1

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

For more information

Consultez notre site web

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Electrophysiological and Morphological
Investigation of the Perirhinal Cortex
in vitro

Robin A. Battye, B. A. Honours

Carleton University

Ottawa, Ontario

Neuroscience

Supervisor: D. C. McIntyre

© Copyright 1995

Robin A. Battye

**National Library
of Canada**

**Acquisitions and
Bibliographic Services Branch**

**395 Wellington Street
Ottawa, Ontario
K1A 0N4**

**Bibliothèque nationale
du Canada**

**Direction des acquisitions et
des services bibliographiques**

**395 rue Wellington
Ottawa (Ontario)
K1A 0N4**

Author's Address

Author's Address

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-612-08999-1

Canada

ABSTRACT

The perirhinal cortex has recently become a prominent cortical structure in both the memory/learning and the kindling literature. The purpose of this study was to identify the morphology, physiology, and synaptic properties of the cells in this area in order to establish a better understanding of its function and interconnectivity. Intracellular recordings in fifty cells from five different areas of the perirhinal cortex were obtained and the cells' morphology was assessed after with the intracellular stain biocytin. From these injections, it was possible to clearly differentiate 5 distinct cell types and classify their laminar orientation. Layer 3 type I cells were pseudo-pyramidal neurons that discharged doublet spikes at threshold and accommodated with no membrane rectification, whereas the type II layer 3 cells were single spiking, nonaccommodating pyramidal cells that exhibited many of the membrane properties of bursting cells but would not burst when activated synaptically. The large layer 5 pyramidal type I neurons clearly were excitable and burst fire but most often produced doublet spikes and a large graded anode break response with threshold spikes, and sizeable inward rectification. The stellate layer 5 type II cells were bursting neurons with non-accommodating trains of action potentials. They showed no inward rectification during anodal injections of current, but generated considerable depolarizing shifts near the threshold for action potential generation. Synaptic stimulation indicated that very few subcortical sites activate the perirhinal cortex in this slice preparation, except for the lateral and basolateral amygdala. The cellular properties, morphology, and interconnectivity are discussed with reference to other similar cortical studies.

Acknowledgements

I would like to thank my supervisor Dr. McIntyre for his support and encouragement throughout my Masters degree. Without his guidance and patience it would not have been possible to see this project through to it's completion.

I would also like to express my appreciation to Dr. Wu, Dr. Fu. Dr. Kelly, Dr. Ito, Brian Van Adel, Patrice Jacobson, Glenda MacNeil, and Christine McGregor for their technical expertise, and for the endless hours they set aside to train me in both the histological and physiological methods that were the basis of my thesis.

On a lighter note, the emotional support offered by my labmates and friends will never be forgotten, but I would like to especially recognize Brian Van Adel, Chris Davidson, Ralph Skilton, Jenny Grabowski, Leanne Felstead, Heather McNeelly, and Cathy Croteau for sticking by me through both the tough times and the good. Their friendship enriched my soul as well as my mind.

Finally, I would like to recognize the love and friendship of my mother Rosamund Battye, father David Battye, and brother Jeremy Battye and thank them for believing in me and supporting me unconditionally throughout my University education. There is no greater love offered than that of a family and friends, and I have been amply blessed with both.

TABLE OF CONTENTS

	Page
Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	vi
List of Figures	viii
 Introductory Comments	 1
Learning and Memory	1
Epilepsy & Kindling	3
Kindling Properties	6
Perirhinal Projections	7
<i>In vitro</i> Studies	9
Purpose of the Present Study	13
 Experiment	
Methods	13
Results	24
 Discussion	
Grouping of Neuronal Types	105

Layer 3 Type I Neurons	107
Layer 3 Type II Neurons	110
Layer 5 Type I Neurons	114
Layer 5 Type II Neurons	118
Layer 6 Type I Neurons	122
Perirhinal Electrophysiology Literature	124
General Comments and Functional Significance	125
References	130
Appendix A	144
Appendix B	148

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
1	Summary of the morphological attributes of each cell type found in the four defined perirhinal areas. A description of cell type, size, soma characteristics (SC), spine density, axonal projections (layers of projections), and dendritic field is provided for the cell type in each area. As only one cell type was found in each area no statistics will be given.	17
2	Cell classification and membrane discharge type (MDT) including % of responses showing regular firing (RF), doublet (D), and burst spiking (BS); the discharge pattern (DP) showing the % of cells that accommodate; the membrane inward rectification behaviour (IR) showing the % of cells that had inward rectifying currents; the % of cells showing an anode break rebound depolarization (ABRD); the % of cells showing rebound hyperpolarization (RH), and the % of cells showing afterdepolarizations following action potentials (ADEP) for each of the perirhinal areas.	68
3	The mean ($\pm$ SEM) equilibrium membrane voltage (E_m , mV), input resistance (R_i , M Ω), action potential amplitude from E_m (AP_{amp} , mV), action potential threshold from E_m (AP_{thres} , mV), action potential half amplitude duration ($AP_{1/2}$, msec.), and	69

membrane time constant (τ , msec.) from the five different areas of the perirhinal cortex.

- 4 Descriptive summary of the cell types within each of the 103
different perirhinal areas that share the same morphological,
physiological and synaptic attributes.

LIST OF FIGURES

- FIGURE 1.** Schematic diagram of the amygdala/piriform/perirhinal slice preparation. The probe shows a typical recording site in the perirhinal cortex (PRC) and other structures include the: rhinal fissure (RF), piriform cortex (PC), external capsule (EX), stria terminalis (ST), and the lateral (L), basolateral (ABL), central (CE), medial (M), and cortical (CO) nuclei of the amygdala (from McIntyre & Plant, 1989).
..... 15
- FIGURE 2.** Photomicrograph montage of a cresyl violet stained coronal section showing the anterior perirhinal cortex and its laminae. The cytoarchitectures in this section is similar to plate 27 in Swanson (1993) (from Kelly, 1995 thesis).
..... 18
- FIGURE 3.** Composite of schematic representations of the perirhinal/piriform slice adapted from Swanson (1993) showing the laminar placement of each recording electrode in four different areas of the perirhinal cortex. Figure 3A shows the distribution and number of recording sites within layer 3 area I, 3B shows the distribution and number of recording sites within layer 3 area II, 3C shows the distribution and number of recording sites within layer 5 area I, and Figure 3D shows the distribution and number of recording sites within layer 5 area II of the perirhinal cortex.
..... 25
- FIGURE 4.** Photomicrographs of Rapid Golgi impregnations in the perirhinal cortex (sections 120 μ m.). Plate A shows a lone layer 3 area I perirhinal cell overlaying the shadow of a second. The high dendritic

spine density can clearly be seen in the processes of the layer 3 area I cell in the middle of plate B. Plate C illustrates the orientation of the layer 3 area I (middle) and area II (bottom right) cells among the layer 5 area I apical dendrites. Finally, plate D shows two layer 3 area II cells with a clear display of the axons and axon collaterals. Scale bar is 100 μm .

..... 27

FIGURE 5. Photomicrographs of Rapid Golgi impregnations in the perirhinal cortex (sections 120 μm). Plate A shows a group of layer 3 area I cells and the dense canopy of apical arbours that spread under the perirhinal fissure. The base of a layer 5 area I cell can be seen in plate B along with an example of the clusters of apical dendrites projecting into layers 1 and 2. C, illustrates a grouping of layer 3 area I and II cells, and plate D shows another representative view of cells in the layer 3 area I and area II cells with one layer 3 area II stellate cell in the middle uppermost section of the photomicrograph just bellow the layer 3 area II pyramidal cell. Scale bar is 100 μm .

..... 29

FIGURE 6. Photomicrograph of Rapid Golgi Impregnations in the perirhinal cortex (sections 120 μm .). Plate A (left & right) is a layer 5 area I cell depicting the typical axonal networks found in these cells. The same cell in plate B (left & right) shows more of the axonal processes emanating from a typical layer 5 area I cell body with a clear image of the spiny dendritic arbours. Scale bar is 40 μm .

..... 33

FIGURE 7. Camera lucida reconstruction depicting two biocytin stained layer 3 area I perirhinal cortex cells from a 400 μm tissue slice. Cells were drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon). The physiological trace inset is representative of the typical membrane properties of cells in this area. Scale bar is 80 μm .

..... 38

FIGURE 8. Photomicrographs of two biocytin stained layer 3 area I perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. In this case, both plates show double stained pairs of identical cells, which occurred in 3 out of 9 cases. Scale bar is 80 μm .

..... 40

FIGURE 9. Camera lucida reconstruction depicting two biocytin stained layer 3 area II perirhinal cortex cells from a 400 μm tissue slice. Cells were drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon, ac-axon collateral). The physiological trace inset is representative of the typical membrane properties of cells in this area. Scale bar is 80 μm .

..... 44

FIGURE 10. Photomicrographs of two biocytin stained layer 3 area II perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

..... 46

FIGURE 11. Camera lucida reconstruction depicting two biocytin stained

layer 5 area I perirhinal stained cells from a 400 μm tissue slice. Cells were drawn at 400 times magnification detailing the dendritic arbour's, soma, and axonal projection (a-axon, ac-axon collateral) with small arrows marking a number of the axon collaterals. The physiological inset is representative of the typical membrane properties of cell in this area. Scale bar is 80 μm .

..... 50

FIGURE 12. Photomicrographs of two biocytin stained layer 5 area I perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

..... 52

FIGURE 13. Camera lucida reconstruction depicting one biocytin stained layer 5 area II perirhinal cortex cell from a 400 μm tissue slice. Cell was drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon, ac-axon collateral) with small arrows marking a number of the axon collaterals. The physiological inset is representative of the typical membrane properties of cells in this area. Scale bar is 80 μm .

..... 55

FIGURE 14. Photomicrographs of two biocytin stained layer 5 area II perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

..... 57

FIGURE 15. Camera lucida reconstruction depicting one biocytin stained layer 6 perirhinal cortex cell from a 400 μm tissue slice.

Cell was drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon, ac-axon collateral). The physiological inset shows the intrinsic membrane properties of this cell. Scale bar is 80 μm .

..... 61

FIGURE 16. Photomicrograph of one biocytin stained layer 6 perirhinal cortex cell from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

..... 63

FIGURE 17. Composite of Camera lucida drawings representing one of each cell type superimposed on a schematic extraction of the perirhinal cortex from plate 28 in Swanson (1993). Each cell is labelled by area and situated relative to where it was stained in the slice: layer 3 area I (a), layer 3 area II (b), layer 5 area I (c), layer 5 area II (d), and a layer 6 area I (e).

..... 66

FIGURE 18. A compilation of the standard intrinsic membrane properties of a typical layer 3 area I perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. Note the afterdepolarizations (a) following each spike. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the doublets (b) in B and the absence of any inward rectification (c) or anode break (d) in C. The current voltage relationship of this layer 3 area I neuron is seen in Figure D. Finally,

the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

..... 73

FIGURE 19. A compilation of the standard intrinsic membrane properties of a typical layer 3 area II perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. Note the afterdepolarizations (a) following each spike and the depolarizing peak (b) at the subthreshold current injection. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the inward rectification (c) and the anode break with a threshold spike (d) in C. The current voltage relationship of this layer 3 area II neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

..... 77

FIGURE 20. A compilation of the standard intrinsic membrane properties of a typical layer 5 area I perirhinal cell. In A, the membrane responses to

depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the multiple spikes (a) on the large depolarizing current injection in B, and the inward rectification (b) present with the hyperpolarizing current step in C. Also, note the large anode break response with a pair of threshold spike (c) also in C. The current voltage relationship of this layer 5 area I neuron is seen in Figure L. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

..... 82

FIGURE 21. A compilation of the standard intrinsic membrane properties of a typical layer 5 area II perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. Note the burst responses (a) occurring at spike threshold and the depolarizing peak (b) at the subthreshold current injection. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the lack of any inward rectification (c) and the absence of an anode break (d) in C. The current voltage relationship of

this layer 5 area II neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

..... 86

FIGURE 22. A compilation of the standard intrinsic membrane properties of the only perirhinal layer 6 cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. The current voltage relationship of this layer 6. neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is presented for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

..... 91

FIGURE 23. Composite schematic of a perirhinal/piriform slice from Swanson (1993) containing blackened circles representing the positions of the recording electrodes for the perirhinal layer 3 area I cells that evoked responses were recorded from. Superimposed are hollowed circles indicating the various sites at which synaptic drive of layer 3

perirhinal area I cells were attempted and containing a number indicating the number of different cells tested. For sites at which synaptic responses were recorded, a trace representing the most typical type of recording was provided and identified with an arrow and lower case letters. The rostral caudate putamen (CP) produced responses in 2 different cells (a), the medial CP produced responses in 3 different cells (b), the posterior basolateral amygdala nucleus produced weak responses in 3 different cells (c), the lateral amygdala nucleus produced responses in 4 different cells (d), and the basolateral nucleus of the amygdala produced responses in 3 different cells (e). A calibration bar is provided representing all traces.

..... 95

FIGURE 24. Composite schematic of a perirhinal/piriform slice from Swanson (1993) containing blackened circles representing the positions of the recording electrodes for the perirhinal layer 3 area II cells that evoked responses were recorded from. Superimposed are hollowed circles indicating the various sites at which synaptic drive of layer 3 perirhinal area II cells were attempted and containing a number indicating the number of different cells tested. For sites at which synaptic responses were recorded, a trace representing the most typical type of recording was provided and identified with an arrow and lower case letters. The lateral amygdala nucleus produced responses in 2 different cells (a), the basolateral nucleus of the amygdala produced responses in 3 different cells (b), and the basomedial amygdala nucleus produced responses in 2 different cells. A calibration bar is provided for each unique trace.

..... 98

FIGURE 25. Composite schematic of a perirhinal/piriform slice from Swanson (1993) containing blackened circles representing the positions of the recording electrodes for the perirhinal layer 5 area II cells that evoked responses were recorded from. Superimposed are hollowed circles indicating the various sites at which synaptic drive of layer 5 perirhinal area II cells were attempted and containing a number indicating the number of different cells tested. For sites at which synaptic responses were recorded, a trace representing the most typical type of recording was provided and identified with an arrow and lower case letters. One response was produced in the lateral amygdala nucleus (a), two different cells responses were recorded in the posterior basolateral amygdala nucleus (b), two different cells responses were recorded in the anterior basolateral amygdala nucleus (c), and one response was recorded in the anterior basomedial amygdala nucleus (d). A calibration bar is provided for each unique trace.

..... 101

Introductory Comments

In the present thesis, we were interested in learning more about the electrophysiological properties and morphological features of the neurons in the perirhinal cortex. This interest stems from two recent lines of evidence concerning the behavioural role of the perirhinal cortex. First, the perirhinal cortex has been implicated as a critical structure in certain forms of learning and memory. And, second, the perirhinal also has been shown to possess several characteristics that are likely critical to the development and/or maintenance of convulsive epileptic seizures.

In the following introduction, I review selected studies supporting an important role for the perirhinal cortex in learning and memory and kindled epilepsy. The introduction concludes with *in vitro* experiments related to temporal lobe electrophysiology, which leads directly to the purpose of the present investigation.

Learning and Memory

Recently, the perirhinal cortex has assumed an important position in the memory and learning literature. This outcome results from studies showing considerable performance deficits in both rats and monkeys on a variety of different tasks as a result of perirhinal lesions.

Previously it was believed that in order to produce temporal lobe

amnesia in animals it was necessary to produce bilateral damage to the hippocampal formation, amygdala and the adjacent cortex (Squire, Zola-Morgan, & Chen, 1988). Under detailed examination however, Zola-Morgan, Squire, Amaral, and Suzuki (1989) found that more discrete damage to the perirhinal and parahippocampal cortex, which avoided the amygdala and hippocampal formation, produced equally severe impairments to those previously witnessed in early studies. The perirhinal and parahippocampal lesions produced severe and long-lasting performance deficits in delayed nonmatching to sample (both tactile and visual), object retention, 8-pair concurrent discrimination, and pattern discrimination tasks (Squire, *et al.*, 1989; Suzuki, Zola-Morgan, Squire, & Amaral, 1993). These results prompted the revised view that it is mainly perirhinal and parahippocampal cortices that are critical in the medial temporal lobe memory systems (Squire & Zola-Morgan, 1991; Suzuki *et al.*, 1993).

As the lesions became more specific to the perirhinal cortex, and in some cases both the perirhinal and entorhinal cortices, severe memory problems continued to be seen on odour-guided delayed nonmatching to sample (Otto & Eichenbaum, 1992), fear potentiated startle (Rosen, Hitchcock, Miserendino, Falls, Campeau, & Davis, 1992), visual delayed nonmatching to sample (Meunier, Bachevalier, Mishkin, & Murray, 1993), and Morris Water Maze

place learning tasks (Nagahara, Otto, & Gallagher, 1995). Further, damage limited only to the perirhinal cortex was sufficient to produce a memory loss that was far greater than that produced from damage to any other single temporal lobe structure in both rats and monkeys (Meunier *et al.*, 1993). Thus, the perirhinal cortex appears to be critical to temporal lobe memory networks.

It is likely no accident that temporal lobe seizure activity can produce memory impairments in humans that are similar in nature to those produced by temporal lobe lesions. This observation would reinforce the prediction that the perirhinal cortex could be critically involved in temporal lobe seizure activity. Such a suggestion also would dictate that certain cellular or network properties within the perirhinal cortex likely assume similar roles in both memory and epileptogenic behaviour.

Epilepsy and Kindling

Epilepsy is a chronic neurological disorder consisting of periods of abnormal brain behaviour that are usually characterized by paroxysmal attacks of electrographic discharges. These discharges are often accompanied by motor responses that range from mild striated muscle contractions to violent motor convulsions, usually resulting in a loss of consciousness. Epileptogenic behaviour can usually be isolated to a causative period of neuronal stress and/or damage, which persists either as an ongoing abnormality or appears after

extended periods of dormancy (years) depending on the severity and pervasiveness of the damage (Kandel, Schwartz, & Jessel, 1991; Shouse, 1994; Kolb & Whishaw, 1990).

Seizures are marked by ictal or rhythmic physiological activity, and are derived from the synchronous discharge of large populations of neurons, creating waveforms consisting of slow waves, sharp waves, and spikes. Sometimes this activity generalizes or spreads into the motor convulsive networks. The frequency, duration, severity, and generalization of these discharges, accompanied by the associated behavioural reactions, define the different types of epilepsy described in both humans and other animals (Goddard *et al.*, 1969; Kolb & Whishaw, 1990).

The mechanisms underlying both epileptogenesis and acute discrete seizures have been difficult to study in the human due to the inaccessibility, sensitivity, and complexity of the many factors involved (Kandel, Schwartz, & Jessell, 1991; Kolb & Whishaw, 1990). Consequently, the means to control this aberrant behaviour have been restricted by a limited understanding of the behaviour and the lack of an appropriate model on which to test hypotheses.

An ideal means by which to examine the development and neural basis of the epileptic behaviour is through the use of an animal model of epilepsy. The most commonly used model, as developed by Goddard, McIntyre, and

Leech (1969) is called kindling. This model describes the slow modification of an animal's behaviour by progressive daily stimulation of a discrete focal forebrain structure using a low-intensity current. This focal stimulation triggers a local electrographic afterdischarge that progressively increases over time and results ultimately in secondary generalization accompanied by motor convulsions (for detailed review see Racine, 1978 & Goddard, 1983). The relevance of this model to human complex partial seizures is attested to by its compliance with most of the criteria proposed by Wada (1976) for a good experimental model of epilepsy. For example, kindling gives precise experimental control over the location of the epileptogenic focus while producing little or no damage at that site. The induction of the seizure is controlled by a fixed current that produces an ictal afterdischarge that is sustained for several seconds after the stimulus offset. Also, as development of the kindled focus is continued, interstimulus intervals can be quite long (weeks) with virtually no attenuation in previously established kindled levels. After extended periods of kindling, spontaneous seizures ultimately develop, appearing at different time periods based on the type of animal and the kindling focus. Thus, kindled tissue provides a very good model of an epileptic network on which to study and test hypotheses.

Kindling Properties

During the kindling of a particular brain site, several measures of epileptogenicity may be gathered. These include the rate of progression to generalized seizure manifestation (# of stimulations); the threshold intensity of current necessary to trigger a local afterdischarge, and the duration of that discharge. There are also several other useful measures once convulsive seizures are evident. These other measures include the length of time from stimulus onset to the appearance of the convulsion (latency), the duration of the convulsion, and the duration of the associated afterdischarge.

It is very clear from the kindling literature that until very recently, the amygdala and piriform cortices provided the fastest rates of kindling in the forebrain (Goddard *et al.*, 1969; McIntyre, 1975; Racine, 1978; Holmes, *et al.*, 1992; McIntyre & Kelly, 1993). More recently, however, the perirhinal cortex has shown ever faster rates of epileptogenesis. The number of stimulations required to kindle the perirhinal cortex is approximately 3-5, as compared to 5-8, and 6-12 for the piriform cortex and amygdala, respectively. This result suggests that the perirhinal cortex can access the motor systems supporting convulsive behaviour faster than all other forebrain sites (McIntyre & Kelly, in progress).

Additional support for this suggestion comes from the clonus onset

latency data from the various kindling experiments. The clonus latencies from the perirhinal kindling sites are approximately 1 second, compared to 20 and 10 seconds from the piriform cortex and amygdala, respectively (McIntyre, Kelly, & Armstrong, 1993). Thus, a seizure triggered from the perirhinal cortex gains access and control over motor seizure networks in fewer sessions of triggered afterdischarge, and with greater speed once a sufficient afterdischarge has been triggered, then other forebrain sites (McIntyre & Kelly, in progress).

In spite of this rapid kindling development, the perirhinal cortex has a relatively high local afterdischarge threshold. Such high local thresholds are quite typical of neocortical sites and are likely related to strong local inhibitory mechanisms (Burnham, 1978). Over the course of kindling, the afterdischarge threshold drops precipitously to much lower values in all structures (McIntyre, Kelly, & Armstrong, 1993). Such a drop likely reflects a change in inhibitory tone in the stimulated structure, which presumably, allows local afterdischarge to increase in duration and recruit additional networks of cells.

The speed with which the perirhinal cortex is able to recruit the motor networks into convulsive seizures begged the question - "to what structures does the perirhinal cortex project?"

Perirhinal Projections

Various studies have been conducted that examine the afferent and

efferent connections to and from the perirhinal cortex using a number of different tracers. It is beyond the bounds of this review to examine thoroughly all these connections, but I will address in general the most prominent of these connections.

Efferent projections determined by an injection of the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PhAl) into anterior perirhinal cortex of rats are found both in neocortical and subcortical targets (McIntyre, Kelly, & Staines, in review). Perhaps most important are the very strong neocortical projections to layers 1, 2, & 6 of the frontal motor cortex. Injections of the retrograde tracer Fluorogold (FG) into both the ipsilateral and contralateral frontal cortex confirmed the perirhinal projection and identify the layer 5 cells as the main source of this cortical projection.

Other less prominent neocortical projections include the insular, infralimbic, orbital, parietal, and temporal cortices. Subcortical targets that are heavily innervated include the basolateral, basomedial and lateral nuclei of the amygdala, the nucleus accumbens, fundus striati, and several thalamic nuclei (Kosel, Van Hoesen, & Rosene, 1983; McIntyre, *et al.*, in review). Less numerous projections include the substantia nigra compacta, anterior pretectal nucleus, central grey, dorsolateral midbrain reticular formation, parabrachial nucleus, lateral hypothalamus, and the laterodorsal tegmental area, which

demonstrate the vast number of outputs arising from the perirhinal cortex. In stark contrast to the prolific perirhinal output, fewer subcortical afferents have been determined. The most prominent subcortical afferent, however, is the lateral nucleus of the amygdala (Krettek & Price, 1974; Deacon, *et al.*, 1983).

Summarizing from the anatomical information, it is clear that there are ample neocortical projections from the perirhinal cortex to support a behavioural motor seizure pathway as suggested by the kindling data. Furthermore, it is clear that a majority of these projections, particularly those accessing the frontal motor networks, emanate from the layer 5 perirhinal cells. Although, very few limbic perirhinal afferents or inputs are presently determined, reciprocal interactions do appear to occur between the lateral nucleus of the amygdala and regions of the perirhinal cortex.

***In vitro* Studies**

As was indicated earlier, the neural networks in the temporal lobe that comprise the piriform and perirhinal areas are of considerable interest to both learning-memory researchers and to epileptologists (Leech & McIntyre, 1976; McIntyre, 1978; Hoffman & Haberly, 1989). Analysis of these areas currently is providing both models of cellular learning and giving insight into the neuronal mechanisms of epilepsy. With this in mind, a number of studies have involved *in vitro* analysis of previously kindled tissue in order to examine

epileptic neurons and their networks in the piriform lobe. McIntyre and Wong (1985) found that in amygdala-piriform-perirhinal slices that were taken from amygdala kindled rats, the normally triggered discharges in the piriform cortex were significantly prolonged compared to the discharges in control tissue. Further, by suppressing the synaptic events with increased Mg^{2+} , it was obvious that the epileptic "burst" responses in the piriform cortex were riding on a depolarizing shift (Prince & Wong, 1981; Jessen & Yaari, 1988; Burke & Hablitz, 1994) that was much greater in the kindled tissue (McIntyre & Wong, 1986). Such burst responses are triggered most typically by networks of neurons, but occasionally can be observed as an important intrinsic response in hyperexcitable neurons.

In this regard Tseng and Haberly (1989) have noted that some types of multipolar neurons in the deep piriform cortex showed spontaneous bursting patterns. These cells were sensitive to both intracellular depolarizing and hyperpolarizing current injections, where their rates of bursting increased and decreased, respectively. However, these neurons were not representative of the majority of cells in this region, and in fact, they appeared to be isolated to a small area deep in layer 3. This type of spontaneous burst response in normal tissue is not unique to the piriform cortex, but it appears in several other regions. For example, Scharfman (1993a, 1993b, 1994a, 1994b) has found that

in the hippocampus, aspiny hilar neurons exhibit bursting or fast spiking patterns that are very similar to those described by Tseng and Haberly (1989). In the hippocampus she also found that CA1 and CA3 cells could show bursting properties but first must be rendered hyperexcitable by GABA blockade.

In a recent elegant study, Stewart and Wong (1993) identified many bursting cells in the subiculum that exhibited unique intrinsic physiological responses. A large number of these subicular neurons burst spontaneously. In addition, when current was injected into the cells, some cells burst and then began single spiking during the depolarizing injection. On the other hand, several other cells showed burst responses, but only following a hyperpolarizing current injection (depolarizing anode break response). Most importantly, they also observed that bursting and non-bursting cells received different synaptic inputs. Clearly this type of experiment can identify significant intrinsic and synaptic behavioural difference between cells, and relate some of those differences to variations in the organization of the neuronal network.

With the presently limited information available on the cellular properties of the perirhinal cortex, it is difficult to postulate on its functional organization. Our lab has shown that in paired extracellular *in vitro* recordings

of the perirhinal and piriform cortex, 0 Mg^{2+} induced bursting, a hallmark of epilepsy, begins first in the perirhinal cortex and then spreads to the piriform cortex in control animals. However, in previously amygdala kindled animals the bursting first occurs in the piriform cortex, then spreads to the perirhinal cortex (McIntyre & Plant, 1991; 1993). This simple demonstration shows how kindling can induce changes in the neural network either within the perirhinal cortex, or between it and other areas, or both, and suggests that the cells or the properties of the cells in the perirhinal area may be changing during epileptogenesis in response to amygdala/piriform inputs. It therefore becomes important to understand the cell types and their physiology within the perirhinal cortex in order to further describe what changes are occurring during limbic kindling.

With one exception, nothing is known about the intrinsic and morphological properties of the cells in the perirhinal cortex. That exception involved a recent study in which an attempt was made to classify the various perirhinal neurons based on their morphological and intrinsic properties (Beggs & Kairiss, 1994). To determine the morphology, ten perirhinal cells were stained intracellularly. This provided a terse morphological identification of two cell types, which were described as pyramidal cells with either thick, or thin, apical dendrites. Based on their intrinsic characteristics, the cells were

classified into three traditional groups: (a) prototypical 'regular spiking' cells, or singlet spikers, (b) intrinsically bursting, or doublet spikers, and (c) fast spiking cells. In that study no synaptic properties were reported for any cell, nor was the cell's location in the perirhinal laminar structure described.

Purpose of the Present Study

The purpose of the present investigation was to further analyze the intrinsic and synaptic electrophysiological properties of cells in the perirhinal cortex. In addition, we morphologically identified the cells with high resolution fluorescent and/or biotin intracellular staining techniques (bio-imaging), indicating the lamina in which they resided. Thus the membrane properties, synaptic responses and firing patterns were compared between cells and layers to better understand the cellular and local network properties of the perirhinal cortex.

Methods

Subjects

Forty-five male Charles River Outbred Long-Evans Hooded rats (75-300 g) were decapitated in accordance with Carleton Animal Care guidelines.

Slice Preparation

The brains were rapidly (1-2 minutes) removed and placed in oxygenated (95% O₂/5% CO₂ mixture), ice chilled (0-2 °C) sucrose-artificial

cerebral spinal fluid (ACSF), consisting of (in mM): 5.0 KCl, 2.0 MgSO₄, 26.0 NaHCO₃, 1.25 NaH₂PO₄, 9.4 D-glucose, 2.0 CaCl₂, 262.0 sucrose, and 0.68 kynurenic acid for 30-90 seconds (pH 7.4). A 3-4 mm block of tissue containing the amygdala, piriform, and perirhinal cortex was removed from either the left or right temporal lobe cortex using a number 10 scalpel blade on a chilled glass plate (0-2 °C). The tissue was cut first with a deep horizontal incision dorsal to the rhinal fissure and penetrating to the hypothalamus. This was followed by a coronal cut anterior to the entorhinal cortex, orthogonal to the first cut. The third cut was coronal, and anterior to the amygdala, at the posterior curvature of the lateral olfactory tract. Finally, the tissue block was excised by a parasagittal section immediately lateral to the optic tract (see Fig. 1 for a cartoon representation of a standard slice). The entorhinal coronal surface of the tissue block was glued to a cutting stone using a generic instant super glue adhesive. The tissue and stone were placed in a chilled ACSF bath (0-2 °C) while being continuously oxygenated and several 400 µm coronal sections were taken through the perirhinal cortex using a series 1000 Vibratome (Pellico 101). Then 2-4 slices were transferred in serial order to a recording chamber with an open interface supplying oxygenated (95% O₂-5% CO₂) humidified air and superfused with warmed (32.0 °C) oxygenated sucrose

FIGURE 1. Schematic diagram of the amygdala/piriform/perirhinal slice preparation. The probe shows a typical recording site in the perirhinal cortex (PRC) and other structures include the: rhinal fissure (RF), piriform cortex (PC), external capsule (EX), stria terminalis (ST), and the lateral (L), basolateral (ABL), central (CE), medial (M), and cortical (CO) nuclei of the amygdala (from McIntyre & Plant, 1989).

ACSF (pH 7.4). The tissue was maintained at a constant flow rate (1.8 ml/min) throughout the experiment and supported on a porous lens paper for easy removal. After 30-45 minutes of perfusion with the sucrose ACSF, the perfusion medium was switched to a normal medium consisting of (in mM): 124.0 NaCl, 5.0 KCl, 2.0 MgSO₄, 26.0 NaHCO₃, 1.25 NaH₂PO₄, 9.4 D-glucose, and 2.0 CaCl₂, and was allowed to equilibrate for a further 15 minutes before recording commenced (pH 7.4). All solutions were prepared daily with fresh (<12 hours) double distilled H₂O stored in a glass receptacle. ACSF was further filtered with a Wattmans number 1 filter paper before the addition of CaCl₂, and then oxygenation.

Recording Preparation

Intracellular recordings were made from at least 15 labelled cells in each of the two main perirhinal layers (3 & 5, see Fig. 2 for the general anatomy of the perirhinal layers), and a few of recordings were obtained from layer 6 cells. An accurate representation of each slice and its anatomy was catalogued, and distinguishing features that could aid in determining electrode placements were noted.

The electrodes were composed of borosilicate glass pipettes (WPI, O.D. 1.0mm, I.D. 0.58mm), pulled horizontally on a Flaming Brown Micropipette puller (Sutter Instruments, Model P-87) to a resistance of 60-150 MΩ.

FIGURE 2. Photomicrograph montage of a cresyl violet stained coronal section showing the anterior perirhinal cortex and its laminae. The cytoarchitectures in this section is similar to plate 27 in Swanson (1993) (from Kelly, 1995 thesis).

Intracellular electrodes were then backfilled with either 2-2.5% Biocytin in 2M potassium acetate (Sigma B4261) or 3% Lucifer Yellow in 1M LiCl (Sigma LO259). The high resistance electrodes were completely filled with 2M potassium acetate and then mounted in electrode holders (WPI, MEH2RW) to fit an AM Systems high-input impedance amplifier (model 1600). At this time, the initial bridge balance was performed and the electrode resistance tested. Stimulator calibrations (Grass SD9) were taken regularly and recorded digitally (see below).

The intracellular electrode was then visually positioned on the recording site by means of a dissecting microscope (Scientific Instruments, AO-580) and an Aus Jena micromanipulator. The electrode position was recorded for every placement whether a recording was made or not along with the duration of the placement. Using a Nicolet Digital Oscilloscope (model 3091), the electrode was guided through the cell fields until an appropriate cell could be held with an equilibrium membrane voltage E_m of -70 ± 10 mV, and input resistance (R_i) greater than $20\text{ M}\Omega$, with overshooting action potentials (> 65 mV).

Data Collection

A standard collection of intrinsic membrane measures were taken including input resistance, capacitance, spike threshold, and certain membrane manipulations (Appendix A). The data were digitized and stored in an

analogue format using an A-D system from Medical Systems Corp. (model PCM4/8, 22222 Hz digitizing frequency) and a Sony Video FM Recorder (model SLV-555UC) with Hi-Fi stereo FM tapes (T120HG-ES SP). The bridge balance was reaffirmed each time current was passed, and during the hyperpolarizing step currents. Following collection of the intrinsic cellular data, cells were stained with either biocytin or lucifer yellow (see below). These methods of cell staining have been shown to have no obvious effect on the intrinsic cellular properties (Libri, *et al.*, 1994; Scharfman, 1993a, 1994b) and are designed to provide single unit staining of soma, dendrites, and axons (Kitai, Penny, & Chang, 1989; Liu, Behbehani, & Shipley, 1993). With this in mind and because of the need to morphologically identify these cells, intracellular staining prior to synaptic activation seemed most reasonable. The reason for this stems from the fact that often cells are "lost" when moving the extracellular stimulating electrode.

For those cells in which successful penetration and recordings were maintained, synaptic activity was determined using a Grass stimulator (model SD9) and a paired set of tungsten microelectrodes (WPI, TST53A05KT, tip separation 0.5mm). The range of stimulation sites included the medial, central, lateral, and basolateral amygdala nuclei, and the ventral piriform cortex followed by the dorsal piriform cortex (see Figures 6-8). After the synaptic

activation phase of the experiment, the intracellular electrode was carefully withdrawn from the cell with an initial forward thrust and a steady slow withdrawal. The quality of the withdrawal was noted along with any consequent changes in cell behaviour. Intracellular data were obtained from only one cell in a cortical area per slice.

Dye Injection and Histology

A complete description of the dye injection methods and stain processing procedure for both biocytin and lucifer yellow can be found in Appendix B.

Additionally, in order to better identify the injected cells in their orientation, classification, and interconnectivity, a series of Golgi stained sections through the perirhinal area were also prepared (Valverde, 1965; Snodgrass, 1970; Millhouse, 1979; Kalsbeck, *et al.*, 1989; Freund & Somogyi, 1989; Ogawa, 1990). Intracellular injection morphological results will be compared to the Golgi stained cells to enhance the descriptive information provided by the injected material (Figs. 20-22).

Data Analysis

All data were analyzed off-line, using a microcomputer and a spreadsheet (Microsoft Excel 5.0) with statistical capabilities. Results were downloaded in binary format from the FM recorder, redigitized (22222 Hz)

using the A-D convertor, and stored in ASCII files for spreadsheet compatibility. Using the data log, all relevant intracellular and synaptic traces for each cell were sorted, edited and truncated, graphed to scale, and then the appropriate quantitative measures were recorded and analyzed (see appendix A).

Intracellular membrane and synaptic properties were determined from the graphed data in combination with the derived values. The cells were then classified into groups based on similarities in their intrinsic, synaptic, and morphological properties and orientation within the laminae. Pictures of the individual cells were evaluated and two cells of each morphologically distinct group were traced under high magnification using a camera lucida system (Zeiss) at a 10X objective and 40X magnification.

Photography

Photographic records were taken from each stained cell at a 200X magnification using a Kodak black and white film (T-Max, Tri-X 400) and a Zeiss MC100 35 mm camera mounted on a Zeiss light microscope. Film was developed and enlarged at Carleton University using Kodak products, and the scale was determined using a 1 mm standard scale bar processed along-side each print.

Results

A complete intrinsic physiological data set was gathered from 50 cells in the perirhinal cortex, 31 of which were stained sufficiently to identify their morphology. Minimum criteria for acceptable neurons were an equilibrium membrane potential (E_m) of at least -68 mV, an action potential of > 65 mV from rest, and a sufficient input resistance (R_i) to show clear charging of the membrane (~20 M Ω).

Grouping and Classification of Cell Types

Each recording electrode was positioned in one of four different areas within the perirhinal cortex, illustrated in Figure 3. These areas were defined by specific aspects of the perirhinal cytoarchitecture seen in the Figure 2 Nissl stain and the Golgi stains described below.

The perirhinal layer 3 was divided into two areas. The superficial area I was representative of the densely packed ribbon of cell bodies composing the most lateral aspects of layer 3. Golgi impregnations in this area suggested that it consisted mainly of small pyramidal cells with large round cell bodies, short densely spined apical dendrites, spiny basilar dendrites, and a large apical dendritic field (Figs. 4AB & 5A). These cells appeared tightly grouped and projected into the crux of the perirhinal fissure with dendritic processes that dominated the superficial aspects of layer 3. No other cell type has been

FIGURE 3. Composite of schematic representations of the perirhinal/piriform slice adapted from Swanson (1993) showing the laminar placement of each recording electrode in four different areas of the perirhinal cortex. Figure 3A shows the distribution and number of recording sites within layer 3 area I, 3B shows the distribution and number of recording sites within layer 3 area II, 3C shows the distribution and number of recording sites within layer 5 area I, and Figure 3D shows the distribution and number of recording sites within layer 5 area II of the perirhinal cortex.

A **B**

• laminar stimulation sites

C **D**

FIGURE 4. Photomicrographs of Rapid Golgi impregnations in the perirhinal cortex (sections 120 μm .). Plate A shows a lone layer 3 area I perirhinal cell overlaying the shadow of a second. The high dendritic spine density can clearly be seen in the processes of the layer 3 area I cell in the middle of plate B. Plate C illustrates the orientation of the layer 3 area I (middle) and area II (bottom right) cells among the layer 5 area I apical dendrites. Finally, plate D shows two layer 3 area II cells with a clear display of the axons and axon collaterals. Scale bar is 100 μm .

FIGURE 5. Photomicrographs of Rapid Golgi impregnations in the perirhinal cortex (sections 120 μm). Plate A shows a group of layer 3 area I cells and the dense canopy of apical arbours that spread under the perirhinal fissure. The base of a layer 5 area I cell can be seen in plate B along with an example of the clusters of apical dendrites projecting into layers 1 and 2. C, illustrates a grouping of layer 3 area I and II cells, and plate D shows another representative view of cells in the layer 3 area I and area II cells with one layer 3 area II stellate cell in the middle uppermost section of the photomicrograph just below the layer 3 area II pyramidal cell. Scale bar is 100 μm .

clearly distinguished in this area, although it is clear in the literature that other smaller stellate cell morphologies are commonly present in other cortical layer 3 tissue (Connors & Gutnick, 1990).

The remaining area II, constituted the loosely packed cell field lateral to the layer 5 border. Golgi impregnations in this region showed two morphologies. Primarily, the medium sized spiny pyramidal cells seen in Figures 4D and 5C&D had longer apical processes than the layer 3 area I cells and appeared sporadically in the tissue between the layer 5 border and layer 3 area I. These neurons had a smaller cell body with a simple apical dendrite that extended into the perirhinal layer 1 laminae. The cell body produced a rather small and thin basal dendritic field that extended mainly within area II bellow the area I cell body fields. The secondary stellate cell type seen in Figure 5D was even less common and appeared at random sites throughout the layer 3 area II pyramidal cell field. Both layer 3 areas can be seen in Figures 5C & D.

The perirhinal layer 5 was divided in a similar manner; area I being defined by the large, loosely distributed cell bodies immediately adjacent to the layer 3/5 border, and area II comprised the tightly grouped cells along the layer 5/6 border. The only clear Golgi pictures of layer five showed the large extremely spiny pyramidal cells that defined layer 5 area I (Figs. 5B, middle)

and the unrecognizable clumps of processes that occupied the deeper layer five area II (Fig. 5B, bottom).

The giant pyramidal cells in layer 5 area I had wide reaching complex basal dendritic fields that dominated most of the layer 5 cell field (Fig. 6). The long apical processes branched numerous times as they projected through all the perirhinal layers and terminated just below the perirhinal fissure. These cells had a very large network of axon and axon collaterals that projected throughout all the perirhinal layers and medially through the external capsule giving the cell access to most of the perirhinal cortex. Although these cells were less densely packed than any of the layer 3, 5 area II, and layer 6 cells they appeared to have the largest dendritic and axonal fields. In contrast, the Nissl stains (Fig. 2) suggest that the perirhinal layer 5 area II consists of a motley collection of smaller cell types packed tightly against the layer 5/6 border. The thin band of closely packed cells in layer 6 were not further divided and treated as one distinct area.

The boundaries of these areas were not definitive, and therefore attention was focused on regions well within these boundaries that were best representative of the cytoarchitecture. Thus, four different recording sites were tested, defined as follows: perirhinal layer 3 area I, perirhinal layer 3 area II, perirhinal layer 5 area I, and perirhinal layer 5 area II (Fig. 3).

FIGURE 6. Photomicrograph of Rapid Golgi Impregnations in the perirhinal cortex (sections 120 μm .). Plate A (left & right) is a layer 5 area I cell depicting the typical axonal networks found in these cells. The same cell in plate B (left & right) shows more of the axonal processes emanating from a typical layer 5 area I cell body with a clear image of the spiny dendritic arbours. Scale bar is 40 μm .

Placement of Electrodes: The placement of the recording electrode within each of these areas was determined by several landmarks. In each slice the external capsule and the perirhinal fissure were the most distinctive features. Care was also taken to place the tissue slices in serial order and in an orientation identical to that of their extraction. Using the Swanson atlas (1993) and the Nissl stains described by Kelly (1995, thesis), the laminae and the areas between the perirhinal fissure and the external capsule were estimated and the electrode tip was placed within the desired area with the aid of a dissecting microscope (Fig. 3). Furthermore, the electrode placements were restricted to a narrow column of the perirhinal cortex directly between the crux of the perirhinal fissure and the division of the external capsule. In situations where cells could not be attained within these delineations, some sites were acquired slightly remote to these areas. Every placement was subsequently recorded on a schematic reproduction of the slice and the position of each acquired cell was compiled as seen in Figure 3.

In total, 14 cells were recorded in the perirhinal layer 3 area I, 18 cells were recorded in perirhinal layer 3 area II, 10 cells were recorded in perirhinal layer 5 area I, 5 cells were recorded in perirhinal layer 5 area II, and 3 cells were recorded in layer 6. Of these recorded cells, morphology was attained for 6 layer 3 area I cells, 11 layer 3 area II cells, 9 layer 5 area I cells, 3 layer 5

area II cells, and 2 layer 6 cells and will be described below.

Morphology

Thirty-one perirhinal cells were stained sufficiently to clearly identify their morphology and their orientation within the perirhinal laminae. Based on a number of descriptive measures including cell size, type, spine density, axonal projections, dendritic fields, and the type of apical dendrite, five distinct types of morphologies were clearly identified (see Table 1). These morphologies corresponded directly with the area from which they were recorded and the Golgi impregnations in those areas (Figs. 7-16). The detailed morphology of the cells in each of the four previously defined perirhinal areas will be outlined below along with the morphology attained in layer 6.

Layer 3 area I neurons

Six neurons with this distinct morphology were stained, three of which stained as one of a pair, the second cell presumably through gap junctions. These cells were found in the superficial portion of layer 3 (Figs. 7 & 8) and appeared as small pyramidal-like cells with a flat but wide field of very spiny dendrites limited to the uppermost horizontal plane of layer 3 (see Figs. 4B & 5A for similar Golgi impregnated examples). The apical dendrites were also very spiny and typically divided into two main branches very close to the soma, which then distributed a wide field of processes into layer 1 around the

TABLE 1

Summary of the morphological attributes of each cell type found in the four defined perirhinal areas. A description of cell type, size, soma characteristics (SC), spine density, axonal projections (layers of projections), and dendritic field is provided for the cell type in each area.

Perirhinal Area	Cell Type	Cell Size	Soma (SC)	Spine Density	Axonal Projection	Dendritic Field
Layer 3 Area I n=6	pyramidal	small compact 2 layers	large round	high over entire cell	layers 3, 2, & 1 back to EC	wide apical, wide compact basal, very dense
Layer 3 Area II n=11	pyramidal	medium fine simple 3 layers	small round	moderate, high on apical	layers 3 & 2 back to EC	narrow apical, sparse wide basal
Layer 5 Area I n=9	pyramidal	large complex 4-5 layers	large oblong triangle	high over entire cell	all layers & back through the EC	narrow dense apical, wide & deep basal, dense
Layer 5 Area II n=3	stellate	medium wide 2 layers	large triangular	moderate over processes	layers 5 & 6, & along lateral EC	long thin dendrites within layer 5
Layer 6 n=2	pyramidal like	small compact	small triangular	sparse over entire cell	layers 6 & toward amygdala	wide compact basal within layer 6, short apical with wide field

FIGURE 7. Camera lucida reconstruction depicting two biocytin stained layer 3 area I perirhinal cortex cells from a 400 μm tissue slice. Cells were drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon). The physiological trace inset is representative of the typical membrane properties of cells in this area. Scale bar is 80 μm .

FIGURE 8. Photomicrographs of two biocytin stained layer 3 area I perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. In this case, both plates show double stained pairs of identical cells, which occurred in 3 out of 9 cases. Scale bar is 80 μm .

perirhinal fissure with a progressively increasing density of spines (Fig. 8).

The biocytin stained layer 3 area I neurons all appeared to have a very simple axonal projection with a minimal number of bifurcations back into the deeper layers of perirhinal cortex (Fig. 8). However, the Golgi stains of these type of cells (Figs. 13A,C) suggest that this may not be the complete story, since many axon collaterals of these cells could be traced superficially into layer 1 and/or between other similar layer 3 cells.

Layer 3 area II neurons

These are moderately sized pyramidal cells (n=11) with long, thin apical dendrites that penetrate into the perirhinal layer 1 where they branch in a limited manner around the perirhinal fissure (Figs. 9 & 10). The dendritic field is contained mainly within the deeper regions of layer three and consists of a rather unremarkable group of moderately spined processes that form a compact radius around a round soma, similar to that of the area I cell's. The cells soma is situated just above the border of layer five, and sends an axon back towards the external capsule, which bifurcates in two directions midway through layer five (Fig. 9). These axon collaterals travel horizontally in both directions through layer 5 as the main axon continues back towards the external capsule. A number of the horizontal axonal projections appear to extend beyond the slice.

FIGURE 9. Camera lucida reconstruction depicting two biocytin stained layer 3 area II perirhinal cortex cells from a 400 μm tissue slice. Cells were drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon, ac-axon collateral). The physiological trace inset is representative of the typical membrane properties of cells in this area. Scale bar is 80 μm .

FIGURE 10. Photomicrographs of two biocytin stained layer 3 area II perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

Layer 5 area I neurons

The soma of layer 5 area I cells (n=9) rests just below the border of layer 5 and 3 sending a very large apical dendrite into layers 1 and 2 that branches numerous times within a very narrow region of the perirhinal fissure, subpially (Figs. 11& 12). A very complex and large array of moderately spiny dendritic processes extend from the soma and in some cases along the apical dendrite. These processes divided and crossed each other numerous times as they formed a field that encompassed large regions of layer 5 and the deeper portions of layer 3 (Fig. 11). In contrast to the layer 3 area II cells, the axon of these neurons bifurcates numerous times, sending collaterals vertically through all the perirhinal layers, and radially throughout layer 5 (Fig. 11). A large number of the collaterals project within the neurons own dendritic field, and others project back through the external capsule in an rostral direction. In this experiment, these were the most morphologically complex of the perirhinal pyramidal cells that were identified.

Layer 5 area II neurons

Only three of these stellate-type cells were stained and each was found at a slightly different depth in layer 5. All had many large dendritic extrusions that penetrated deep into layer 6 and spread horizontally within layer 5 (Figs. 13 & 14). The orientation of the major dendritic arbours in relation to the

FIGURE 11. Camera lucida reconstruction depicting two biocytin stained layer 5 area I perirhinal stained cells from a 400 μm tissue slice. Cells were drawn at 400 times magnification detailing the dendritic arbour's, soma, and axonal projection (a-axon, ac-axon collateral) with small arrows marking a number of the axon collaterals. The physiological inset is representative of the typical membrane properties of cell in this area. Scale bar is 80 μm .

FIGURE 12. Photomicrographs of two biocytin stained layer 5 area I perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

FIGURE 13. Camera lucida reconstruction depicting one biocytin stained layer 5 area II perirhinal cortex cell from a 400 μm tissue slice. Cell was drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon, ac-axon collateral) with small arrows marking a number of the axon collaterals. The physiological inset is representative of the typical membrane properties of cells in this area. Scale bar is 80 μm .

FIGURE 14. Photomicrographs of two biocytin stained layer 5 area II perirhinal cortex cells from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

soma gave these cells the appearance of a flattened 'X'. Furthermore, the dendritic branching was such that most areas immediately around the neuron were involved. Axonal projections from these cells formed a highly complex network of collaterals in the immediate vicinity of the cell and one major axon that projected rostrally along the most immediate surface of the external capsule.

Layer 6 neurons

This very unique satellite-like cell has a major dendritic process that gives the impression this cell is orientated towards the external capsule (n=2). The remaining dendrites form around this process creating a rather narrow and long dendritic field spread through layer 6 (Figs. 15 & 16). The major axon projects through layer 6 toward the amygdala portion of the slice with a number of collaterals projecting to the space immediately adjacent to the recorded neuron.

Perirhinal area morphology

The morphology of the different perirhinal areas corresponded exactly to that suggested by the Golgi impregnation data with the exception of the failure to inject a perirhinal layer 3 area II stellate cell and the limited information available on layer 5 area II cells. The Golgi data indicated that relatively few cell types existed in each of these perirhinal areas and that the areas were morphologically distinct. This was further confirmed with the biocytin

FIGURE 15. Camera lucida reconstruction depicting one biocytin stained layer 6 perirhinal cortex cell from a 400 μm tissue slice. Cell was drawn at 400 times magnification detailing the dendritic arbours, soma, and axonal projection (a-axon, ac-axon collateral). The physiological inset shows the intrinsic membrane properties of this cell. Scale bar is 80 μm .

FIGURE 16. Photomicrograph of one biocytin stained layer 6 perirhinal cortex cell from a 400 μm tissue slice taken in one focal plane. Scale bar is 80 μm .

injections. The intracellular morphological data summarized in Figure 17, shows the morphology of each cell type that was readily stained in each of the defined perirhinal areas. The morphology was consistent for every cell stained in each of the perirhinal layers and no other cell types could be readily identified.

Physiology of Neurons in the perirhinal areas

Physiology was gathered for all of the laminar and area stimulation sites indicated in Figure 3. From these data a number of descriptive and quantitative measures were described and compared for the cells in each area, shown in Tables 2 and 3. Although some of these measures were the same for each area of the cortex, a number of distinctive physiological characteristics presented themselves and will be described below for each area.

In general, the more superficial layer 3 area I cells were all doublet spiking cells that accommodated and usually showed no form of inward rectification or rebound phenomena. In cases where inward rectification was present it was not clear whether it was an artifact or membrane channel properties as there were no anode break responses and the traces were inconsistent on the hyperpolarizing current steps in those cells. In most cases the layer 3 area I cells also showed afterdepolarizations following each action potential, again, some traces were inconsistent and only included in the analysis because they exhibited a doublet spiking pattern identical to the other

FIGURE 17. Composite of Camera lucida drawings representing one of each cell type superimposed on a schematic extraction of the perirhinal cortex from plate 28 in Swanson (1993). Each cell is labelled by area and situated relative to where it was stained in the slice: layer 3 area I (a), layer 3 area II (b), layer 5 area I (c), layer 5 area II (d), and a layer 6 area I (e).

TABLE 2

Cell classification and membrane discharge type (MDT) including % of responses showing regular firing (RF), doublet (D), and burst spiking (BS); the discharge pattern (DP) showing the % of cells that accomodate; the membrane inward rectification behaviour (IR) showing the % of cells that had inward rectifying currents; the % of cells showing an anode break rebound depolarization (ABRD); the % of cells showing rebound hyperpolarization (RH), and the % of cells showing afterdepolarizations following action potentials (ADEP) for each of the perirhinal areas.

Cell Type	MDT	DP	IR	ABRD	RH	ADEP
Layer 3 Area I n=14	D & RF 100 %	86 %	33 %	0 %	0 %	79 %
Layer 3 Area II n=18	RF 100 %	0 %	89 %	89 %	0 %	89 %
Layer 5 Area I n=10	D & RF 100 %	0%	100 %	100 %	100 %	0 %
Layer 5 Area II n=5	BS & RF 80 %	0%	0 %	0 %	0 %	0 %
Layer 6 n=3	RF	100 %	0 %	0 %	0 %	0 %

TABLE 3

The mean ($\pm$ SEM) equilibrium membrane voltage (E_m , mV), input resistance (R_i , $M\Omega$), action potential amplitude from E_m (AP_{amp} , mV), action potential threshold from E_m (AP_{thres} , mV), action potential half amplitude duration ($AP_{1/2}$, msec.), and membrane time constant (τ , msec.) from the five different areas of the perirhinal cortex.

Cell Type	E_m	R_i	AP_{amp}	AP_{thres}	$AP_{1/2}$	τ
Layer 3 Area I	-73.8 $\pm$ 2.0	36.2 $\pm$ 4.3 ^d	82.7 $\pm$ 3.6	22.0 $\pm$ 6.5	0.7 $\pm$ 0.2 ^a	12.1 $\pm$ 2.7
Layer 3 Area II	-75.2 $\pm$ 3.4	46.5 $\pm$ 2.6 ^d	84.0 $\pm$ 5.1	15.9 $\pm$ 3.1	3.9 $\pm$ 1.2 ^a	20.0 $\pm$ 2.4 ^d
Layer 5 Area I	-75.2 $\pm$ 1.7	26.4 $\pm$ 3.2 ^b	82.3 $\pm$ 6.5	3.3 $\pm$ 1.1 ^d	17.2 $\pm$ 7.6 ^b	10.8 $\pm$ 0.8
Layer 5 Area II	-73.6 $\pm$ 4.5	21.9 $\pm$ 2.3 ^b	92.0 $\pm$ 3.9 ^c	16.2 $\pm$ 2.8	15.1 $\pm$ 3.7 ^b	10.6 $\pm$ 1.6
Layer 6	-75.2	45.0	82.1	8.3	3.75	25

a = Significantly different from both layer 5 type cells, $p < 0.01$

b = Significantly different from both layer 3 type cells, $p < 0.01$

c = Significantly different from all other groups, $p < 0.05$

d = Significantly different from all other groups, $p < 0.01$

layer 3 area I cells.

The deeper layer 3, area II cells were all regular spiking and in most cases did not accommodate. A large number of these cells also exhibited the afterdepolarizations following each action potential - a feature unique to most of the layer 3 cell types. The membrane of these layer 3 area II cells showed inward rectification on both depolarizing and hyperpolarized current steps in most recordings, and in almost all cases exhibited a graded anode break response on the hyperpolarizing step, the latter occasionally was accompanied by an action potential. In the two case were no inward rectification, anode break, and afterdepolarization were present, it was assumed that these were representative of another cell type of which only this small representative sample were acquired.

The only recorded type of layer 5, area I neurons exhibited some properties that were analogous to the layer 3, area II cells, but these cells fired doublets, did not accomodate, and demonstrated no afterdepolarizations following action potentials. In contrast four of the very deep layer 5, area II cells were bursting cells and were nothing like the other recorded cell types. The layer 5, area II bursting cells did not accommodate, but also showed no inward rectification or anode break response as seen in the other non-accommodating cells. Classification for a bursting cell was based on a neurons ability to discharge (burst) at least 3 spikes with interspike intervals (ISI) of

less than 6 ms, while cresting the peak of a depolarizing shift. Although many bursting classifications exist, we chose this one based on the research by Beggs and Kairiss (1994), since theirs was the only other intracellular work done in the perirhinal cortex. For this reason the fifth layer 5 area II cell could not be classified as bursting although it appeared to exhibit all the other physiological behaviour as the other bursting cells.

Information on the layer 6 cells was restricted to one documented case. This cell had physiological properties that were analogous to layer 3 area I cells, but it did not demonstrate an afterdepolarizing peak following each action potential. This cell also became very unstable with strong injection currents and often began to show a rough inward rectification on both the depolarizing and hyperpolarizing current injection.

As there were a number of physiological membrane characteristics that were unique to most of the cells in each perirhinal area, I have described each of the perirhinal areas individually in greater detail below. Although some variability exists between magnitude of physiological characteristics within these areas, the characteristics themselves are robust and will be discussed on those merits alone. In later research we will attempt to delve into the properties of those characteristic by more appropriate means.

Characteristics of layer 3 area I neurons

Fourteen of the fifty cells were recorded from layer 3 area I and twelve

of those were clearly distinguished by their demonstration of accommodation, inward rectification, anode break response (Table 2) and their superficial location in the slice (see Fig. 3). Both the input resistance (R_i) and the action potential half width duration ($AP_{1/2}$) in these cells were significantly different from both areas of layer 5 (Table 3), and the R_i was different from the layer 3 area II cells. The two remaining cell physiologies were difficult to analyze, apart from their ability to double spike at threshold depolarizing input, due to the quality of recording.

The I-V graph in Figure 18D, representing a standard layer 3 area I cell's behaviour, shows a typical slope, that is confirmed in the current injection I-V traces (Fig. 18A). This cell type shows the doublet spiking pattern and an afterdepolarization following each action potential (Fig. 18Aa). The high intensity hyperpolarizing current input (Fig 18C) shows no inward rectification (c) or evidence of an anode break response (d) as in the case in layer 3 area II cells. Finally, the membrane potential manipulations seen in Figure 18E show that a large hyperpolarizing holding current change in the membrane potential is necessary before any change in firing pattern is witnessed in these layer 3 area I neurons. This is again unique to this type of neuron, whereas the other neurons appear to be very sensitive to small changes in their E_m . Depolarizing membrane manipulations were not possible as they tended to cause spontaneous discharges that precluded any useful current injections.

FIGURE 18. A compilation of the standard intrinsic membrane properties of a typical layer 3 area I perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. Note the afterdepolarizations (a) following each spike. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the doublets (b) in B and the absence of any inward rectification (c) or anode break (d) in C. The current voltage relationship of this layer 3 area I neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

Characteristics of layer 3 area II neurons

Of the remaining 18 layer 3 area II cells, sixteen were non-accommodating, inward rectifying, regular spiking, and exhibited an anode break response (Table 2). These neurons also had an afterdepolarization following each spike (Fig. 19Aa) analogous to that seen in area I cells but, in addition, demonstrated a clear depolarizing peak (Fig. 19Ab) following depolarizing stimulation that, during the current injection, returns to steady state with an ensuing inward rectification. At membrane threshold, a single spike is generated in all layer 3, area II cells, whereas the layer 5 area I doublet cells and the layer 5 area II bursting cells all discharge multiple spikes. The duration of the layer 3 area II AP_{1/2} were significantly shorter than either of the layer 5 area cell types (Table 3), which in addition tended to single spike following each doublet or burst response, something not found in the layer 3 area II cells at threshold.

A hyperpolarizing current regularly produced a hyperpolarizing peak followed by a slight inward rectification in these cells. This was associated with a significantly high input resistance and long membrane time constants seen in Table 2 and the input current of Figure 19Cd. These hyperpolarizing peaks were graded and increased as the hyperpolarizing current injections increased, often causing a parallel magnification of the inward rectification.

Most of the recorded layer 3 area II cells demonstrated an anode break

FIGURE 19. A compilation of the standard intrinsic membrane properties of a typical layer 3 area II perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. Note the afterdepolarizations (a) following each spike and the depolarizing peak (b) at the subthreshold current injection. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the inward rectification (c) and the anode break with a threshold spike (d) in C. The current voltage relationship of this layer 3 area II neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

2 OF/DE 2

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

following each hyperpolarizing current injection (Table 2). As previously mentioned, the two cells that did not demonstrate these membrane characteristics are likely representative of another cell type. These two cases are likely standard regular spiking cell that do not demonstrate any of the excitable membrane properties observed in the other recorded layer 3 area II cells. Due to the limited amount of information available on these two cells we will reserve a detailed analysis of this physiological type for later study.

In the 16 remaining layer 3 area II neurons the anode break responses as compared to those seen in the layer 5 area I cells showed a less pronounced afterdepolarization and only spiked at high intensity hyperpolarizing current injections (Fig. 19Cc). However, in the layer 3 area II cells, with graded responses that reached spike threshold, an afterdepolarization was produced following the spike (Fig. 19Cc). This was not seen in layer 5 area I cells.

In the membrane potential manipulations (Fig. 19E), an approximately 10 mV change hyperpolarizing the membrane was required to block all action potentials to previously superthreshold stimuli. These spike subthreshold responses exhibited all the properties of an equilibrium response, clearly showing the depolarizing peak, and less clearly the inward rectification, identical in nature to the layer 5 area II cell behaviour.

Characteristics of layer 5 area I neurons

Eight of the fifteen layer 5 cells were recorded in area I. In all cases

these cells doublet spiked and exhibited this distinct short interspike interval double spiking pattern at a significantly lower membrane threshold than any of the other cells studied (Table 2). Additionally, these were the only cells to fire a rapid train of single action potentials at spike threshold stimulation (Fig. 20A). There were no apparent afterdepolarizations following any of the action potentials as opposed to the layer 3 cell behaviour. Also, each spike was followed by an afterhyperpolarization using low current intensities (Fig. 20A), while higher intensity depolarizing current injections did not have this same effect (Fig. 20B). As the interval between the action potentials reduced with higher current intensities, the membrane's steady state merged into the next action potential without stabilizing (Fig. 20B).

Analogous to the layer 3 area II cells, these layer 5 area I cells had exhibited pronounced hyperpolarizing peaks followed by a rectifying return to steady state as seen in Figure 20Cc. In these layer 5 area I cells, however, this inward rectification was clearly larger than in the layer 3 area II cells, and occurred definitively at every hyperpolarizing current step (Fig. 20A). Whereas, the inward rectification (seen in Fig. 19A) of the layer 3 area II cells was not typically as clearly pronounced until the third or fourth depolarizing current step.

As seems to be the case with all the studied cells that demonstrate inward rectification with hyperpolarizing current input (Table 2), layer 5 area I

FIGURE 20. A compilation of the standard intrinsic membrane properties of a typical layer 5 area I perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the multiple spikes (a) on the large depolarizing current injection in B, and the inward rectification (b) present with the hyperpolarizing current step in C. Also, note the large anode break response with a pair of threshold spike (c) also in C. The current voltage relationship of this layer 5 area I neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

E

+0.3 nA Stimulation

40 mV
50 msec.

neurons also exhibit an anode break. In keeping with the dramatic differences found between specific properties of layer 3 area II and layer 5 area I neurons, the layer 5 cells are again more reactive to lower levels of current input (Table 3). All the layer 5 graded responses appear to be much greater than those seen in the layer 3 cells (Fig. 19A). In addition, likely as a result of the significantly lower membrane threshold and R_i , the afterdepolarizing anode break produces multiple action potentials at almost every current input level (Figs. 20A & 20Cb).

During the membrane potential changes with different holding currents seen in Figure 20E, it is clear that as the rate of membrane spiking is reduced by hyperpolarizing the membrane, it is possible to produce a response without spiking that is similar to that seen in the layer 3, type II cells. For example the depolarizing peak is now present, and the afterhyperpolarizing rebound potential following the single AP at the -80.9 mV membrane potential is clear.

Characteristics of layer 5 area II neurons

Of the remaining five layer 5 cells recorded in area II, four had a propensity to burst multiple spikes at a depolarizing spike threshold without returning to steady state (Table 2 & Fig. 21A). They also produced significantly larger action potentials (Table 3) and tended to have a very low R_i . There was no evidence of any inward rectification (Figs. 21A & Cc) on either the depolarizing or hyperpolarizing current steps, but large rebound

FIGURE 21. A compilation of the standard intrinsic membrane properties of a typical layer 5 area II perirhinal cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. Note the burst responses (a) occurring at spike threshold and the depolarizing peak (b) at the subthreshold current injection. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. Note the lack of any inward rectification (c) and the absence of an anode break (d) in C. The current voltage relationship of this layer 5 area II neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is graphed for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

40 mV
50 msec

afterhyperpolarizations followed each burst (for a typical example see Fig. 21Aa) and many of the following single spikes (Fig. 21B), yet there was no afterhyperpolarizations following the current injection offset. Of the single case that did not burst by our classification

Unlike the layer 5, area I cells, there was no inward rectification, as evidenced in the I-V traces (A) of Figure 21. However, the penultimate spike threshold stimulation produced an identical depolarizing shift response to that of the layer 3 area II cells (Fig. 19A), and that demonstrated by the layer 5 area I cells (Fig. 20Ab). This depolarizing peak potential appears as a prelude to most of the burst responses found in these layer 5 area II neurons, and is witnessed again when the suprathreshold response is suppressed by hyperpolarizing the membrane potential in Figure 21E (trace 4).

The membrane potential manipulations in Figure 21E also show that when the membrane potential is held depolarized below the E_m (-80mV in Fig. 21A), the cell will no longer burst spike and changes to the single spiking mode. Conversely, none of the hyperpolarizing or depolarizing (data not shown) membrane holding current manipulations shown in the other three cell types from the different perirhinal areas managed to produce a bursting cell, and thus it appears that intrinsic bursting is unique to this layer 5 type of cell.

Characteristics of the layer 6 neuron

Only one valid case was gathered for layer 6, although three separate

cells were stained. Figure 22 shows a regular or single spiking cell that appears to accommodate (Table 2) and has no afterdepolarizations or afterhyperpolarizations following the action potentials. It also appears to respond negatively (unstable baseline) to any intensity of current input greater than ± 0.5 nA, showing a possible hyperpolarizing peak at -0.6 nA (Fig. 22D).

Furthermore, it was not possible to suppress the membrane spiking by hyperpolarizing the membrane potential as much as 30 mV from E_m . Although this cell stands alone, it appears to have some properties unique from the other types of cells studied. Therefore, the morphological data will be required before any further assessment can be made.

Synaptic Properties

Synaptic information was gathered from 10 of the 50 cells recorded. Several of these cells were activated antidromically (determined by spikes arising directly from baseline E_m), seen in Figures 23-24. Data were obtained from five layer 3 area I cells, three layer 3 area II cells, and two layer 5 area II cells. Nothing was known previously of the projection networks of these cells and, therefore, the attempts to synaptically drive these cells from various areas on the slice and the areas that produced evoked responses are outlined below for each of the perirhinal areas. The emphasis in the synaptic recording was purely centred on identifying areas of this slice preparation that evoked responses with little attention being paid to the type of response evoked.

FIGURE 22. A compilation of the standard intrinsic membrane properties of the only perirhinal layer 6 cell. In A, the membrane responses to depolarizing and hyperpolarizing current injections are graphed, including the spike threshold response. In B, the membrane response to a single large depolarizing current injection is seen and in C, the response to a hyperpolarizing current provides a clear image of the membrane characteristics. The current voltage relationship of this layer 6, neuron is seen in Figure D. Finally, the membrane response to a fixed suprathreshold depolarizing current injection is presented for a series of different negative holding membrane potentials down to a level where no spike threshold response is observed (Fig. E). A calibration bar is provided for Figures A and E, and distinguishing features are indicated with lower case letters and arrows.

Activation of layer 3 area I neurons

Synaptic activation of these layer 3 area I cells was attempted from 15 different sites in the slice (Fig. 23). Although 11 of the sites were negative, four of the sites produced a consistent antidromic response followed by a synaptically evoked EPSP. The EPSPs were activated by stimulation of the basolateral and lateral amygdala (Fig 23c-e) and the rostral caudate/putamen (Fig. 23a). The fifth site in the caudal caudate/putamen (Fig. 23b, n=3) evoked a robust threshold slow EPSP eliciting two action potentials with no antidromic spike. The layer 3 area I cells were the only cells to receive inputs from the caudate/putamen.

Of the three responsive sites in the amygdala, the lateral amygdala (n=4) was the only one to evoke an action potential (Fig 23d). The anterior basolateral amygdala (Fig. 23e, n=3) elicited a strong EPSP at a moderate stimulus intensity, whereas the more rostral posterior basolateral amygdala (n=3) site required a very strong intensity stimulus to evoke even a weak EPSP (Fig. 23c). The best projection areas within the amygdala appeared to reside along the medial aspects of the lateral and basolateral amygdala nuclei.

Other areas within the slice, including the medial amygdala, piriform cortex, endopiriform cortex, and the deep entorhinal cortex failed to evoke any response even when stimulated with a pulse as high as 30 V.

FIGURE 23. Composite schematic of a perirhinal/piriform slice from Swanson (1993) containing blackened circles representing the positions of the recording electrodes for the perirhinal layer 3 area I cells that evoked responses were recorded from. Superimposed are hollowed circles indicating the various sites at which synaptic drive of layer 3 perirhinal area I cells were attempted and containing a number indicating the number of different cells tested. For sites at which synaptic responses were recorded, a trace representing the most typical type of recording was provided and identified with an arrow and lower case letters. The rostral caudate putamen (CP) produced responses in 2 different cells (a), the medial CP produced responses in 3 different cells (b), the posterior basolateral amygdala nucleus produced weak responses in 3 different cells (c), the lateral amygdala nucleus produced responses in 4 different cells (d), and the basolateral nucleus of the amygdala produced responses in 3 different cells (e). A calibration bar is provided representing all traces.

Activation of layer 3 area II neurons

Moderate intensity stimulations in the lateral amygdala (n=2), anterior basolateral amygdala (n=3), and high intensity stimulation of the anterior medial amygdala (n=3) produced an antidromic activation in a number of the layer 3 area II cells (Fig. 24). Unlike the layer 3 area I cells, these antidromic activations were not followed by any clear synaptic response. On the other hand the lateral amygdala (Fig. 24a) produced a spike potential on several trials in both recorded cells, but this did not occur from E_m , and was delayed from the stimulation artifact, which may suggest that an underlying EPSP was present but partially masked.

No other responses could be driven from any of the 12 remaining stimulation sites, which included: the superficial entorhinal cortex, endopiriform cortex, piriform cortex, remaining amygdala nuclei and the caudate putamen. Once again, the projection areas within the amygdala would appear to reside along the lateral wall of the medial horn of the external capsule, with a distinct emphasis in the lateral amygdala.

Activation of the layer 5 area II neurons

For the two layer 5 area II cells recorded in this experiment, all stimulations were restricted to the amygdala nuclei. The high intensity currents necessary to drive the synaptic responses from the amygdala also tended to disrupt the membrane steady state before stimulation of other sites could be

FIGURE 24. Composite schematic of a perirhinal/piriform slice from Swanson (1993) containing blackened circles representing the positions of the recording electrodes for the perirhinal layer 3 area II cells that evoked responses were recorded from. Superimposed are hollowed circles indicating the various sites at which synaptic drive of layer 3 perirhinal area II cells were attempted and containing a number indicating the number of different cells tested. For sites at which synaptic responses were recorded, a trace representing the most typical type of recording was provided and identified with an arrow and lower case letters. The lateral amygdala nucleus produced responses in 2 different cells (a), the basolateral nucleus of the amygdala produced responses in 3 different cells (b), and the basomedial amygdala nucleus produced responses in 2 different cells. A calibration bar is provided for each unique trace.

attained.

However, despite this shortcoming, evoked potentials were found in the lateral amygdala (Fig. 25a, n=1), anterior basolateral amygdala (Fig. 25c, n=2), posterior basolateral (Fig. 25b, n=2), as with both other cell types, but also the anterior basomedial amygdala (Fig. 25d, n=1). In all cases except one (posterior basolateral amygdala), the first current injection capable of producing a response in these bursting neurons was an EPSP with a threshold spike. Conversely, the posterior basolateral amygdala required a much greater stimulus intensity to evoke a response, as was also the case in layer 3 area I neurons, and still a spike threshold was not obtained.

In keeping with the other perirhinal areas, the remaining amygdala nuclei failed to evoke responses in the layer 5 area II cells at stimulus intensities exceeding 40 V. In those stimulation sites where a response was evoked, the stimulation intensities were much higher than in similar stimulation sites for either the layer 3, area I or II neurons.

Laminar, Morphological, Physiological, and Synaptic Commonalities

Based on the morphological, physiological, and synaptic data gathered from the four major areas of the perirhinal cortex, a number of distinguishing properties from each of these attributes have been used to define four different cell types, one occurring in each of the four major perirhinal areas (see Table 4). Although the morphological and physiological data suggest that another

FIGURE 25. Composite schematic of a perirhinal/piriform slice from Swanson (1993) containing blackened circles representing the positions of the recording electrodes for the perirhinal layer 5 area II cells that evoked responses were recorded from. Superimposed are hollowed circles indicating the various sites at which synaptic drive of layer 5 perirhinal area II cells were attempted and containing a number indicating the number of different cells tested. For sites at which synaptic responses were recorded, a trace representing the most typical type of recording was provided and identified with an arrow and lower case letters. One response was produced in the lateral amygdala nucleus (a), two different cells responses were recorded in the posterior basolateral amygdala nucleus (b), two different cells responses were recorded in the anterior basolateral amygdala nucleus (c), and one response was recorded in the anterior basomedial amygdala nucleus (d). A calibration bar is provided for each unique trace.

TABLE 4

Descriptive summary of the cell types within each of the different perirhinal areas that share the same morphological, physiological, and synaptic attributes.

Perirhinal Area	Cell Types	Morphology	Physiology	Synaptic Sites
Layer 3 Area I	1 n=12	densely packed small pyramidal cells with large round soma very spiny dendrites, large apical dendritic field, wide basal dendritic field, and axons within layer 1-3 and back through the EC n=6 of 6	doublet spiking cell that accommodates, has no inward rectification, anode break response or rebound hyperpolarization, and exhibits an afterdepolarization on each action potential n=12 of 14	lateral amygdala n=4 anterior basolateral n=3 anterior medial n=3 caudate putamen n=3
Layer 3 Area II	1 n=16	sparsely grouped medium pyramidal cell with small round soma, moderately spiny, thin apical dendrite with small field in layer 1, simple basalar dendrites and axons in layers 3 & 5 and back to EC n=11 of 11	single spiking cell that does not accommodate, inward rectifies, has an afterdepolarization following each action potential and has an anode break response n=16 of 18	lateral amygdala n=2 anterior basolateral n=3 anterior basomedial n=3
Layer 5 Area I	1 n=10	sparsely grouped very large pyramidal cell with a large oblong soma, heavily spined dendrites, thick apical process with a small field in layer 1, large basalar dendritic field in layer 5, and extensive axonal projections in perirhinal n=10 of 10	doublet spiking cell that does not accommodate, inward rectifies, has no afterdepolarizations following each action potential, and has an anode break response n= 10 of 10	none
Layer 5 Area II	1 n=4	spiny stellate cells, medium triangular soma long thin dendrites in layer 5 & 6, axon projections in 5 & 6, and back to EC n=4 of 4	burst firing cell that does not accommodate or inward rectify and does not have an anode break response or an afterdepolarization n=4 of 5	lateral amygdala n=1 anterior basolateral n=2 posterior basolateral n=2 anterior basomedial n=1

cell type exists within layer 3 area II, not enough information was gathered to make a case for this assumption. The data presented in Table 4, represent a compilation of descriptive measures from each recording site that describe the typical properties of similar types of cells in each perirhinal area.

As the electrodes were positioned in each cortical area in order to acquire specific types of cells, it is not surprising that the majority of cells recorded and stained in each area were the same. Additionally, as both staining techniques and the method of intracellular recording tended to bias towards larger cell bodies, this would further limit the range of cell types we were likely to identify in each area.

The correspondence between the morphology, physiology and the different laminar electrode placements outlined in Table 4, sufficiently differentiate four cell types in the perirhinal cortex that will be discussed as follows: the layer 3 neurons in area I and area II will be referred to as perirhinal layer 3 type I and type II neurons respectively, and the area I and area II neurons in layer 5 will be referred to as perirhinal layer 3 type I and type II neurons respectively.

Discussion

The purpose of this thesis was to further identify the morphology, physiology, and synaptic properties of neurons in the perirhinal cortex. After

the injection of 50 cells in five different perirhinal areas it was possible to distinguish five different cell types, each with their own unique morphological and physiological properties. As is commonly the case with intracellular recording, these data showed a distinct bias towards larger cell types (Ghosh, Fyffe, & Porter, 1988), suggesting that a number of the smaller cell types likely remain unexplored. Still, a representative sample of the morphology and physiology was found for each of the identified cell types, which included a modest amount of synaptic information.

Grouping of Neuronal Types

Of five the cell types that were identified, two were distinctly pyramidal cells, appearing in the deeper layer 3 (layer 3 area II, Fig. 9) and shallow layer 5 (layer 5 area I, Fig. 11) laminae. Both had a prominent inward rectification with depolarizing and hyperpolarizing peaks, including an anode break response. These characteristics are typical of this type of neuron (Takahshi, 1965; Deshchênes, Labelle, & Landry, 1979). Although the two pyramidal cell types were similar in some respects, more detailed examination of their morphology and their threshold discharges as well as a number of different membrane properties revealed many important differences. For these reasons, the two pyramidal cell types were treated individually.

The pyramidal-like cells, found in the very superficial layer 3 cell field

(layer 3 area I, Fig. 7), were anatomically and physiologically similar to the motor cortex slow pyramidal tract neurons found by Sakai and Woody (1988). Not only were they similar in shape but both had a high density of dendritic spines. Like the motor cortex cells, the accommodating, doublet spiking threshold discharge of these layer 3 type I cells, in combination with a lack of inward rectification and the presence of afterdepolarizations following each action potential, clearly distinguished them from the other cell types in our study.

Also unique were the layer 5 type II (Fig. 13) neurons, as evidenced by their stellate morphology and unique burst response. These cells were the only cells identified in layer 5 that produced a clear burst response to threshold stimulation and had no inward rectification. In cases where the cell membranes were hyperpolarized, the burst responses of these cells disappeared and were replaced by single spiking discharges. The opposite was not observed when non-bursting cell membranes were depolarized, i.e., single spiking did not turn into burst discharging. The only other stellate-like neuron was found in layer 6 (layer 6, Fig. 15), however, they all had neither a bursting discharge pattern nor an expansive dendritic arbour like the layer 5, type II cells.

It is clear from the present findings that at least five diverse neuronal populations exist within the perirhinal cortex, each behaving in a different

manner and likely interacting with different parts of the cortex. Based on this assumption, each one of the cell types will be discussed in turn in an attempt to better understand their unique qualities.

Layer 3 Type I Neurons

Morphology: Layer 3, type I neurons were situated along the lateral border of layer 3 immediately surrounding the perirhinal fissure. They extended a primary apical dendrite directly into layer I, subpially, where numerous spiny process created a wide reaching network of arbours. Typically, staining of these cells produced a paired set of identical neurons in close proximity to one another (see Figs. 7 & 8). This would suggest that a relatively dense population of these neurons exists in a band running parallel to the layer 2/3 border (as can be witnessed in Fig. 4C). Their slightly offset round cell bodies produced a web of spiny dendrites that provided a dense region of processes in the uppermost horizontal plane of layer 3. The structure and branching of a single layer 3 type I cell interconnected broad areas of perirhinal layers 1, 2, and 3, giving these cell a large source of input from axons entering the upper cortical perirhinal layers. The major axonal output projected downward into the underlying white matter. These cells were very similar to the layer 3 pyramidal cells described by Ghosh, Fyffe, and Porter (1988) in the cat's cortex, only in those neurons very extensive networks of axon collaterals were

found projecting to all lamina of the cortex. This may be the case as well with the layer 3 type I cells found in this study, but only a limited portion of the descending axon could be visualized in any of the staining attempts.

It is generally accepted that spiny cells have excitable terminals (Colonnier, 1981) and that the middle cortical layers supporting a majority of the cortical spiny cells are also the centre of most burst responses (Connors, 1984). Therefore, it would be expected that these spiny pyramidal-like cells lying in the central tissue of layer 3 might burst.

Intrinsic Membrane Properties: Behaviourally, these cells exhibited a normal cortical R_i and produced a typical I-V graph as seen in Figure 18. At spike threshold, however, the membranes discharged with doublet spikes and an ensuing afterdepolarization. Furthermore, with high stimulus intensities this doublet was converted transiently into a triplet (Fig. 18Bb). This type of response supports the assertion made by McCormick, Connors, Lighthall and Prince (1985) regarding the bursting behaviour of spiny cells in cortical tissue. They believed that pyramidal neurons actually have a continuous distribution of spiking modes that includes bursting, but the latter disposition is limited or inhibited in normal cortical tissue. Under appropriate conditions, however, cells such as these doublet spikers theoretically could be induced to burst, but this behaviour can be permanently suppressed depending on where the cell lies in

the cortex (McCormick *et al.*, 1985). In some cases, depolarizing the membrane of suppressed bursting cells can induce them to burst (Rainnie, Asprodini, & Shinnick-Gallagher, 1993). However, as this was not the case here, it is likely that these layer 3 type I cells are truly non-bursting spiny cells that fire simple doublets and accommodate in normal tissue. Disinhibiting them with a GABAergic antagonist, however might unmask a burst response mode.

Synaptic Properties: Layer 3 cells could be activated antidromically from sites within the rostral caudate, basolateral amygdala, and lateral amygdala. This suggests that the descending axon observed in most of the stained layer 3 type I cells, likely bifurcated and sent projections medially through the amygdala and rostr dorsally into the caudate (see Fig. 23). A number of anterograde tracer studies have also demonstrated projections to several of the thalamic nuclei, basolateral amygdala, and the lateral amygdala from perirhinal layer 3 cells, which would further substantiate this observation (Krettek & Price, 1974; Deacon, *et al.*, 1983; McIntyre, *et al.*, in review). The lack of antidromic responses found from the numerous other stimulation sites, implies that the layer 3 type I axonal projections are quite restricted in this slice preparation, and may be confined largely to the perirhinal cortex itself.

The presence of EPSPs following many of these stimulations, suggests

that some regions send projections into the perirhinal layer 3 area. These inputs were largely excitatory, often producing threshold EPSPs at moderate stimulus intensities. The only exception to this occurs in the posterior basolateral amygdala, where a very high intensity stimulation was required to produce even a poor excitatory response. This response was possibly a result of the high stimulus intensity recruiting fibres projecting from the lateral amygdala.

In summary, the layer 3 type I neurons could best be described as small spiny pyramidal-like cells with round cell bodies that receive excitatory inputs from the lateral amygdala, anterior basolateral amygdala, and parts of the caudate. Although, certain evidence suggests that these cells should have the propensity to burst fire, they appear in pharmacologically normal medium as accommodating doublet spikers, with no form of membrane rectification or anode break response.

Layer 3 Type II Neurons

Morphology: The second type of layer 3 cell also had a large round soma with a nominal number of primary basal dendritic processes, a common characteristic of pyramidal cells from the same laminae (Ghosh, *et al.*, 1988). The type II cells, however, had one distinctly larger apical dendrite that arose from the soma directly towards the perirhinal fissure, giving these cells their pyramidal appearance (Fig. 9). The apical dendrite branched sparsely in layer

2 and sent a narrow column of processes to the cortical surface of layer 1, directly into the crux of the perirhinal fissure. The limited surface area this provided in combination with the lack of processes along the apical shaft suggested that these cells received input mainly in layer 1 and deeper in parts of layers 3 and 5. The lightly spined basal dendrites formed a relatively tight radius around the cell body, branching closely to the soma and forming a compact field covering both the deep layer 3 and the superficial aspects of layer 5.

The primary axon projected out of layer 3 medially or to deeper layers, the towards the external capsule, and sent wide reaching collaterals horizontally through layer 5 and others deeper into layer 5 and 6 immediately beneath the soma. This cell morphology exactly described that seen by Chagnac-Amitai, Luhmann, and Prince (1990) in the medial layer 5 bursting cells of the somatosensory neocortex. Thus, it might be expected that similar bursting behaviour would be seen in these cells.

Intrinsic Membrane Properties: As it turned out, however, these were not bursting cells, but exhibited a number of properties commonly associated with bursting cells. Although these cells had a similar input resistance to the type I cells and demonstrated the same afterdepolarization following each action potential, the membrane of the type II cells showed a marked inward

rectification on both the hyperpolarizing and depolarizing steps. Furthermore, the depolarizing steps produced a depolarizing peak or depolarizing shift like the subicular cells described by Stewart & Wong (1993), which was crested by only a single action potential at threshold. As in most cells with depolarizing/hyperpolarizing peaks and inward rectification, the layer 3 type II cells followed each hyperpolarizing current injection with an anode break response (Fig. 19Cc). This anode break was small compared to the layer 5 type I cells, but did produce an action potential at high intensity stimulations, indicating that the responses were graded.

Cells that show a marked inward rectification are often more excitable (Jones, 1994) and, in most cases, as was previously mentioned, are followed by an anode break on the hyperpolarizing steps. Characteristics and morphology such as these repeatedly have been associated with bursting type cells of the somatosensory cortex, piriform cortex, amygdala nuclei, endopiriform cortex, and the hippocampus (McCormick, *et al.*, 1985; Chagnac-Amitai, *et al.*, 1990; Rainnie, Asprodini, & Shinnick-Gallagher, 1993; Stewart & Wong, 1993), so it was unexpected that bursting responses were not found here. Additionally, these cells did not begin to show any evidence of bursting when subjected to membrane potential changes as seen in Figure 19E.

McCormick *et al.* (1985) have found that many rectifying cortical cells

possess slowly activating depolarizations generated by voltage-dependent calcium conductances which are associated with the membrane properties of bursting cells. However, these depolarizing shifts and rectifying properties often were not large enough, or lacked a sufficient outward potassium current to support burst spiking in normal tissue (Connors, Gutnick, & Prince, 1982). This would appear to be the case here, evidenced in the small anode break response and the lack of bursting behaviour despite the apparent presence of the necessary membrane conductances. It is unlikely that these cells remain non-bursting in epileptic or stressed tissue, as suggested by the 0 Mg^{2+} studies conducted by McIntyre and Plant (1989, 1991, & 1993), and the complimentary studies in piriform layer 2 cells conducted by Hoffman and Haberly (1989).

Synaptic Properties: As in the layer 3 type I cells, the type II cells appear to have axonal projections through the lateral amygdala and anterior basolateral amygdala, based on antidromic activation from these amygdala sites. Stimulations in the lateral amygdala produced a delayed spike on the descending phase of the antidromic response that was difficult to isolate as a synaptic EPSP. Due to the small sample size and obscurity of these data, it was impossible to make any inferences about the synaptic properties of these cells.

The layer 3 type II cells can best be described as regular spiking, non-

accommodating pyramidal cells with long apical dendrites that collateralize in layer 1 of the perirhinal cortex. The typically round cell body sends axonal projections down through the lateral amygdala and anterior basolateral amygdala with a number of collaterals that cover extensive regions of layers 5 & 6. They exhibit all the membrane properties of bursting cells but appear to lack the necessary conditions to burst in normal medium.

Layer 5 Type I Neurons

Morphology: These pyramidal cells were by far the largest and most complex of the identified cell types, and very closely resembled the deep layer 3 piriform cells found by Tseung and Haberly (1989). From the soma, a large thick apical dendrite ascended into layers 1 and 2, extruding many branches within layer 3. The actual dendritic field in layer 1 was very narrow, but contained a high density of arbours that terminated subpially along the perirhinal fissure.

The soma of these pyramidal cells had a more typical triangular shape and a very complex network of overlapping and multi branched basilar dendrites. These moderately spiny dendrites extended into large areas around the soma, which provided a location for input from axons in the surrounding layers 6, 5, & 3. The somas themselves were commonly situated slightly below the layer 5 border, and were found anywhere along its dorsoventral

extent.

The main axon of the layer 5 type II cell left the basal soma and projected directly through the external capsule, a feature common to all the perirhinal pyramidal neurons. The truly unique feature of these particular cells was their extensive network of axonal collaterals. Immediately upon exiting the soma, the axon collaterals sprouted in all directions, particularly projecting along the apical dendrite and through layers 1 & 2, and extensively around layer 5. The axons also projected to more distant regions of layer 3 and 6 (Fig. 11). These were the only cells to show this degree of interconnectivity with other regions of the perirhinal cortex. For this reason, and based on their general morphology, these cells can be compared directly to the somatosensory layer 5 cells described by Chagnac-Amitai, *et al.* (1990). Those somatosensory neurons were called bursting cells, but actually were more similar to our layer 5 type I doublet spikers, a point that we will make more clearly when discussing the intrinsic membrane properties below.

Intrinsic Membrane Properties: Based on the subthreshold records, these layer 5 type I cells had almost identical membrane properties to those of the layer 3 type II neurons just discussed (compare Figs. 19 & 20). Thus, they exhibited all the behaviour that was predictive of bursting type neurons described in the previous section. They differed, however, in that they had a significantly lower

peak R_i and membrane spike threshold, as seen in Table 3. This was evidenced in the spike threshold discharge of the anode break response and the larger inward rectification, suggestive of a faster Na^+/K^+ conductances and a greater disposition for destabilization (Jones, 1994).

The depolarizing threshold potential in these neurons produced a doublet spikes followed by a series of non-accommodating single spikes, but lacked the afterdepolarizations seen in both the layer 3 cells. Although our classification precludes these cells from being regarded as bursting cells, numerous authors consider this an acceptable bursting response (McCormick, *et al.*, 1985; Chagnac-Amital, *et al.*, 1990; Connors & Gutnick, 1990; Rainne, *et al.*, 1993; Avoli, Hwa, Lacaille, Olivier, Villemure, 1994). These experimenters typically describe a bursting cell as one where the first interspike interval is shorter than 6 ms and must have two spikes riding a depolarizing shift. Regardless, of the classification method used, our layer 5 type I cells clearly fit the description of most bursting layer 5 pyramidal cells discussed in the literature.

The most obvious difference between our layer 5 type I cells and the layer 3 type II cells is in the size of the graded potentials during the depolarizing shift and the cells relative spike threshold. The depolarizing shift in the layer 5 type I cells was sufficiently large to elicit a doublet spike on the

first threshold response, whereas the layer 3 type II cells merely discharged single spikes and at much higher thresholds. This layer 5 excitatory behaviour is suggestive of faster Ca^{2+} dependent membrane shifts and faster K^{+} currents that presumably the layer 3 type II cells somehow lack (Jones, 1994). This implies that the layer 3 cells have slightly different membrane conductances to certain ions that prevent them from burst discharging under normal conditions. Yet, the relatively morphologically and physiologically similar layer 5 type I cells have membrane properties that are conducive to bursting in normal tissue. The reason for these differences between otherwise similar cells could stem from a number of factors, for example, the cells laminar position (environment) and/or genomic differentiation (innate). The difference, however, remains unclear.

Although the previously described layer 3 type I neurons did demonstrate doublet spiking at threshold stimulations, those neurons had no inward rectification, depolarizing shifts, or anode break responses. This would suggest that the membrane properties of the layer 3, type I neurons are substantially different from the layer 5 type I cells we have just discussed. For these reasons, the basis of the layer 3 doublet spikes are presumably different from that of the layer 5 type I neurons.

The layer 5 type I cells can be summarized as large pyramidal cells

with a larger, long apical dendrite and very complex and long moderately spiny basal dendrites, whose soma lies immediately medial to the layer 3/5 border. At threshold, these cells discharge a 'burst-like' doublets and a non-accommodating train of action potentials. These also manifest strong inward rectification with an anode break response. These cells are interconnected with all laminae via a vast network of local axon collaterals, and the primary axon projects medially into the external capsule. The excitatory nature of this cell type and its presumed strong interaction with other perirhinal cells suggest an ability to recruit other cell populations within the perirhinal cortex.

Layer 5 Type II Neurons

Morphology: These neurons, located approximately in the mid-region of layer 5, were the first stellate cells found in the perirhinal cortex. They were relatively aspiny, and typically had four major dendritic processes that extended horizontally through layer 5 and medially across layer 6 (Fig. 13) with relatively few other arbours as compared to the layer 5 type I neurons. The triangular soma gave the impression of a pyramidal cell, but the lack of an apical dendrite, in combination with the simple but long dendritic processes, clearly defined these as stellate cells. Their location and morphology suggest that they primarily interact within the confines of layer 5 and layer 5 cells.

Tseng and Haberly (1989a) have described several multipolar neurons in

the endopiriform or deep layer 3 piriform cortex that are similar to these stellate cells. Some of these cells had a pyramidal shaped body and branched in an analogous manner to our stellate cells. The data suggest that a number of stellate type morphologies exist, yet each exhibit response properties that are similar, as described below (Tseng & Haberly, 1989b).

The primary axon arose from the basal soma and bifurcated, sending one branch dorsally along the most lateral surface of the external capsule, while the main axon projected ventrally, also appearing to send a few collaterals rostrally where they disappeared out the surface of the slice. The remaining collaterals apparently innervated a number of regions in the immediate vicinity of the cell body. This included one collateral in the deep regions of layer 3 and a number of collaterals deep in layer 6. This observation further emphasizes the deeper layer confines of this cell and its probable site of interactions.

Intrinsic Membrane Properties: The stellate cells were the first to produce a true burst response, consisting of four rapid spikes occurring within approximately 12 ms and riding a depolarizing shift (see Table 3 & Fig. 21A). Of particular interest here is the absence of any form of inward rectification or anode break response. Furthermore, the apparent voltage-dependent Ca^{2+} conductance did not appear until the penultimate depolarizing current step,

whereas all other current steps, both hyperpolarizing and depolarizing were steady state with no paroxysmal shifts.

From the membrane potential manipulations seen in Figure 21E, the active depolarization or hyperpolarization of the E_m can be observed to eliminate the burst response in these cells. This is indicative of endogenous bursting cells (McCormick, *et al.*, 1985) or, in other words cells that burst to depolarizing stimulation (Tseng & Haberly, 1989a; Stewart & Wong, 1993). Therefore, the multipolar stellate neurons found here can best be described as endogenous bursting cells with non-accommodating single spikes and no membrane rectification.

It has been suggested that this type of cell is ideal for the integration of synaptic information coming from the thalamus as it has the ability to vigorously respond to strong synaptic input, but in a transient manner (McCormick, *et al.*, 1985). This burst activity is capable of synchronizing cellular activity within the cortex (Connors, 1984) and, in some cases, may be responsible for encoding sensory information (Silva, Amitai, & Connors, 1991). Stewart and Wong (1993) have described a number of different types of bursting cells based on the conditions under which they burst. These neurons can be clearly categorized as cells that burst and spike to direct depolarizing input as opposed to cells which require dendritic input or environmental

changes in order to burst discharge. Rhythmic activity in the neocortex can be produced by a number of different behavioural and pathological conditions, but likely is supported by endogenous bursting cells under normal cellular conditions. As previously mentioned, the location and connectivity of our layer 5 type II stellate cells likely allows them to directly interconnect with the larger layer 5 type I cells, which have a more prolific interaction with the rest of the cortex and are also prone to excitatory burst responses.

Synaptic Properties: Synaptic data were acquired for two of the five burst spiking neurons, and were limited to stimulation sites in the amygdala. As with the other attempts to synaptically drive the layer 3 type II cells, the different amygdala nuclei stimulation elicited responses in the layer 5 type II cells that were activated antidromically from the lateral amygdala, anterior basolateral amygdala, and anterior basomedial amygdala. The only differences in stimulation sites found were in the stimulus intensities required to elicit these responses. Stimulus intensities were three times greater than those required to drive the layer 3 type II cells (Fig. 25). In addition to the antidromic spike, in almost all cases an additional spike occurred on the descending phase of the antidromic spike. This would suggest that these amygdala simulations also drove some excitatory afferents to these layer 5 cells, but the true nature of the underlying synaptic responses were buried

beneath the immense antidromic artifact, and thus were impossible to distinguish.

Despite the relative simplicity of the membrane properties and morphology of these cells, they undoubtedly serve a functionally significant role governing the interaction of cells in the perirhinal cortex. As previously suggested in the morphological data, and from analogous cases studied by Rainnie, *et al.* (1993), these stellate cells appear to be configured so as to provide facile communication with the neighbouring layer 5 type I neurons, and thus influence the entire perirhinal cortex through the extensive axon collateral branching observed in the layer 5 type I cells. In combination with their endogenous bursting nature these other data suggest that the layer 5 type II cells possibly drive the synchronous discharge patterns described by Silva, *et al.* (1991), which are presumably responsible for certain types of memory encoding.

Layer 6 Neurons

Morphology: This cell type has been described as a spiny pyramidal neuron (Rainnie, *et al.*, 1993), modified pyramidal neuron (Ghosh, *et al.*, 1985), asymmetrical pyramidal neuron (Lima, Voigt, & Morrison, 1990), or even a multipolar neuron (Tseng & Haberly, 1989), but all authors describe a pyramidal-like cell with a short, compact apical dendrite projecting down into

the deeper cortical layers rather than superficially to layer 1. Branches of the apical extended across distances horizontally through layer 6. This attribute gives these cells their multipolar appearance (Fig. 15). The basilar dendrites parallel the apical process's, leaving a vacant region above the soma just below the layer 5/6 border. From its somatic surface a single axon arose and bifurcated, sending a main axon and a collateral ventrally along the external capsule, through the endopiriform nucleus and into the amygdala. The remaining axon collaterals terminated in the cell field immediately adjacent to the soma.

Based on the morphological attributes of this cell, it appears that it serves primarily to interconnect cells within layer 6, and additionally provides an output to other ventral subcortical structures. Anatomical studies have suggested this output not only is to the amygdala, but prominently to the thalamus (McIntyre, *et al.* in review).

Intrinsic Membrane Properties: Only one complete data set was available for the layer 6 cells, providing a rather terse description of its membrane properties. At best this neuron can be described as a single spiking, accommodating cell with no membrane rectification or anode break response. As input current intensity increased, the membrane potential became very unstable and, during hyperpolarizing current steps, began to exhibit a rough

inward rectification with a hyperpolarizing peak (Fig. 22A&D). Although, Jones (1994) has found a similar current dependent inward rectification in layer 2 stellate cells of the entorhinal cortex, he was able to suggest nothing specific in terms of its' normal cellular activity.

Due to the limited information available on this cell type, the data can serve most usefully as a catalogue of these cells' morphology and laminar orientation.

Perirhinal Electrophysiology Literature

Only one other study, by Beggs and Kairiss (1994), has investigated the intrinsic membrane properties of cells in the perirhinal cortex. Based on the present results, it is now possible to more clearly describe and differentiate the three cell types proposed in their experiment. A majority of their data were obtained from what they referred to as singlet spiking cells, which most closely corresponded to our layer 3 type II neurons. Because that cell type occupies a relatively large area in the middle part of the perirhinal cortex, it is easy to conceive how Beggs and Kairiss' pioneering investigation of the perirhinal cortex would favour such electrode placements.

This hypothesis is further supported in that the doublet spikers described by Beggs and Kairiss (1994) are clearly analogous to our layer 5 type I neurons, which exist immediately subadjacent to the layer 3 type II neurons.

On the other hand, the fragmented information concerning their small sample of intrinsically bursting cells permits us only to suggest that these cells are perhaps similar to our layer 5 type II cells. We believe, therefore, that their findings best provide a detailed physiological portrayal of our layer 3 type II and layer 5 type I neurons. If this is the case, the results in our two studies for those two cell types are identical.

General Comments and Functional Significance

The present study set about to define the morphology, physiology and synaptic properties for the perirhinal cortex in an established perirhinal/piriform slice preparation (McIntyre & Wong, 1985). The results described neocortical-like cells that had many properties and morphology similar to the cells previously explored in the somatosensory cortex (McCormick, *et al.*, 1985, Connors, *et al.*, 1982; Chagnac-Anitai, *et al.*, 1990; Connors & Gutnick, 1990), motor cortex (Ghosh, *et al.*, 1988; Pockberger, 1991), and the temporal cortex (De Lima, *et al.*, 1990; Avoli, *et al.*, 1994), that were organized architecturally in cellular layers minus layer 4. Each layer presented cells with a distinct physiologies and morphologies.

In layer 1, the terminals of all apical dendrites developed an extensive arborization that spread laterally under the and around the perirhinal fissure. The variably spiny dendrites branched extensively and likely formed synapses

with terminals projecting from cortical and subcortical cells (Deacon, *et al.*, 1983; Deschênes, *et al.*, 1979).

Perirhinal layer 2 consisted mainly of tight bundles of apical dendrites projecting from layers 3 and 5 (Fig. 5B) neurons. The spines density of the various processes differed depending on from what cells they arose, but all were consistent within each bundle. The layer 3 type I (Fig. 4b) and layer 5 type II (Fig. 6B) neurons had the greatest spine density suggesting that both these cell types received a greater input from projections into the level of layer 2, as compared to the relatively sparse spine density of the layer 3 type II cells (Fig. 5D).

In this, study only pyramidal cells were found in layer 3, which by no means suggests these are the only cell types present, but emphasizes the higher packing density and size of these types of neurons (Gosh. *et al.*, 1988). The identified layer 3 cells were all regular or doublet spikers which is characteristic of neocortical layer 3 cells in normal tissue. Typically, the role of these suppressed cells is to attenuate prolonged excitatory, stimuli while promoting the transmission of acute phasic events (Connors & Gutnik, 1990). This disposition is very conducive to rhythmicity often created by the underlying bursting cells, which is usually associated with memory encoding (Silva, *et al.*, 1991) or pathologies such as epilepsy (Hoffman & Haberly, 1989;

Burke & Hablitz, 1994).

The layer 3 type II cell membrane characteristics suggested that they had a high propensity to burst, but failed to do so with membrane potential manipulations or strong synaptic input. All data implied that normally these cells were not endogenously bursting (which was possibly due to insufficient Ca^{2+} and K^{+} dependent conductances e.g. McCormick, *et al.* 1985). However, the 0 Mg^{2+} studies conducted by McIntyre and Plant (1989, 1991, & 1993) have demonstrated that extracellular bursting can be recorded from layer 3 perirhinal cells in this adjusted environment.

NMDA receptor mediated bursting is a fundamental characteristic of the synchronized burst responses recorded in endogenous bursting cells of layer 5 (Silva, *et al.*, 1991). It is likely therefore that the enhanced glutamate receptor sensitivity caused during 0 Mg^{2+} perfusion is sufficient to transform these layer 3 cells into rhythmically bursting neurons by virtue of their innate membrane properties. Connors, Gutnick, and Prince (1982) have proposed that cortical neurons harbour the characteristics of many neurons in different combinations, and that these neurons express themselves based on the functional requirements of the neocortical circuits. The implications that this could have on behavioural or pathological states is beyond the bounds of this experiment, but the question arises, "Do the same inhibitory or control mechanisms that

regulate endogenously bursting neurons govern exogenously induced bursting in regular spiking cells?" Obviously a more careful investigation of these cells is necessary and warranted.

The layer 5 cells were no less interesting, and fit well into the typical cortical layer 5 format (Connors & Gutnick, 1990). The large pyramidal cells, which were very similar to the layer 3 type II cells, were situated just below the layer 5 border with a basal dendritic arborization that encompassed large portions of this region. Interspersed between these large complex cells were a series of multipolar stellate cells that interacted mainly within the confines of this layer.

Both of these cell types generated a burst-like response, but it was the stellate cells that produced the more typical burst events associated with synchronized rhythmicity of layer 5 (Silva, *et al.*, 1991). These same cell types have also been strongly associated with integration occurring between deep layer 5 pyramidal cells (Rainnie, *et al.*, 1993). It is likely that these burst generating type II cells use the larger pyramidal type I cells, with their extensive network of axonal projections and low threshold excitatory nature, to create synchronous oscillations within the cortex. The function of this type of network is not completely understood, but it is presumably typical of information encoding behaviour in many cortical systems.

Clearly, the perirhinal has been demonstrated to have a physiological disposition and morphological architecture that is similar to a number of neocortical areas, and exhibits behavioural responses that are typical of these areas. A very limited number of synaptic inputs have been found within this slice preparation, but the sample size was small, and further attempts will be required to draw any firm conclusions. Sufficient data have been compiled, however, to conclusively identify 5 different cell types that can now be the focus of more detailed investigations into the function for the perirhinal cortex.

References

- Avoli, M., Hwa, G. G. C., Lacaille, J., Olivier, A., & Villemure, J. (1994). Electrophysiological and repetitive firing properties of neurons in the superficial/middle layers of the human neocortex maintained *in vitro*. Experimental Brain Research, **98**, 135-144.
- Beggs, J. M. & Kairiss, E. W. (1994). Electrophysiology and morphology of neurons in rat perirhinal cortex. Brain Research, **665**, 18-32.
- Burke, P. J. & Hablitz, J. J. (1994). Metabotropic glutamate receptor activation decreases epileptiform activity in rat neocortex, Neuroscience Letters, **174**, 29-33.
- Burnham, W. M. (1978). Cortical and limbic kindling: Similarities and differences. In K. E. Livingston & O. Hornykiewicz (Eds.), Limbic mechanisms: The continuing evolution of the limbic system concept (pp.507-519). New York: Plenum Press.
- Chagnac-Amitai, Y., Luhmann, H. J., & Prince, D. A. (1990). Burst generating and regular spiking layer 5 pyramidal neurons of rat neocortex have different morphological features. Journal of Comparative Neurology, **296**, 598-613.

- Colonnier, M. (1991). The electron microscopic analysis of the neuronal organization of the cerebral cortex. In F. O. Schmitt, F. G. Worden, G. Adelman, & S. G. Dennis (Eds.), The organization of the cerebral cortex (pp. 125-152). Cambridge: MIT Press.
- Connors, B. W. (1984). The initiation of synchronized neuronal bursting in neocortex. Nature London, 310, 685-687.
- Connors, B. W. & Gutnick, M. J. (1990). Intrinsic firing patterns of diverse neocortical neurons. Trends in Neuroscience, 13, 99-104.
- Connors, B. W., Gutnick, M. J., & Prince, D. A. (1982). Electrophysiological properties of neocortical neurons *in vitro*. Journal of Neurophysiology, 48, 1302-1320.
- Deacon, T. W., Eichenbaum, H., Rosenberg, P., & Eckmann, K. W. (1983). Afferent connections of the perirhinal cortex in the rat. Journal of Comparative Neurology, 220, 168-190.
- Deschênes, M., Labelle, A., & Landry, P. (1979). Morphological characterization of slow and fast pyramidal tract cells in the cat. Brain Research, 178, 251-274.
- De Lima, A. D., Voigt, T., & Morrison, J. H. (1990). Morphology of the cells within the inferior temporal gyrus that project to the prefrontal cortex in the macaque monkey. The Journal of Comparative Neurology, 296, 159-172.

- Dingledine, R. (1984). Brain slices. New York: Plenum Press.
- Freund, T. F. & Somogyi, P. (1989). Synaptic relationships of golgi-impregnated neurons as identified by electrophysiological or immunocytochemical techniques. In L. Heimer & L. Záborszky (Eds.). Neuroanatomical Tract-Tracing Methods 2: Recent Progress, Plenum Press: New York, pp. 201-238.
- Gellman, R. L. & Aghajanian, G. K. (1994). Serotonin₂ receptor-mediated excitation of interneurons in piriform cortex: Antagonism by atypical antipsychotic drugs. Neuroscience, 58(3), 515-525.
- Ghosh, S., Fyffe, R. E. W., & Porter, R. (1988). Morphology of neurons in area 4 γ of the cat's cortex studied with intracellular injection of HRP. The Journal of Comparative Neurology, 269, 290-312.
- Goddard, G. V. (1967). Development of epileptic seizures through brain stimulation at low intensity. Nature, 214, 1020-1221.
- Goddard, G. V. (1983). The kindling model of epilepsy. Trends in Neurosciences, 6(7), 275-279.
- Goddard, G. V., McIntyre, D. C., & Leech, C. K. (1969). A permanent change in brain function resulting from daily electrical stimulation. Experimental Neurology, 25, 295-330.

- Grace, A. A. & Limas, R. (1984). Electrophysiology and morphology of four classes of prefrontal neocortical neurons studied *in vitro*. Society of Neuroscience Abstracts, 10, 302-320.
- Guildin, W. O. & Markowitsch, H. J. (1983). Cortical and thalamic afferent connections of the insular and adjacent cortex of the rat. The Journal of Comparative Neurology, 215, 135-153.
- Haberly, L. B. & Bower, J. M. (1984). Analysis of association fibre system in piriform cortex with intracellular recording and staining techniques. Journal of Neurophysiology, 51(1), 90-112.
- Haberly, L. B. & Presto, S. (1986). Ultrastructural analysis of synaptic relationships of intracellularly stained pyramidal cell axons in piriform cortex. The Journal of Comparative Neurology, 248, 464-474.
- Hoffman, W. H. & Haberly, L. B. (1989). Bursting induces persistent all-or-none EPSPs by an NMDA-dependent process in piriform cortex. The Journal of Neuroscience, 9(1), 206-215.
- Holmes, K. H., Bilkey, D. K., & Lavery, R. (1992). The infusion of an NMDA antagonist into perirhinal cortex suppresses amygdala-kindled seizures. Brain Research, 587, 285-290.

- Jensen, M. S. & Yaari, Y. (1988). The relationship between interictal and ictal paroxysms in an *in vitro* model of focal hippocampal epilepsy. Annals of Neurology, 24(5), 591-598.
- Jones, R. S. G. (1994). Synaptic and intrinsic properties of neurons of origin of the perforant path in layer II of the rat entorhinal cortex *In Vitro*. Hippocampus, 4(3), 335-353.
- Kalsbeck, A., Matthijssen, M. A. H., & Uylings, H. B. M. (1989). Morphometric analysis of prefrontal cortical development following neonatal lesioning of the dopaminergic mesocortical projection. Experimental Brain Research, 78, 279-289.
- Kandel, E. R., Schwartz, J. H., & Jessell, T. M. (1991). Principles of neural science (3rd ed.). New York: Elsevier.
- Kelly, M. E., McIntyre, D. C., & Staines, W. A. (1993). Perirhinal connections to the orbital and frontal cortex: Anatomy and kindling. Society for Neuroscience Abstracts, 19, 1467.
- Kitai, S. T., Penny, G. R., & Chang, H. T. (1989). Intracellular labelling and immunocytochemistry. In L. Heimer & L. Záborszky (Eds.). Neuroanatomical tract-tracing methods 2: Recent Progress (pp. 173-199). New York: A Division of Plenum Publishing Corporation.

- Kolb, B., & Whishaw, I. Q. (1990). Fundamentals of human neuropsychology (3rd ed.). New York: W. H. Freeman and Company.
- Kosel, K. C., Van Hoesen, G. W., & Rosene, D. L. (1983). A direct projection from the perirhinal cortex (area 35) to the subiculum in the rat. Brain Research, 269, 347-351.
- Krettek, J. E. & Price, J. L. (1974). Projections from the amygdala to the perirhinal and entorhinal cortices and the subiculum. Brain Research, 71, 150-154.
- Lacaille, J. (1991). Postsynaptic potentials mediated by excitatory and inhibitory amino acids in interneurons of stratum pyramidale of the CA1 region of rat hippocampal slices *in vitro*. Journal of Neurophysiology, 66(5), 1441-1454.
- Leech, C. K. & McIntyre, D. C. (1976). Kindling rates in inbred mice: An analog to learning? Behavioral Biology, 16, 439-452.
- Libri, V., Constanti, A., Calaminici, M., & Nistico, G. (1994). A comparison of the muscarinic response and morphological properties of identified cells in the guinea-pig olfactory cortex *in vitro*. Neuroscience, 59(2), 331-347.
- Liu, W., Behbehani, M. M., & Shipley, M. T. (1993). Intracellular filling in fixed brain slices using Miniruby, a fluorescent biocytin compound. Brain Research, 608, 78-86.

McCormick, D. A., Connors, B. W., Lighthall, J. W., & Prince, D. A. (1985).

Comparative electrophysiology of pyramidal and sparsely spiny stellate neurons of the neocortex. Journal of Neurophysiology, 54(4), 782-806.

McIntyre, D. C. (1975). Split-brain rat: Transfer and interference of kindled amygdala convulsions. The Canadian Journal of Neurological Sciences, 429-437.

McIntyre, D. C. (1978). Kindling and memory: The adrenal system and the bisected brain. In K. E. Livingston & O. Hornykiewicz (Eds.). Limbic mechanism (pp. 495- 505). New York: Plenum Publishing Corporation.

McIntyre, D. C., & Goddard, G. V. (1973). Transfer, interference and spontaneous recovery of convulsions kindled from the rat amygdala. Electroencephalography and Clinical Neurophysiology, 35, 533-543.

McIntyre, D. C. & Wong, R. K. S. (1985). Modification of local neuronal interactions by amygdala kindling examined *in vitro*. Experimental Neurology, 88, 529-537.

McIntyre, D. C. & Wong, R. K. S. (1986). Cellular and synaptic properties of amygdala-kindled pyriform cortex *in vitro*. Journal of Neurophysiology, 55(6), 1295-1307.

- McIntyre, D. C. & Plant, J. R. (1991). Alteration of *in vitro* excitability after kindling. In F. Morrell (ed.), Kindling and synaptic plasticity: Legacy of Graham Goddard (pp. 239-251). Boston: Birkhauser.
- McIntyre, D. M. & Kelly, K. E. (1993). Are differences in dorsal hippocampal kindling related to amygdala-piriform are excitability? Epilepsy Research, **14**, 49-61.
- McIntyre, D. C. & Plant, J. R. (1993). Long-lasting changes in the origin of spontaneous discharge: from amygdala-kindled rats: Piriform vs. perirhinal cortex *in vitro*. Brain Research, **624**, 268-276.
- McIntyre, D. C., Kelly, M. E., & Armstrong, J. N. (1993). Kindling in the perirhinal cortex. Brain Research, **615**, 1-6.
- McIntyre, D. C., Kelly, M. E., & Staines, W. A. (1995, in review). Efferent projections of the anterior perirhinal cortex in the rat.
- Meunier, M., Bachevalier, J., Mishkin, M., & Murray, E. A. (1993). Effects on visual recognition of combined and separate ablations of the entorhinal and perirhinal cortex in rhesus monkeys. The Journal of Neuroscience, **13**(12), 5418-5432.
- Millhouse, E. O. (1979). The golgi methods. In L. Heimer & M. J. Robards (Eds.). Neuroanatomical tract-tracing methods (pp. 331-344). New York: Plenum Press.

- Miller, M. W. (1987). The origin of corticospinal projection neurons in rat. Experimental Brain Research, 67, 339-351.
- Nagahara, A. H., Otto, T., & Gallagher, M. (1995). Entorhinal-perirhinal lesions impair performance of rats on two versions of place learning in the morris water maze. Behavioral Neuroscience, 109(1), 3-9.
- Nicholls, J. G., Martin, A. R., & Wallace, B. G. (1992). From neuron to brain (3rd ed.). Massachusetts: Sinauer Associates, Inc.
- Ogawa, Y. (1990). Silver impregnation method for neurons using synthetic surfactants: A contribution to golgi method. In M. P. Conn (Ed.). Quantitative and Qualitative Microscopy (Vol. 3)(pp. 335-369). New York, Academic Press.
- Otto, T. & Eichenbaum, H. (1992). Complementary roles of the orbital prefrontal cortex and the perirhinal-entorhinal cortices in an odour-guided delayed-nonmatching to sample task. Behavioural Neuroscience, 106(5), 762-775.
- Pockberger, H. (1991). Electrophysiological and morphological properties of rat motor cortex neurons *in vivo*. Brain Research, 539, 181-190.
- Prince, D. A. & Wong, R. K. S. (1981). Human epileptic neurons studied *in vitro*. Brain Research, 210, 232-333.
- Racine, R. (1978). Kindling: The first decade. Neurosurgery, 3, 234-252.

- Rainnie, D. G., Asprodini, E. K. & Shinnick-Gallagher, P. (1993). Intracellular recordings from morphologically identified neurons of the basolateral amygdala. Journal of Neurophysiology, 69(4), 1350-1362.
- Rosen, J. B., Hitchcock, J. M., Miserendino, M. J. D., Falls, W. A., Campeau, S., & Davis, M. (1992). Lesions of the perirhinal cortex but not of the frontal, medial prefrontal, visual, or insular cortex block fear-potentiated startle using a visual conditioned stimulus. The Journal of Neuroscience, 12(12), 4624-4633.
- Sakai, H. & Woody, C. D. (1988). Relationships between axonal diameter, soma size, and axonal conduction velocity of HRP-filled, pyramidal tract cells of awake cats. Brain Research, 460, 1-7.
- Saunders, R. C. & Rosene, D. L. (1988). A comparison of the efferents of the amygdala and the hippocampal formation in the Rhesus Monkey: I. convergence in the entorhinal, prethinal, and perirhinal cortices. The Journal of Comparative Neurology, 271, 153-184.
- Shouse, N. M. (1994). Epileptic seizure manifestations during sleep. In M. H. Kryger, T. Roth, & W. C. Dement (Eds.). Principles and practice of sleep medicine (pp. 801-814). Philadelphia: W. B. Saunders Company.

- Scharfman, H. E. (1993a). Characteristics of spontaneous and evoked EPSP's recorded from dentate spiny hilar cells in rat hippocampal slices. Journal of Neurophysiology, 70(2), 742-757.
- Scharfman, H. E. (1993b). Activation of dentate hilar neurons by stimulation of the fimbria in rat hippocampal slices. Neuroscience Letters, 156, 61-66.
- Scharfman, H. E. (1994a). Paradoxical enhancement by bicuculline of dentate granule cell IPSP's evoked by fimbria stimulation in rat hippocampal slices. Neuroscience Letters, 168, 29-33.
- Scharfman, H. E. (1994b). Synchronization of area CA3 hippocampal pyramidal cells and non-granule cells of the dentate gyrus in bicuculline-treated rat hippocampal slices. Neuroscience, 59(2), 245-257.
- Silva, L. R., Amitai, Y., & Connors, B. W. (1991). Intrinsic oscillations of neocortex generated by layer 5 pyramidal neurons. Science, 251, 432-435.
- Snodgrass, A. B. (1970). Surfactant treatment of frozen section: its use with modifications of the bodian and of the holmes method. Brain Research, 24, 113-119.
- Squire, L., R., Zola-Morgan, S., & Chen, K. S. (1988). Human amnesia and animal models of amnesia: Performance of amnesic patients on tests designed for the monkey. Behavioral Neuroscience, 102, 210-221.

- Squire, L. R. & Zola-Morgan, S. (1991). The medial temporal lobe memory system. Science, 253, 1380-1386.
- Stewart, M. & Wong, R. K. S. (1993). Intrinsic properties and evoked responses of guinea pig subicular neurons *in vitro*. Journal of Neurophysiology, 70(1), 232-245.
- Suzuki, W. A. & Amaral, D. G. (1994). Topographic organization of the reciprocal connections between the monkey entorhinal cortex and the perirhinal and parahippocampal cortices. The Journal of Neuroscience, 14(3), 1856-1877.
- Suzuki, W. A., Zola-Morgan, S., Squire, L. R., & Amaral, D. G. (1993). Lesions of the perirhinal and parahippocampal cortices in the monkey produce long-lasting memory impairment in the visual and tactual modalities. Journal of Neuroscience, 13, 2430-2451.
- Swanson, L. W. (1992). Brain Maps: structure or the rat brain. Elsevier: New York.
- Swanson, L. W. & Köhler, C. (1986). Anatomical evidence for direct projections from the entorhinal area to the entire cortical mantle in the rat. The Journal of Neuroscience, 6(10), 3010-3023.

- Taghert, P. H., Bastiani, M. J., Ho, R. K. & Goodman, C. S. (1982). Guidance of pioneer growth cones: Filopodial contacts and coupling revealed with an antibody to Lucifer yellow. Developmental Biology, 94, 391-399.
- Takahashi, K. (1965). Slow and fast groups of pyramidal tract cells and their respective membrane properties. Journal of Neurophysiology, 28, 908-924.
- Tseng, G. & Haberly, L. B. (1989a). Deep neurons in piriform cortex. I. morphology and synaptically evoked responses including a unique high-amplitude paired shock facilitation. Journal of Neurophysiology, 62(2), 369-385.
- Tseng, G. F. & Haberly, L. B. (1989b). Deep neurons in piriform cortex. II. Membrane properties and regenerative calcium potentials. Journal of Neurophysiology, 62, 386-400.
- Valverde, F. (1965). Studies on the piriform lobe. Harvard University Press: Cambridge.
- Wada, J. A. (1976). Kindling. New York: Plenum Press. Wong, R. K. S. & Prince, D. A. (1979). Dendritic mechanisms underlying penicillin-induced epileptiform activity. Science, 204, 1228-1231.

Zola-Morgan, S., Squire, D. G., Amaral, D. G., & Suzuki, W. A. (1989).

Lesions of perirhinal and parahippocampal cortex that spare the amygdala and hippocampal formation produce severe memory impairment. Journal of Neuroscience, 9, 4355-4370.

Zola-Morgan, S., Squire, L. R., Clower, R. P., & Rempel, N. L. (1993).

Damage to the perirhinal cortex exacerbates memory impairment following lesions to the hippocampal formation. Journal of Neuroscience, 13, 251-265.

Appendix A

Intrinsic and Synaptic Measurements

Membrane Potential (V_m):

A measure of the unequal distribution of ions between the extracellular and intracellular fluids at a steady state potential. This measure is mechanically derived through our high input resistance recorder, but is representative of the equation:

$$V_m = 58 \log \frac{r [K]_o + b [Na]_o}{r [K]_i + b [Na]_i}$$

b = the ratio of potassium permeability (p_{Na}/p_K)

r = the coupling ratio of the transport system ($\text{transport}_{Na}/\text{transport}_K$)

o = outside

i = inside

Conductance (g_{ion}):

The sum of the conductance (permeability) of all the open channels permeable to that ion. eg. g_{Na} , conductance of the membrane due to sodium channels

$$g_{ion} = \frac{I_{ion}}{V_m - E_{ion}}$$

E_{ion} = the equilibrium potential for that ion

Current (I_{ion}):

Represents the inward leak of an ion through the resting membrane, which is proportional to the net ionic flux through the channel permeable to that ion.

$$I_{ion} = g_{ion} (V_m - E_{ion})$$

E_{ion} = the equilibrium potential for that ion

In one portion of this experimental manipulation, we define exactly how much current is injected into the cell and observe the natural response.

Ohm's Law:

A given amount of current I passed through a resistor r produces a voltage $V=Ir$.

$$V=V_A(1-e^{-t/\tau})$$

V_A =initial charge of the membrane

t =the time from the beginning of the pulse

τ =time constant, RC:

R =membrane resistance in ohms (Ω)

C =capacitance coulombs/volt or FARADS (F)

a capacitor is defined by how much charge (q) can be stored for each volt applied to it $C = q/V$

Time Constant (τ):

Describes the rising phase of a pulse.

$$V=iR(1-e^{-t/\tau})$$

t =the time from the beginning of the pulse

τ =the product of the resistance and capacitance in the circuit, the time for the potential to rise to a fraction $(1 - 1/e)$, or 63 percent of its final value

Length Constant (λ):

This is the distance over which the potential falls to $1/e$ (37 percent) of its maximum value.

$$\lambda = (r_m/r_i)^{1/2}$$

r_m =membrane resistance, the resistance outward across the axon

r_i = input resistance, the internal resistance along the axoplasm from the midpoint of one axon segment to the midpoint of the next

Input Resistance (r_{input})

The resistance measured by injecting current into a cell or fibre.

$$r_{input} = 0.5 \sqrt{r_m r_i}$$

$$V_o = I r_{input}$$

V_o = the peak potential change

r_m = membrane resistance, the resistance outward across the axon

r_i = input resistance, the internal resistance along the axoplasm from the midpoint of one axon segment to the midpoint of the next

Step Current's

An electrical current of either positive or negative polarity delivered manually into the cell in .1 nA increments through the glass recording electrode for varying durations of time (50 - 200 ms) will be taken for each record. A series of step current's (± 1 - ± 1.6 nA) will be obtained from each impaled neuron and graphed both individually and in combination in order to observe capacitive changes. Graphs will consist of a 250 ms or greater trace with a 50 ms prestimulus baseline.

Spike Threshold

The current level at which the first action potential is acquired during a set of depolarizing step current's of 200 ms duration.

Membrane Manipulations

These are a series of manual membrane adjustments in which the baseline membrane potential is artificial forced in either a positive or negative direction in increments of ± 5 mV. The membrane is then stimulated at each increment with an current level .2 nA greater than the spike threshold until AP disappear with depolarizing increments or the tissue becomes spontaneous with hyperpolarizing increments. This series is graphed in the same manner as the step current data.

Current-voltage Graphs

The time constant (τ) was used to derive the value from each step current to be plotted on an IV curve for each cell in order to complement the step current data. From this IV curve the input resistance was calculated and further used to compare the different types and classifications of neurons studied. The IV data was plotted for the full range of step current's and usually consisted of and average from three recordings.

Action Potential Amplitude and Spike Threshold Data (mV):

Both action potential amplitude and spike threshold amplitude were measured from the resting baseline membrane potential and based on the first overshooting spike (>80.0 mV) produced at the spike threshold.

Refer to Dingleline (1985); Eckert, Randall, & Augustine (1988); Nicholls, Martin & Wallace (1992); Kandel, Schwartz, & Jessel (1991)

Appendix B

Intracellular Biocytin Staining Procedure

Biocytin (intracellular, mol. wt. 372.48)

Biocytin is a compound of biotin and lysine (negative valance) that can bind to avidin and thus be visualized with any of the various probes commonly conjugated with avidin. As an intracellular label, biocytin can be combined with a number of anatomic tracers (FITC, rhodamine, neutral red, Texas Red, Fluoro-gold, or Phal) in order to determine its spatial relationship or anterograde/retrograde interconnectivity. It is also well suited to be processed for immunocytochemistry providing useful neurotransmitter determination. However, due to the large molecular weight of the typical probes conjugated to avidin, a detergent such as Triton-X must be used to enhance the probe penetration of the membrane, and this will sometimes compromise the high resolution morphology of the cell (electron microscopy). Avidin itself is a glycoprotein with a molecular weight of 68000 kD, and four active binding sites for biotin that bind virtually irreversibly and therefore is ideal for double labelling.

The most common conjugate for avidin is Horse Radish Peroxidase (HRP) giving a detailed visualization of the entire neuron, including soma, dendrites, dendritic spines and a great extent of the axon and its collateral's with terminals. It has the advantage of being robust and permanent. Typically, in an intracellular stain, HRP is introduced as an avidin and biotinylated horseradish peroxidase macromolecular complex, otherwise referred to as an ABC complex. This is the goal of the intracellular injection of biocytin, as it is possible to avoid unlabelled primary and secondary antibody reactions, and reduces background and indiscriminant staining providing a very specific stain of the injected cell.

The chromogen that best reacts peroxidase is diaminobenzidine tetrahydrochloride (DAB) producing a reddish brown precipitate in sensitive areas. It can also be intensified to a blue-black colour in the presence of divalent cations such as cobalt chloride (CoCl_2) combined with nickel ammonium sulphate. The advantage of DAB, is its insolubility in alcohol and clearing agents such as xylene, making it very permanent and pervasive.

Other chromogens that react with peroxidase in tissue slices include 3-amino-9ethyl carbazole (AEC) or two new Vector substrate kits, VIP, producing an intense purple precipitate and an SG substrate, giving a gray blue reaction product.

The procedure below focuses on reacting a biocytin injected cell using an Vectastain Elite ABC kit, visualized with a DAB reaction in a 400 μm tissue slice. For reacting thinner tissue slices ranging from 2-399 μm , simply reduce the time and number of baths where indicated in the below procedure and further modify concentrations to your own tastes and needs.

Biocytin with Cobalt-Nickel Intensification

Injection

Once you have acquired and recorded from the cell you wish to stain, determine a stable (does not alter baseline) stimulation intensity between 0.6 and 1.0 nA depolarizing current and balance the bridge. Stain the cell with a series of pulses consisting of 100 msec., 5-6 Hz stimulations applied 1 minute on and 15 secs. off for the first five trials and 2 minutes on and 20-30 secs. off for another 3-6 trials. This constitutes a good basis for staining any cortical neuron. If the cell remains stable beyond these parameters and the physiological recording is complete, continue to inject the biocytin for as long as possible as this will only intensify your staining.

Step One

Remove and fix tissue in a 4% paraformaldehyde solution (0.2 - 15% picric acid optional) or a formaldehyde-glutaraldehyde (low concentration, 0.2-.5%) overnight at 4°C in preparation for staining. The tissue can be stored from 1-5 days, although early staining seems to produce the most reliable results and overfixation will cause distortion in cell morphology. When longer storage is necessary overfixation can be avoided by transferring the tissue to a buffer (10 mM PBS, pH 7.2) after one day or into cryoprotectant (Chapter 2). If further immunocytochemistry is intended, it is recommended that the tissue not be fixed for more than 3 hours before being processed or transferred to a buffer.

Step Two (not applicable to large uncut slices)

Embed the tissue in one of a number of embedding agents and cut to desired thickness on a sliding microtome or vibrotome in 10 mM PBS (pH 7.2). The embedding agent should be prepared with paraformaldehyde (<4%) and kept below 60 °C and then cooled in a refrigerator while not being used. Suitable embedding agents include: 2.5-3% agar, polyethylene glycol, or paraffin usually dripped onto the slice resting on a petri dish.

Step Three (not applicable to large uncut slices)

If you use paraffin, remove the embedding agent thoroughly through a series of Clearene or Xylene baths, being careful to use unpolluted solutions. Continue to hydrate the tissue with a graded alcohol series of your choice ending with dH₂O. ie. 30sec - 5 minutes (depending on thickness of slice) 100% ethanol followed by 90%, 70%, 50%, and dH₂O.

Step Four

Rinse tissue briefly (~1 min) in 10 mM PBS (pH 7.2) for three consecutive baths.

Step Five

Eliminate all endogenous peroxidase activity with a 20 minute (40 μ m) to 2 hour (400 μ m) incubation in 0.2-0.5% hydrogen peroxide (H_2O_2) solution in 10 mM PBS (pH 7.2). This will eliminate a lot of background and nonspecific staining, however, if background is not important you may ignore this step. Agitate slowly at room temperature.

Step Six

Rinse tissue 3-4 times in separate baths of 10 mM PBS (pH 7.2) for 15-30 minutes each depending on the thickness of the slice(s). Agitate slowly at room temperature (Very Important Rinse)

Step Seven

In order to allow penetration of the avidin & biotinylated horse radish peroxidase complex (ABC) in slices thicker than 20 μ m, the cell membranes must be weakened with a detergent. At this point it is may also appropriate to block nonspecific staining (blocking) with the primary antibody if you intending on latter immunocytochemistry and are having background problems, however, this is not a necessary process when using an intracellular stain such as biocytin as there is no secondary antibody in this procedure. If you are going to incubate in a primary and secondary antibody as a part of another procedure, then blocking may be appropriate here (2-12 hours in 2-20% normal serum, same species).

Incubate the tissue in 0.3-0.5% Triton X-100 in 10 mM PBS (pH 7.5) for 1 (very thin slices) to 16 hours (400 μ m, ~ 8 hours) and agitate at 4°C, usually overnight. If appropriate add the necessary normal serum (150 μ l , 3 drops) of stock to 10 ml of buffer or in this case buffered detergent) to reduce background. Agitate slowly at 4°C.

*****Remember you need 30 minutes to allow the ABC reagents to preincubate*****

Step Eight

Prepare ABC reagents 30 minutes prior to incubation, this will allow the avidin and biotinylated-HRP to bind, creating the avidin-biotin-HRP complex that will label the injected biotin. As this procedure will sometimes use an ionic salt (cobalt-chloride) in later steps, the ABC reagents will be mixed in a 10 mM phosphate buffer (PB) pH 7.5, without the 0.9% NaCl or KCl.

Incubate the slices in the Elite ABC reagent, prepared with exactly (very important) 2 drops (100 μ l) solution A (avidin) and exactly 2 drops (100 μ l) of solution B (biotinylated HRP) from the same kit, in 5 ml 0.1 % Triton X-100 in 10 mM PB (pH 7.4) for at least 1 hour, extending to 16 hours depending on the thickness of the tissue and previous success. With adequate incubation in Triton X-100, I find 1.5 - 2 hours incubation to be more than adequate for most tissue. Agitate slowly at room temperature. I suspect that too long an incubation period will cause a build-up of ABC on the surface of thick slices, resulting in a general overstaining on the immediate surface of the slices. It is also important to use exact proportions of A and B from the same kit, as improper mixing will cause such problems as background staining, or staining failure.

Step Nine

Rinse the tissue in 3 baths of 10 mM PB (pH 7.4) for 15-30 minutes each. Agitate slowly at room temperature.

Step Ten

Prepare the chromogen, in this case a DAB solution consisting of 1 mg DAB per 10 ml of 10 mM PB pH 7.4 (0.1% DAB). Filter the DAB with a fast, light filter to remove the granular particles often left undissolved. Proportion 3 * 5 ml wells of DAB for each pair of 400 μ m slices, or for thinner slices, ensure that you can observe each piece clearly during the reaction. To the second chamber add 1 drop/ ml of a mixture consisting of 2 ml 1% cobalt chloride in dH₂O (CoCl₂) and 1.6 ml of 1% nickel ammonium sulphate in dH₂O (NiNH₃SO₄). In the last well prepare to add 1 drop/min for 3 minutes of the 0.03% H₂O₂ solution in dH₂O, this will catalyze the DAB-HRP (peroxidase) reaction producing the dark purple precipitate in the presence of the cobalt/nickel solution.

Incubate the slice(s) in the first two wells respectively for 20 minutes each, while gently agitating the solutions. For the final 20 minutes pipette 1 drop/min of 0.03% H₂O₂ into each well for a total of 3 minutes and 3 drops each. Watch carefully, as a colour

reaction can occur instantly, depending on the thickness of the slice and the appropriateness of the time in the previous steps. You can increase the amount of 0.03% H_2O_2 to 5 or even 10 drops total, but this will accelerate the reaction and leave you with darker tissue. When the desired stain is reached remove the tissue to the phosphate buffer rinse in step eleven to stop the reaction. The surface of the tissue in thick slices will appear to colour a great deal more quickly than the desired cell. In these cases, trial and error will tell you how far to let the background stain, and still let you visualize the cell. There is always an option to slice or trim the tissue before dehydration and this will typically remove any surface staining. Be warned, this may also remove your cell! Agitate slowly at room temperature.

In the ideal situation there will be no background at all, and therefore there will be no indication as to whether the cell has stained or not. If the above procedures were followed accurately and the biocytin injection was good, you should feel confident that the cell has stained appropriately despite no visible reaction.

Remember that DAB is a mutagen, and considered very toxic. Follow the safety and waste disposal methods

Step Eleven

Rinse the tissue in 3 baths of 10 mM PB for 15 minutes each. Agitate slowly at room temperature. It is often difficult to resolve the injected cells at this point as the tissue has not been dehydrated. Don't be discouraged, wait until the process is complete and the tissue is mounted and dried, this procedure seems to improve with age.

Step Twelve

Mount the tissue directly onto gelatin coated or natural slides and analyze wet under the microscope. The tissue will not be clear, but try and determine the placement, depth, and intensity of staining in the desired cell(s). For poor penetration and staining it is feasible to repeat steps five through to the end, or from any rinse step thereafter. For poor visibility due to excessive surface staining, considered the practicality of cutting a vanier from the face of each slice or sectioning the slice completely, be sure to sugar the sections in 20% sucrose before cutting. In the case of adequate penetration and background, continue with the tissue dehydration and clearing in the following steps. If you are preparing thick slices, be careful to monitor the sections as they are dried onto the slide, and make sure that the edges do not lift or the surfaces crack. It is not recommended to leave the tissue overnight to dry as this will almost definitely result in section damage. I prefer to free float all sections through the

dehydration and clearing, and mount directly onto a slide and coverslip from xylene with DPX.

You may also consider many other staining protocols at this point which could compliment the intracellular staining, including a basic cresyl violet or complete immunocytochemistry.

Step Thirteen

Dehydrate the tissue on slides or free floating through a standard dehydration regime.

For example: 5 minutes 50 % ethanol
 5 minutes 60 % ethanol
 5 minutes 70 % ethanol
 5 minutes 80 % ethanol
 5 minutes 90 % ethanol
 10 minutes 100 % ethanol

15-30 minutes xylene (clean & fresh)

Coverslip with DPX or Micromount using rather generous amounts due to the thickness of the slice. Allow to dry 1-5 days before photography.

The stained material will become more defined and easier to resolve as the DPX dries over the first few days, hence, any detailed analysis should be left for several days. I have found that there is some shrinkage to the tissue around the cells causing the distortion of long thick process such as the apical dendrite. This is likely due to the type of fixation used and the short fixation periods in combination with the type of dehydration regime. I am currently investigating ways to alleviate this problem.

Lucifer Yellow Staining Procedure

Lucifer Yellow was injected from the intracellular recording electrode by passing a hyperpolarizing DC current through the electrode at intermittent periods (0.2-1.0 nA at 10 Hz, 200ms for 60s, ISI 15s) for a total of 10-30 minutes depending on the stability of the cell. Upon completion of experiment the electrode was withdrawn slowly, and after a poststaining period of at least one hour, the tissue was removed and sandwiched between two pieces of filter paper soaked in fixative (3.5% paraformaldehyde & 0.5% glutaraldehyde) for 1-5 days of refrigeration at 4°C. The slice was then removed and dehydrated (0.1 M NaH_2PO_4 buffer, 5 min; 50% ethanol, 5 min; 75% ethanol, 5 min; 90% ethanol, 5 min; 95% ethanol, 10 min; 100% ethanol, 10min) before being cleared and mounted on a slide in methyl salicylate as a 400 μm section. The wet mounted sections were then examined by epifluorescence using a

high power Zeiss fluorescent microscope (EL-Eiuslaz) and photographed with 35-mm camera (Zeiss MC100, Tri-X black and white print film, 100 ASA: Kodak). The tissue was transferred to a cryoprotectant and stored at sub-zero temperatures (-40°C) for further processing. We also examined the possibility of using an antiserum to fix the LY for an indefinite period (Tseng & Haberly, 1989; Taghert, *et al.*, 1982).

END

1 1-0 6-9/6

FIN