HIPPOCAMPAL ASSOCIATIONAL AND COMMISSURAL PATHWAYS;
ANATOMICAL AND ELECTROPHYSIOLOGICAL STUDIES IN THE RAT

by

Colin Anthony Zappone

A Dissertation Submitted to the Faculty of the
COMMITTEE ON NEUROSCIENCE
In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2004
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI Microform 3132277
Copyright 2004 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Colin Anthony Zappone entitled Hippocampal associational and commissural pathways: anatomical and electrophysiological studies in the rat and recommend that it be accepted as fulfilling the dissertation requirement for the degree of Doctor of Philosophy.

Carol A. Barnes, Ph.D.  
Date: 4/8/04

Edward D. French, Ph.D.  
Date: 4/8/04

Nathaniel T. McMullen, Ph.D.  
Date: 4/8/04

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director: Robert S. Sloviter, Ph.D.  
Date: April 8, 2004
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

Signed: A. Zappone
ACKNOWLEDGMENTS

Over the course of my doctoral training, I have been fortunate to work with several talented individuals who have all helped me along my journey. These people are: Leila Akbarian, Argyle Bumanglag, Brian Harvey, Kathy Horvath, Hemant Kudrimoti, Aaron Leetch, Kent Menkins, Braxton Norwood, and Jeremy Novey. I am indebted to them for their friendship and technical assistance during my graduate work.

I would like to acknowledge all of the staff members who have helped me with a variety of tasks ranging from registration to ordering supplies. The Program in Neuroscience graduate coordinators appointed during my graduate career were always ready to help me in completing the necessary steps towards my degree. These people are: Teri Lamour, Jennifer Lawrence, and Erin Johnson. In addition, Jane Ageton, Karla Hayes, and Terri Vorholzer in the Pharmacology Department assisted me on many occasions. I sincerely appreciate all of your help.

I would also like to express my gratitude to the members of my dissertation committee: Drs. Carol Barnes, Ed French, Nate McMullen, and Hank Yamamura.

Finally, I would like to thank my mentor, Dr. Robert Sloviter. Bob has not only taught me how to think and conduct myself as a scientist, he has taught me to enjoy the process. His enthusiasm for science has been a joy to experience.
DEDICATION

This dissertation is dedicated to my mother Karen, father Ron, and sister Alida who, with their love and support, have given me the confidence to pursue my dreams.
# TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................... 9

LIST OF TABLES............................................................................................................. 12

ABSTRACT......................................................................................................................... 13

CHAPTER 1: INTRODUCTION............................................................................................. 15

The associational and commissural projection pathway of the dentate gyrus.......................... 18
The “lamellar” hypothesis.................................................................................................. 20
Mossy cells....................................................................................................................... 23
The “granule cell association” hypothesis.......................................................................... 25
The “dormant basket cell” hypothesis.............................................................................. 26
GABA \(_A\) mediated inhibition............................................................................................. 27
Lateral inhibition in the mammalian central nervous system............................................. 28
Lateral inhibition in the rat hippocampus......................................................................... 31
Lesions of the dentate gyrus in temporal lobe epilepsy..................................................... 33
Hilar cell loss in animal models....................................................................................... 35
Summary of research goals............................................................................................... 36

CHAPTER 2: MATERIALS AND METHODS....................................................................... 38

Animal treatment.............................................................................................................. 38
Dorsal hippocampal injection of Fluoro-Gold for contralateral labeling.......................... 38
Septal, neocortical, and ventral hippocampal injections of Fluoro-Gold........................... 39
Perfusion fixation and tissue treatment.............................................................................. 40
Immunocytochemical methods for commissural transport studies................................. 41
Kainic acid-status epilepticus and perforant path stimulation........................................... 43
Assessment of translamellar influences........................................................................... 44
Dorsal hippocampal injection of Fluoro-Gold for ipsilateral labeling.............................. 46
TABLE OF CONTENTS - CONTINUED

Perfusion-fixation, tissue treatment, and immunocytochemistry methods used for translamellar studies .................................................. 47
Cell counting methods .................................................................. 48

CHAPTER 3: COMMISSURAL PROJECTIONS STUDIES .......................... 50

Introduction .................................................................................. 50
Contralateral Fluoro-Gold labeling after dorsal hippocampal injection ........................................................................... 52
Longitudinal distribution of Fluoro-Gold labeling after dorsal hippocampal injection ......................................................... 55
“Control” Fluoro-Gold injections .................................................. 60
Ventral hippocampal injection of Fluoro-Gold ................................... 60
Neocortical injection of Fluoro-Gold .............................................. 62
Septal injection of Fluoro-Gold ...................................................... 64
Immunocytochemical identification of retrogradely labeled hippocampus principal cells and interneurons ......................... 68
Co-localization of Fluoro-Gold and glutamate receptor-2 subunit ...... 70
Co-localization of Fluoro-Gold and somatostatin ......................... 72
Co-localization of Fluoro-Gold with parvalbumin and GABA .......... 73
Co-localization of Fluoro-Gold and calretinin .................................. 75
Lack of Fluoro-Gold labeling of interneurons in the ventral hippocampus ........................................................................ 76
Granule cell innervation of granule cell layer but not dentate molecular layer interneurons ....................................................... 76
Summary ....................................................................................... 80

CHAPTER 4: TRANSLAMELLAR INHIBITION STUDIES ......................... 85

Introduction .................................................................................. 85
Translamellar influences in the normal rat dentate gyrus ................... 87
C-Fos expression in normal rats after bicuculline methiodide induced focal discharges .................................................... 91
Translamellar disinhibition in kainic acid-treated and perforant path stimulated rats ...................................................... 96
Summary ....................................................................................... 106
# TABLE OF CONTENTS - CONTINUED

## CHAPTER 5: ASSOCIATIONAL PROJECTIONS STUDIES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>109</td>
</tr>
<tr>
<td>Longitudinal associational projections of hippocampal inhibitory</td>
<td>109</td>
</tr>
<tr>
<td>interneurons</td>
<td></td>
</tr>
<tr>
<td>Septal Fluoro-Gold injections</td>
<td>117</td>
</tr>
<tr>
<td>Summary</td>
<td>120</td>
</tr>
</tbody>
</table>

## CHAPTER 6: CONCLUSIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>123</td>
</tr>
</tbody>
</table>

## APPENDIX - LIST OF PUBLICATIONS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>136</td>
</tr>
</tbody>
</table>

## REFERENCES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>137</td>
</tr>
</tbody>
</table>
CHAPTER 1

1.1 Coronal rat hippocampus section immunocytochemically stained for the neuronal marker NeuN and, subsequently, artificially color coded to illustrate the various hippocampal subfields. ........................................ 16

CHAPTER 3

3.1 Patterns of hippocampal Fluoro-Gold-like immunoreactivity in two rats receiving small and large Fluoro-Gold injections into the contralateral hippocampus ........................................ 53

3.2 Septo-temporal distribution of Fluoro-Gold-like immunoreactivity after Fluoro-Gold injection into the contralateral dorsal hippocampus .......... 56

3.3 Selective Fluoro-Gold labeling of dentate gyrus non-principal cells after contralateral Fluoro-Gold injection ........................................ 58

3.4 Demonstration that all hippocampal neuron populations are capable of transporting Fluoro-Gold ........................................ 59

3.5 Retrograde transport of Fluoro-Gold-like immunoreactivity after Fluoro-Gold injection into the ventral hippocampus .......................... 61

3.6 Retrograde transport of Fluoro-Gold after injection into the neocortex or septum ........................................ 63

3.7 Comparison of hippocampal Fluoro-Gold-like immunoreactivity after Fluoro-Gold injection into the dorsal hippocampus or septum .... 65

3.8 Fluorescence co-localization of Fluoro-Gold and somatostatin-like immunoreactivity in dentate hilar neurons following Fluoro-Gold injection into the medial septum ........................................ 67

3.9 Immunocytochemical identification of Fluoro-Gold-containing neurons of the rat dentate gyrus after homotopic contralateral Fluoro-Gold injection ........................................ 71
LIST OF FIGURES - CONTINUED

3.10 Fluorescence co-localization of parvalbumin-, GABA-, and Fluoro-Gold-like immunoreactivity in the rat dentate gyrus after homotopic contralateral Fluoro-Gold injection................................................................. 74

3.11 Fluorescence co-localization of Fluoro-Gold-, glutamate receptor-2 subunit-, parvalbumin-, and somatostatin-like immunoreactivity in the ventral dentate gyrus after contralateral Fluoro-Gold injection into the dorsal hippocampus ........................................................................ 77

3.12 Granule cell innervation of interneurons of the granule cell layer, but not the molecular layer, of the normal rat ................................................................. 78

3.13 Schematic diagram of the commissural projections of hippocampal principal cells and dentate interneurons ........................................................................ 81

CHAPTER 4

4.1 Translamellar inhibition in the normal rat dentate gyrus under urethane anesthesia.............................................................................................................. 88

4.2 Progressive and reversible translamellar inhibition in the normal rat dentate gyrus .............................................................................................................. 90

4.3 C-Fos expression following bicuculline methiodide-induced granule cell disinhibition............................................................................................... 93

4.4 C-Fos expression beyond the region of focal granule cell activation evoked by bicuculline methiodide diffusion ............................................................. 95

4.5 Translamellar inhibition or disinhibition 3 days after kainic acid-induced status epilepticus or 24 hr intermittent perforant pathway stimulation............................... 98

4.6 Hilar neuron loss and c-Fos expression 3 days after kainic acid-induced status epilepticus or 24 hr intermittent perforant pathway stimulation......................................................... 100
LIST OF FIGURES - CONTINUED

4.7 Hilar cell loss correlates with failure of translamellar inhibition........................................ 101
4.8 Loss or survival of dorsal hippocampal hilar neurons corresponds
to loss or preservation of translamellar inhibition........................................................................ 103
4.9 Correlation between hilar neuron loss and loss of translamellar
inhibition.............................................................................................................................................. 105

CHAPTER 5

5.1 Retrograde transport of Fluoro-Gold from the saline electrode
site to the bicuculline methiodide electrode site................................................................. 111
5.2 Primarily hilar neurons retrogradely transport Fluoro-Gold
long distances.......................................................................................................................... 112
5.3 Limited longitudinal transport of the retrograde tracer Fluoro-
Gold by hippocampal interneurons......................................................................................... 113
5.4 Longitudinal transport of Fluoro-Gold by dentate hilar
neurons.......................................................................................................................................... 114
5.5 Retrograde transport of Fluoro-Gold by dentate hilar neurons
after Fluoro-Gold injection into the rostral hippocampus or the
medial septum.............................................................................................................................. 118
CHAPTER 3

3.1 Percentage of dentate interneuron subpopulations containing retrogradely transported Fluoro-Gold (FG) in the dorsal and ventral hippocampus......................................................... 69

CHAPTER 4

4.1 Translamellar inhibition at different BMI and saline electrode tip separations in control animals................................................................. 92

4.2 Translamellar granule inhibition 3 days after kainic acid (KA)-induced status epilepticus or 24hr intermittent perforant path stimulation......................................................... 99
ABSTRACT

Hippocampal associational and commissural pathways of the dentate gyrus include inhibitory and excitatory elements, but which inhibitory and excitatory neurons contribute to this pathway remain unclear. In addition, it is not known whether hilar mossy cells establish a recurrent excitatory network with dentate granule cells or generate lateral inhibition by activating inhibitory neurons. Improved methods for detecting neuronal markers and the retrograde tracer Fluoro-Gold (FG) were used to identify associationally and commissurally projecting neurons of the rat hippocampus and describe their three-dimensional organization. FG-positive, commissurally projecting interneurons of the dentate granule cell layer and hilus were detected in numbers greater than previously reported. FG labeling of interneurons was as high as 96% of hilar somatostatin-positive interneurons, 84% of parvalbumin-positive cells of the granule cell layer and hilus, and 33% of hilar calretinin positive cells. Whereas hilar mossy cells and CA3 pyramidal cells were FG-labeled throughout the contralateral longitudinal axis, FG-positive interneurons exhibited a relatively homotopic distribution contralaterally. In addition, retrograde transport revealed that few inhibitory interneurons were among the many retrogradely labeled hilar neurons 2.5-4.5 mm longitudinal and ipsilateral to the FG injection site. Conversely, GluR2-positive hilar mossy cells were the only with significant longitudinal associational projections. During perforant pathway stimulation in urethane-anesthetized rats, diffusion of the GABA<sub>A</sub> receptor antagonist bicuculline methiodide from the tip of a glass recording electrode evoked granule...
cell discharges and c-Fos expression in granule cells, mossy cells, and inhibitory interneurons within a ~400 μm radius of the tip. This focally-evoked activity powerfully suppressed distant granule cell responses recorded ~2.5-4.5 mm longitudinally. Three days after kainic acid-induced status epilepticus or prolonged perforant pathway stimulation, longitudinal inhibition was intact in rats with < 40 % hilar neuron loss, but was consistently abolished after extensive (> 85 %) hilar cell loss. These data suggest that hilar mossy cells establish surround inhibition by activating inhibitory neurons and that mossy cell loss, which is observed in the disease process of temporal lobe epilepsy, may represent a network-level mechanism underlying post-injury hippocampal dysfunction and epileptic network hyperexcitability.
The hippocampus has been studied for its role in memory and a variety of neurological disorders including Alzheimer’s disease and epilepsy. The laminar structure (Fig. 1.1) of the hippocampus is a highly conserved in mammalian species. This laminar arrangement of the hippocampal subfields has facilitated both electrophysiological and anatomical studies, which have furthered the understanding of normal and abnormal neuronal network function. Although much has been learned about hippocampal anatomy and physiology, the three dimensional functional organization of this archicortical structure remains unclear.

The hippocampus includes the dentate gyrus and the hippocampus proper. The hippocampal neurons of these regions can be divided into two broadly defined groups, principal and non-principal cells. Principal cells are excitatory neurons that include hilar mossy cells and the tightly packed neurons of the dentate gyrus (granule cells) and the hippocampus proper (CA1-CA3 pyramidal cells). Non-principal cells are inhibitory, and include GABAergic interneurons of all hippocampal subfields. Anatomical data suggest that non-principal cells mediate principal cell feed-forward and feedback inhibition, and modulate afferent input to principal cells. The classical description of the hippocampal organization is referred to as the “trisynaptic circuit”. The first synaptic connection in this circuitry is made by perforant path fibers arising from the entorhinal cortex, which form synapses in the outer two thirds of the dentate molecular layer on the dendrites of granule cells and
Figure 1.1. Coronal rat hippocampus section immunocytochemically stained for the neuronal marker NeuN and artificially color coded to illustrate the various hippocampal subfields. The dentate gyrus includes the molecular layer, granule cell layer, and hilus. The hippocampus proper consists of the pyramidal cell layers (CA1-CA3), stratum radiatum, stratum lucidum, stratum lacunosum moleculare, and stratum oriens. Hippocampal afferent inputs from the entorhinal cortex travel via the perforant pathway and synapse on granule and hilar cell dendrites in the outer molecular layer. The granule cell axons (mossy fibers) synapse on hilar neurons which send associational and commissural projections to the inner and outer molecular layers. Mossy fibers also synapse on CA3 pyramidal cell dendrites located in stratum lucidum. CA3 axons form the Schaffer collateral pathway, which sends axons to CA3 and CA1 pyramidal cells bilaterally, synapsing in the corresponding dendritic areas in strata radiatum and oriens. CA1 and CA3 pyramidal cells also receive direct input from entorhinal afferents, which terminate in stratum lacunosum-moleculare.
interneurons. The second synapse is formed by granule cell axons, the mossy fibers, which terminate on the dendrites of hilar neurons and CA3 neurons in stratum lucidum. Finally, the third synaptic connection is between CA3 pyramidal cell axons, the Schaffer collaterals, and CA1 pyramidal cell dendrites in stratum radiatum. Although this representation is helpful to understand the basic circuitry of the hippocampal formation, it is an oversimplification of the modern understanding of this structure. The trisynaptic circuit concept was based on the anatomical studies of Ramón y Cajal and Lorente de Nó, who used the Rapid Golgi method to visualize neurons. Advances in immunocytochemistry and tracing methods have, however, elaborated on the connectivity of virtually every hippocampal subfield. The actual circuitry of the hippocampus is not two-dimensional as this simplified schematic representation implies. For example, the perforant pathway has a longitudinally widespread projection to the dentate gyrus, and the CA3 projection is highly collateralized in which subsets of CA3 pyramidal cells preferentially synapse on subsets of CA1 pyramidal cells along the hippocampal septo-temporal axis (Amaral, 1993). Another pathway that is not described in the “trisynaptic circuit” schematic is the extensive associational and commissural pathway of the dentate gyrus. Although the precise hippocampal functional organization remains unclear, this projection pathway is thought to be involved (Amaral and Witter, 1989, Sloviter, 1994, Buckmaster and Schwartzkroin, 1994). The experiments described in this dissertation address the heterogenous nature of the associational and commissural pathways and the role of this pathway in the functional organization of the hippocampal formation. In addition, selective death of neurons participating in this pathway has been implicated in the
disease process of hippocampal sclerosis, which is thought to be causally related to temporal lobe epilepsy. Thus, experiments were conducted to examine the effect of selective hilar cell loss on hippocampal network excitability.

The associational and commissural projection pathway of the dentate gyrus

Anatomical studies of the dentate gyrus have suggested that neurons participating in the associational projection also contribute to the commissural projection (Deller et al., 1996). This general principle of hippocampal organization is based on the observation that the axonal projections of associational and commissural hilar neurons have similar termination patterns in the dentate molecular layer (Deller, 1998). Although the hippocampal associational and commissural connections undoubtedly have functional significance, the septo-temporal extent of these projections and the cells from which they originate is unclear. Dentate hilar mossy cells (Amaral, 1978; Soriano and Frotscher, 1994) and CA3 pyramidal cells (Hjorth-Simonsen and Laurberg, 1977; Laurberg, 1979; Berger et al., 1980; Laurberg and Sørensen, 1981; Swanson et al., 1980; Swanson et al., 1981; Voneida et al., 1981) of the hippocampus proper form the major associational and commissural pathways of the hippocampus. The axons of hilar and CA3 cells establish termination zones in the inner molecular layer of the dentate gyrus (Blackstad, 1956) and the dendritic regions (strata oriens and radiatum) of CA1-CA3 respectively. In addition to the contribution of mossy cells and CA3 pyramidal cells, other groups of hilar neurons have been shown to form commissural projections in this pathway: 1) GABAAergic neurons immunoreactive for the neuropeptides
somatostatin (Zimmer et al., 1983, Bakst et al., 1986) and neuropeptide Y (Deller and Leranth, 1990), 2) GABAergic neurons containing the calcium binding proteins calretinin (Miettinen et al., 1992) and parvalbumin (Goodman and Sloviter, 1992).

Retrograde tracer studies have indicated that inhibitory cells have a much smaller commissural projection density than mossy cells (Zimmer et al., 1983; Bakst et al., 1986; Ribak et al., 1986; Miettinen et al., 1992; Deller and Leranth, 1990). Such relatively sparsely detected projections of commissurally projecting GABAergic neurons have suggested to some that not all neuron types project contralaterally, and that the associational and commissural pathways are therefore not homologous (Deller, 1998). However, Goodman and Sloviter (1992) identified a previously undetected contralateral projection of parvalbumin-immunoreactive basket cells of the dentate granule cell layer using the retrograde tracer FG, combined with the then newly developed FG antibody. Their study suggested a greater inhibitory commissural contribution than previously estimated and that more sensitive tracer methods might reveal new projections. In a complementary study, Deller and colleagues used the anterograde tracer *Phaseolus vulgaris-leucoagglutinin* (PHAL) to identify commissural axon projections to different laminae of the dentate gyrus, including those innervated by inhibitory interneurons (Deller et al., 1995; Deller et al., 1996; Deller, 1998). This anterograde tracer labels individual axons and their collaterals, and revealed a previously undetected component of commissural projection extending into the outer molecular layer (Deller, Nitsch, and Frotscher, 1995). A follow-up study revealed the heterogenous nature of the commissural pathway, demonstrating a variety of axonal termination patterns in the
outer molecular layer (Deller et al., 1996). These termination patterns were observed to correspond to known termination patterns of ipsilaterally projecting GABAergic neurons, further supporting the idea that the associational and commissural pathways are homologous. Another significant finding in their studies was the observation that PHAL-positive fibers in the contralateral outer molecular layer were restricted to the septo-temporal level of the injection site, whereas the commissural innervation of the inner molecular layer was longitudinally extensive (Deller et al., 1995). The precise role of this pathway in the functional organization of the hippocampus is unknown. However, the available anatomical data have influenced several well established hypotheses.

The "lamellar" hypothesis

The "lamellar" hypothesis was one of the earliest models of hippocampal functional organization. In addition, the lamellar hypothesis has had a significant influence on the conceptualization of hippocampal information processing, and was largely responsible for the development of the in vitro slice preparation, which has contributed to our understanding of hippocampal function (Amaral and Witter, 1989). This idea posits a topographically arranged system in which focal excitation is conveyed sequentially to target neurons within parallel functional slices or "lamellae" (Andersen et al., 1971; 2000; Moser and Moser, 1998; Hampson et al., 1999). The idea that the hippocampus is organized into functional lamellae was first proposed by Per Andersen and colleagues in 1971, in their studies of the rabbit hippocampus. Andersen and colleagues directly stimulated hippocampal fiber pathways to
determine the spread of such stimulation. Orthodromic and antidromic simulation of the mossy fibers, Schaffer collaterals (CA3 axons), and alvear fibers (CA1 axons) resulted in limited spread of the electrical impulse along the hippocampal longitudinal axis. Andersen and colleagues concluded that the three pathways in question were oriented in the same plane, transverse to the longitudinal axis. They proposed therefore that the hippocampus is organized into independent parallel functional lamellae, and that excitatory and inhibitory transverse connections may modify the behavior of the neighboring lamellae. These experiments in the rabbit were replicated in the rat hippocampus (Rawlins and Green, 1977). The lamellar arrangement of granule cell axons has been verified by a variety of axon tracing techniques. Gaarskjaer (1978) used the Fink-Heimer silver impregnation method to visualize the longitudinal spread of mossy fibers in the rat. Lesions to the granule cell layer resulted in limited spread of degenerating terminals septal and temporal to the lesion. Buckmaster and Dudek (1999) analyzed the axonal arbors of biocytin filled granule cells, also showing that granule cell axons have limited spread in the septo-temporal plane.

Amaral and Witter (1989) subsequently proposed that the existence of longitudinally-projecting hippocampal pathways is antithetical to the concept of lamellar function. Andersen et al. (1971) had previously concluded that a localized activation of the entorhinal cortex would result in activation of a hippocampal lamella. Amaral and Witter, however, pointed out that the existence of widespread perforant path projections to the dentate gyrus is inconsistent with lamellar function. They further critiqued the experimental design of Andersen and colleagues, suggesting that placement of the stimulating electrode in the
hippocampus activated perforant path collaterals, resulting in the observed lamellar activation. Amaral and Witter theorized that a focal activation of entorhinal cortical cells would result in more widespread, non-lamellar activation of the hippocampus. In addition, they provided anatomical evidence from anterograde tracer studies showing widespread, associational projections of CA3 pyramidal and dentate mossy cells. They inferred that mossy cells establish a recurrent excitatory circuit and that such network connections are antithetical to a lamellar organization in the hippocampus. However, they provided no new electrophysiological evidence that mossy cells generate recurrent excitation.

Andersen and colleagues (2000) re-examined the lamellar organization of the Schaffer collateral projection from CA3 to CA1 using electrophysiological quantification of axon collateral density. The activity in this pathway was assessed by stimulating CA3 cells and measuring the compound action potentials in various longitudinally distributed points along CA1. In addition, the investigators charted the positions at which Schaffer collateral stimulation antidromically activated CA3 cells. Whereas anatomical data show that CA3 Schaffer collateral axons have a wide septo-temporal spread (Ishizuka et al., 1990), these stimulation experiments revealed that CA3 Schaffer collaterals maximally activated CA1 cells in a narrow band. Thus, CA3 neurons may act functionally in a lamellar fashion despite having extensive longitudinal projections. However, the role of mossy cells in establishing hippocampal network excitability remains controversial.
Mossy cells

The most common and largest (25-35 μm) in diameter cell type of the dentate hilar region is the mossy cell (Amaral, 1978). These large neurons have three or more proximal dendrites that are covered with complex spines called “thorny excrescences”, which are the sites of mossy fiber termination (Ribak et al., 1985; Frotscher et al., 1991). This monosynaptic input produces large-amplitude excitatory postsynaptic potentials in mossy cells, indicating that these cells are relatively excitable (Scharfman and Schwartzkroin, 1988; Scharfman et al., 1990). Mossy cell dendrites are largely constricted to the dentate hilar region (Amaral, 1978), although a minority of the mossy cells extend a dendrite to the molecular layer, where it may receive direct input from the perforant path (Buckmaster et al., 1992). Mossy cells receive inhibitory synaptic input (Ribak et al., 1985; Scharfman, 1992; Buckmaster and Schwartzkroin, 1995) from cholecystokinin- and parvalbumin-immunoreactive basket cells (Acsády et al., 2000). The associational projections of mossy cells extend several millimeters along the septo-temporal axis of the hippocampus (Soltesz et al., 1993) and, as mentioned above, mossy cells project commissurally to the contralateral dentate gyrus. The ipsilateral projections of mossy cells preferentially terminate in the inner molecular layer approximately 1 mm away from the cell body of origin, resulting in a gap of mossy cell innervation both in the septal and temporal directions (Amaral and Witter, 1989; Buckmaster et al., 1992). The commissural termination field of mossy cells does not, however, have such a gap (Deller et al., 1995).

Mossy cells have been intensely studied because they form the major projection
pathway of the dentate gyrus and they have been shown to be highly vulnerable to seizure activity. A consistent feature of hippocampal sclerosis in patients with temporal lobe epilepsy is a loss of hilar cells, including mossy cells (Blümcke et al., 2000). In addition, mossy cells have been shown to be vulnerable in animal models that induce seizure activity, which often reproduces the damage observed in human epileptics (Nadler, 1978; Sloviter, 1983; 1987; 1991). The apparent vulnerability of mossy cells has suggested to some that mossy cell injury may be responsible for the hippocampal hyperexcitability associated with temporal lobe epilepsy (Sloviter, 1994; Ratzliff et al., 2002).

Mossy cells form asymmetric synapses (Ribak et al., 1985; Buckmaster et al., 1992) and they are immunoreactive for glutamate (Soriano and Frotscher, 1994). Thus, they are presumed to be excitatory in nature. Hilar mossy cells innervate both granule cell dendritic spines and dentate GABAergic neurons (Wenzel et al., 1997). Even though mossy cells appear to have more numerous synaptic contacts with granule cells than interneurons (Buckmaster et al., 1996), it is not known which connection dominates functionally. The role of mossy cells in normal hippocampal function has been addressed by two competing theories. The “granule cell association” hypothesis suggests that mossy cells, receiving convergent input from granule cells, spread excitatory information to spatially separated groups of granule cells (Buckmaster and Schwartzkroin, 1994). Conversely the “dormant basket cell” hypothesis suggests that mossy cells excite inhibitory neurons, establishing surround inhibition (Sloviter, 1991; 1994; 2003).
The "granule cell association" hypothesis

The "granule cell association" hypothesis proposes that the synaptic connections from mossy cells on granule cell dendritic spines functionally serve to spread excitatory impulses throughout the septo-temporal axis of the hippocampus. This functional arrangement has been suggested to be consistent with hippocampal circuitry models, used to explain theoretical networks involved in spatial memory (Buckmaster and Schartzkroin, 1994). These models have the common feature of sparse interconnections among neuronal units (Buckmaster and Schartzkroin, 1994). This arrangement may allow simultaneously active neurons to become coupled in such a way that subsequent activation of a few members of the original set results in activation of the rest of the set (McNaughton and Morris, 1987). This idea is called "the collateral effect" (originally proposed by Marr in 1971), and CA3 associational projections have been suggested to mediate this type of network mechanism (McNaughton and Morris, 1987). Buckmaster and Schwartzkroin (1994) proposed that mossy cells establish a similar recurrent excitatory network producing a "collateral effect" in the dentate gyrus. Hetherington and colleagues (1994) have provided electrophysiological evidence that direct stimulation of the hilar region activates granule cells up to 3 mm away from the stimulating electrode. However, the observed activation was weak, and the use of direct dentate stimulation makes it difficult to determine precisely which pathway was activated.
The "dormant basket cell" hypothesis

Another perspective on hippocampal lamellar function is the "dormant basket cell" hypothesis. This idea was originally proposed by Sloviter (1987; 1989; 1991) to explain the hippocampal hyperexcitability that resulted following prolonged perforant path (PP) stimulation of urethane-anesthetized rats. Immediately following the induced seizure discharges, these animals displayed principal cell hyperexcitability that was closely correlated with selective injury of hilar neurons and CA3 pyramidal cells. The apparent survival of GABAergic basket cells led Sloviter (1991) to suggest that the observed principal cell disinhibition, following prolonged PP stimulation, was primarily caused by the loss of mossy cell excitation of dentate basket cells and the loss of CA3 activation of area CA1 basket cells. Although hilar mossy cells densely innervate granule cell dendritic spines (Buckmaster et al., 1996), direct excitation of mossy cells in vitro inhibits granule cells disynaptically, via activation of inhibitory interneurons (Scharfman, 1995). Even when slices were bathed in the GABA\(_A\) antagonist bicuculline, mossy cell discharges only weakly depolarized granule cells (Scharfman, 1995). These in vitro data are consistent with earlier in vivo studies showing that dentate gyrus stimulation produced contralateral granule cell inhibition via commissural activation of contralateral inhibitory interneurons (Buzsáki and Eidelberg, 1982; Douglas et al., 1983). Thus, despite dense innervation of granule cell dendritic spines (Buckmaster et al., 1996), mossy cell innervation of inhibitory interneurons (Wenzel et al., 1997) may predominate functionally. Thus, if longitudinal associational projections primarily inhibit distant granule cells, they may constitute a disassociative system.
that establishes functional segregation within the granule cell layer (Sloviter, 1994; Sloviter and Brisman, 1995).

\textit{GABA}_A \textit{mediated inhibition}

A fundamental organizing principle of the mammalian central nervous system is the regulation of principal cell excitability by inhibitory neurons. Interneurons have a high degree of heterogeneity in morphology and connectivity compared to the principal cells, which are much more uniform in appearance (Maccaferri and Lacaille, 2003). This high variability in afferent and efferent connectivity is thought to give interneurons an important role in shaping response properties of other neurons (Freund and Buzsaki, 1996).

The most prevalent inhibitory mechanism in the central nervous system is that mediated by the release of the neurotransmitter gamma-aminobutyric acid (GABA) onto \textit{GABA}_A receptors. \textit{GABA}_A receptors belong to a group of receptors known as ionotropic receptors, which rapidly alter the conductance of Cl$^-$ ions. GABA binds to the \textit{GABA}_A receptor increasing the conductance of Cl$^-$ ions through the receptors associated channel pore. This effectively shunts any depolarizing current, reducing the chances that an excitatory post-synaptic potentials will reach the cell’s action potential threshold. \textit{GABA}_A receptors are composed of multiple subunits which are thought to form a transmembrane heteropentameric complex with a central water-filled pore (Nayeem et al., 1994). In mammalian species, the components of these receptors have been named \(\alpha\), \(\beta\), \(\delta\), \(\rho\), and \(\gamma\) subunits (Macdonald and Olsen, 1994). These subunit groups are found throughout the brain,
except for subunit p which is found only in the retina (Nayeem et al., 1994). Neurons express different combinations of these subunits resulting in a heterogenous population of GABA_A receptor subtypes with varying pharmacological properties. The α subunit is essential, however, for binding GABA and producing a functional channel (Macdonald and Olsen, 1994). Structural diversity of these receptors does not differentiate inhibitory GABAergic function, but rather appears to be associated with modulation of the gating potency of GABA (Costa, 1998). The inhibitory mechanism of GABA_A receptors is much faster than that of metabotropic receptors therefore GABA_A mediated inhibition is necessary for maintaining the overall level of network excitability. Without effective GABA_A-receptor-mediated inhibition, the hippocampal network generates epileptic seizures because normal excitatory stimuli evoke prolonged depolarizations and repetitive discharges.

Lateral inhibition in the mammalian central nervous system

On a systems level, GABA_A mediated inhibition is thought to participate in lateral or surround inhibition. Lateral inhibition is a computational neuronal mechanism in which neurons compete to generate a response to a given stimulus, the success of these neurons depends on the strength of the stimulation they receive and their ability to suppress activity in surrounding neurons (Spratling and Johnson, 2001). Lateral inhibition has been observed or proposed to be a mechanism of functional organization in several regions of the central nervous system. However, there are variations in the neuronal circuitry responsible for establishing surround inhibition.
One of the earliest examples of lateral inhibition was described in the cerebellum (Eccles et al., 1967). The cerebellum has a relatively simplistic structural organization constructed of similar units described as cerebellar modules. The major output cells of these modules are the Purkinje cells which receive excitatory input from parallel fibers and climbing fibers. The parallel fibers and climbing fibers originate from cerebellar granule cells and inferior olivary nucleus neurons, respectively. Bundles of parallel fiber axons excite Purkinje cell dendrites along a beam of activation. In addition, these parallel fibers activate inhibitory basket cells and stellate cells of the cerebellum's molecular layer. The long axons of these inhibitory interneurons are thought to establish lateral inhibition that further sharpens parallel fiber activation (Eccles et al., 1967; Cohen and Yarom, 2000). This inhibitory circuitry allows complex motor coordination tasks to be compartmentalized within the relatively homogenous structure of the cerebellum.

The discovery of columnar organization in the somatosensory cortex and the primary visual cortex in cats and monkeys provided another early example of segregation of function in the central nervous system. (Mountcastle, 1957; Powell and Mountcastle, 1959; Hubel and Wiesel, 1977). The alternating columns of the primary visual cortex, labeled with 2-deoxyglucose (Sokoloff et al., 1977), represent monocular information originating from the retina. The basic framework of these columns is established by axons originating from the lateral geniculate nucleus of the thalamus. Although the anatomical circuitry underlying these functional columns is not “visibly columnar”, the boundaries of these columns are thought to be maintained by local inhibitory interneurons (Lund et al., 2003). That is, these anatomical
columns are reinforced by disinhibition within the column and inhibitory impulses spreading beyond the columns boundaries (Lund et al., 2003).

In the olfactory system, the olfactory bulb is composed of large spherical structures called glomeruli, which serve as a convergence point for specific odorant signals transmitted from receptors located in the olfactory epithelium. Mitral, tufted, and granule cells within the glomeruli establish circuitry that generates feedback inhibition and lateral inhibition of neighboring mitral and tufted cells (Mori et al., 1999). Lateral inhibition between glomerular modules is thought to enhance the difference between strongly activated and weakly activated glomeruli, thus the sharpening individual tuning specificity of mitral and tufted cell responses to various odor molecules (Mori et al., 1999; Laurent, 1999). GABAergic neurons in the striatum and globus pallidus of the basal ganglia have been suggested to establish focal activation through lateral inhibition as well (Bar-Gad and Bergman, 2001). Computational models used to explain basal ganglia function incorporate lateral inhibition as a mechanism involved in processing motor, cognitive, and motivational information (Bar-Gad and Bergman, 2001). As illustrated by these examples, lateral or surround inhibition is a ubiquitous mechanism in the central nervous system. Lateral inhibition is a common inhibitory mechanism to focus excitatory activity across a variety of neuroanatomical and neurophysiological systems thereby serving as a mechanism to establish a structure-function relationship where computational operations can be performed without affecting activity in surrounding areas.
Lateral inhibition in the rat hippocampus

The presence of surround inhibition in the hippocampus was first indicated by experiments in which a penicillin seizure focus on the surface of the hippocampus established a “ring” of inhibition. (Dichter and Spencer, 1969). This hippocampal inhibition was interpreted as a mechanism to prevent the spread of epileptiform discharges from the site of seizure origin. Hippocampal lateral inhibition was further characterized using passive diffusion of the GABA<sub>δ</sub> receptor antagonist bicuculline methiodide (BMI) from a glass microelectrode (Sloviter and Brisman, 1995). This method of BMI application results in focal granule cell discharges in response to low frequency perforant path stimulation. This experiment established that focal discharges induced in one segment of the dentate granule cell layer remained restricted to that segment in both the longitudinal and transverse directions. In addition, the focal discharge generated by the focal BMI application resulted in effective inhibition of granule cell activity recorded at a saline electrode located approximately 1 mm away. Even though the identity of the cells mediating lateral inhibition was not determined, this study established lateral inhibition as an operant physiological mechanism in the dentate gyrus. Although this study examined lateral inhibition at a maximal distance of distance a distance of 1.5 mm, it is difficult to determine precisely which cells are involved in mediating lateral inhibition at this distance. The axon arbors of granule cells have a total septo-temporal length of up to 1.32 mm (Buckmaster and Dudek, 1999) and in the lateral inhibition study by Sloviter and Brisman (1995), the BMI was diffused at least 200 µm from the electrode tip. Thus, the possibility remains that direct
granule cell to inhibitory cell connections are responsible for the observed lateral inhibition at 1 mm. In addition, several studies have created the impression that inhibitory interneurons have long extensive projections and have a far reaching influence on hippocampal excitability (Strube et al., 1978; Qiu and Han, 1995; Buckmaster and Schwartzkroin, 1995; Sik et al., 1997). An intracellular fill study, based on a small number of dentate neurons, indicated that labeled interneuron axon arbors were most dense close to the somata but also minimally innervated regions greater than 1.5 mm longitudinally (Buckmaster and Schwartzkroin, 1995). Thus, it is possible that lateral inhibition recorded at the distance of 1.5 mm involves direct actions of interneurons excited by granule cells. Conversely, as proposed by the "dormant basket cell" hypothesis, this surround inhibition could be established by mossy cells activating basket cells. Therefore, at the distance of 1-1.5 mm, virtually all hilar cell types could mediate lateral inhibition.

Buckmaster and Dudek (1997) using the same BMI filled electrode technique replicated the findings of Sloviter and Brisman (1995) with a tip separation of 1 mm. In addition, they tested lateral inhibition in rats that had been treated with systemic injections of kainic acid. Kainic acid is a glutamate agonist that induces status epilepticus, typically lasting several hours, and results in widespread brain damage, including the hippocampus. These investigators observed a preservation of lateral inhibition following seizure-induced cell loss, but they reported an average of 60% loss of the total hilar neuron population. Despite the incomplete cell loss, the authors concluded that surviving hilar inhibitory neurons were responsible for the preserved lateral inhibition. However, the authors did not rule out
that surviving mossy cells could be responsible for the preserved LI. In addition, these studies were conducted in animals that had undergone kainate induced status epilepticus, on average, 169 days earlier. Kainate induced status epilepticus results in an immediate failure of hippocampal inhibition, followed by a recovery of inhibition which is correlated with synaptic reorganization (Sloviter, 1992). Thus, the investigators’ choice to evaluate lateral inhibition after synaptic reorganization further confounds their interpretations. Therefore, to understand the role of hilar cells in surround inhibition, it is important to evaluate animals with complete hilar cell loss before synaptic reorganization has occurred.

Lesions of the dentate gyrus in temporal lobe epilepsy

Determining the neuronal architecture of the hippocampus is crucial to understanding both, how the hippocampus functions normally, and how neurological conditions that damage this structure affect network excitability. The disease process of temporal lobe epilepsy (TLE) involves a variety of changes to temporal lobe structures, including significant structural and functional changes in the dentate gyrus. However, whether these changes are the passive result of seizure activity or are epileptogenic in nature remains the subject of much debate. In temporal lobe epilepsy patients, the hippocampus often has a characteristic pattern of cell loss and shrinkage, referred to as hippocampal sclerosis (HS). Bouchet and Cazauvieilh (1825) initially described the gross atrophy of the medial temporal lobes, however microscopic studies of hippocampal sclerosis were initiated several decades later (Sommer 1880), indicating specific cell loss in hippocampal subfields. The epileptic
hippocampus showed a stereotypical pattern of cell loss that included hilar neurons (endolium sclerosis) in addition to CA3 and CA1 (Sommer sector) pyramidal cells. The preservation of CA2 pyramidal neurons earned this region the name “resistant zone.” The next several decades of research were focused on the etiology and pathogenesis of hippocampal sclerosis, and on determining the cause-effect relationships of temporal lobe seizures and hippocampal sclerosis (Gloor, 1991). Initially, it was assumed that hypoxia injured the hippocampus during repetitive seizures (Spielmeyer, 1927), and that temporal lobe damage might result in behavioral and memory disturbances, but did not cause epilepsy. However, the advent of the EEG allowed surgeons to detect electrical disturbances emanating from the temporal lobe, suggesting that this region was the source of seizures. These observations led to the idea that if the temporal lobe was the focus of seizure activity, then the removal of these structures could be used as a treatment for temporal lobe epilepsy (TLE). En bloc resection of the anterior temporal lobe has proved to be an effective treatment for intractable TLE (Falconer et al., 1964; Gloor, 1991). The success of this surgical procedure increased interest in temporal lobe structures as the source of spontaneous seizures. Magerison and Corsellis (1966) examined autopsy tissue from patients with TLE. They found that out of 55 epileptics, more than half had hippocampal sclerosis. In addition, in 14 of these patients, the only lesion found was hilar cell loss. This finding suggested that specific hilar cell loss may be sufficient to cause temporal lobe epilepsy.
Hilar cell loss in animal models

Animal models have been developed to understand better how the pattern of hippocampal damage observed in humans affects hippocampal network excitability. Prolonged focal electrical stimulation of the perforant pathway (PP) in anesthetized rats produced acute pathology that replicated the human pattern of hippocampal cell loss and survival (Sloviter and Damiano, 1981). This stimulation paradigm damaged neurons in the precise postsynaptic region innervated by excitatory terminals of the mossy fiber pathway and produced permanent granule cell disinhibition and hyperexcitability that was closely associated with loss of dentate hilar neurons (Sloviter, 1983; 1987; 1991). This focal hilar cell loss induced by PP stimulation has been shown to be sufficient to induce a spontaneous epileptic state (Shirasaki and Wasterlain, 1994). The excitotoxins kainic acid (KA) (Nadler, 1978) and domoic acid (Zaczek and Coyle, 1982) provoke prolonged status epilepticus and hilar cell loss similar to human hippocampal sclerosis. Other investigators looked for similar anatomical and functional changes following head trauma, which often precedes the development of TLE. Using the fluid percussive-injury as a model for head trauma, Lowenstein and colleagues (1992) demonstrated that hilar cells are vulnerable to a brief, unilateral impact to the surface of the brain. Similar to PP and KA treatments, this treatment resulted in granule cell hyperexcitability that was correlated with hilar cell loss. Hilar neurons have also been shown to be vulnerable to experimental animal treatments that model stroke (Johansen et al., 1987). These studies indicate that the dentate gyrus and, in particular, the hilar region is susceptible to damage in the disease process of a variety of neurological
conditions. As mentioned previously, the hilar neurons damaged in the above mentioned conditions participate in a major associational and commissural pathway of the hippocampus. Therefore, understanding the precise anatomical organization of this pathway and its role in normal hippocampal function is crucial to determine how neurological conditions, that are associated with specific hilar cell lesions, could affect hippocampal functioning and disease progression.

Summary of research goals

An understanding of normal hippocampal network function, and of hippocampal dysfunction in a variety of neurological disorders, requires accurate information regarding the axonal projections and interconnections existing among hippocampal principal cells and interneurons (Patton and McNaughton, 1995; Freund and Buzsáki, 1996). The associational and commissural pathways of the dentate hilus appear to be important in the functional organization of the hippocampus. In addition, the neurons that comprise this pathway are lesioned in humans with TLE and in animal models of TLE. This damage has been hypothesized to play a role in hippocampal dysfunction and hyperexcitability associated with TLE. However, whether this pathway spreads excitation or inhibition along the hippocampal septo-temporal axis remains controversial. Therefore, the focus of this dissertation is to characterize further the role of the associational and commissural pathway in the functional organization of the hippocampus. Recently improved immunocytochemical methods and high sensitivity fluorescence detection will be used to provide a more accurate description
of these hippocampal projection neurons. In addition, translamellar influences generated by focal granule cell discharges will be studied to determine how the hilar post-synaptic targets of granule cells affect network excitability. Since the failure of dentate inhibitory circuitry has been suggested to underlie the pathophysiology observed in TLE, translamellar influences will be examined following animal treatments that result in hilar cell loss. Finally, the associational projection of hilar neuron subpopulations will be examined to determine which cells have the requisite projections to mediate translamellar influences.
CHAPTER 2: MATERIALS AND METHODS

*Animal treatment*

All animals were treated in accordance with the guidelines set by the National Institutes of Health for the humane treatment of animals, and their use was approved by the University of Arizona Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 250-400 g were anesthetized with urethane (1.25 g/kg subcutaneous (sc) injection; Sigma) and placed in a Kopf stereotaxic apparatus. Rectal temperature was automatically maintained at 37.0 ± 0.2° C (Harvard Apparatus).

*Dorsal hippocampal injection of Fluoro-Gold for contralateral labeling*

A combination recording electrode/injection syringe was used to inject retrograde tracer reproducibly into specific hippocampal target sites. Glass microelectrodes were pulled on a Sutter electrode puller and glued with cyanoacrylate onto the tip of a 1 μl Hamilton syringe (#7001). By soldering a gold pin connector to the metal hub of the syringe, the syringe acted as a recording electrode through which minute volumes could be injected. This syringe/recording electrode, containing a 4% solution in saline of the retrograde tracer hydroxystilbamidine (Fluoro-Gold; FG; Fluorochrome, Englewood, CO; Schmued and Fallon, 1986; Schmued, 1990; Wessendorf, 1991) was lowered into the brain (approximately 2mm lateral from the midline suture, 3 mm rostral to the lambda, and 3.5 mm below the brain surface). A bipolar stainless steel stimulating electrode was placed in the angular
bundle of the perforant pathway (4.5 mm lateral from the midline suture and immediately rostral to the lambdoid suture). The final recording electrode tip location in, or just above, the dorsal blade of the dentate granule cell layer was reached by optimizing the characteristic shapes of the evoked potentials evoked by perforant path stimulation (Andersen et al., 1966). Monophasic current pulses (0.1 ms duration) were generated by a Grass S88 stimulator with a Grass stimulus isolation unit. Evoked potentials were amplified by a Grass preamplifier, and displayed on a Nicolet Series 400 digital oscilloscope. After the target location was reached, 10-50 nl of the FG solution was injected through the recording electrode to examine the commissural pathway. 30-50 nl injections were used to maximally label this pathway. To prevent ablation of neural tissue when more than 10 nl volumes were injected, each 10 nl was injected at 10 minute intervals until the total volume was delivered. The injection electrode was left in place for an additional 10 minutes following the final injection. After electrode removal, the skull was washed with Betadine and the skin was sutured using surgical staples. Animals were kept warm during recovery from urethane anesthesia, and allowed to survive for at least 5 days (usually 7-21 days) before perfusion-fixation to permit retrograde transport of FG (Schmued and Fallon, 1986).

*Septal, neocortical, and ventral hippocampal injection of Fluoro-Gold*

Injections of the same concentration and volumes of the FG solution used for hippocampal injection were made into the neocortex, septum, and ventral hippocampus to address several control issues. The burr hole for FG injections into the left ventral
hippocampus was 6 mm posterior to the bregma and 4.7 mm lateral from the midline (coresponding approximately to Plate 104 of the atlas by Paxinos and Watson, 1998). Injections of 30-50 nl of FG solution into the ventral hippocampus were made stereotaxically, with the syringe/electrode tip approximately 6 mm below the brain surface. The injection site for similar FG injections into the septum were also determined stereotaxically (0-0.5 mm posterior to the bregma, and ~5 mm below the brain surface). For neocortical injection of FG, identical methods were used as for hippocampal injection described above, except that 30-50 nl of FG was injected with the tip of the syringe/recording electrode placed in the neocortex above the corpus callosum (~1.5 mm below the brain surface).

Perfusion-fixation and tissue treatment

After recovery for a period allowing retrograde transport (≥5 days), rats were anesthetized with urethane (1.25 g/kg ip) and perfused through the heart by gravity-feed using one of two fixation protocols. One protocol, which was used to preserve FG and peptide immunoreactivity optimally, involved perfusion with saline for 2 min, followed by 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for 10 min. The second protocol, which preserves both GABA and peptide immunoreactivity (Sloviter et al., 2001), involved perfusion with saline for 2 min, followed by 2% paraformaldehyde in 0.1 M sodium acetate buffer, pH 6.5, for 2 min, followed by 2% paraformaldehyde (Sigma)/0.1% purified glutaraldehyde (Ladd) in 0.1 M sodium borate buffer, pH 8.5, for 1 hr. The descending aorta
was clamped to reduce the volume of fixative used. After storage in situ overnight at 4°C, brains were removed from the skull, and cut on a Vibratome. For horizontal sections of the ventral hippocampus, the brain was glued by the dorsal surface to a cutting block. When horizontal sectioning reached the level of the joining of the fimbria, the brain was removed from the chuck, re-glued in coronal orientation, and then cut coronally. All sections were cut at a thickness of 40 μm and collected in 0.1 M TRIS buffer, pH 7.6.

*Immunocytochemistry methods for commissural transport studies*

Sections were mounted on Superfrost Plus slides, air dried for 15 min, and placed in 0.1M TRIS buffer, pH7.6 (TRIS). Slides were then immersed in 85-87°C TRIS for 1min, washed in room temperature TRIS, and placed in TRIS containing 0.25% bovine serum albumin (BSA; fraction V from Sigma) and 0.1% Triton X-100. Slides were incubated overnight at room temperature in primary antisera diluted in the same solution. After primary antibody incubation, slides were washed in TRIS-BSA-Triton X buffer (2 x 5 min).

Polyclonal rabbit antisera used in this study included anti-Fluoro-Gold (Chemicon AB153; 1:50,000; Chang et al., 1990); anti-calretinin (CR) (AB5054; 1:100,000; Chemicon), and anti-GluR2 (AB1768; 1:3000; Chemicon). Monoclonal antibodies used included mouse anti-NeuN (MAB377; 1:10,000; Chemicon), mouse anti-GABA (A-0310; 1:300,000 dilution; Sigma), mouse anti-parvalbumin (PV)(P3171; 1:1,000,000; Sigma), and mouse anti-somatostatin (SS) (1:25,000; kindly provided by A.J. Malcolm of the MRC Regulatory Peptide Group, University of British Columbia).
For light microscopic visualization with diaminobenzidine (DAB) as the chromagen, slides were incubated in biotinylated secondary antibody solution (1:2000 dilution of goat anti-mouse or rabbit IgG (Vector Labs) in TRIS-BSA-Triton X buffer for 2 hr, followed by washing in the same buffer, and then incubation for 1 hr in avidin-biotin-HRP complex (Vector Labs Elite kit diluted 1:1000 in TRIS-BSA-Triton X buffer). Slides were then washed in TRIS (3x5 min minimum) and incubated in a hydrogen peroxide-generating DAB solution (100 ml TRIS containing 50 mg DAB (Sigma), 40 mg ammonium chloride, 0.6 mg glucose oxidase (Type VII; Sigma), and 200 mg β-D+ glucose). After incubation in DAB solution for a period that produced optimal staining (usually 20-30 min), slides were rinsed in TRIS, dehydrated in graded ethanols and xylene, and coverslipped with Permount.

For immunofluorescence co-localization, after primary antibody incubation, slides were incubated overnight at room temperature in species-appropriate fluorescent secondary goat antibodies (Alexa Fluor 488 or 594 from Molecular Probes; diluted 1:10,000 in TRIS-BSA buffer). Slides were then washed in TRIS buffer and coverslipped with Vectashield (Vector Labs).

For light microscopic immunocytochemistry, controls included sections in which the primary antisera and/or the secondary antibodies were omitted, or the primary antiserum was substituted with normal serum of the same species at the same dilution. For the fluorescence methods, controls included omission of one of the two primary antisera, both primary antisera, both primary and secondary antisera, and all combinations of omission of one or both secondary antisera. This was done to determine non-specific background fluorescence...
and the degree of “bleed-through” of one fluorochrome at the emission wavelength of the second fluorochrome, and to confirm that each species-selective secondary antibody to which a fluorochrome was attached produced no artifactual fluorescent results by cross-reactive binding to the primary antibodies of the other species.

Brightfield and fluorescent images were acquired digitally on a Nikon E800M microscope with a Hamamatsu C5180 camera, using Adobe Photoshop 5.0. This program was used to optimize contrast and brightness, but not to remove blemishes, or to enhance or change the image content in any way. All comparison photographs were taken under identical conditions of image acquisition, and all adjustments of brightness and contrast were made uniformly to all images.

*Kainic acid-status epilepticus and perforant path stimulation*

Rats were briefly anesthetized with ether, a small incision was made in the skin overlying the saphenous vein, and kainic acid (KA; 9-12 mg/kg; 10 mg/ml saline; Ocean Produce International, Shelburne, Canada) or saline was injected intravenously. After the end of status epilepticus, animals were given saline periodically by sc injection until each animal was eating and drinking spontaneously. Until then, apple slices were provided as a source of food and water in a combined, digestible form. Four saline-treated rats and 14 rats that survived KA-induced SE lasting 3-6 hr were subsequently evaluated as described below. Intermittent PP stimulation for 24 hr was performed in 6 urethane-anesthetized rats to produce extensive hilar neuron loss throughout the dorsal hippocampus, as previously
described (Sloviter et al., 2003). All stimulated animals received urethane at a dose of 1.25 g/kg sc prior to the start of stimulation, and were given additional doses of 0.25 g/kg sc approximately 8 and 14 hr later to ensure the maintenance of surgical anesthesia throughout the procedure. This anesthesia regimen, involving urethane by the subcutaneous route, produced no lethality and full recovery within 1 day after the last urethane dose. After electrode removal, the incised area was washed with Betadine and the skin was sutured using surgical staples. Animals were given saline periodically by sc injection, apple slices, and kept warm during recovery from anesthesia.

Assessment of translamellar influences

Normal (n = 23), KA-treated- (n = 14), saline-treated- (n = 4), and PP-stimulated rats (n = 6) were anesthetized with urethane (1.25 g/kg sc) 3 days after saline or KA injection, or 3 days after the end of 24 hr intermittent perforant pathway stimulation, and placed in a stereotaxic apparatus. Rectal temperature was maintained at 37.0 ± 0.2° C. A bipolar stimulating electrode (NE-200; Rhodes Medical Instruments, Summerland, CA) was placed in the left angular bundle of the perforant pathway (~4.5 mm lateral from the midline suture and immediately rostral to the lambdoid suture). Two glass recording microelectrodes were used to assess translamellar inhibition, one of which contained 10 mM bicuculline methiodide dissolved in saline (BMI; Sigma, St. Louis, MO) and was attached to a Hamilton microsyringe, as previously described (Sloviter and Brisman, 1995). Although granule cell discharges were produced by passive leakage of BMI from the recording electrode tip (tip
diameter ~50-80 μm), a microsyringe was used so that the BMI solution could be ejected just before insertion into the brain to ensure that the tip was open. The other recording electrode was filled with saline and attached directly to a microelectrode holder.

Translamellar influences were assessed by first lowering a saline-filled recording electrode into the hippocampus (~2 mm lateral from the midline, ~3 mm caudal to bregma, and 3.5 mm below the brain surface) at the approximate site of Plate 32 in the atlas of Paxinos and Watson (1998). The final tip location in the dorsal blade of the granule cell layer was reached by observing the potentials evoked by PP stimulation (Andersen et al., 1966). Stimuli were biphasic current pulses (0.1 msec duration) generated by a Master 8 stimulator and stimulus isolation unit (AMPI; Jerusalem, Israel). Potentials were amplified, displayed on a Nicolet 400 oscilloscope, and recorded digitally at a 20-40 kHz sampling rate (AD Instruments, Mountain View, CA). Following optimization of the evoked potentials, at least ten responses were recorded to paired-pulse stimulation at 0.3 Hz at an interpulse interval of 60 msec. The BMI-containing recording electrode was then lowered into the brain ~2.5-4.5 mm posterior to the saline-filled electrode along the longitudinal axis of the dentate gyrus at the location shown in Plates 41-42 in the atlas of Paxinos and Watson (1998). A 60 msec interpulse interval was chosen because normal rats do not exhibit suppression of the 2nd population spike at a 60 msec interval. Therefore, the 2nd population spikes evoked in both control and experimental animals were of similarly large amplitudes prior to insertion of the BMI-filled recording electrode. Average spike amplitudes were measured using an automated program written using LabVIEW 7.0 (National Instruments; Austin, Texas).
Dorsal hippocampal injection of Fluoro-Gold for ipsilateral labeling

FG was injected in naive rats in the same hippocampal location in which the saline-filled electrode was inserted in the physiological experiments (n = 9). This was done to determine which neurons at the distant BMI electrode site possess axonal projections capable of directly affecting granule cells at the anterior saline electrode site. The methods used to inject FG have been described above. In brief, under urethane anesthesia, and after placement of the electrode tip in the dorsal blade of the granule cell layer under physiological guidance during perforant path stimulation, 20-30 nl of a 4% solution of FG in saline was injected using a syringe/electrode identical to that used for BMI diffusion. For control comparisons, FG (20-30 nl of the same FG solution) was injected into the medial septum (0-0.5 mm posterior to the bregma and ~5 mm below the brain surface; n = 6), the neocortex above the dentate injection site (n = 3), and the CA1 region above the dentate injection site (n = 5). The injection electrode was left in place for 10 minutes following injection. After electrode removal, the skull was washed with Betadine and the skin was sutured using surgical staples. Animals were allowed to survive for 7-21 days before perfusion-fixation as described below. FG injections in the volumes used deposited FG throughout the laminae of the dentate gyrus and the overlying area CA1. Thus, the FG injections encompassed all axonal termination zones in the dentate molecular-, granule cell-, and hilar layers, and are not represented as being focal deposits of retrograde tracer in any specific hippocampal subregion.
Perfusion-fixation, tissue treatment, and immunocytochemistry methods used for translamellar studies

After 1 hr of BMI-induced granule cell discharges at the posterior recording site, all electrodes were removed, and the distance between the BMI and saline electrode tips was measured. In 11 additional rats, electrodes were removed after 7 hr of BMI-induced discharges, a duration used to determine whether prolonged discharges would evoke c-Fos expression in distant target cells. All animals were perfused with saline for 2 min, then 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 10 min. After storage of the intact rat overnight at 4°C, brains were removed from the skull and placed in perfusate. Coronal sections 40 μm-thick were cut on a Vibratome in 0.1 M Tris (hydroxymethyl) aminomethane (TRIS) buffer, pH 7.6. Sections were mounted on gelatin and chrom alum-coated slides for subsequent Nissl (cresyl violet) staining or for staining acutely degenerating neurons with Fluoro-Jade B (Schmued and Hopkins, 2000). After staining, all slides were dehydrated in graded ethanols and xylene, and then coverslipped with Permount or DPX (for Fluoro-Jade B).

For immunocytochemistry, sections were mounted on Superfrost Plus slides, air dried for 15 min, placed in 0.1 M TRIS buffer, pH 7.6, and processed as previously described in detail (Sloviter et al., 2003). Antisera used included rabbit anti-c-Fos (Ab H125; 1:5,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Fluoro-Gold (Chemicon AB153; 1:50,000; Chemicon, Temecula, CA), guinea pig anti-vesicular glutamate transporter 1 (Ab5905; 1:50,000; Chemicon, Temecula, CA), mouse anti-parvalbumin (P3171;
1:1,000,000; Sigma), mouse anti-somatostatin (V1169; 1:300; Biomed, Foster City, CA), mouse anti-NeuN (MAB377; 1:10,000; Chemicon), and mouse anti-glutamate receptor subunit 2 (MAB397; 1:25,000; Chemicon). Images were acquired digitally on a Nikon E800M microscope with Hamamatsu C5180 camera, using Adobe Photoshop 7.0.

To determine the extent of hilar neuron loss at the BMI site in KA-treated- and PP-stimulated rats, three non-consecutive Nissl-stained sections were selected from within the c-Fos-positive region of the BMI electrode. The numbers of surviving hilar neurons were counted in 3 sections from each animal, and expressed as means ± standard deviations. Although hilar cell loss or survival was relatively uniform throughout the dorsal hippocampus, only sections at the BMI electrode site were assessed quantitatively for hilar neuron loss or survival because the primary assumption of the experimental design was that it was the activated neurons at the BMI electrode site that mediated the distant effects of the focal granule cell discharges. Statistical comparisons of spike suppression and hilar cell number were made using the Mann-Whitney non-parametric test, that does not make assumption about the distribution of data.

Cell counting methods

The primary immunocytochemical and cell loss related findings of these studies are qualitative in nature, and the conclusions derived from the histological results are not dependent on the semi-quantitative data provided in Table 3.1. The results of cell counting therefore provide corroborative semi-quantitative information in support of the primary
qualitative findings. These data were regarded as semi-quantitative in nature because a subjective judgement must be made regarding the threshold for counting a cell as positive for specific staining. Therefore, a conservative judgement that differentiates specific staining from non-specific staining must underestimate the actual number of immunoreactive cells present. As a result, the numbers presented must be regarded as minimum percentages of co-localization or cell loss, and not as an accurate count of the total number of cells in these studies.

For co-localization of native FG fluorescence, as conducted in the commissural projection studies, with peptide immunoreactivity, the number of peptide-containing cells that were in focus in the focal plane of the section surface were counted. Then, without changing the focal plane, the number of those peptide-positive cells also containing FG were counted. This counting method was used because incubation of sections in antibody solutions resulted in partial penetration of reagents into the section, whereas FG-fluorescent cells were visible throughout the section.

For co-localization of FG- and peptide immunoreactivities, in which both epitopes were localized by immunocytochemistry, a similar method was used. Once a peptide-positive cell was judged to be countable, the fluorescence filter cubes were switched without altering the focal plane to determine whether the same cells were also immunoreactive for FG-like immunoreactivity (LI). Control sections, which were not exposed to primary antisera, but were otherwise processed identically, were run in every experiment to differentiate artifactual fluorescence and autofluorescence from specific staining.
CHAPTER 3: COMMISSURAL PROJECTIONS STUDIES

Introduction

The extent of the commissural projections originating from different subpopulations of hippocampal principal cells and interneurons, and their functional significance, is unclear. Blackstad’s pioneering anatomical studies utilized silver degeneration staining after commissurotomy to identify the termination patterns of commissurally-projecting axons (Blackstad, 1956). The results of his study, and that of Gottlieb and Cowan (1973), revealed that the most dense commissural projections innervated the inner molecular layer of the dentate gyrus and the dendritic regions (strata oriens and radiatum) of areas CA1-CA3. Subsequent studies utilizing retrograde tracers unequivocally identified excitatory hilar mossy cells (Amaral, 1978; Soriano and Frotscher, 1994) and CA3 pyramidal cells as the cells of origin of these commissural projections (Hjorth-Simonsen and Laurberg, 1977; Laurberg, 1979; Berger et al., 1980; Laurberg and Sørensen, 1981; Swanson et al., 1980; Swanson et al., 1981; Voneida et al., 1981).

Although CA3 pyramidal cells and dentate hilar mossy cells clearly form the predominant commissural projections, hippocampal non-principal cells (interneurons) have also been reported to transport retrograde tracers. Zimmer et al. (1983) reported that only 4-5% of dentate hilar somatostatin-immunoreactive interneurons were retrogradely labeled by granular blue, a finding that was supported by Bakst and colleagues (1986), who reported that only a few hilar somatostatin-positive interneurons retrogradely transported wheat germ
agglutinin-horseradish peroxidase (WGA-HRP). The apparently minor role of inhibitory hippocampal commissural projections was reinforced by the observations of Ribak and colleagues (1986), who showed that relatively few hilar GABA neurons (~5%) retrogradely transported Fluoro-Gold (FG) or WGA-HRP. Similarly, only a few (<2%) neuropeptide Y- and calretinin-positive neurons of the dentate gyrus were retrogradely labeled after hippocampal injection of WGA-HRP (Deller and Leranth, 1990) or HRP (Miettinen et al., 1992), respectively.

Using a then newly developed antibody to FG, Goodman and Sloviter (1992) identified parvalbumin-immunoreactive basket cells of the dentate granule cell layer as among the cells that retrogradely transported FG, suggesting a greater inhibitory commissural contribution than previously detected. In a complementary study, Deller and colleagues used the anterograde tracer *Phaseolus vulgaris-leucoagglutinin* (PHAL) to identify commissural axon projections to different laminae of the dentate gyrus, including those innervated by inhibitory interneurons (Deller et al., 1995; Deller et al., 1996; Deller, 1998). A significant finding in their studies was the observation that PHAL-positive fibers in the contralateral outer molecular layer were restricted to the septo-temporal level of the injection site, whereas the commissural innervation of the inner molecular layer was longitudinally extensive (Deller et al., 1995). Thus, commissural projections of hippocampal interneurons may have a different pattern of topographic distribution compared to the extensive projections of hilar mossy cells and CA3 pyramidal cells (Gottlieb and Cowan, 1973). Nonetheless, the relatively minimal retrograde and anterograde labeling of hippocampal inhibitory interneurons obtained
in all previous studies has influenced, possibly in a misleading manner, the current views of hippocampal circuitry and network function (Patton and McNaughton, 1995; Freund and Buzsáki, 1996).

Although retrograde FG-labeling of parvalbumin-positive dentate basket cells were previously detected (Goodman and Sloviter, 1992), it is possible that the relatively insensitive methods that were used at the time (rapidly fading FG fluorescence, conventional immunocytochemistry, and standard photographic methods) had detected only those cells containing the highest concentration of transported FG, thereby underestimating the true number of commissurally-projecting neurons. The purpose of the present experiments is to describe the hippocampal commissural pathway using recently improved immunocytochemical methods and high sensitivity fluorescence detection (Sloviter et al., 2001), to provide a more accurate description of the hippocampal neurons that retrogradely transport FG, and to describe the septo-temporal extent of these cells axonal projections.

Contralateral Fluoro-Gold labeling after dorsal hippocampal injection

Injection of 10-50 nl of the fluorescent retrograde tracer Fluoro-Gold (FG) into the dorsal hippocampus resulted in the deposition of FG in relatively large areas of the injected hippocampus (Fig. 3.1). The neocortex above the hippocampus was frequently labeled after electrode insertion through the neocortex, requiring neocortical control injections described below. Relatively large FG volumes were intentionally used in order to label the maximum number of contralateral hippocampal neurons. However, unintended injections of small
Figure 3.1. Patterns of hippocampal Fluoro-Gold (FG)-like immunoreactivity (LI) in two rats receiving small and large FG injections into the contralateral hippocampus. A1, B1: Small and large FG injection, respectively, into the left hippocampus. A2,B2: The dentate gyri contralateral and homotopic to the small and large injections, respectively. A3,B3: Higher magnification of A2 and B2. Note that a small FG injection into the dentate gyrus (A1) primarily labeled dentate hilar neurons, whereas the larger injection (B1) labeled hilar neurons, CA3 pyramidal cells (sp), and non-principal cells exhibiting morphological features and location of dentate basket cells (arrows in B3) in the granule cell layer (sg). Note also that even relatively large FG injections involving areas CA3 and CA1 (B1) did not produce labeling of interneurons in the dentate molecular layer (sm) or in the dendritic layers of the hippocampus proper (B2). Survival: 8 days after FG injection for both animals. Magnification: 6.2X in A1,B1; 25X in A2,B2; 124X in A3, B3. Calibration bar = 2 mm in A1,B1; 500 μm in A2,B2; 100 μm in A3,B3.
volumes provided additional information about commissurally-projecting cells. Small FG injections limited to the medial aspects of the dentate gyrus (Fig. 3.1 A1) produced contralateral FG-labeling of dentate hilar neurons exclusively (Fig. 3.1 A2, A3), as shown in previous studies (Berger et al., 1980; Laurberg and Sørensen, 1981). Larger FG injections that were confirmed to encompass the dentate gyrus as well as areas CA3 and CA1 (Fig. 3.1 B1) produced more extensive contralateral FG-labeling, which included prominent FG labeling of CA3 pyramidal cell somata and their surrounding dendrites (Figs. 3.1 B2, B3; 3.2 A). It should be noted that the FG staining of the hilar neuropil was darker after large FG injections (Fig. 3.1 B2) than after smaller injections (Fig. 3.1 B1). This is apparently due to the presence of detectable FG-LI in the dendrites of hilar neurons and CA3c pyramidal cells, and is not non-specific or "background" staining.

FG immunocytochemistry revealed that neurons exhibiting FG-LI also included dentate gyrus neurons of the granule cell layer and hilar region (Fig. 3.1 B3). FG-immunopositive neurons of the granule cell layer exhibited the pyramidal-shaped somata and ascending dendritic features of dentate basket cells (arrows in Fig. 3.1 B3), whereas granule cells and interneurons of the dentate molecular layer were never observed to exhibit detectable FG-LI. In the hilus, FG-positive cells exhibited a variety of morphological features (Fig. 3.1 B3). In animals in which FG injection inadvertently involved small injections encompassing both the dentate gyrus and area CA3 (n = 5), contralateral FG-labeled cells included presumed hilar mossy cells and CA3 pyramidal cells. Dentate basket cells and other presumed interneurons were not FG-labeled by small injections of FG contralaterally.
FG-labeling of dentate gyrus interneurons in the dentate granule cell layer and hilus was detected by evaluating both native FG fluorescence and FG-immunoreactivity. FG-like fluorescence in presumed interneurons of the granule cell layer and hilus was not observed in sections from naive animals perfusion-fixed by identical methods, and FG-LI was not detected in sections in which the primary FG antiserum was omitted. Thus, the FG fluorescence and FG-LI detected in interneurons after contralateral FG injection was not due to non-specific autofluorescence or artifactual immunocytochemical staining.

**Longitudinal distribution of Fluoro-Gold labeling after dorsal hippocampal injection**

An analysis of sections throughout the septo-temporal axis after FG injection into the contralateral dorsal hippocampus revealed topographic distributions of different cell types (Fig. 3.2). Homotopic to the injection site (Fig. 3.2 A), FG-labeled cells included all of the cells described above (dentate granule cell layer interneurons, hilar neurons, and CA3 pyramidal cells). Sections of the most anterior hippocampus also exhibited FG-labeling of CA1 pyramidal cells, as described by Swanson et al. (1981), but few area CA1 interneurons. Progressively ventral sections (Fig. 3.2 B-F) consistently exhibited fewer FG-positive interneurons in the granule cell layer, and less intense FG-LI in CA3 pyramidal cells. In the most ventral sections, which were cut in the horizontal (Fig. 3.2 D-F), rather than the coronal plane (Fig. 3.2 A-C), only hilar cells were FG-positive. The relatively homotopic labeling of dentate interneurons was a consistent finding in ten rats in which FG was injected into the dorsal hippocampus and the contralateral granule cell layer exhibited FG-labeled...
Figure 3.2. Septo-temporal distribution of Fluoro-Gold (FG)-LI after FG injection into the contralateral dorsal hippocampus. A-F: sequential sections of the dentate gyrus proceeding in the ventral direction. A: a coronal section homotopic to the contralateral FG injection site. B,C: Coronal sections progressively farther along the longitudinal axis. D-F: Horizontal hippocampal sections of the same hippocampus proceeding in the ventral direction. Note the presence of FG-immunopositive, pyramidal-shaped non-granule cells of the dentate granule cell layer in the homotopic section shown in A, and the absence of similarly labeled cells in D-F. Also note decreasing intensity of FG-LI in CA3 pyramidal cells in the ventral direction. Despite the progressive loss of CA3 pyramidal cell labeling, dentate hilar neurons remain labeled at the farthest ventral sites. Survival: 11 days after FG injection. Magnification: 62X in all panels. Calibration bar = 200 µm in all panels.
interneurons (pyramidal-shaped neurons with ascending dendrites).

A consistent and unexpected finding was that despite an extensive labeling of dentate interneurons lying at the base of the granule cell layer, interneurons of the adjacent dentate molecular layer were never labeled in hundreds of sections that exhibited extensive FG labeling of other dentate gyrus non-principal cells. In addition, despite extensive labeling of CA3 pyramidal cells, interneurons in the stratum oriens and radiatum of areas CA3 and CA1 were rarely seen to be FG-labeled. Figure 3.3 shows a comparison of adjacent sections from an FG-injected animal that were immunostained for the neuronal marker NeuN (Mullen et al., 1992), or for FG. The lack of FG-labeled dentate molecular layer cells is apparent in Figure 3.3 A2, and the rare labeling of area CA3 interneurons is shown in Figure 3.3 B2. In area CA1, the many NeuN-positive interneurons present in strata radiatum and lacunosum-moleculare (Fig. 3.3 C1) were consistently devoid of FG-LI, although a few FG-labeled cells were occasionally detected in stratum oriens (arrows in Fig. 3.3 C2). Thus, even large injections of FG confirmed to encompass all hippocampal regions resulted in contralateral FG labeling that was mainly limited to non-principal cells of the dentate granule cell layer, a variety of dentate hilar cells, and CA3 pyramidal cells.

The possibility that interneurons of the dentate molecular layer and hippocampus proper might be consistently FG-negative because of an inability to transport FG was addressed by evaluating sections on the FG-injected side that were adjacent to the injection site. On the FG-injected side, FG-labeled interneurons were observed in all dendritic regions, including the dentate molecular layer, stratum radiatum, and stratum oriens (Figure 3.4 A).
Figure 3.3. Selective Fluoro-Gold (FG) labeling of dentate gyrus non-principal cells after contralateral FG injection. A1-C1: NeuN-LI (a neuronal marker protein) in the hippocampus of a naive rat. Note numerous NeuN-positive interneurons outside the principal cell layers of all hippocampal subregions. A2-C2: FG-LI in the hippocampus contralateral and homotopic to the FG injection site. Note that FG-labeled cells included dentate hilar neurons (h; A2), presumed dentate basket cells (arrows in A2) in the granule cell layer (sg), and CA3 pyramidal cells (sp in A2 and B2). Note also that with rare exceptions (arrows in B2 and C2), NeuN-positive interneurons of the dentate molecular layer (sm in A1), and virtually all non-principal cells in the hippocampus proper were conspicuously FG-negative. Survival: 5 days after FG injection. Magnification: 62X in all panels. Calibration bar = 200μm in all panels.
Figure 3.4. Demonstration that all hippocampal neuron populations are capable of transporting Fluoro-Gold (FG). A: a coronal hippocampal section from the FG-injected side approximately 0.4 mm from the outer edge of the FG injection site. Note that dentate granule cells (sg), CA1 pyramidal cells (sp), and some interneurons in all strata (arrows) exhibit FG-LI. B: the contralateral hippocampus from the same section of the anterior dorsal hippocampus showing that despite FG-labeling of presumed dentate basket cells, hilar neurons, CA3 pyramidal cells, and a small number of CA1 pyramidal cells, interneurons of the dentate molecular layer and all strata of the hippocampus proper only rarely exhibited detectable FG-LI (arrow). Survival: 5 days after FG injection. Magnification: 62X in both panels. Calibration bar = 200 μm in both panels.
In addition, CA1 pyramidal cells and granule cells were FG-labeled in the section shown in Figure 3.4 A, which was approximately 400 µm from the outer edge of the injection zone. On the contralateral side of the same brain section, despite extensive labeling of the dentate gyrus and the CA3 pyramidal cell layer, and occasional FG-positive cells in the CA1 pyramidal cell layer, all interneurons of the dentate molecular layer and all but one interneuron in the CA1-CA3 dendritic subfields were unequivocally FG-negative (Figure 3.4 B).

"Control" Fluoro-Gold injections

Injections of FG into the ventral hippocampus, neocortex, or septum addressed several issues of interpretation. Dentate gyrus interneurons could theoretically be labeled after contralateral hippocampal injection via FG uptake from the extracellular fluid, via septal neurons that may innervate both hippocampi, or from the neocortex above the hippocampus, which was frequently FG-labeled minimally after insertion and removal of the injection electrode. Septal FG injections, which have been shown to label hippocampal interneurons (Toth and Freund, 1992), addressed the ability of hippocampal interneurons to transport FG from known projection sites.

Ventral hippocampal injection of Fluoro-Gold

FG injections into the ventral hippocampus that were confirmed to encompass all hippocampal fields produced homotopic FG labeling (Fig. 3.5) that was much less extensive
Figure 3.5. Retrograde transport of Fluoro-Gold (FG)-LI after FG injection into the ventral hippocampus. A: a horizontal section showing the FG injection site (arrow) and the contralateral homotopic FG-labeling. B: homotopic labeling at higher magnification contralateral to the FG injection site. C and D: less extensive FG-labeling in the dentate hilus and CA3c pyramidal cell layer only ~0.2 mm and ~0.6 mm, respectively, from the homotopic location. Note that the longitudinal contralateral labeling is much less extensive after ventral hippocampal FG injection than after dorsal hippocampal injection shown in Figure 2. Also note the absence of FG-labeled interneurons of the granule cell layer after ventral hippocampal injection. Survival: 8 days after FG injection. Magnification: 6.2X in A; 62X in B-D. Calibration bar = 2.0 mm in A; 200 µm in B-D.
than the labeling observed after dorsal hippocampal injection (Figs. 3.1-3.3). In 5 animals injected in the ventral hippocampus with 30-50 nl of FG solution, with all animals exhibiting areas of FG deposition as large as that shown in Figure 3.1 B1, contralateral FG-LI was detected in relatively few dentate hilar neurons and CA3 pyramidal cells at the homotopic site (Fig. 3.5). Progressively rostral (septal) sections exhibited fewer FG-positive cells (Fig. 3.5 C, D), even in horizontal sections relatively close (approximately 0.5-1 mm) to the homotopic sections. Dorsal hippocampal sections exhibited no detectable FG-positive cells, even after extensive contralateral ventral hippocampal injections. Thus, the rostro-caudal (longitudinal) distribution of FG-labeled cells was minimal after ventral hippocampal injection compared to the pattern observed after dorsal hippocampal injection. Our observation that the commissural projection to the ventral hippocampus is less dense than that to the more rostral hippocampus is consistent with the observation that labeling of the dorsal hippocampus with an anterograde tracer produced minimal labeling of the contralateral ventral hippocampus (Gottlieb and Cowan, 1973).

**Neocortical injection of Fluoro-Gold**

After injection of 30-50 nl of FG solution into the neocortex above the hippocampus, ipsilateral and contralateral FG labeling was prominent in the homotopic neocortex, thalamic neurons (Fig. 3.6 A), and other nuclei (e.g. periaqueductal gray, hypothalamus, and associational neocortex). The hippocampus on the injected side, which was between the FG-injected neocortex and the retrogradely labeled thalamus, exhibited no detectable FG-LI.
Figure 3.6. Retrograde transport of Fluoro-Gold (FG) after injection into the neocortex or septum. All FG injections into the hippocampus unavoidably involved some FG injection into the neocortex above the hippocampus. This necessitated "control" FG injections restricted to the neocortex to determine whether hippocampal labeling could be due to FG transport from the neocortex. Similarly, FG was injected into the septum to determine whether the pattern of hippocampal FG labeling after contralateral hippocampal FG injection could be due to uptake by septo-hippocampal fibers or non-specific FG uptake of extracellular FG unrelated to the hippocampal injection site. A: FG-LI 16 days after FG injection into the neocortex above the hippocampus. Note that FG labeling was restricted to the neocortex on the injected side. FG staining was prominent in the ipsilateral thalamus (arrows in A) and the homotopic neocortex contralaterally (arrows in B). Neither hippocampus exhibited detectable FG-LI after neocortical injection. C: FG-LI 7 days after FG injection into the medial septum. Note transport of FG into the horizontal limb of the diagonal band (arrows). Hippocampal FG labeling from this injection is shown in Figure 7, B1 and B2. Survival: 16 days in (A) and (B); 7 days in (C). Magnification: 13X in A and B; 6.2X in C. Calibration bar = 947μm in A and B; 2.0 mm in C.
Similarly, the contralateral hippocampus exhibited no FG-positive cells after contralateral neocortical injection (Fig. 3.6 B). Thus, hippocampal labeling after injection of FG into the contralateral dorsal hippocampus and neocortex was not the consequence of FG uptake from the neocortex above the hippocampus.

*Septal injection of Fluoro-Gold*

FG injections that primarily involved the medial septum bilaterally, but also the lateral septum, resulted in FG-labeling of neurons in both hippocampi, as well as in the hypothalamus (Fig. 3.6 C). Figure 3.7 contrasts the pattern of dorsal and ventral hippocampal FG-LI after FG injection into the contralateral dorsal hippocampus or into the septum. FG injection into the hippocampus produced contralateral FG-labeling in the dorsal hippocampus that included CA1-CA3 pyramidal cells and dentate hilar neurons (Fig. 3.7 A1), as described above. FG labeling in the more ventral hippocampus of the same animal exhibited FG-labeled hilar neurons and CA3 pyramidal cells, but not CA1 pyramidal cells or area CA1 interneurons (Fig. 3.7 A2). In contrast to the pattern of hippocampal labeling after contralateral dorsal hippocampal FG injection, injection of FG into the medial and lateral septum primarily labeled presumed interneurons of the stratum oriens in area CA1 and in all layers of area CA3 of the anterior hippocampus (Fig. 3.7 B1). Minimal FG labeling of hippocampal principal cells was observed in the most septal pole of the hippocampus after septal injection (Fig. 3.7 B1). Conversely, in the more ventral hippocampal sections of the same animal, FG-labeled hippocampal neurons included CA1 pyramidal cells and a variety
Figure 3.7. Comparison of hippocampal Fluoro-Gold (FG)-LI after FG injection into the dorsal hippocampus or septum. A1, B1: FG-LI in the anterior rat hippocampus after contralateral hippocampal or septal FG injection, respectively. Note that all anterior hippocampal pyramidal cells were FG-positive after contralateral dorsal hippocampal injection, whereas septal FG injection (shown in Figure 3.6,B) labeled non-principal cells exclusively in all anterior hippocampal subregions. A2,B2: FG-LI in the posterior hippocampus of the same animals showing that FG injection into the dorsal hippocampus labeled contralateral hilar neurons and CA3 pyramidal cells, but not CA1 pyramidal cells (B1), whereas septal FG injection labeled dentate hilar neurons, CA1 pyramidal cells, and putative interneurons in stratum oriens and stratum radiatum (arrows in B2), but not CA3 pyramidal cells. Thus, retrograde transport of FG by hippocampal neurons after FG injection into the hippocampus is site specific. In addition, CA1 pyramidal cells and interneurons of the hippocampus proper are capable of transporting FG, indicating that the failure of interneurons in areas CA1-CA3 to transport FG after FG injection into the contralateral hippocampus is not due to an inability to do so. Survival: 5 days after FG injection in (A), 7 days in (B). Magnification: 25X in all panels. Calibration bar = 500 μm in all panels.
of interneurons in strata oriens and radiatum of area CA1, in area CA3, and interneurons in
the hilus of the dentate gyrus (Fig. 3.7 B2). This pattern of retrograde hippocampal labeling
after septal injection is consistent with the known projections of hippocampal neurons to the
septal nuclei (Swanson and Cowan, 1977; 1979; Alonso and Köhler, 1982; Risold and
Swanson, 1997).

The dorsal hippocampal neurons labeled by FG injection into the septum exhibited
the location and morphological characteristics of somatostatin (SS)-positive interneurons
(Bakst et al., 1986; Sloviter and Nilaver, 1987), whereas FG-labeled cells after dorsal
hippocampal FG injection included CA3 pyramidal cells and presumed hilar mossy cells
(Fig. 3.8 A, B). Therefore, fluorescence co-localization methods were used to determine
whether the hippocampal interneurons labeled by septal FG injection were SS-positive
interneurons. This was done mainly to confirm that interneurons that are FG-negative after
contralateral hippocampal FG injection are nonetheless capable of transporting FG from a
distant injection site. In six hippocampal sections from an animal that received a septal FG
injection, at least 131 of 140 FG-positive interneurons in the dentate hilus, stratum oriens of
area CA1, and the strata oriens, radiatum, and lucidum of area CA3 were confirmed to be
unequivocally SS-immunoreactive (Fig. 3.8 B1-C2). Unlike the SS-positive dentate hilar
cells labeled by contralateral hippocampal injection, which were usually less intensely FG-
fluorescent (see below), SS-positive interneurons were intensely FG-fluorescent after
identical injection into the septum. The specificity of FG/SS co-localization in hippocampal
interneurons after septal FG injection was confirmed in the same brain sections by the
Figure 3.8. Fluorescence co-localization of Fluoro-Gold (FG) and somatostatin (SS)-LI in dentate hilar neurons following FG injection into the medial septum. A1, A2: FG-LI in the dentate gyrus 5 days after FG injection into the dorsal hippocampus (A1) or 8 days after FG injection into the septum (A2). Note that septal injection labels a smaller number of hilar neurons than hippocampal FG injection. B1, C1: FG-LI (green) in the dentate hilar region after septal FG injection. B2, C2: co-localized SS-LI (red) in the same sections indicating that virtually all FG-labeled neurons after septal FG injection are SS-positive hilar interneurons (arrows). Magnification: 124X in A1-B2; 248X in C1,C2. Calibration bar = 100 μm in A1-B2; 50 μm in C1,C2.
observation that intensely SS-immunoreactive (red) cells of the periventricular hypothalamic nucleus were entirely FG-negative (no native or green fluorescence), and intensely FG-immunoreactive (native and green fluorescence) cells of the lateral hypothalamus were entirely SS-negative (no red fluorescence). Thus, CA1 pyramidal cells and area CA1-CA3 interneurons of all strata clearly have the capacity to transport FG from a distant injection site, and to appear brightly FG-fluorescent.

**Immunocytochemical identification of retrogradely labeled hippocampal principal cells and interneurons**

Several neuronal markers were co-localized with FG to identify the hippocampal interneurons that were FG-immunopositive after injection of FG into the contralateral dorsal hippocampus. These studies were performed in twelve animals exhibiting FG labeling of various degrees. The semi-quantitative results are presented in Table 3.1. It should be emphasized that the quantification of immunocytochemical results is inherently problematic, and underestimates the degree of co-localization, as discussed in detail previously (Sloviter et al., 2001). Therefore, the quantitative data should be regarded only as estimates of the minimum degree of co-localization. That is, a statement that 82% of cells, for example, were found to be FG-positive means that at least 82% were immunoreactive, and not that 18% were confirmed to be immunonegative.
Table 3.1. Percentage of dentate interneuron subpopulations containing retrogradely transported Fluoro-Gold (FG) in the dorsal and ventral hippocampus. Quantitative co-localization analysis of FG and somatostatin (SS), or of FG and calretinin (CR) included the area between the two blades of the granule cell layer, but excluded cells in the CA3c pyramidal cell layer. Therefore, the analysis included SS- and CR-positive hilar cells and adjacent interneurons intermingled with the dendrites of CA3c pyramidal cells. Co-localization of FG and parvalbumin (PV) was limited to PV-positive non-principal cells of the dentate granule cell layer.
Co-localization of Fluoro-Gold and the glutamate receptor-2 subunit

FG-LI in the contralateral hippocampus was co-localized first with the glutamate receptor-2 (GluR2) subunit because most FG-positive cells exhibited the locations and morphological features of hilar mossy cells or CA3 pyramidal cells, which express the GluR2 receptor subunit constitutively (Leranth et al., 1996; He et al., 1998; Sloviter et al., 2001). Because both the anti-FG antiserum and the highest quality anti-GluR2 antiserum available to us were raised in rabbits, native FG fluorescence, which bleeds through to green wavelengths, was co-localized with GluR2 using a secondary antibody emitting red fluorescence. As expected, most brightly FG-fluorescent cells of the CA3 pyramidal cell layer and dentate hilus (Fig. 3.9 A1) were also GluR2-positive (Fig. 3.9 A2). Frequently, however, less intensely FG-fluorescent neurons, usually exhibiting the locations and morphological features of interneurons, appeared only weakly GluR2-immunoreactive or were devoid of detectable GluR2-LI, consistent with the observations that hippocampal inhibitory interneurons express minimal GluR2 (Leranth et al., 1996; He et al., 1998; Sloviter et al., 2001). In animals in which FG injection inadvertently involved small injections (n = 5), which produced limited contralateral labeling (Fig. 3.1 A2, A3), virtually all of the relatively small population of FG-labeled cells were GluR2-positive, suggesting that when the tissue concentration of injected FG is relatively low, only the cells with the most dense axonal projections to the contralateral hippocampus, i.e. dentate hilar mossy cells and CA3 pyramidal cells, transport a detectable concentration of FG.
Figure 3.9. Immunocytochemical identification of Fluoro-Gold (FG)-containing neurons of the rat dentate gyrus after homotopic contralateral FG injection. A1,A2: native FG fluorescence and glutamate receptor 2 subunit (GluR2-LI), respectively. Note that most brightly fluorescent FG-positive hilar neurons (arrows) are also GluR2-immunoreactive. B1,B2: co-localization of FG- and somatostatin (SS)-LI, respectively, showing that hilar neurons exhibiting diffuse FG-LI are SS-immunopositive in B2. Note that the brightly fluorescent hilar neurons in B1 (asterisks) are SS-negative, demonstrating that bright green fluorescence (B1) does not bleed through at the red wavelengths (B2). Survival: 21 days after FG injection. Magnification: 62X in A1,A2; 124X in B1,B2. Calibration bar = 200 μm in A1, A2; 100 μm in B1,B2.
Co-localization of Fluoro-Gold and somatostatin

FG and SS were co-localized using antisera of different species, which allowed the use of both red- and green-fluorescence displaying secondary antibodies generated in different species. Anti-FG immunostaining revealed that FG-positive hilar cells approximately homotopic to the FG injection site included intensely immunoreactive cells exhibiting FG-positive cytoplasmic granules, and less intensely immunoreactive cells exhibiting diffuse cytoplasmic fluorescence, as reported previously (Goodman and Sloviter, 1992). Co-localization of FG with SS-LI revealed that the most brightly fluorescent hilar cells containing FG-positive cytoplasmic granules (Fig. 3.9 B1 and B2) were SS-immunonegative, whereas the less brightly fluorescent cells exhibiting diffuse FG staining were often SS-positive (Fig. 3.9 B1 and B2). In four animals in which FG injection produced extensive contralateral FG-labeling, the average frequency of FG/SS co-localization was high (51-81%; Table 3.1). In the most extensively FG-labeled hippocampi, virtually all (as many as 96%) hilar SS-positive cells exhibited FG-LI (Table 3.1; animals 3 and 4). Conversely, in animals exhibiting less extensive labeling, in which case most contralateral FG-labeled cells were GluR2-positive, the average frequency of FG/SS co-localization was relatively low (3-20%; Table 3.1; rats 5 and 10). As described above, the presence of FG-positive cytoplasmic granules in brightly fluorescent cells suggests that the cells that transport high concentrations of FG package it intracellularly (Schmued et al., 1989), whereas lower concentrations of intracellular FG remain diffusely distributed throughout the cytoplasm. Although it might be concluded that different cell types may handle cytoplasmic FG
differently, this was not the case because some brightly FG- and SS-positive cells contained FG-positive granules after contralateral hippocampal injection (Fig. 3.9 B1), and most FG-labeled, SS-positive interneurons after septal injection exhibited bright FG fluorescence and FG-positive cytoplasmic granules (Fig. 3.8 C1). Thus, the presence of FG-positive cytoplasmic granules may reflect lysosomal packaging of high concentrations of FG (Schmued et al., 1989), which in turn may reflect a relatively dense axonal projection to the injection site.

Co-localization of Fluoro-Gold with parvalbumin and GABA

PV-positive neurons, which include basket cells and chandelier cells of both the inner and outer aspects of the granule cell layer (Soriano et al., 1990; Martínez et al., 1996), were found to exhibit both FG- and PV-LI approximately homotopic to the contralateral FG injection site, as previously described (Goodman and Sloviter, 1992; Fig. 3.10 A1). However, immediately adjacent interneurons of the dentate molecular layer were consistently devoid of detectable FG in the same sections. PV-positive hilar neurons were also FG-positive (Fig. 3.10 B1, B2), which were not detected previously using less sensitive methods (Goodman and Sloviter, 1992). In the most intensely FG-labeled sections, as many as 84% of PV-positive cells of the granule cell layer and hilus combined were also FG-positive, although the co-localization rates varied between animals. For PV-positive neurons with somata within the granule cell layer, as many as 38% were judged to be clearly FG-positive (Table 3.1).
Figure 3.10. Fluorescence co-localization of parvalbumin (PV)-, GABA-, and Fluoro-Gold (FG)-LI in the rat dentate gyrus after homotopic contralateral FG injection. A1, A2: PV- and FG-LI, respectively, showing that PV-positive interneurons of the granule cell layer (arrows) are FG-positive. B1, B2: PV- and FG-LI, respectively, showing labeled PV-positive interneurons of the granule cell layer and hilus (arrows) in a different animal. C1, C2: GABA- and FG-LI, respectively, showing that two GABA-immunoreactive neurons at the base of the granule cell layer (arrows) are FG-positive. A GABA-negative cell in the hilus (arrowhead) is marked for comparison. Survival: 13 days in (A), 24 days in (B), and 23 days in (C). Magnification: 248X in all panels. Calibration bar = 50 μm in all panels.
FG- and PV-immunopositive cells of the dentate granule cell layer were confirmed to be GABA-immunoreactive in a limited number of experiments. The scope of this analysis was limited for two reasons. First, the glutaraldehyde needed to preserve GABA immunoreactivity also decreases FG- and peptide immunoreactivity significantly. This necessitated using a low concentration of glutaraldehyde for this experiment, which produced suboptimal preservation of GABA-LI. Second, GABA-LI was primarily restricted to the section surface, where relatively few FG-positive basket cells lie cut and exposed on the single stainable surface of any section. Nonetheless, Figure 3.10 C shows two FG-labeled non-principal cells of the granule cell layer and hilus, both of which are GABA-immunoreactive (arrows in Fig. 3.10 C1, C2).

**Co-localization of Fluoro-Gold and calretinin**

Although the frequency of co-localization of FG and calretinin in dentate hilar neurons was found to be higher than that reported in the study by Miettinen et al. (1992), FG was detected in a minority of calretinin (CR)-positive cells (Table 3.1), even in the animals exhibiting a high frequency of FG- and PV- or SS co-localization. FG- and calretinin-positive cells were only observed in the dentate hilus, and only in animals with extensive labeling of SS- and PV-positive cells. Whether only a subset of CR-positive cells have commissural projections, or all CR-positive cells have commissural projections but transport an amount of FG that was detected in only a fraction of CR-positive cells cannot be differentiated by these methods.
Lack of FG labeling of interneurons in the ventral hippocampus

The co-localization profile changed dramatically in ventral locations that were longitudinally distant from the dorsal hippocampal FG injection site. Although FG injection labeled contralateral granule cell layer interneurons homotopically, FG-positive cells in the ventral hippocampus were restricted to the hilus and area CA3c (Fig. 3.1 D-F; Fig. 3.11 A1, B1, C1). Virtually all of these FG-labeled neurons in the horizontal sections were immunoreactive for GluR2 (Fig. 3.11 A1, A2), and immunonegative for SS-, PV-, or calretinin-LI (Fig. 3.11 B, C; Table 3.1).

Granule cell innervation of granule cell layer but not dentate molecular layer interneurons

The results described above suggested to us that virtually all cells labeled by FG injection into the contralateral hippocampus shared the property of being direct targets of dentate granule cells. This correlation was most striking when interneurons of the granule cell layer were compared to interneurons of the adjacent molecular layer. The former were often FG-positive, whereas the latter were consistently FG-negative (Fig. 3.12 A). To address this correlation, sections from normal rat brains produced in previous studies involving GABA- and 67-kDa isoform of glutamic acid decarboxylase (GAD67)-immunoreactive neurons of the dentate gyrus (Sloviter et al., 1996), and the innervation of dentate interneurons by mossy fiber axon collaterals (Sloviter, 1992) were examined. Figure 3.12 A shows the pattern of extensive FG labeling of granule cell layer interneurons contrasted with the normal distribution of PV-(Fig. 3.12 B), GAD67- (Fig. 13C), and GABA-
Figure 3.11. Fluorescence co-localization of Fluoro-Gold (FG)-, glutamate receptor 2 subunit (GluR2)-, parvalbumin (PV)-, and somatostatin (SS)-LI in the ventral dentate gyrus after contralateral FG injection into the dorsal hippocampus. A1,A2: native FG fluorescence and GluR2-LI, respectively, in horizontal sections of the ventral hippocampus 11 days after FG injection. Note that virtually all FG-positive cells are restricted to the dentate hilus, and are GluR2-positive (arrows on three representative cells). B1,B2: FG and SS-LI, respectively, in an adjacent section from the same animal. Several FG-positive cells are arrowed in B1. Note that unlike the homotopic dorsal hippocampus in this animal, the heterotopic ventral hippocampus exhibits no FG-labeled SS-positive neurons. C1,C2: FG and PV-LI in an adjacent section from the same animal. Note that FG-labeled cells in C1 (arrows) are PV-negative, and that no PV-positive cells are FG-labeled. Survival: 11 days in (A), 21 days in (B) and (C). Magnification: 124X in all panels. Calibration bar = 100 μm in all panels.
Figure 3.12. Granule cell innervation of interneurons of the granule cell layer, but not the molecular layer, of the normal rat. A: the Fluoro-Gold (FG)-labeled dentate gyrus after contralateral FG injection into the dorsal hippocampus. Same animal as shown in Figure 3.2. Note that the dentate molecular layer (sm) is devoid of detectable FG-LI. B-D: parvalbumin (PV)-, GAD67-, and GABA-like immunoreactivity (LI), respectively, in the same region of a normal hippocampus. Note that FG-labeled interneurons in (A) are those of the granule cell layer, and do not include the PV-positive interneurons of the molecular layer (arrows in B), or the numerous GAD67- and GABA-immunoreactive neurons of sm. E-G: Timm-stained sections of normal rat hippocampi showing that Timm-stained granule cell axon collaterals outline interneurons of the granule cell layer (arrows), but do not extend to surround molecular layer interneurons. Note that the section in (F) was counterstained with cresyl violet to indicate that pyramidal-shaped basket cells (arrows) of the granule cell layer, covered with Timm-stained granule cell axon collaterals, are surrounded by uninnervated granule cells. Survival: 5 days in (A). Magnification: 124X in A-D; 248X in E-G. Calibration bar in (F) = 100 µm in A-D; 50µm in E-G.
immunopositive neurons (Fig. 3.12 D) of both the dentate granule cell- and molecular layers. Figure 3.12, E-G show the distribution of Timm-stained granule cell axon collaterals in the granule cell- and inner molecular layers (Laurberg and Zimmer, 1981; Ribak and Peterson, 1991; Sloviter, 1992). Timm-stained fibers surround and outline pyramidal-shaped basket cells with ascending dendrites, and other non-principal cells of the granule cell layer, but do not similarly innervate or outline interneurons of the molecular layer (Fig. 3.12).
SUMMARY

The principal original finding of this chapter is that improved methods for detecting retrogradely transported Fluoro-Gold (FG) have revealed that many inhibitory interneurons of the rat dentate gyrus are among the hippocampal neurons that form commissural projections to the contralateral hippocampus. The results indicate that a significant proportion of PV-positive basket cells, which form the axo-somatic innervation of the granule cell layer (Kosaka et al., 1987; Sloviter, 1989; Freund and Buzsáki, 1996), and virtually all dentate hilar SS-positive interneurons, which innervate the dentate molecular layer (Bakst et al., 1986; Sloviter and Nilaver, 1987; Freund and Buzsáki, 1996), are FG-labeled after contralateral hippocampal injection. This finding contrasts with the observation that interneurons in the dentate molecular layer and the dendritic regions of the hippocampus proper were consistently and conspicuously FG-negative, even in sections exhibiting extensive labeling of dentate interneurons and virtually all CA3 pyramidal cells.

The second original finding is that after focal injection of FG into the dorsal hippocampus, the FG-labeled excitatory dentate mossy cells and CA3 pyramidal cells were much more widely distributed along the longitudinal axis of the hippocampus than the FG-labeled inhibitory interneurons. The heterotopic distribution of excitatory commissural projections (Gottlieb and Cowan, 1973; Amaral and Witter, 1989; Deller et al., 1995; 1996), and the homotopic distribution of fibers from inhibitory interneurons is consistent with the organizing principle of parallel commissural and associational systems (Berger et al., 1980; Swanson et al., 1981; Deller et al., 1996). A schematic diagram (Fig. 3.13) illustrates the
Figure 3.13. Schematic diagram of the commissural projections of hippocampal principal cells and dentate interneurons. This simplified diagram shows that after injection of Fluoro-Gold (FG) into the dorsal hippocampus, contralateral dentate hilar mossy cells (MC) are FG-immunopositive along the longitudinal axis of the hippocampus. In the most temporal region containing FG-labeled cells, hilar mossy cells are the only labeled cells. CA3 pyramidal cells (PC) are similary FG-labeled far along the longitudinal axis, but do not extend as far in the temporal direction as the mossy cells. Parvalbumin-positive dentate basket cells (BC), somatostatin-positive hilar interneurons (SS), and calretinin-positive cells (not shown) exhibited FG-labeling primarily at relatively homotopic levels.
heterotopic projections of hilar mossy cells and CA3 pyramidal cells, and the relatively homotopic projections of dentate interneurons.

The third original finding, which is speculative in nature, is our inference that the pattern of retrogradely labeled FG-positive cells appears to represent a labeling of neurons, both inhibitory and excitatory, that share the property of being direct postsynaptic targets of dentate granule cells. In this regard, we were influenced by the finding that PV-positive basket cells of the dentate granule cell layer were clearly FG-positive, but that the immediately adjacent interneurons of the dentate molecular layer were consistently devoid of detectable FG in hundreds of sections evaluated. One significant difference between these two inhibitory interneuron subpopulations is that FG-positive basket cells possess dendrites within the hilus and granule cell layer that are the targets of granule cell axon collaterals (Laurberg and Zimmer, 1981; Ribak and Peterson, 1991; Sloviter, 1992; Acsády et al., 1998). Conversely, dentate molecular layer interneurons are not innervated by granule cell axon collaterals.

Consistent with the observation that virtually all FG-labeled neurons are direct targets of dentate granule cells was the paucity of FG-labeled interneurons of the contralateral hippocampal areas CA1-CA3, despite their demonstrated ability to transport FG from the septum. Thus, we hypothesize a principle of hippocampal organization according to which the hippocampal commissural projections primarily serve to convey granule cell information. According to this view, focal discharge of dentate granule cells, which innervate their exclusively ipsilateral target cells in a lamellar pattern (Blackstad et al., 1970; Andersen et
al., 1971; Gaarskjaer, 1978; 1981; Frotscher et al., 1994), may produce heterotopic excitation of the targets of hilar mossy cells and CA3 pyramidal cells, and homotopic inhibition of the contralateral granule cell layer.

Consistent with this view, Deller and colleagues (1995) found that PHAL injections restricted to the dentate gyrus produced contralateral fiber staining in the inner molecular layer (innervated by mossy cells) that was longitudinally extensive, whereas PHAL stained fibers to the outer molecular layer (innervated by SS-positive hilar interneurons) were restricted to the “lamella” contralateral to the PHAL injection site. Thus, the present results involving retrograde transport, taken together with the results of Deller and colleagues (1995; 1996) involving anterograde transport, suggest that dentate gyrus interneurons may serve a previously underappreciated role in producing relatively homotopic “lamellar” inhibition. This hypothetical homotopic inhibition might serve to silence the corresponding “twin” granule cell lamella in the contralateral hippocampus, thereby lateralizing and amplifying the influence of the initiating granule cell discharge.

Although nearly all FG-labeled cells are granule cell targets, not all granule cell target cells were labeled by contralateral FG injection. In this regard, SS-positive interneurons of the stratum lucidum, which were intensely labeled by FG injection into the septum, were not labeled by contralateral hippocampal FG injection, although some hilar SS neurons were labeled by FG injections into either site. This pattern of FG-labeling suggests the possibility that some interneurons that are targets of the mossy fibers (e.g. hilar SS-positive cells), may convey granule cell information to the septum and the contralateral hippocampus, whereas
other target cells, e.g. SS-positive cells and other interneurons of the stratum lucidum (Gulyás et al., 1992; Soriano and Frotscher, 1993; Spruston et al., 1997; Acsády et al., 1998) may convey lamellar information derived from granule cells both locally and to the septum or other target regions, but not to the contralateral hippocampus. Clearly, the full axonal projections of different hippocampal interneuron subpopulations remain to be determined in a manner similar to that done for CA3 pyramidal cells (Swanson et al., 1980; 1981). Thus, further functional speculation is not warranted on the basis of the limited anatomical findings presented in this chapter.
CHAPTER 4: TRANSLAMELLAR INHIBITION STUDIES

Introduction

Despite decades of study, the nature of the three-dimensional functional organization of the mammalian hippocampus remains controversial. The lamellar hypothesis posits a system in which focal excitation is conveyed sequentially to target neurons within functionally separated slices or "lamellae" (Andersen et al., 1971; 2000; Moser and Moser, 1998; Hampson et al., 1999). The available physiological data suggest that the net effect of mossy cell discharges may be longitudinal, translamellar, inhibition rather than excitation. Although hilar mossy cells innervate granule cell dendritic spines (Buckmaster et al., 1996), direct excitation of mossy cells in vitro inhibits granule cells disynaptically via excitation of inhibitory interneurons (Scharfman, 1995). Even when slices were bathed in bicuculline, mossy cell discharges only weakly depolarized granule cells (Scharfman, 1995). These in vitro data are consistent with earlier in vivo studies showing that activation of excitatory commissural fibers had a net inhibitory effect on contralateral granule cells via excitation of contralateral inhibitory interneurons (Buzsáki and Eidelberg, 1982; Douglas et al., 1983). Thus, despite a numerically extensive innervation of granule cell dendritic spines, mossy cell excitation of inhibitory interneurons (Wenzel et al., 1997; Sloviter et al., 2003) may dominate functionally. Thus, excitatory associational projections may constitute a disassociative system that establishes lateral inhibition and lamellar functional segregation within the granule cell layer (Sloviter, 1994; Sloviter and Brisman, 1995).
Earlier studies have examined hippocampal surround inhibition at a distance of 1-1.5 mm (Sloviter and Brisman, 1995; Buckmaster and Schwartzkroin, 1995). However, at this distance, determining the circuitry involved in this inhibitory mechanism is difficult. Direct granule cell activation of inhibitory neurons could be responsible for the observed inhibitory response because the total septo-temporal distance traversed by granule cell axons is as far as 1.3mm in the adult rat (Sutula et al., 1998; Buckmaster and Dudek, 1999). Inhibitory interneurons also have longitudinal projections that traverse the 1-1.5 mm distance (Struble et al., 1978; Qiu and Han, 1995; Buckmaster and Schwartzkroin, 1995). In addition, the method of focal BMI application used in these studies results in passive diffusion of the GABA<sub>A</sub> antagonist, with a longitudinal spread of at least 200 µm from the bicuculline methiodide (BMI) electrode site (Sloviter and Brisman, 1995). In this chapter, the nature of the influence of granule cell discharges on distant granule cells located ~2.5-4.5 mm along the longitudinal axis is determined. This distance is important because mossy cell axons minimally innervate the ~1 mm radius around their somata, which inhibitory neurons preferentially target (Buckmaster et al., 1996), and because the associational projections of mossy cells preferentially target the dentate inner molecular layer beginning approximately 1.2 mm in the septal direction from an anterograde tracer injection (Amaral and Witter, 1989). Translamellar influences were assessed at distances that both: a) exclude those traversed by the mossy fibers, and; b) include those innervated by the longitudinal associational projections formed by excitatory hilar mossy cells.

One strategy used to understand the normal structure and function relationship in the
brain is to study network behavior when anatomical structures are altered by pathological conditions. Animal models of disease states provide a valuable tool to study the affects of experimentally controlled brain insults. Prolonged perforant path (PP) stimulation (Sloviter, 1987; 1991) and administration of the glutamate agonist kainate (KA) (Sloviter et al., 2003) are treatments that lesion dentate hilar neurons, providing an opportunity to study the functional consequences of associational pathway damage. To address whether vulnerable hilar neurons are possible mediators of the observed translamellar influences, the same network inhibition experiments described above were performed in animals with hilar cell loss produced by KA-induced status epilepticus or prolonged PP stimulation.

Translamellar influences in the normal rat dentate gyrus

In normal (n = 12) and saline-treated control rats (n = 4), paired-pulse stimulation of the perforant pathway (see drawing in Fig. 4.1) at 0.3 Hz and an interpulse interval of 60 ms evoked large-amplitude population spikes at the saline-filled recording electrode that exhibited potentiation, i.e. the amplitude of the second of two evoked population spikes was larger than the amplitude of the first population spike (Fig. 4.1 A2). While these potentials at the saline electrode site were continuously recorded, and without changing the stimulus parameters, the BMI-filled recording electrode was lowered into the granule cell layer ~2.5-4.5 mm along the longitudinal axis. Immediately after reaching the dorsal granule cell layer, and prior to any appreciable diffusion of BMI from the recording electrode tip, both the saline- and BMI-filled recording electrodes simultaneously recorded relatively normal
Figure 4.1. Translamellar inhibition in the normal rat dentate gyrus under urethane anesthesia. The influence of focal granule cell discharges evoked at a bicuculline methiodide (BMI)-filled recording electrode in response to slow (0.3 Hz) perforant pathway (PP) stimulation (PP stim site in the drawing above) was assessed simultaneously at a distant segment of the granule cell layer via a saline-filled recording electrode. A1,A2: Prior to passive leakage of BMI from the tip of a glass recording electrode, PP stimulation at 0.3 Hz simultaneously evoked single population spikes at both the BMI- (Al; pre = pre-diffusion of BMI) and saline-filled recording electrodes (A2), which were separated ~3.5 mm along the longitudinal hippocampal axis. As BMI diffused from the tip of the BMI-filled electrode, the same afferent stimuli evoked multiple population spikes (post = post-diffusion of BMI) at the BMI site (Bl) and simultaneously produced, at the saline electrode (B2), a selective inhibition of the 2nd evoked response (compare waveforms in the shaded box in A2-B2). Distant inhibition included suppression of both the negative-going population spike (asterisk) and the amplitude of the evoked field EPSP. Calibration bars: 10 mV and 10 msec in A1-B2.
evoked responses (Fig. 4.1 A1, A2). Within seconds, however, as BMI diffused from the recording electrode tip, the BMI-filled electrode began to record multiple population spikes (Fig. 4.1 B1), and the distant saline electrode simultaneously recorded selective suppression of the 2nd evoked population spike and field EPSP (fEPSP; Andersen et al., 1966) amplitudes (Fig. 4.1 B2; Fig. 4.2). Figure 4.2 shows a series of evoked responses in a normal animal in which BMI apparently diffused relatively slowly from the tip of the BMI-filled recording electrode. This series of evoked potentials illustrates the gradually progressive, selective inhibition of the distant second granule cell population spikes (right shaded box in Figure 4.2) caused by the first of two multiple discharges evoked at the BMI-filled recording electrode (left shaded box in Figure 4.2). The selective inhibition of the second evoked response at the saline-filled electrode was presumably initiated by the first evoked discharge at the BMI-filled electrode and required the interpulse interval to manifest its longitudinal influence on the second evoked response at the distant site. Figure 4.2 also demonstrates the reversibility of the effect that occurred reliably at the saline electrode site within minutes after the BMI-filled electrode was removed (Fig. 4.2, bottom traces). Focal granule cell discharges generated at the BMI electrode did not increase population spike amplitudes or the number of spikes recorded at the saline electrode in any animal tested (n = 16). Nor did they evoke any detectable positive-going field potentials indicative of longitudinal excitation that was in any way comparable to even weak perforant path stimulation. That is, if the second of two multiple discharges recorded at the BMI-filled electrode produced a synchronous depolarization or discharge of the distant granule cells being simultaneously
Figure 4.2. Progressive and reversible translamellar inhibition in the normal rat dentate gyrus. In this series of simultaneously recorded evoked responses to 0.3 Hz perforant pathway stimulation, diffusion of bicuculline methiodide (BMI) from one recording electrode produced gradually increasing responses (multiple granule cell population spikes) to the same slow afferent stimuli. Coincident with these focal discharges recorded at the BMI-filled electrode (left shaded box), responses recorded simultaneously exhibited selective and progressive suppression of the second population spike and the field EPSP amplitudes (right shaded box). This selective inhibition of the second of two evoked responses at the saline-filled electrode was presumably initiated by the first of two discharges at the BMI-filled electrode, which required the interpulse interval to manifest its longitudinal influence. Note also that neither of the two multiple granule cell discharges at the BMI electrode evoked any detectable excitatory response after the second response recorded at the saline electrode site (arrowed line in trace 7). That is, no distant longitudinal excitatory influences of focal granule cell discharges were detected. Granule cell responses to perforant path stimulation recorded during the 10 min period after removal of the BMI-filled electrode from the granule cell layer (left trace 8), showed the decline of translamellar inhibition at the saline electrode (right trace 8), apparently reflecting recovery from the influence of the distant BMI-induced discharges as BMI was cleared from the tissue. Calibration bars: 5 mV and 10 msec.
monitored at the saline electrode site, an evoked potential would presumably be detectable after the second evoked potential, during the period marked by the arrowed line in Figure 4.2, trace 7.

In a comparison of 2^n spike suppression in control animals at different distances between the BMI- and saline-filled electrodes (Table 4.1), no statistically significant difference in 2^n spike suppression was observed in 6 controls in which the mean distance was 4.03 mm (range: 3.60-4.89 mm) between the electrodes and 4 controls in which the mean distance was 2.79 mm (range: 2.52-3.24). In these two groups, 2^n spike suppression was 67.67 ± 4.24% in the larger separation group and 73.90 ± 10.13% in the smaller separation group. Thus, the magnitude of 2^n spike suppression in control animals was not significantly different over the range of distances evaluated.

C-Fos expression in normal rats after bicuculline methiodide induced focal discharges

C-Fos expression was assessed immunocytochemically to determine the extent of focal neuronal activation evoked at the BMI-filled recording electrode. BMI diffusion during slow (0.3 Hz) afferent stimulation consistently evoked c-Fos expression within a radius of ~400 μm from the electrode tip in all animals (Fig. 4.3 A; range of diameter of c-Fos activation: 500-1050 μm; mean: 798.8 μm± 78.2μm s.e.m.; n = 8). In addition to c-Fos-positive granule cells, glutamate receptor subunit 2 (GluR2)-positive dentate hilar neurons (presumed mossy cells; Leranth et al., 1996; Fig. 4.3 B), and somatostatin (SS)- and parvalbumin (PV)-positive inhibitory interneurons (Fig. 4.3 C, D) were consistently c-Fos
Table 4.1. Translamellar inhibition at different BMI and saline electrode tip separations in control animals. In a comparison of 2nd spike suppression in control animals at different distances between the BMI- and saline-filled electrodes, no statistically significant difference in 2nd spike suppression was observed in 6 controls in which the mean distance was 4.03 mm (range: 3.60-4.89 mm) between the electrodes and 4 controls in which the mean distance was 2.79 mm (range: 2.52-3.24). In these two groups, 2nd spike suppression was 67 ± 4.24% in the larger separation group and 73.90 ± 10.13% in the smaller separation group. The magnitude of 2nd spike suppression in control animals was not significantly (Mann-Whitney test) different over the range of distances evaluated.
Figure 4.3. C-Fos expression following BMI-induced granule cell disinhibition. A.: c-Fos immunoreactivity at the BMI site after 1 hr of BMI-induced granule cell discharges, showing the extent of granule cell activation (region between arrows; c-Fos in red; vesicular glutamate transporter 1 (vGluT1) in green for contrast). B: co-localization of c-Fos (red) and glutamate receptor subunit 2 (GluR2; green). C: c-Fos (red) and somatostatin (green). D: c-Fos (red) and parvalbumin (green). Note that c-Fos-expressing neurons include large GluR2-positive-hilar neurons (presumed mossy cells), and somatostatin- and parvalbumin-positive inhibitory interneurons. Rats were perfusion-fixed 1 hr after the end of 1 hr BMI diffusion. Scale bar: 200 μm in A; 50 μm in B-D. Magnification: 33X in A; 130X in B-D.
positive in the region surrounding the BMI electrode tip. C-Fos expression in the BMI diffusion zone was entirely dependent on afferent stimulation-induced granule cell discharges because identical placement of the BMI electrode for 1 hr, in the absence of afferent stimulation, produced no detectable c-Fos expression greater than that produced in local granule cells by placement of a saline-filled glass electrode (n = 3; data not shown). In these unstimulated control animals, an obstructed electrode lumen would not be detectable without delivery of afferent stimuli. Therefore, a single stimulus pair was delivered immediately before perfusion-fixation to confirm that BMI had been diffusing from the electrode tip and that afferent stimuli would have evoked focal granule cell discharges during the preceding one hour period had they been delivered.

C-Fos expression in dentate hilar neurons and presumed basket cells of the granule cell layer was consistently evident longitudinally, beyond the region of focal granule cell activation (Fig. 4.4 A1). That is, at relatively short longitudinal distances from the outer limit of c-Fos-positive granule cells (~300-500 μm), hilar GluR2-, SS-, and PV-positive neurons were c-Fos-positive. This presumably occurred as a result of mossy fiber activation of hilar neuron dendrites that extended into the mossy fiber axon projection zone from somata located slightly beyond this zone. C-Fos-immunostained sections at increasing longitudinal distances were systematically evaluated to determine whether focal granule cell discharges at the BMI-filled electrode evoked c-Fos expression in granule cells or non-principal cells farther along the longitudinal axis. Consistent with the lack of evidence of excitation of distant granule cells, there was observed no instance in which distant granule cells were
Figure 4.4. C-Fos expression beyond the region of focal granule cell activation evoked by bicuculline methiodide (BMI) diffusion. A1: in sections at a longitudinal distance of ~200-400 μm from the outer edge of the granule cell c-Fos activation zone, c-Fos-positive cells included hilar neurons (h) and presumed interneurons of the granule cell layer (sg). Note that the section shown in A1 was ~240 μm from the outer edge of the c-Fos-positive granule cell layer. A2: farther from the BMI electrode site (~3.6 mm longitudinally from the c-Fos-positive granule cell layer), c-Fos-positive cells included hilar neurons (h) and presumed basket cells of the granule cell layer (sg; arrows). B1, B2: immunocytochemical co-localization confirmed that c-Fos immunostained neurons (red) were primarily somatostatin (SS)- and parvalbumin (PV)-positive (green) interneurons (~2.2 mm longitudinally in B1 and ~2.5 mm longitudinally in B2). Thus, when BMI diffusion produced translamellar c-fos expression, the distantly activated neurons included presumed basket cells of the granule cell layer and hilar SS- and PV-positive hilar interneurons, but not distant dentate granule cells. Scale bar: 100 μm. Magnification: 97X.
c-Fos-positive after 1 hr of BMI-induced granule cell discharges. A separate group of normal animals (n = 11), in which the BMI electrode remained in place for 7 hr before perfusion-fixation, was used to increase the possibility of evoking c-Fos expression in distant target cells. Although evidence of c-Fos expression in distant SS- and PV-positive inhibitory interneurons was observed in a minority of animals (~2.5-3.9 mm longitudinally from the edge of the c-Fos-positive BMI activation zone), which was consistent with the observed distantly evoked granule cell inhibition (Fig. 4.4 A2, B1, B2), most animals exhibited no detectable c-Fos induction at the distant site of the saline-filled recording electrode. Thus, although distant c-Fos expression, whenever it was detected, was present only within inhibitory interneurons, this effect was observed in only 2 of 11 animals tested, and no other stimulation or duration parameters tested in pilot experiments (0.3-2.0 Hz; 1-20 hr duration) were found to produce this effect consistently.

Translamellar disinhibition in kainic acid-treated- and perforant path stimulated rats

Translamellar inhibition was assessed in KA-treated rats 3 days after all 14 animals had exhibited prolonged SE lasting at least 3 hr. This was done to determine whether prior hilar neuron loss would abolish BMI-induced translamellar inhibition. Analysis of each KA-treated animal, which was blinded in the sense that the extent of hilar neuron loss was not known during the electrophysiological assessment, revealed two distinct responses to BMI diffusion. In 10 of the 14 KA-treated rats, BMI-induced granule cell discharges evoked relatively normal suppression of both the population spike and the fEPSPs recorded at the
saline electrode (Type 1 response, Fig. 4.5 A4, Table 4.2). However, in 4 of the 14 identically KA-treated rats, BMI-induced granule cell discharges produced minimal or no distant inhibition at the saline-filled electrode, despite similar BMI-induced granule cell discharges (Type 2 response; Fig. 4.5B). Thus, the loss of translamellar inhibition in KA-treated rats appeared to be a relatively “all-or-none” phenomenon, with 10 KA-treated rats appearing similar to normal animals, and 4 identically KA-treated rats nearly devoid of translamellar inhibition.

Histological analysis (Fig. 4.6,4.7) revealed that, in KA Type 1 rats (translamellar inhibition preserved), the number of hilar neurons counted in sections at the BMI electrode site was 20% lower than controls (saline-treated controls: 62.8 ± 12.0 hilar neurons per section; n = 4; KA Type 1: 50.0 ± 3.5 neurons per section; range:10-38% fewer neurons; n=10; p > 0.05), which was not statistically significant. In contrast, KA Type 2 animals (translamellar inhibition abolished) exhibited an average hilar cell loss of 85%, which was significantly different from Type 1 rats and controls (9.5 ± 3.7 neurons per section; range: 68-94% fewer hilar neurons; n = 4; p < 0.05). Although the extent of hilar cell loss in the dorsal hippocampus was highly variable among identically-treated KA rats, extensive CA3 pyramidal cell layer damage, ventral hippocampal hilar cell loss, and temporal cortex damage were present in both KA groups. Thus, only extensive dorsal hippocampal hilar neuron loss was consistently associated with a failure of BMI-induced granule cell discharges to evoke translamellar granule cell inhibition (Figure 4.7). The close correlation between hilar neuron loss and translamellar disinhibition in KA-treated rats was also addressed in rats previously
Figure 4.5. Translamellar inhibition or disinhibition 3 days after kainic acid (KA)-induced status epilepticus or 24 hr intermittent perforant pathway (PP) stimulation. A: KA Type 1 response. Rats subsequently found to have an average of 20% fewer hilar neurons compared to controls (Fig. 4.7) exhibited intact translamellar inhibition of the evoked granule cell population spike (asterisk in A4; n = 10). B: KA Type 2 response. In rats subsequently found to have an average of 85% fewer hilar cells than controls (Fig. 4.7; n = 4), BMI-induced discharges (B3) evoked minimal translamellar inhibition (asterisk in B4). C: Similar failure of translamellar inhibition in PP-stimulated rats (asterisk in C4), which exhibited an average of 93% fewer hilar cells (range: 89-95%; n = 6) than controls (Fig. 4.7). Calibration bars: 10 mV and 10 ms.
<table>
<thead>
<tr>
<th></th>
<th>Spike amp before (mV)</th>
<th>Spike amp after (mV)</th>
<th>% Spike suppression</th>
<th>Distance between electrodes (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=4)</td>
<td>10.76 ± 2.59</td>
<td>2.62 ± 1.11</td>
<td>73.90 ± 10.13</td>
<td>2.79 ± 0.17</td>
</tr>
<tr>
<td>KA Type 1 (n=10)</td>
<td>12.51 ± 1.85</td>
<td>2.34 ± 0.47</td>
<td>79.54 ± 4.39</td>
<td>2.70 ± 0.13</td>
</tr>
<tr>
<td>KA Type 2 (n=4)</td>
<td>10.30 ± 2.98</td>
<td>9.84 ± 3.04</td>
<td>5.71 ± 2.77</td>
<td>2.52 ± 0.29</td>
</tr>
<tr>
<td>24hr PP Stim (n=6)</td>
<td>14.06 ± 0.57</td>
<td>13.78 ± 0.62</td>
<td>1.92 ± 1.86</td>
<td>2.64 ± 0.21</td>
</tr>
</tbody>
</table>

Table 4.2. Translamellar granule inhibition 3 days after kainic acid (KA)-induced status epilepticus or 24 hr intermittent perforant path stimulation. Significantly different from the control (*) and KA Type 1 group (+) means; * p < 0.05, ** p < 0.02, +++ p < 0.01, ++++ p < 0.001 (Mann-Whitney test).
Figure 4.6. Hilar neuron loss and c-Fos expression 3 days after kainic acid (KA)-induced status epilepticus or 24 hr intermittent perforant pathway (PP) stimulation. A1: NeuN immunoreactivity (a neuronal marker) in the dentate gyrus of a saline-treated control rat, illustrating the normal neuronal constituents of the dentate granule cell layer (stratum granulosum; sg), the hilus (h), and the adjacent end of the CA3c pyramidal cell layer (stratum pyramidale; sp). A2: c-Fos expression in the region of the BMI electrode, showing c-Fos-positive nuclei in cells of all dentate subregions. B1,B2: NeuN and c-Fos expression in a KA-treated rat that exhibited intact translamellar inhibition (Type 1 response). Note the persistence of NeuN- and c-Fos-immunoreactivity in the hilus, which in 10 KA Type 1 animals involved an average of 20% fewer hilar neurons counted (range: 10-38%) compared to controls. C1,C2: NeuN and c-Fos expression in an identically KA-treated rat that exhibited a loss of translamellar inhibition (Type 2 response). Note extensive hilar neuron loss (an average of 85% hilar cell loss; n=4; range: 68-94%). D1, D2: NeuN and c-Fos expression 3 days after 24 hr of PP perforant path stimulation, which resulted in a failure to evoke translamellar inhibition despite effective BMI-induced activation of granule cells (Fig. 4.5, C3). In 6 PP-stimulated animals, the average hilar neuron loss was 93% (range: 89-95%). Scale bar = 100 μm. Magnification: 64X.
Figure 4.7. Hilar cell loss correlates with failure of translamellar inhibition. Graph showing the correlation between BMI-induced suppression of the 2nd evoked population spike amplitude recorded at the saline electrode, and the number of hilar neurons surviving at the septo-temporal level of the BMI electrode. The group means for cell number (*) and the degree of second spike suppression (+) in both the KA type 2 and PP stimulation groups were significantly different from the control and KA type 1 groups (p < 0.05); the latter were not significantly different from each other (p > 0.05) (Mann-Whitney test).
subjected to prolonged PP stimulation because this paradigm reliably produces extensive hilar cell loss throughout the dorsal hippocampus without involving behavioral SE or producing any significant extra-hippocampal damage (Sloviter, 1991; Sloviter et al., 2003). Thus, if dorsal hippocampal hilar cell loss per se caused the observed translamellar disinhibition in KA-treated rats, then PP-stimulated rats with relatively selective hilar cell loss should exhibit the same loss of translamellar inhibition seen in the more extensively damaged KA Type 2 animals.

Three days after the end of 24 hr of intermittent PP-stimulation, BMI-induced granule cell discharges consistently failed to evoke distant inhibition (n = 6; Fig. 4.5, C2 and C4). Compared to controls (n = 4), PP-stimulated rats exhibited an average of 93% fewer hilar neurons (4.6 ± 0.7 hilar neurons per section; range: 89-95% fewer neurons; n = 6; p < 0.01) at the dorsal hippocampal BMI electrode (Fig. 4.6 D1,D2). Injury was also apparent in area CA3c of PP-stimulated animals, but no obvious injury was detected in the temporal neocortex or in any other brain regions. Thus, 24 hr intermittent PP stimulation previously shown to produce primarily unilateral hilar neuron loss was as effective in abolishing translamellar granule cell inhibition as widely brain-damaging KA-induced SE. The complementary structural features of these two animal models that are most relevant to the physiological results are illustrated in Figure 4.8, which contrasts a PP-stimulated rat with extensive hilar cell loss and no apparent extrahippocampal damage, with a KA-treated rat with minimal hilar cell loss, but extensive brain damage. In contrast to the PP-stimulated animal, which exhibited a loss of translamellar inhibition (Fig. 4.8 A1), the KA-treated rat
Figure 4.8. Loss or survival of dorsal hippocampal hilar neurons corresponds to loss or preservation of translamellar inhibition. A: in a rat subjected to prolonged perforant path (PP) stimulation 3 days previously, distant translamellar inhibition evoked at the saline electrode by focal granule cell discharges was abolished (A1); upper trace: before distant BMI-induced granule cell discharges; lower trace: after BMI diffusion. Note selective and extensive loss of hilar neurons (Nissl-stained sections in A2), but survival of adjacent CA3 pyramidal cells (A3). B: in a rat subjected to kainic acid (KA)-induced status epilepticus 3 days previously, translamellar inhibition (B1) and dorsal hilar neurons (B2) were preserved, whereas CA3 pyramidal cells had degenerated (B3). C1 and C2: FluoroJade-B-stained dorsal (C1) and ventral (C2) sections from a KA-treated animal. Note that brightly fluorescent cell bodies and processes reflect acute degeneration. Despite extensive injury to dorsal hippocampal CA1-CA3 pyramidal cells (C1), ventral dentate hilar neurons (h) and CA1 pyramidal cells and temporal cortical nuclei including the lateral entorhinal cortex (LEnt; C2), translamellar inhibition was preserved, and the only apparently undamaged hippocampal region in this animal was the dorsal dentate gyrus (asterisk in C1). Calibration bars: 5 mV and 10 ms in A1-B2; scale bar: 100 μm in A2,B2; 356 μm in A3,B3; 237 μm in C1, C2. Magnification: 46X in A2,B2; 14X in A3, B3; 19X in C1,C2.
with intact translamellar inhibition (Fig. 4.8 B1) exhibited selective survival of hilar neurons (Fig. 4.8 B2), but extensive damage to CA1 and CA3 pyramidal cells (Fig. 4.8 B3, C1), and temporal cortical nuclei (Fig. 4.8 C2). In Fluoro-Jade B-stained sections of this KA-treated rat, the only hippocampal region lacking acutely degenerating neurons was the dentate gyrus (asterisk in Figure 4.8 C1).

Figure 4.9 illustrates the correlation between hilar neuron loss and the loss of translamellar inhibition. The clustering together of all 10 disinhibited animals (6 perforant path-stimulated rats and 4 KA Type 2 rats) near the origin of the X-Y axis, and the more scattered distribution of all controls and KA Type 1 animals suggests that translamellar disinhibition is uniquely associated with extensive hilar cell loss, whereas minimal hilar cell loss produces no obvious disinhibition (Fig. 4.9). Table 4.2 presents the quantitative data showing that at longitudinal distances which were greater than 2.5 mm and not significantly different between groups, the 4 KA Type 2 rats and all 6 PP stimulated rats exhibited statistically significant differences in 2nd population spike suppression compared to controls and KA Type 1 animals.
Figure 4.9. Correlation between hilar neuron loss and loss of translamellar inhibition. The average number of Nissl-stained hilar neurons per section from each of 31 rats tested is plotted against the percent of suppression of the second of two granule cell population spikes evoked by perforant path stimulation at 0.3 Hz in control, kainate (KA)-treated, and 24 hr perforant path (PP)-stimulated rats. There was a correlation between the number of hilar neurons and the degree of second spike suppression ($r^2 = 0.68$). Although the data display a somewhat linear relationship, there was a distinct clustering of data points. All rats that exhibited a failure of translamellar inhibition (4 KA Type 1 and all 6 PP-stimulated rats) were subsequently found to have extensive hilar neuron loss, and were clustered close to the origin of the X-Y axis. Conversely, all control and KA Type 1 rats exhibited intact second spike suppression and relative hilar neuron preservation.
SUMMARY

The main original findings of these experiments are, first, that the net translamellar influence of focal granule cell discharges on distant granule cells was inhibition, not excitation. Second, translamellar inhibition was abolished by KA-induced status epilepticus whenever extensive dorsal hilar neuron loss resulted, and by prolonged perforant path stimulation, which consistently produced extensive hilar neuron loss but minimal extra-hippocampal damage.

Although mossy cells have been hypothesized, mainly on anatomical grounds, to constitute a recurrent excitatory system that links distant granule cells together (Amaral and Witter, 1989; Buckmaster and Schwartzkroin, 1994), only distant granule cell inhibition was detected after focal granule cell discharge. Relatively weak longitudinal excitation has been reported (Bekenstein and Lothman, 1991; Hetherington et al., 1994), but the use of electrical stimulation in these studies makes it difficult to identify the intrinsic pathways or fibers of passage that mediate responses recorded within the same stimulated structure. These interpretative difficulties led to the utilization of the BMI diffusion method (Sloviter and Brisman, 1995) to evoke focal granule cell excitation. If synchronously-activated, longitudinally-projecting mossy cells directly excite distant granule cells, a summated excitatory potential analogous to that evoked by perforant path stimulation should have been generated at the appropriate latency at the distant recording site. However, the same focal discharges that powerfully inhibited distant granule cells produced no concomitant evidence of distant granule cell excitation.
These results are consistent with previous conclusions that inhibitory interneurons are the primary targets of the excitatory associational/commissural pathway, which paradoxically produced commissural granule cell inhibition, rather than excitation (Buzsáki and Eidelberg, 1982; Douglas et al., 1983). The mechanism by which mossy cells might excite inhibitory neurons more powerfully than they excite granule cells may be similar to that reported in recent studies of CA1 interneurons, which express GluR5 and GluR6 subunits of KA-preferring glutamate receptors. Area CA1 interneurons were shown to be powerfully activated by KA, resulting in pyramidal cell inhibition (Bureau et al., 1999; Cossart et al., 1998; Frerking et al., 1998). Conversely, CA1 pyramidal cells exhibit minimal GluR5 subunit expression, and were not directly excited by KA receptor activation (Melyan et al., 2002). In the dentate gyrus, KA receptors are concentrated in the exact location where mossy cell axons terminate (Monaghan and Cotman, 1982), and, like CA1 pyramidal cells, granule cells lack GluR5 subunits, whereas dentate inhibitory interneurons express both subunits (Bureau et al., 1999; Paternain et al., 2000). If the differential effects of KA receptor activation in the CA1 region are similarly manifested in the dentate gyrus, mossy cells may preferentially excite distant inhibitory neurons, causing granule cell inhibition. This scenario is consistent with the location and expression pattern of KA receptor subunits in both dentate granule cells and interneurons, and our results, demonstrating transcortical inhibition, rather than excitation.

That the transcortical disinhibition observed in lesioned animals was due to hilar neuron loss, rather than other effects of prolonged SE, is supported by the observation that
all KA-treated rats exhibited SE lasting > 3 hr, and all exhibited hippocampal and extrahippocampal damage. The main difference detected between KA-treated rats with intact or impaired translamellar inhibition was the extent of hilar cell loss. More conclusively, identical translamellar disinhibition was reproduced by prior perforant pathway stimulation, which reliably produced extensive hilar neuron loss, but little other damage. However, other undetected effects of prolonged granule cell discharges that might uniquely parallel hilar neuron injury, such as downregulation of GABA<sub>A</sub> receptors in granule cells (Brooks-Kayal et al., 1998), cannot be excluded as alternate explanations.

The finding that only extensive hilar cell loss was reliably associated with translamellar disinhibition explains why a previous study failed to observe translamellar disinhibition in KA-treated rats (Buckmaster and Dudek, 1997). These authors used animals that exhibited only partial hilar neuron loss (corresponding to our KA Type 1 animals), and assessed lateral inhibition only 1 mm from the BMI electrode, two factors that preclude their conclusion that mossy cells do not activate longitudinally distant inhibitory neurons. Given that bicuculline diffuses ~400 µm toward the saline electrode, inhibitory responses recorded only 1mm from the BMI electrode apparently reflect local *intralamellar* inhibition, rather than *translamellar* inhibition (Sloviter, 1994). Any activity recorded at the short distance assessed by Buckmaster and Dudek (1997) is unlikely to involve associational projections of mossy cells, regardless of the extent of mossy cell loss, because mossy cell innervation of the dentate inner molecular layer specifically avoids the ~1 mm radius surrounding their somata (Amaral and Witter, 1989; Buckmaster et al., 1996).
CHAPTER 5: ASSOCIATIONAL PROJECTIONS STUDIES

Introduction

The previous chapter demonstrated that translamellar inhibition can be evoked up to a distance of ~4.5 mm. The hilar neurons forming the neuroanatomical substrate for this phenomenon remain unknown. Although it is generally accepted that mossy cells have extensive associational projections (Amaral and Witter, 1989; Soltesz et al., 1993), recent studies have proposed that inhibitory interneurons have far-reaching influence (Qiu and Han, 1995; Buckmaster and Schwartzkroin, 1995). Buckmaster and Schwartzkroin (1995) characterized the projections of dentate interneurons that were intracellularly labeled with biocytin. The axonal arbors of these cells were found to extend long distances along the septo-temporal axis and the authors concluded that dentate interneurons have far-reaching effects on distant hippocampal lamellae. This study, however, reconstructed a total of two neurons and the majority of the axonal branches were relatively close to the cell body.

The purpose of the following experiments was to characterize the longitudinal associational projections of hilar neuron subpopulations using the retrograde tracer FG to determine which neurons have the requisite projections to mediate translamellar inhibition.

Longitudinal associational projections of hippocampal inhibitory interneurons

The simplest explanation for the observation that focal granule cell discharges evoked translamellar inhibition is that locally activated, c-Fos-positive inhibitory interneurons
innervated and directly inhibited distant granule cells. Therefore the relative extent of the longitudinal projections of hilar mossy cells and hippocampal inhibitory interneurons was determined by focally injecting the retrograde tracer Fluoro-Gold into the saline electrode site, and assessing the subsequently labeled neuron populations at the BMI site ~2.5-4 mm in the posterior (temporal) direction.

FG injection into the saline recording electrode site produced a large FG-positive region that involved all dentate gyrus laminae and the CA1 region above the injection target site, as previously illustrated (Zappone and Sloviter, 2001). In sections at the outer edge of the FG injection site (Fig. 5.1 A), all hippocampal principal cells and interneuron subpopulations were FG-positive, indicating an ability of all neuronal subpopulations to sequester FG intracellularly. The most notable and consistent feature of associational transport of Fluoro-Gold was the precipitous decline in FG staining of inhibitory interneurons in all hippocampal subregions at longitudinal distances >500 μm from the outer edge of the FG injection site (Figs. 5.1 C, 5.4). Although sections progressively farther from the FG injection site exhibited extensive FG-labeling of dentate hilar neurons (Figs. 5.1 C, 5.2 B, 5.4), GAD67-positive inhibitory interneurons of the dentate molecular and granule cell layers were conspicuously and consistently FG-negative only several hundred microns from the outer edge of the FG injection site. The consistent lack of FG transport (Figs. 5.1 C, 5.3 B2, 5.4 B) by any presumed dentate basket cells of the granule cell layer, or by axo-axonic chandelier cells of the molecular layer (Freund and Buzsáki, 1996), was assessed quantitatively after co-localization of FG and GAD67 immunoreactivities. Of 573 GAD67-
Figure 5.1. Retrograde transport of Fluoro-Gold (FG) from the saline electrode site to the bicuculline methiodide (BMI) electrode site. The retrograde tracer FG was used to identify the hippocampal neuron subtypes with axonal projections spanning the longitudinal distance over which translamellar inhibition was evoked. A: at the approximate outer edge of the FG injection zone, all neuronal subpopulations in the dentate gyrus were FG-immunopositive, including granule cells, hilar neurons (h), and presumed interneurons of the granule cell layer (arrows in stratum granulosum; sg). B and C: GAD67- and FG-immunostained sections, respectively, at the ~3.0 mm longitudinally distant site showing the location of GAD67-positive interneurons in the granule cell and molecular (stratum moleculare; sm) layers (arrows), which were consistently FG-negative (C). Scale bar = 100 μm. Magnification: 65X.
Figure 5.2. Primarily hilar neurons retrogradely transport Fluoro-Gold (FG) long distances. (FG) long distances. A: immunocytochemical localization of the neuronal marker NeuN shows the normal location and number of hippocampal principal cells and interneurons in the dorsal hippocampus. B: in a section ~3.2 mm distant from the rostral FG injection site, FG-immunopositive cells consistently included numerous hilar neurons (h) and a small number of interneurons of the stratum lacunosum-moleculare (slm) and stratum oriens (so) but not interneurons of the principal cell layers, stratum radiatum (sr) or the dentate molecular layer (sm). Note that the three slm interneurons shown in A2 was the largest number of area CA1 interneurons per section seen in any section analyzed. Scale bar = 200 μm, Magnification: 33X.
Figure 5.3. Limited longitudinal transport of the retrograde tracer Fluoro-Gold (FG) by hippocampal interneurons. A1: close to the FG injection site (~360 μm longitudinally), FG-labeled cells (green) include somatostatin (SS)-positive hilar interneurons (red), which appear orange or yellow when co-localized (arrows). A2: sections ~3.6 mm from the FG injection site rarely exhibited co-localization of SS and FG (see graph in Fig. 10). B1: co-localization of FG and parvalbumin (PV) (red) similarly indicates that although PV-positive inhibitory interneurons immediately adjacent to the FG injection site co-localize FG (arrows), even slightly more distant PV-positive cells within the same section do not transport detectable FG. B2: ~720 μm from the FG injection site, PV-positive interneurons were not co-localized with FG. Scale bar = 100 μm. Magnification: 65X.
Figure 5.4. Longitudinal transport of Fluoro-Gold (FG) by dentate hilar neurons. A: co-localization of FG (green) and glutamate receptor subunit 2 (GluR2; red) immunoreactivities ~2.9 mm from the FG injection site. Note that most intensely GluR2-positive hilar neurons (presumed mossy cells) contain FG immunoreactivity relatively far from the FG injection site. B: graph showing that somata of dentate parvalbumin- and GAD67-positive inhibitory interneurons transported detectable Fluoro-Gold (FG) immunoreactivity only a short distance (~500 µm) from the FG injection site, and only a small proportion of hilar somatostatin-positive interneurons transported detectable FG more than 1 mm longitudinally. Conversely, GluR2-positive hilar neurons (presumed mossy cells) transported FG > 3 mm longitudinally in high proportion. Note that the outer edge of the FG injection site (the green sphere at the origin of the X-Y axis) served as the zero distance from the FG injection site. Scale bar = 50 µm. Magnification: 192X.
positive interneurons of the dentate granule cell and molecular layers counted in 18 sections that were ~480-1920 μm from the FG injection sites in 3 rats, only 1 cell was judged to be FG-positive, and this cell was located ~480 μm from the injection site. Thus, no GAD67-positive interneurons more than 0.5 mm from the FG injection site were FG-labeled in 3 animals in which most hilar neurons were intensely FG-positive >4 mm longitudinally.

In the hippocampus proper, the steep longitudinal decline in FG labeling of interneurons included the large populations of hippocampal interneuron somata in stratum oriens, radiatum, and lacunsum-moleculare (Fig. 5.2 A, B). One consistent exception was a small number of intensely FG-immunolabeled interneurons at the stratum radiatum/lacunosum-moleculare border and in the stratum oriens (Fig. 5.2 B). Thus, our ability to detect intense FG immunoreactivity in a small number of interneurons relatively far (>2 mm) from the FG injection site indicates the effectiveness of the methods used to detect FG in interneurons, and demonstrates that the vast majority of interneurons, including all basket cell somata of all hippocampal principal cell layers did not transport even a minimally detectable concentration of FG 2-4 mm from the injection site. Results of the FG control injections indicated that the FG-labeling of dentate gyrus neurons was the result of site-specific transport of FG from the dentate gyrus injection site. Neither FG injection into area CA1 above the dentate gyrus, or into the neocortex above the dentate gyrus, labeled any dentate gyrus neurons.

As previously described after injection of retrograde tracer into the anterior hippocampus (Qiu and Han, 1995; Buckmaster and Jongen-Rêlo, 1999), the principal
location of the vast majority of retrogradely-labeled, longitudinally-projecting neurons was the dentate hilus (Fig. 5.1 C), which contains excitatory mossy cells (Soriano and Frotscher, 1994) and a variety of intermingled inhibitory interneurons (Amaral, 1978; Freund and Buzsáki, 1996). To determine whether FG-labeled hilar neurons included a significant number of inhibitory interneurons capable of directly mediating translamellar inhibition, FG was co-localized with GluR2, which primarily labels mossy cells (Leranth et al., 1996), with SS, which labels the largest population of hilar inhibitory interneurons (Bakst et al., 1986; Sloviter and Nilaver, 1987), and with PV, which labels several inhibitory interneuron populations in all dentate subregions (Freund and Buzsáki, 1996; Sloviter et al., 2003).

Quantitative analysis of sections progressively distant from the FG injection site revealed that the proportion of hilar FG-positive neurons that were also GluR2-positive increased as the distance from the tracer injection site increased, whereas the proportion of FG-labeled neurons that were SS- or PV-positive decreased progressively and precipitously (Fig. 5.3, 5.4). Within the region ~2.2-2.9 mm from the edge of the FG injection site, 81% of all GluR2-positive hilar neurons (465 of 573 neurons) were FG-labeled. Within this same region, ~6% of all FG-labeled neurons were SS-positive. These few FG-labeled hilar interneurons constituted ~5% of the SS-positive hilar neuron population (36 of 679 SS-positive hilar neurons) in the sections from this longitudinal segment. In addition, virtually all of the few FG-labeled, SS-positive hilar neurons that were only weakly FG-positive were detected, whereas GluR2-positive neurons in adjacent, co-processed sections were intensely FG-immunopositive (Fig. 5.4 A). Farther along the longitudinal axis (~3.2-3.9 mm), the
percentage of SS-positive neurons that was FG-labeled declined to ~2% (22 of 965 SS-positive neurons). Consistent with these results indicating minimal FG-labeling of distant hilar SS- and GAD67-positive dentate interneurons, PV-positive neurons were consistently FG-negative >0.5 mm from the FG injection site (Fig. 5.4).

**Septal FG injections**

Given the consistently infrequent and weak FG-labeling of SS-positive hilar interneurons after intrahippocampal FG injection, whether these interneurons lack the ability to transport FG in concentrations that result in intense labeling was determined. This experiment addressed the possibility that SS-positive hilar interneurons might have extensive associational projections, as suggested by Buckmaster and Schwartzkroin (1995), yet be incapable of transporting FG in detectable quantities for unknown biological reasons. In the previous chapter on the commissural pathway the hippocampal projection to the medial septum was examined. However, in the context of these associational studies, a more thorough analysis of the septal projection was conducted. Injection of FG into the medial septum (n = 4) consistently resulted in intense FG-labeling of virtually all SS-positive interneurons (720 of 743, 96.6% ± 1.3) throughout the longitudinal axis of the hippocampus (Fig. 5.5 B1). A total of 1982 SS-positive cells were detected throughout the septo-temporal extent of the hilar region and 720 of these cells were FG-positive. Thus the FG injections into the medial septum strongly labeled 36.1% ± 0.9 of the hilar SS-positive neurons.

However, no PV-positive neurons were labeled by these medial septal injections.
Figure 5.5. Retrograde transport of Fluoro-Gold (FG) by dentate hilar neurons after FG injection into the rostral hippocampus or the medial septum. A1 and A2: after FG injection into the rostral hippocampus, hilar neurons were labeled ~2-4 mm caudally. However, hilar somatostatin (SS)-positive interneurons (A3; red) were rarely FG-positive (green) at longitudinal distances at which translamellar inhibition was evoked. B1 and B2: after injection of FG into the septum, numerous hilar interneurons throughout the longitudinal axis of the hippocampus (arrows) were intensely FG-immunopositive, as were CA1 pyramidal cells and interneurons. B3: After FG injection into the septum, virtually all FG-labeled hilar neurons were SS-immunoreactive (red). These results indicate that hilar SS-positive interneurons have the capacity to transport FG from a distant site (medial septum; ~6-10 mm distant) in concentrations that result in intense FG labeling, but that these same interneurons do not transport even minimally detectable FG from significantly closer injection sites within the hippocampus. Scale bar = 508 μm in A1,B1; 100 μm in A2,B2; 50 μm in A3,B3. Magnification: 13X in A1,B1; 64X in A2, B2; 130X in A3,B3.
Thus, these hilar SS-positive interneurons, few of which transported even minimally detectable FG from relatively nearby hippocampal injection sites, were nonetheless capable of transporting easily detectable quantities of FG from the septum, which, for ventral hilar SS-positive somata (Fig. 5.5 B1-B3), represents transport ~10 mm from the FG injection site. Therefore, the lack of detectable FG in hilar interneuron somata after relatively nearby FG injection was not the result of an inability of these hilar interneurons to take up or transport FG (Fig 5.5 A3).
SUMMARY

The experiments described in this chapter indicate that: 1) mossy cells form extensive longitudinal axon projections spanning the distance over which translamellar inhibition was evoked; 2) basket cells and chandelier cells appear to have extremely limited longitudinal axonal projections, and; 3) with rare exceptions, SS-positive interneurons form sparse and longitudinally limited axonal projections, despite sending extensive projections to the more distant septum.

The identity of neurons capable of mediating translamellar inhibition was addressed by retrograde transport studies, which confirmed that mossy cells are the only dentate neuron population with significant longitudinal projections spanning a range of ~2.5-4.5 mm longitudinally (Amaral and Witter, 1989; Buckmaster and Jongen-Rêlo, 1999). Although the exceedingly small population of longitudinally-projecting inhibitory interneurons that we and others (Buckmaster and Schwartzkroin, 1995; Sik et al., 1997) have identified could conceivably contribute to translamellar granule cell inhibition, the results of the present and previous anterograde and retrograde tracing studies suggest that associational projections of inhibitory interneurons are minimal beyond a longitudinal distance of 1 mm (Amaral and Witter, 1989; Qiu and Han, 1995; Buckmaster and Jongen-Rêlo, 1999). This appears particularly true for basket cells and chandelier cells, which presumably mediate powerful inhibition of the principal cell somata and axon initial segments (Freund and Buzsáki, 1996), because these interneurons of the granule cell and molecular layers only transported FG locally. Consistent with the present results, studies in which tracer-filled interneurons were
reconstructed indicate that dentate inhibitory interneurons most densely innervate targets close to their somata. In addition, total axon length decreases between 0.5 and 1 mm from the soma, and interneurons only sparsely innervate regions more than 1 mm longitudinally (Buckmaster and Schwartzkroin, 1995; Sik et al., 1997). In this regard, the observation that inhibitory interneurons project to target cells close to their somata seems of greater functional significance than the observation that a small minority of interneuron axon can sometimes project slightly beyond 1 mm from the soma. The view of complementary patterns of interneuron/ mossy cell axon termination, involving concentration of inhibitory interneuron terminals within the region that is preferentially avoided by hilar mossy cell axons (Sik et al., 1997), appears to be consistent with lamellar organization. It does not appear that hilar inhibitory interneurons have significant, far-reaching translamellar effects, as suggested by Buckmaster and Schwartzkroin (1995). Thus, lamellar function may be established by inhibitory interneurons acting locally to focus strong excitation to local targets (intralamellar lateral inhibition), and by mossy cells establishing lateral inhibition in surrounding lamellae to increase the “signal-to-noise” ratio. The present finding that many hilar SS-positive interneurons were intensely FG-labeled after distant septal FG injection, but few were even weakly labeled by relatively nearby hippocampal FG injections, supports this conclusion because hilar SS-positive interneurons apparently convey information received from a lamellar granule cell input directly to the distant medial septum, but not to much closer segments of the granule cell layer. However, given the finding that individual inhibitory interneurons can have widely divergent influences on large numbers of principal cells (Cobb
et al., 1995), inferences drawn primarily from anatomical data cannot eliminate the possibility that small numbers of inhibitory interneurons may directly and significantly contribute to translamellar inhibition.
CHAPTER 6: CONCLUSIONS

The stated purpose of the experiments described in this dissertation was to describe the septo-temporal extent of excitatory and inhibitory neuronal projections that participate in the associational and commissural pathways of the dentate gyrus. Another goal was to determine precisely how these pathways might be involved in the functional organization of the hippocampus. The “lamellar” hypothesis suggests that the hippocampus is organized into functional slices or “lamellae” (Andersen et al., 1971), a scenario similar to the columnar organization observed in other brain regions. Because it has been assumed that mossy cells form a recurrent excitatory network with granule cells, the existence of extensive longitudinal projections of excitatory mossy cells has been suggested to be antithetical to a lamellar organization in the dentate gyrus (Amaral and Witter, 1989; Buckmaster and Schwartzkroin, 1994). Whereas mossy cells form synaptic contacts on both granule cells and inhibitory neurons (Wenzel, 1997), it is not clear which of these synaptic connections dominates functionally. The discovery of lateral inhibition in the hippocampus suggested that excitatory activity may be restricted along the septo-temporal extent of the hippocampus (Sloviter and Brisman, 1995). However, it is unknown which neurons mediate surround inhibition in the hippocampus. The findings of this dissertation work include: 1) the observation that hippocampal inhibitory interneurons have much more limited longitudinal ipsilateral projections than known previously, but more prominent homotopic commissural projections 2) focal granule cell activation inhibits rather than excites distant segments of the
granule cell layer, 3) a failure of translamellar inhibition occurs following significant hilar cell loss, and finally 4) mossy cells appear to be the only neurons with the appropriate projections to mediate translamellar inhibition and, possibly, dentate lamellar function.

Experiments conducted to study the commissural pathway of the dentate gyrus revealed several original findings. Improved methods for detecting retrogradely transported Fluoro-Gold (FG) demonstrated that many inhibitory interneurons of the rat dentate gyrus are among the hippocampal neurons that form commissural projections to the contralateral hippocampus. These results indicate that a significant proportion of PV-positive basket cells, which form the axo-somatic innervation of the granule cell layer (Kosaka et al., 1987; Sloviter, 1989; Freund and Buzsáki, 1996), and virtually all dentate hilar SS-positive interneurons, which innervate the dentate molecular layer (Bakst et al., 1986; Sloviter and Nilaver, 1987; Freund and Buzsáki, 1996), are FG-labeled after a contralateral hippocampal injection. Earlier studies indicated that few inhibitory neurons projected commissurally (Zimmer et al., 1983; Bakst et al., 1986; Ribak et al., 1986; Deller and Leranth, 1990; Miettinen et al., 1992). These findings seemed to contradict the idea that neurons contributing to the associational pathway also project commissurally (Deller, 1998). However, the commissural studies of this dissertation support this idea as a general principle of hippocampal organization. The next original finding is that after focal injection of FG into the dorsal hippocampus, the FG-labeled excitatory dentate mossy cells and CA3 pyramidal cells were much more widely distributed along the longitudinal axis of the hippocampus than the FG-labeled inhibitory interneurons. The heterotopic distribution of excitatory
commissural projections (Gottlieb and Cowan, 1973; Amaral and Witter, 1989; Deller et al., 1995; 1996), and the homotopic distribution of fibers from inhibitory interneurons are also consistent with the organizing principle of homologous commissural and associational systems (Berger et al., 1980; Swanson and Cowan, 1981; Deller et al., 1996). These commissural studies led to the inference that the pattern of retrogradely labeled FG-positive cells appears to represent a neuronal population, both inhibitory and excitatory, that share the property of being direct postsynaptic targets of dentate granule cells. Thus, the present results involving retrograde transport, taken together with the results of Deller and colleagues (1995; 1996) involving anterograde transport, suggest that dentate gyrus interneurons may serve a previously underappreciated role in producing relatively homotopic “lamellar” inhibition. This hypothetical homotopic inhibition might serve to silence the corresponding “twin” granule cell lamella in the contralateral hippocampus, thereby lateralizing and amplifying the influence of the initiating granule cell discharge.

The next set of original findings are from experiments conducted to study translamellar influences in the dentate gyrus. The net translamellar influence of focal granule cell discharges on distant granule cells was inhibition, not excitation. This translamellar inhibition was abolished by KA-induced status epilepticus whenever extensive hilar neuron loss resulted, and by prolonged perforant path stimulation, which consistently produced extensive hilar neuron loss but minimal extra-hippocampal damage. Finally anatomical experiments examining the associational pathway of the dentate gyrus revealed the following: 1) mossy cells form extensive longitudinal axon projections spanning the distance
over which translamellar inhibition was evoked; 2) basket cells and chandelier cells appear to have extremely limited longitudinal axonal projections, and; 3) with rare exceptions, SS-positive interneurons form sparse and longitudinally limited axonal projections, despite sending extensive projections to the more distant septum.

These findings suggest that mossy cells are involved in establishing surround inhibition in the hippocampus by exciting distant inhibitory neurons, which supports the "dormant basket cell" hypothesis. In addition, translamellar inhibition could be a mechanism for establishing a lamellar organization in the dentate gyrus. Even though these results suggest that mossy cells are involved in establishing translamellar inhibition, these findings do not rule out the possibility that interneurons also mediate surround inhibitory influences. Mossy cell axons minimally innervate the neuropil within a 1 mm radius of a region preferentially targeted by inhibitory neurons (Buckmaster et al., 1996). However, the associational projections of mossy cells preferentially target the dentate inner molecular layer beginning approximately 1.2 mm in the longitudinal direction from an anterograde tracer injection (Amaral and Witter, 1989). Locally projecting inhibitory neurons may establish lateral inhibition in this gap of mossy cell innervation. Thus, lateral inhibitory influences in the hippocampus may involve multiple populations of specialized hilar neurons that mediate inhibition at different distances. For example, granule cell activated somatostatin and/or basket cells might mediate relatively short distance, intralamellar inhibition. Furthermore, inhibitory neurons activated by mossy cells may mediate long distance, translamellar, inhibition. In addition, mossy cells axons have been shown to locally (within ~1 mm)
synapse on inhibitory hilar neurons (Buckmaster et al., 1996). This mossy cell, hilar projection may reinforce granule cell activation of local inhibitory interneuron in establishing lamellar boundaries. Specialization of laterally projecting neurons, in maintaining surround inhibition, has been observed in the cerebellum. On-beam and lateral inhibition are two different types of inhibitory mechanism in the cerebellum (Cohen and Yarom, 2000). Whereas these mechanisms are both GABA\(_A\) mediated, they are considered two physiologically distinct components (Cohen and Yarom, 2000). In the cerebellum, a single pulse of stimulation to the surface of the cerebellar cortex evokes lateral inhibition on both sides of the activated beam module and is thought to be mediated by molecular layer stellate and basket cells (Eccles et al., 1967; Cohen and Yarom, 2000). Conversely, on-beam inhibition, thought to be mediated by Golgi cell inhibition of granule cells, remains sequestered within the borders of an activated beam following a train of stimulation (Cohen and Yarom, 2000). Thus, like the cerebellum, the dentate gyrus may be arranged into a series of parallel lamella that serve as the functional units.

Regardless of which cell types and synaptic mechanisms mediate translamellar inhibition in the dentate gyrus, the present data clearly support the concept of functional separation in the granule cell layer as a consequence of inhibitory translamellar impulses (Andersen et al., 1966; Struble et al., 1978; Sloviter, 1994; Sloviter and Brisman, 1995). Thus, the findings of this dissertation do not support the idea that the existence of excitatory longitudinal projections is antithetical to the concept of individual hippocampal lamellae functioning independently (Amaral and Witter, 1989). Presumably, the net functional effect
of the longitudinal projections, not their existence or their excitatory nature, determines their physiological influence. That these projections appear powerfully inhibitory in nature in vivo supports the idea of functional segregation of granule cell activity, possibly resulting in lamellar organization of the dentate gyrus (Andersen et al., 1971; 2000; Moser and Moser, 1998; Hampson et al., 1999). Furthermore, an extensive loss of hilar inhibitory interneurons (deLanerolle et al., 1989) and excitatory mossy cells (Blümcke et al., 2000) occurs in many patients with temporal lobe epilepsy who have hippocampal sclerosis, which is, in some cases, the only pathology evident anywhere within the epileptic human brain (Margerison and Corsellis, 1966). The loss of translamellar inhibition that reliably follows extensive hilar neuron loss suggests that the loss of both local and longitudinal inhibitory influences may disrupt lamellar function and underlie post-injury hippocampal network dysfunction related to amnestic conditions and temporal lobe epilepsy.

The discovery of lateral inhibition, and now translamellar inhibition, in the hippocampus indicates that this structure has neuronal mechanisms to establish functional segregation. The hippocampus receives a variety of extrinsic inputs and thus is a convergence point for multi-modal inputs (Amaral, 1993). However, the structure-function relationship involved in processing this information is unknown. Septo-temporal differences in the projection pathways and neuronal constituents of the dorsal and ventral hippocampal networks has been suggested as evidence for hippocampal functional differentiation (Moser and Moser, 1998). The major cortical projection to the hippocampus arises from three cytoarchitecturally defined zones in the entorhinal cortex which then project to the dentate
gyrus, in a topographical manner, to three partially overlapping zones (Witter et al., 1989). In addition, there appears to be a disconnect between the dorsal two-thirds of the hippocampus and the ventral one-third based on the anatomical finding that hippocampal projection cells do not cross a boundary between these two regions (Fricke and Cowan, 1978). The number of calretinin-immunoreactive (IR), nitric oxide synthase-IR, somatostatin-IR, and mossy cell populations are significantly larger in the ventral hilar region of the dentate gyrus than the dorsal hilus (Nomura et al., 1997; Fujise and Kosaka, 1999; Blasco-Ibanez and Freund, 1997). These anatomical differences between the dorsal and ventral hippocampus suggest that these regions are involved in different processes.

Behavioral experiments also suggest hippocampal functional segregation (Moser and Moser, 1998). The hippocampus appears to be necessary for memory formation, however the nature of its contribution remains controversial. Human studies suggest that the hippocampus is involved in declarative memory, conversely animals studies indicate that the hippocampus is primarily involved in spatial mapping (Wilson and McNaughton, 1993; Eichenbaum, 1999). Lesioning studies have indicated that the dorsal and ventral hippocampus are involved in different forms of memory (Moser and Moser, 1998). Rats are capable of learning spatial tasks with only a small tissue block remaining in the anterior hippocampus, however equally large blocks in the ventral hippocampus are not capable of supporting spatial learning (Moser et al., 1993, 1995). A similar polarized functional differentiation was detected in hippocampal neurons recorded in primates performing a spatial delayed matching-to-sample task (Colombo et al., 1998). In addition, a human functional MRI study provided evidence
of differential activity between the anterior and posterior hippocampus while the subjects were performing a perceptual novelty task (Strange et al., 1999). These anatomical and behavioral studies suggest that there is a difference in hippocampal function along the septo-temporal axis.

Multi-neuron recording studies in freely behaving rats have provided conflicting data regarding correlated firing patterns in anatomically neighboring neurons. These studies have primarily involved recordings of CA1 and CA3 pyramidal cells, which exhibit characteristic response properties to spatial behavior tasks. Hampson et al. (1999) provided data supporting lamellar segregation of function. These investigators recorded CA1 and CA3 neurons along the septo-temporal extent of the hippocampus of freely behaving rats, performing a delayed nonmatch-to-sample memory task. Different aspects of the animals' behavior, performed during the task, activated alternating cross-sectional clusters of neurons. This segregation of function was compared to the functional columns observed in other areas of the cerebral cortex (Eichenbaum, 1999) and supports the lamellar hypothesis. However, Redish et al. (2001) found no clustering of hippocampal CA1 pyramidal neurons recorded in animals running on narrow, elevated tracks. In addition, they found no clustering of neurons, assessed by the activity-induced gene Arc, in animals that had been freely exploring open environments. The contradictory results between these two groups could be explained by their respective use of different behavioral tasks and different multi-unit recording techniques. The use of the retrograde tracer FG to examine the dentate projection pathways requires a similar consideration of technical issues of interpreting anatomical results.
presented in this dissertation. The primary assumption underlying the interpretation of any study of retrograde transport is that all labeled somata far from the injection site have acquired tracer by axonal uptake directly from the injection site. If the presence of somal FG labeling far from the injection site were due to: 1) artifactual neuron autofluorescence; 2) non-specific immunocytochemical staining; 3) non-specific uptake of FG from the extracellular fluid, or; 4) non-specific transfer of FG from labeled cells to neighboring cells, the conclusions of the study would be invalid. A second assumption of this study is that cells exhibiting intense FG-fluorescence contain more FG than less intensely FG-fluorescent cells, and by inference, that differences in the amount of FG transported reflect differences in the density of axonal innervation into the injection site. A third assumption is that cells exhibiting no detectable FG were nonetheless capable of doing so, indicating that they lack commissural projections to the FG injection zone.

The issues of artifactual autofluorescence and non-specific immunostaining are particularly relevant to the results of this dissertation because one of the primary observations was that most inhibitory interneurons exhibited FG-fluorescence that was generally less intense than that of previously identified cells. Relatively weak immunolabeling is a problem of interpretation in all immunocytochemical studies because weak specific staining must be differentiated from artifactual non-specific staining. The issue of FG-like autofluorescence was addressed by the observation that none was evident in sections from naive animals perfusion-fixed by identical methods. Nor was FG-like fluorescence observed in granule cell layer interneurons of the dorsal hippocampus after FG injection into the ventral
hippocampus. If these interneurons were acquiring FG from the extracellular fluid, they would be expected to be FG-positive after FG injection into the ventral hippocampus. FG-LI was similarly not detected in granule cell layer interneurons in sections stained with the chromagen DAB when the primary FG antiserum was omitted. Furthermore, the FG antibody used for immunodetection produced virtually no non-specific background staining, which facilitates a conclusion that the observed FG immunostaining of interneurons in the granule cell layer and hilus represents specific staining. Thus, dentate gyrus interneuron fluorescence and immunostaining were entirely dependent on injection of FG, and on its injection into the contralateral dorsal hippocampus.

Although it is theoretically possible that weakly FG-fluorescent- or FG-immunoreactive interneurons internalized and transported FG non-specifically from the extracellular fluid, it would have to be concluded that dentate interneurons of the granule cell layer and hilus have that capacity, but that granule cells, inhibitory interneurons of the immediately adjacent molecular layer, and virtually all interneurons of the hippocampus proper do not. Furthermore, the absence of FG labeling of dentate basket cells after similarly large injections into the neocortex, septum, or the ventral hippocampus makes this remote possibility even less likely. Finally, although there is no evidence that FG escapes from cells or is transported from one cell to neighboring cells (Schmued and Fallon,, 1986), the possibility exists that interneurons without commissural projections acquired FG from adjacent, commissurally projecting cells. In this case, however, granule cells adjacent to intensely FG-labeled hilar cells would also be expected to be faintly FG-positive, which was
not the case. Most convincing is the observation that in locations contralateral and heterotopic to the FG injection site, dentate basket cells adjacent to brightly fluorescent dentate hilar neurons were FG-negative. If FG were escaping from hilar mossy cells, for example, and being picked up by adjacent interneurons, interneurons in both heterotopic and homotopic locations would be expected to be FG-positive. The clearly homotopic distribution of FG-positive interneurons argues strongly against the indiscriminate uptake of FG from neighboring cells. Regardless, all studies involving retrograde transport of tracers are subject to the same assumptions regarding somal labeling after distant injection, and no special conditions were extant in this dissertation.

Thus, the primary assumption in this dissertation is that all cells that exhibited detectable FG fluorescence and immunoreactivity after contralateral FG injection acquired it by internalization of FG by their axon terminals within the injection site. A secondary assumption is that the relative amounts of FG fluorescence or immunoreactivity exhibited by FG-positive cells reflect differences in the amount of FG transported, and that this is directly proportional to the relative density of commissural innervation of each cell type. Such an interpretation assumes that all axon terminals are capable of taking up FG at the same rate per unit of presynaptic membrane surface area, and that therefore the most intensely FG-labeled cells form the most dense commissural projections. Although there are no data establishing these assumptions to be accurate, they are the simplest explanation of the observed results.

An obvious point to address is the question of how the interneurons detected, in the
present studies, as being FG-positive could have been missed in all preceding studies involving retrograde transport of tracers. The answer to this question has not changed since Swanson and colleagues addressed these same issues (Swanson et al., 1981). The use of horseradish peroxidase (HRP) as a retrograde tracer in early studies never labeled more than 30%-40% of CA3 pyramidal cells, whereas all were labeled by the subsequently developed tracers true blue and bisbenzamide. Other fluorescent tracers, including Evans blue and nuclear yellow, were found to be less effective in labeling long axonal projections, which would result in false-negative results. The continuous development of better tracers and more sensitive methods for detecting them enables the investigator to see both the cells that all tracers can easily label, as well as the cells that apparently transport smaller amounts of tracer. The use, in this dissertation, of immunocytochemical methods to detect FG, rather than reliance on the detection of rapidly fading native fluorescence alone, was a significant factor in detecting previously undetectable FG in interneurons. The constantly improving methodology for the detection of a variety of substances highlights the danger of the "false-negative" conclusion. Although the presence of specific immunoreactivity by any method is evidence of the presence of a substance, the absence of detectable immunoreactivity is not evidence of the absence of the substance. Thus, with technical improvements, more hippocampal neurons have been found to transport and contain FG (Goodman and Sloviter,, 1992 and the present results). In addition, the commissural axonal projections of hippocampal neurons are apparently less dense than their ipsilateral associational projections (Deller et al., 1996). This may explain why relatively little tracer is picked up by
commissural terminals, and can easily be missed by standard methods. An asymmetry in the density of ipsilateral and contralateral axonal projections may also have significant functional implications, as yet unknown.

Finally, the conclusion that virtually all FG-labeled hippocampal cells share the quality of being direct targets of dentate granule cells assumes that all FG-negative interneurons of the dentate molecular layer and other hippocampal subregions lack commissural axonal projections. Clearly, this conclusion is subject to the assumption that future improvements in tracer detection, development of new tracers, or different injection methods, will not label the cells that remained unlabeled in the present studies.

The anatomical and electrophysiological experimental findings in this dissertation support the lamellar hypothesis (Andersen et al., 1971) as it applies to the dentate gyrus. In addition, these results suggest that translamellar disinhibition may be a network-level mechanism underlying post-injury hippocampal dysfunction and epileptic network hyperexcitability.
APPENDIX - LIST OF PUBLICATIONS


REFERENCES


The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted Final Approval according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:  
#03-139 - “Hippocampal Network Structure and Function in Epilepsy”

PRINCIPAL INVESTIGATOR/DEPARTMENT:  
Robert S. Sloviter, PhD - Pharmacology

GRANTING AGENCY:  
NIH-NINDS

SUBMISSION DATE: September 17, 2003

APPROVAL DATE: October 23, 2003  
APPROVAL VALID THROUGH*: October 22, 2006

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: October 24, 2003

REVISIONS (if any):

MINORITY OPINIONS (if any):

Richard C. Powell, PhD, MS  
Vice President for Research

DATE: October 24, 2003

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.
The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted Final Approval according to the review policies of the IACUC:

**PROTOCOL CONTROL NUMBER/TITLE:**

#00-131 - “Hippocampal Network Structure and Function in Epilepsy”

**PRINCIPAL INVESTIGATOR/DEPARTMENT:**

Robert S. Sloviter, PhD - Pharmacology

**GRANTING AGENCY:**

NIH-NINDS

**SUBMISSION DATE:** August 25, 2000

**APPROVAL DATE:** November 1, 2000

**APPROVAL VALID THROUGH:** October 31, 2003

*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

**REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON:** November 2, 2000

**NOTES:**

THIS PROTOCOL HELD PREVIOUS APPROVAL UNDER CONTROL NUMBER #97-161.

**MINORITY OPINIONS (if any):**

Richard C. Powell, PhD, MS
Vice President for Research

DATE: November 2, 2000

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.
Verification of Review
By The Institutional Animal Care and Use Committee (IACUC)

Final Approval Granted

PHS Assurance No. A-3248-01 -- USDA No. 86-3

TITLE: PROTOCOL CONTROL #97-110
“Hippocampal Network Structure and Function in Epilepsy”

PRINCIPAL INVESTIGATOR/DEPARTMENT:
Robert S. Sloviter, PhD - Pharmacology

SUBMISSION DATE: July 25, 1997
APPROVAL DATE: September 25, 1997

GRANTING AGENCY:
NIH-NINDS

The University of Arizona Institutional Animal Care and Use Committee reviews all sections of proposals relating to animal care and use. The above named proposal has been granted Final Approval according to the review policies of the IACUC.

NOTES:

*** Full approval of this control number is valid through*: September 24, 2000

* When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

*** Continued approval for this project was confirmed: September 26, 1997

*** Revisions (if any), are listed below:

Michael A. Cusanovich, Ph.D.
Vice President for Research

DATE: September 26, 1997