition of the function in the embed.cpp file. Additionally, a new read[axonalttype] function is needed to read the axonal voxels listed in the ./DataFolder/AxonalVoxels-List/ corresponding to that particular axon.

b. Estimating connectivity to interneurons & other axons

If one wants to extend this framework to compute potential synapses to other than CA pyramidal dendrites, some changes need to be done with respect to sampling the voxels for embedding and orienting the interneurons in various layers. Since this framework, primarily focuses on the principal cell network, the cpp files which implement the aligning and orientation (embed_dend.cpp, getaxis.cpp, transform_swc.cpp) can be used to embed and orient the interneuron dendrites in various layers. Compared to the principal neurons, the number of interneurons is considerably less. As a result the voxel sampling ratios will also be much higher than that of the sampling ratios for the principal cells. The programmer/student needs to find the approximate voxel sampling ratios for the interneurons in various layers in the hippocampus. The current framework has many of the interneuron reconstructions mapped to the 3D template and the voxel cell database listed in the ./DataFolder/Sorted_VoxelCellDBSTSlices/ contains these mapping files for all the layers. In order to compute potential synapses on the interneurons, one has to change the getdendswccell.cpp file to sample the appropriate interneuron corresponding to a layer. Additionally, the main function that computes the potential synapses (estimateconnectivity.cpp) needs to be changed to sample the voxels for each layer and
call the embed_dend function that embeds and orients the interneuron. In addition, one needs to create the scripting files similar to those listed in this manual to run, & analyze the raw output files.
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Deepak Ropireddy graduated from Govt. Junior College, Warangal, India, in 1994. He received his Bachelor of Technology in Metallurgical and Material Science Engineering from National Institute of Technology, Warangal, India, in 1999. He received his Master of Science in Computer Science from Texas A&M University-Commerce in 2002.