NEURAL SUBSTRATES OF NAVIGATION AND SPATIAL COGNITION IN THE RAT

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This main purpose of this dissertation is to describe a set of three experiments that were designed to explore neural systems supporting navigation and spatial cognition. Additionally, the theoretical significance of the experiments and their relationship to the current literature will be discussed in detail. This introductory section provides some motivation for the study of navigation and spatial cognition, followed by an overview of the three experiments, a discussion of previous work leading to each experiment, and a brief description of the methodology used for each study. Experiment 1 utilizes high-density neurophysiological recording techniques to examine hippocampal place cell activity in cue-rich environments where the rats are exposed to cue conflict situations, and concludes that the hippocampal representation of spatial location is concordant. Experiment 2 takes advantage of the transynaptic retrograde transport of pseudorabies virus to define the synaptology of a pathway through which cells whose activity code for head direction could receive vestibular sensory information. Experiment 3 also uses pseudorabies virus to define polysynaptic pathways from the hippocampal formation to the hypothalamus via the lateral septum, which could provide the pathways for spatial information to be transported to areas involved in motivated behavior. The last chapter of this dissertation provides an extensive discussion of some issues surrounding the data presented, and brings together the experimental conclusions to construct generalizations regarding neural substrates of navigation and spatial cognition.

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PREFACE

Organization of Dissertation

This dissertation consists of five chapters: a general introduction, three experimental manuscripts, and a general discussion. Each manuscript contains a specific introduction and discussion of relevant issues. The three manuscripts are as follows:

> Brown JE, Skaggs WE (2002) Concordant and discordant coding of spatial location in populations of hippocampal CA1 pyramidal cells. J Neurophys 88:1605-1613.
>
> Brown JE, Card JP, Yates BJ. (2004) Polysynaptic Pathways from the Vestibular Nuclei to the Lateral Mammillary Nucleus of the Rat: Substrates for Vestibular Input to Head Direction Cells. Exp Brain Res (in press).
>
> Brown JE, Yates BJ, Card JP. (2004) Polysynaptic pathways from the entorhinal cortex and hippocampal formation to the lateral hypothalamus via the lateral septum in the rat (in preparation).

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Compiling this document while looking toward my future has helped me appreciate the humbling amount of assistance that I have received from people too numerous to thank at the end of a 45 minute talk. The purpose of this preface is to mention at least some of them. And it is more for the writing, than the reading. With that in mind….

It goes without saying that I would not be here without the unconditional support and love of my parents. I definitely won't understand such things unless Jessica and I have children of our own. I think the most important thing my parents did in regard to my career was to patiently trust that this extended education would be useful and rewarding. I'm sure that my goals, though backed by tremendous energy, didn't seem very clear when I started poking lab rats at UConn. Nevertheless, I hope that I have fulfilled their expectations so far by achieving this milestone.

The best part of my time in Pittsburgh has been every day since early September, 2000, when Jessica Check walked into my lab looking for somebody else. Thanks to our mutual friend, Joy Balcita, I was able to wrangle a date with Jessica, and the rest is history. Jessica, thank you for enduring me; *your* unconditional support and love is indescribable. Your patience and flexibility has certainly made helped me through my graduate school experience.

At the University of Connecticut in the spring of 1996, I had the good fortune to lead a tour through the basement of the Psychology building where I met Dr. Etan Markus. He showed us a video of a hippocampal place cell recording and explained some concepts of spatial cognition. I watched the presentation in awe of both him AND the science, immediately begged him for a job, and…here I am. I am also thankful that he put my best interests first by encouraging me to go elsewhere for graduate school. Thanks to other people at UConn including, but not limited to: Michael Anderson and Maya Zecevic; (finally) Drs. Jonathan Oler,

Jennifer Tropp and Joseph Consiglio; Dr. Heather Bimonte-Nelson; and fellow one-time Pittsburghers Drs. Eric Neisenbaum, Laura Niesenbaum and John Salamone. Special thanks to Dr. Harvey Swadlowe for being the first to teach me that psychology has to do with the brain.

Dr. Bill Skaggs took me into his lab as a high-energy/higher-ego first year student, and gently showed me that there are people out there who are way, way smarter than I'll ever be (like him). His über-cerebral approach to science has been very influential on my progress as a young scientist.

Dr. Bill Yates took me into his lab when after I had completed three years of graduate school. He provided me with the resources necessary to continue my work and I greatly appreciate his support of my projects. More importantly, he has taught me about the business of science. Every graduate student should get such an education from their advisor. Also, Bill has shown me that the personal and professional *organization* is essential to productive scientific research.

Dr. Pat Card, my "other" advisor, has set a very high personal and professional example for me. I would not be able to thank him enough for the time and effort that he invested in my projects, as well as for the encouragement he provided during challenging times. Working with Pat makes me want to be a neuroanatomist, and I hope that some day I can set a similar example for young scientists.

I have been fortunate to have very useful committees in graduate school. I have enjoyed and learned from my interactions with them. The have been composed of: Drs. Bill Skaggs, Jay McClelland, Dave Touretzky, German Barrionuevo, Pat Card, Susan Sesack, Alan Sved, Linda Rinaman, Pat Sharp and Bill Yates.

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No one gets their PhD in our department without the thankless work of the staff. Special thanks to Joan Blaney and Mary Spanodoukis.

I hesitate to mention any of my fellow graduate students and peers here because I would undoubtedly leave too many people out. Exceptional gratitude, however, goes to Big Matt, Jeremy Masters, Fishy, Mike Sabatini, Vince McGinty, Richard Clarke, Andrew Maurer, Decade Dan, my comrade-in-arms Brian Jian and the Master Chief (you know who you are).

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1. GENERAL INTRODUCTION

This main purpose of this dissertation is to describe a set of three experiments that were designed to explore neural systems supporting navigation and spatial cognition. Additionally, the theoretical significance of the experiments and their relationship to the current literature will be discussed in detail. This introductory section will begin by providing some motivation for the study of navigation and spatial cognition. It will then present an overview of the three experiments, a discussion of previous work leading to each experiment, and a brief description of the methodology used for each study.

Most of the time we take spatial cognition and the behavior of navigation for granted – that is, until we get lost! Some people seem to be more prone to navigational errors than others, which can be a source of entertainment or frustration depending on the situation. However, in general we rarely have major problems finding our way from one place to another, and we are flexible enough to use different strategies that can guide our way-finding behavior.

Feats of navigation or way-finding have been reported in both humans and animals. O'Keefe and Nadal (1978) wrote a landmark book that discussed examples of impressive navigation by animals such as migrating birds, homing pigeons. They also described how human inhabitants of South Sea islands (Puluwatans) were known to be able to navigate vast expanses of open ocean using clever navigation techniques. In 1948, E.C. Tolman described what became a famous experiment suggesting that rats had access to "cognitive maps" that allowed them to solve spatial mazes by taking shortcuts.

The concept of a "cognitive map," or a "mental map," is rather intuitive because we often organize our thoughts in ways that could be described as "mapping," whether or not they possess

spatial qualities. Being able to rely on an internal representation of space is very useful when, for example, one is trying to figure out how get to the Pittsburgh International Airport when the Fort Pitt Tunnel is closed. However, tasks involving spatial navigation can not simply be accomplished with an internal sense of place, space or location. The skill of orienteering is based on both representations of space as well as representations of direction, heading or orientation. A map is useful, but infinitely more so when combined with a sense of direction.

When studying the neural basis of navigation and cognition, I believe that it is useful to address both the internal representation of location and the internal representation of direction. This can easily be done in rats as neural correlates of location and heading have been discovered. Briefly, the activity of some neurons in the rat hippocampus were found to correlate with the rat's location in its environment (hippocampal "place cells," O'Keefe and Dostrovsky, 1971), and the neural activity of a more distributed set of neurons have been found to correlate with the animal's head direction ("head direction cells," Taube et al., 1990a). Both of these sets of cells have been studied extensively since they were discovered because they have been thought to be components of the "cognitive map."

Over time, several theories too numerous to describe here have been advanced regarding significance of these cells (for review, see Redish, 1999), even resulting in longstanding feuds between opposing theorists. The present state of the scientific field examining this aspect of spatial cognition is positive, however; it seems that most everyone has realized how complicated these signals can be, and that it is much more important to figure out how they REALLY WORK instead of stubbornly advancing one's own pet theory (myself included).

One of the many reasons why place cells and head direction cells are interesting to me is that, as the reader will hopefully understand throughout the course of this dissertation, they

represent something tangible. The first time that I observed a place cell recording I was both amazed and positive that it coded for the location of the animal. The first time that I observed a head direction cell recording, I was positive that it coded for the head direction of the animal. It is no small thing to listen to a neuron fire every time a rat runs over a specific location or turns its head through a specific angle.

Upon entering graduate school in 1998 I had specific plans to study the physiology of hippocampal place cells, and dreams of studying the physiology of head direction cells. Furthermore, I slowly developed a secret goal of studying the neuroanatomy supporting these systems as I came face-to-face with the knowledge of function can be wonderfully complimented by knowledge of structure. Finally, scientifically immature as I was, I realized that place cells and head direction cells can fire all they want, but what is most interesting is how such signals can be used to support motivated behavior. For reasons best explained over one's alcoholic beverage of choice, at this point in 2004 I have had the opportunity to study a much broader range of neurophysiology and neuroanatomy than I had expected. And I have had the opportunity to address all of the subjects listed above – with the exception of head direction cell physiology, which I will accomplish during post-doctoral work with Dr. Jeffrey Taube at Dartmouth College.

Accordingly, the first of my experiments presented in this dissertation examines the physiology supporting the neural representation of spatial location in the rat, the second experiment examines the anatomy supporting the neural representation of direction, and the third experiment examines neural pathways through which this information could support motivated behavior.

1.1."Sandstorm"

Before diving into the background and significance supporting each of these experiments, I would like to address the following question: is navigation itself a simple task, or is it one that brains are especially good at performing? One way to address this question is to explore the success of robots designed specifically for autonomous navigation. It turns out that programming computers to navigate is a surprisingly complex task. Recently, the Defense Advanced Research Projects Agency (DARPA) established a "Grand Challenge," whose purpose was to encourage the development of autonomous military vehicle technology (see http://www.darpa.mil/grandchallenge/ for complete documents excerpted here). A $1 million cash prize was offered to the team whose autonomous ground vehicle could navigate a pre-determined 300 mile course over desert terrain. Below is an excerpt from the press release:

> January 2, 2003
> **DARPA Plans Grand Challenge for Robotic Ground Vehicles**
>
> **U.S. Department of Defense Agency Calls on Trailblazers and Pioneers from a Variety of Fields to Compete in 2004 Event and Spur New Robotics Innovations for the Battlefield Applications**
>
> Arlington, VA – The Defense Advanced Research Projects Agency (DARPA) of the U.S. Department of Defense (DoD) has announced that next year it will conduct a Grand Challenge for autonomous robotic ground vehicles.
>
> Scheduled for March 2004, the DARPA Grand Challenge for autonomous robotic ground vehicles will cover a course of approximately 300 miles between Los Angeles and Las Vegas, and the team that most quickly completes the route in less than the prescribed time will receive a cash prize of $1 million. This challenge is intended to spur the accelerated development of autonomous robotic ground vehicle technology for military applications, and is the first in a series of Grand Challenges planned by DARPA.
>
> DARPA is calling for trailblazers and pioneers in wide range of fields to become part of Grand Challenge teams, including advertisers and corporate sponsors, artificial intelligence developers, auto manufacturers and suppliers, computer programmers, futurists, inventors, motor sports enthusiasts, movie producers, off-road racers, remote-sensing developers, roboticists, science fiction writers, technology companies, universities, video game publishers, and other trailblazers.

(http://www.darpa.mil/grandchallenge/media/announcement.pdf)

Specifically, the vehicles had to:

> …autonomously traverse the challenge route without human interface or control of any kind. The vehicles must perform general route selection and navigation to follow the Challenge route. Vehicles must sense their environment to perceive terrain features, ground conditions, obstacles, and other Challenge vehicles. They must intelligently control their speed and direction so as to avoid or accommodate all of the above. And they must do these things quickly- overall speed will be the deciding factor and the time limit is designed to push vehicle speeds far beyond current technologies. (http://www.darpa.mil/grandchallenge/faq.htm)

Fifteen teams were selected to participate in the event following a qualification event prior to the challenge event (see http://www.darpa.mil/grandchallenge/media/qid_results5.pdf). They included teams based in both academia and industry, supported by substantial sponsorships allowing the vehicles to sport the latest and greatest computer technology. I'd like to describe in great detail the technological features of one of the vehicles in order to establish how much processing power it takes to even attempt this task.

The team that qualified for the "pole position" and was favored to win was based at Carnegie Mellon University's Field Robotics Institute. They were named "Red Team" after the group's leader, Professor William"Red"Whittaker. Their vehicle, "Sandstorm," was built out of a 1986 Hummer and "of combine[d] the raw power Intel® Itanium® 2 systems with the flexibility of Intel Xeon™ systems, and the mobile performance of handheld systems based on Intel XScale® technology to drive the state of the art in robotics into the 21st century (http://www.intel.com/labs/features/rs12031.htm)." Intel's additional description of Sandstorm is impressive, especially when describing the processing power designed to turn the challenge into a mere set of calculations:

> The Hummer can easily drive 25 miles per hour for ten hours — the limiting factor is the driver. The computer systems driving the vehicle are fundamentally limited by computational performance.

To understand why, observe that video display typically requires processing of about 30 frames-per-second. Creating machine perception with stereo vision means two cameras are needed, hence twice the frame rate. Furthermore, the Red Team will also use laser range-finders to scan the terrain in front of the vehicle. And perceiving terrain is only the start.

A lot of data crunching occurs as the scanned images are analyzed by the system to create a terrain model. Ideally this model incorporates subtleties such as "what is a bush" (something to drive over) and "what is a rock" (something to approach with care).

The terrain model is combined with a physical model of the vehicle and a desired course to simulate the result of the vehicle following the course. A result such as "roll-over" or "spin-out" means the vehicle should try a different course. In the end, the speed of the vehicle is determined by the rate at which it can reason a safe path through its environment.

Current research robots such as the Honda* ASIMO* illustrate the scale of the challenge in this race. The ASIMO uses three Intel® Pentium® III processors to navigate at 1.5 miles per hour down a protected hallway. Multiply by 20 to get into the range of computing performance required by the Red Team.

The modified Hummer chassis on which the race vehicle sits will be driven by a system that combines the raw power of the Intel® Itanium® 2 platform with the flexibility of an Intel Xeon™–based system, and the mobile performance of Intel XScale® technology. Of course, the vehicle will also undergo a number of suspension modifications to soften the ride across the desert, and a second suspension will support the "ebox" where the computer systems will reside

The main compute engine is a four-processor Intel Itanium 2 "Bandera" system. Bandera is an advanced development system of 4 U-rack–mounted servers using next-generation Intel microprocessors, code-named "Madison." The advanced Itanium 2 microarchitecture is ideally suited for the physics and image-processing algorithms required for path simulation, which are loop- and floating-point intensive.

In addition to the Bandera system, four dual-processor Intel Xeon–based systems will host a variety of applications, including route planning, map data access, actuator control, and status monitoring.

The team will also use Intel XScale technology–based mobile systems for field testing and prerace communications. Race rules require the vehicles to "think" for themselves, so computers are carried in the vehicle, with no radio communications or wireless networks allowed during the race (http://www.intel.com/labs/features/rs12032.htm).

The official Sandstorm website, http://www.redteamracing.org/index.htm, provides additional technical details regarding the “sensory” systems of the vehicle:

> Sandstorm utilizes [multiple] perception sensors to "see" terrain, obstacles and other vehicles. It utilizes scanning Radar, SAIC stereo vision, and scanning laser ranging. Position and orientation are estimated by six-axis inertial sensing and axle encoding that are fused with GPS by an Applanix pose estimator. Additionally, position is sensed by OmniStar GPS.
>
> The perception sensors are stabilized and pointed by a 3-axis gimbal that uses actuators from HD systems, contollers from Advanced Motion Controls, and components machined and purchased by Boeing . The sensors and gimbal are protected by a dome created by ALCOA. The stabilization isolates the sensors from vehicle motion while Sandstorm moves over rugged terrain.
>
> Generating models from digital sensor data is not simple. This is one of the challenges of the "grand challenge". Integrating sensor data with machine intelligence is a profound challenge for navigating rough terrain.
>
> Sensor data is imperfect due to sensor calibration errors, visual glare, unusual surface properties, and error induced by vibration. Navigation computers must utilize this data while moving at high speed across rough terrain. It must continually make decisions with limited and imperfect information!
>
> Xeon computers donated by Intel Corporation provide the most powerful computing ever to drive a robot. Mass storage utilizes shock resistant hard drives by Seagate Technology. The speed enables the rich sensor processing and far-horizon dynamic planning. Computers are shock isolated by Lord Corporation in an electronics enclosure fabricated by J&J Truck Bodies.

How quickly did "…the most powerful computing ever to drive a robot…" propel Sandstorm to the finish line to collect the prize? Unfortunately, Sandstorm traveled 7.4 miles in about an hour before it got stuck on the side of the road and burnt out its gearbox trying to extract itself. Surprisingly, Sandstorm was by far the most successful vehicle in the contest! Of all the reasons leading to vehicle failures, most had to do with failures in navigation in one sense or another, although some vehicles had non-navigational problems (for full list, see http://www.darpa.mil/grandchallenge/media/final_data.pdf). DARPA considered the contest a huge success and plans to hold the challenge again next year, doubling the prize money to $2 million.

I believe that this example provides clear evidence that navigation is no simple process! As described above, an impressive array of human intelligence and raw computing power is no

match for the lump of organic material in our skulls. So how do brains do it, and why is it an important subject worthy of study?

The brain's ability to keep track of items, locations and events within a spatial context and generate the behavior of navigation can serve as a model for neural information processing. What aspects of the environment do we use to help guide us along a route? How does the brain process incoming sensory information in such a way that allows us to keep track of where we are, where we're headed, and the best way to get there? How does sensory information of so many different types get integrated such that we can form "cognitive maps?"

Over the last 30 years, advances in neurophysiological and neuroanatomical techniques have propelled the pace at which the study of these neural systems has progressed. Both place cells and head direction cells are very accessible to neuroscientific study, and great progress has been made in understanding of how these neural systems support navigation and spatial cognition.

Solving the code for internal representations of location and direction and their contributions to motivated behavior is important for several reasons, one of which addresses how brains process information. Understanding how brains work is the ultimate goal, but achieving it requires starting at the beginning by understanding how its components work. Because place cells and head direction cells are so accessible and their output is so tangible, they can serve as wonderful model systems for exploring neural information processing and answering questions such as those posed throughout this paper.

1.2.Overview of experiments

Rats have been subjects of experiments examining the brain's role in navigation and spatial cognition for many years. Over this time it has been determined that neural activity in the rat hippocampus is strongly related to the animal's spatial location within an environment. As experimental paradigms examining the hippocampal representation of location have matured, it has become apparent that the hippocampal code is extremely complicated; many questions remain concerning its function in navigation and spatial cognition.

There are several theories of hippocampal function that make predictions regarding how the hippocampal codes for space in complex environments (Nadel and Eichenbaum, 2001; Redish, 1999). Using the proper controls, it is important to test such predictions in order to understand the intricacies of hippocampus function. The first experiment presented in this dissertation (Chapter 2) employed single cell recording in the hippocampus of the behaving rat to test the following hypothesis: the hippocampal representation of spatial location is a cohesive map, whose individual parts respond as one to cue manipulations. This study examined the effect of specific cue-conflict situations on the activity of ensembles of simultaneously recorded pyramidal cells in the hippocampus of the freely behaving rat. The goal of this experiment was to determine whether or not the hippocampal representation remains cohesive during such cue-conflict trials by following cue sets instead of individual cues.

Additionally, the internal representation of location in rats is complimented by an internal sense of direction, coded by "head direction" cells. Head direction cells exist in several parts of the brain, and understanding the factors that influence their activity is essential in understanding spatial cognition and navigation. The circuitry responsible for generating and maintaining the directional signal is being slowly deciphered, and evidence from neural recording and behavioral

experiments suggests that the vestibular system contributes to the directional signal (Stackman and Taube, 1997). Anatomical studies using monosynaptic tracers in the rat suggest that the lateral mammillary nucleus (LMN) may be receiving vestibular input via a polysynaptic pathway (Taube, 1998). The second experiment presented in this dissertation (Chapter 3) employed retrograde transneuronal tracing techniques with pseudorabies virus to test the following hypothesis: vestibular nuclei project to the lateral mammillary nucleus via a polysynaptic circuit that includes the dorsal tegmentum, and potentially other relays. The goal of this experiment was to provide insight into the anatomical basis for the generation and maintenance of the head direction signal in the rat.

Finally, the extent to which the internal representations of location and direction are used to guide behavior is unclear. Behavioral experiments to this point have generated somewhat conflicting results that are difficult to evaluate because current knowledge of basic circuitry linking the hippocampus to brain areas regulating behavior is incomplete. In order to better understand the role of these neural systems in behavior, it is imperative to better understand the neural circuitry supporting them. There is evidence suggesting that there are pathways linking the hippocampal formation to the caudal hypothalamus via the lateral septum, which could be the circuitry through which spatial information is integrated and transported to behavioral regulation centers in the hypothalamus (Risold and Swanson 1996). The third experiment in this dissertation (Chapter 4) employed retrograde transneuronal tracing techniques with pseudorabies virus to test the following hypothesis: the hippocampus projects to the caudal lateral hypothalamus and mammillary complex via a topographically organized polysynaptic pathway via the lateral septum. Monosynaptic tracing studies have provided evidence that the hippocampal formation projects to the lateral septum in a topographically organized manner.

Also, those areas in the lateral septum receiving hippocampal input map onto the longitudinal columns of nuclei of the hypothalamus known to be involved in controlling motivated behavior (Risold and Swanson, 1997b; Swanson, 2000). The goal of this experiment was to further characterize the topographical organization of the polysynaptic hippocampo-septo-hypothalamic pathway.

Taken together, the information generated by these three experiments will advance the current understanding of the complex relationships between the hippocampal code for space, the internal sense of direction, and the pathways linking spatial information to behavioral regulatory areas in the rat.

1.3. Brief background and significance

Navigation based on spatial information requires two types of information: 1) current location in space, and 2) current heading, or direction. Both of these types of information are represented in the rat brain. Location is represented by activity of hippocampal pyramidal cells (place cells), while direction is represented by head direction (HD) cells in several areas including, but not limited to, the lateral mammillary nucleus (LMN), anterior dorsal thalamic nucleus (ADN), and postsubiculum (PoS). Numerous studies examining the firing properties of these types of cells have been published since place cells were first studied in the early 1970's (O'Keefe and Dostrovsky, 1971) and head direction cells were studied in the mid 1980's (Taube et al., 1990). Since then, research on rat spatial cognition and navigation has been extensively linked to these subjects (Sharp, 2002).

1.3.1. Place cells.

The activity of pyramidal cells in the rat hippocampus was thought to represent the animal's current spatial location, so it was originally hypothesized that the global hippocampal activity comprised a "cognitive map" of the rat's environment (O'Keefe and Nadel, 1978). Upon deeper investigation, however, it has been found that this activity can be reliably influenced by other factors. Such factors include changes in the animal's behavioral task (Markus et al., 1995), salient visual cues that indicate relative location rather than absolute location (Muller et al., 1987), vestibular input (Sharp et al., 1990) or goal location (Hollup et al., 2001). As a result, it has become apparent that experiments conducted in simple environments are no longer the ideal manner by which to examine hippocampal activity. Researchers have begun to examine place cell activity in increasingly complicated environments to better determine the details of the hippocampal code for space by using more cues in general (Knierim, 2002; Shapiro et al.,1997; Tanila et al., 1997) or environments with several different reference frames (Gothard et al., 1996; Muller et al, 1999; Redish et al., 2000).

As a result of such work it has become obvious that generating a heuristic for hippocampal activity that satisfies all of the experimental data published over the past 30 years is very difficult. Important questions remain unanswered in spite of numerous experimental manipulations having been performed with these cells. For example, one of the most important experimental issues is the following: it is unclear whether the hippocampal pyramidal cells represent spatial location within an environment with a cohesive mapping system, or whether each hippocampal pyramidal cell is independently representing relationships between individual cues, behavioral sequences or tasks. **Experiment 1** looks specifically at this issue by examining place cell activity in cue-rich environments where the rats are exposed to cue conflict situations.

These cue conflicts are intended to showcase response the hippocampal representation as it follows either sets of cues or individual cues.

This type of experiment is important because the current place cell literature is divided into two main camps: one supporting the cognitive map theory and the idea that the representation of location by the hippocampus is done in a cohesive, map-like manner (O'Keefe, 1999); the other camp supporting the idea that hippocampal activity simply in a reflection of associations between events, places and actions that are associated in an environment (Eichenbaum et al, 1999). Regardless of the outcome, the results of this experiment will add to the current knowledge of hippocampal function and will provide evidence that may serve to better interpret discrepancies in the literature.

1.3.2. Head direction cells.

Interpretation of the head direction signal, on the other hand, has traditionally been more straightforward than that of the place code. Therefore, most remaining questions in the literature are centered on the generation and maintenance of the signal rather than those regarding its characterization. One important question is: which sensory inputs contribute to head direction cell activity? Most current models include some kind input from the vestibular system or internal representation of self motion or position relative to gravity (Sharp and Blair 2001; Taube, 1998). It seems likely that at least some vestibular information contributes to the signal because semicircular canals generate neural activity based on movements of the head. However, there is no direct projection from the vestibular system to areas containing head direction cells. It is possible that vestibular information is transported to head direction cells in the LMN via a polysynaptic circuit, based on information from anatomical studies (for general review, see Taube, 1998; Sharp et al., 2001) and experimental data (Bassett and Taube, 2001; Stackman and

Taube, 1997). **Experiment 2** examines the potential pathway through which vestibular information might reach the LMN, and will identify potential relays connecting these areas.

1.3.3. Hippocampal influence on behavior.

Regardless of the specifics of place cell or head direction cell coding, perhaps the most significant question remains: do rats use these representations of location and orientation to guide behavior, and if so, how? In approaching this question it is important to determine the neural pathways through which spatial information may be transported to areas regulating behavior. There are several ways by which spatial information can be used to regulate behavior, including higher cognitive functions in the posterior parietal association cortex, or lower regulatory functions in the hypothalamus. One pathway connecting hippocampal output to the hypothalamus has been proposed by Swanson and colleagues (Risold and Swanson 1996, 1997b, Swanson 2000) based on experiments performed with monosynaptic tracers. Their studies have begun to examine individually the topographic organization of hippocampal projections to the lateral septum, and the corresponding lateral septal projections to the hypothalamus and mammillary complex. **Experiment 3** builds upon this foundation of data by exploring the topography and synaptology of the ***polysynaptic*** circuit connecting the hippocampus to the caudal lateral hypothalamus and mammillary complex. Based on their work it is unclear exactly how hippocampal information is being carried to these areas, and it is particularly important to understand the role of the lateral septum in transmitting this information. For example, is the lateral septum faithfully transmitting spatial information via a labeled-line relay, or is it performing some sort of the signal before it projects to the hypothalamus? Insight into these issues will be gained by examining the details of the microtopography of the polysynaptic

circuit. The experiment presented here will add considerable information to the current literature because, regardless of the outcome, the results will advance our understanding of spatial information processing and its role in behavior.

1.4. Brief background of methodologies

1.4.1. Methodological background for Experiment 1

Ensemble recording of hippocampal place cells. Advances in technology have allowed researchers to progress from recording a small number of hippocampal neurons simultaneously in freely behaving rats (McNaughton et al., 1983) to being able to isolate over a hundred hippocampal pyramidal cells in a single experiment (Wilson and McNaughton, 1993). This technology was used in the Experiment 1 to examine the activity of large ensembles of hippocampal pyramidal cells in freely behaving rats as they ran for food reward in an elevated maze.

The use of environmental cues to study the activity of place cells. It has been shown that hippocampal place cell activity can be influenced by environmental cues (Muller et al., 1987, Bostock et al., 1991), as well as the by surface texture of the animal's environment (Knierim, 2002). Cue conflict experiments with a range of complexity have been performed with success in rats (Tanila et al, 1997; Shapiro et al 1997; Skaggs and McNaughton, 1998) and provide a methodological basis for this experiment.

1.4.2. Methodological background for Experiments 2 and 3.

Using PRV to trace polysynaptic pathways in the rat brain. Traditional neuroanatomical tracers have the limitation that they do not cross synapses, and thus do not allow the identification of polysynaptic pathways. However, attenuated strains of pseudorabies virus

(PRV) have been shown to be effective transynaptic tracers because of 1) their high affinity for axon terminals, 2) their ability to replicated in neurons and produce infectious progeny, and 3) the demonstrated propensity of newly replicated virons to leave the parent cell at sites of afferent synaptic contact (Card, 2001). PRV therefore fulfills the criteria of a self-amplifying tracer as it moves in a retrograde direction through polysynaptic circuits. Over time the virus passes through chains of synaptically linked neurons; the number of synapses separating an infected neuron from the brain region initially infected with PRV can be estimated by performing a temporal analysis (analyzing labeling patterns at several different time points). Experiment 2 employed the retrograde transynaptic transport of PRV to determine whether areas containing head direction cells receive relatively direct or indirect input from the vestibular nuclei, while Experiment 3 used it to examine the organization of polysynaptic hippocampal pathways to the hypothalamus.

A number of factors must be taken into consideration when considering whether transneuronal viral tracing using PRV was an appropriate technique to test our hypotheses. Intracerebral injections of aphaherpesviruses, including the Bartha strain of swine alphaherpesvirus PRV utilized in the present experiments, have been widely used to identify synaptically connected neurons in rats in a reproducible fashion (see Card 1998 and Enquist & Card 2003 for review). However, false-positive and false-negative results may occur with PRV injections. False-positive results include infected neurons which were not infected via retrograde transsynaptic transport of PRV. False-negative results include the failure of synaptically linked neurons, or neurons located at the injection site, to become infected. Some factors related to these possibilities are discussed below.

PRV is a neurotropic virus which enters neuron terminals around an injection site via a receptor mediated attachment process (Card et al 1991, 1992, 1993; Spear 1993; Vahlne 1978).

Unlike some conventional tracers, PRV does not diffuse far from the injection site (Card 1999; Jasmin et al 1997), making it ideal for small injection targets such as the LMN. Glial cells have also been shown to take up PRV (Vahlne et al 1980) although they effectively sequester PRV rather than transport it to other cells because they are unable to replicate the virus (Card 1995). It is transported transsynaptically, essentially leaving one cell and entering another by crossing a synapse (Card et al 1990). Finally, the Bartha strain of PRV has been shown to move in a retrograde direction thrown synaptically linked chains of neurons (Enquist et al 1993, Jasmin et al 1997, O'Donnel et al 1997).

The microarchitecture of a target region is an important component of the usability of PRV in an experimental situation (Card et al 1999). For example, a region with a high density of terminals from local circuitry will produce a higher number of infected cells around the injection site. Conversely, a region with fewer local circuit terminals but more terminals from axons projecting from other nuclei will have a weaker local infection while neurons projection to the injection site will be more heavily infected. In the case of the LMN, there are relatively few local circuit neurons (Allen and Hopkins 1988). Most of the axon terminals in LMN are from neurons projecting to LMN. Therefore, the infected cells in LMN will be less than those in adjacent control regions, such as SUM where there is a higher density of local circuit synapses. Additionally, it is possible that LMN cells may be infected via transsynaptic transport via reciprocal connections with other nuclei. For example, LMN receives a dense projection from DTN, and DTN in turn receives a strong projection from LMN. It is therefore possible that heavier infections in LMN can be due to PRV uptake by DTN axon terminals in LMN, followed by the retrograde transynaptic transport of PRV from DTN to LMN over a longer survival time.

The extent to which PRV is taken up by fibers of passage adjacent to the injection site is unclear (Aston-Jones and Card 2000) although it has been shown in at least one case that it is possible (Chen et al 1999). However, given the high affinity for axon terminals and the receptor mediated attachment process of PRV at those terminals, it is clear that PRV most easily enters cells at axon terminals. In terms of the current experiments, the most notable fibers of passage that course through the mammillary complex are the mammillothalamic tract (which connects thalamic nuclei to the mamillary nuclei) and the fornix (which connects the hippocampal formation to the mammillary nuclei. In each case, preliminary data from our PRV injections into LMN have shown that second-order infections are limited to areas that project directly to the mammillary complex.

When combined with temporal analysis, PRV can be used to identify the hierarchical relationship of first-order, second-order, third-order, etc, neurons (Card 1998). Obviously, at longer survival times many cells will be infected for one to determine a meaningful relationship between infected cells. However, using multiple sets of animals which live for different lengths of time following the injection of PRV can aid one in identifying the progression of PRV through the synaptically linked circuitry.

2. EXPERIMENT 1: CONCORDANT AND DISCORDANT CODING OF SPATIAL LOCATION IN POPULATIONS OF HIPPOCAMPAL CA1 PYRAMIDAL CELLS

2.1.Introduction.

Currently a major controversy among investigators studying the hippocampus arises from the need to reconcile two substantial bodies of data. One suggests that the hippocampus is heavily involved in encoding the spatial location of an animal in its environment [the "cognitive map" hypothesis, supported by many neural recording, lesion and behavioral experiments (McNaughton et al., 1996; Morris et al., 1982; Muller et al., 1987; O'Keefe and Dostrovsky, 1971; O'Keefe and Nadel, 1978)]. The other suggests that the hippocampus plays a crucial role in certain types of memory [the "declarative memory" hypothesis, supported by many behavioral and lesion studies (Cohen and Eichenbaum, 1993; Marr, 1971; Squire, 1992)]. These two hypotheses make different predictions on a variety of levels; in particular, they arguably make different predictions about the coding properties of hippocampal pyramidal cells. These have sometimes been called "place cells" because their spike activity in many experimental situations is strongly related to the animal's current spatial location. As Eichenbaum has recently written, "If the [hippocampal] representation is a cohesive map, all of the elements should respond together to any manipulation. Alternatively, if place cells are merely memory elements that encode familiar places, they would also change their firing patterns to alterations in the environment but not necessarily in a cohesive way. The really interesting cases are when a large number of salient cues are changed and many others are not, or when the environmental stimuli are held constant while the task is changed, or when the animal moves between two adjacent environments composed of identical spatial cues. Can some place cells follow some of the cues, while other simultaneously recorded cells follow different cues? If so, the 'map' is not a

cohesive spatial framework; rather the hippocampus contains a collection of representations that encode subsets of the cues" (Nadel and Eichenbaum, 1999).

These predictions were examined in a recent study (Tanila et al., 1997a) in which rats were trained to run for brain stimulation reward on a plus-maze with local cues on the arms and distal cues on the curtains surrounding the maze. The rats were trained with all of the cues in a fixed ("standard") configuration, and then the spatial firing properties of ensembles of 2–10 simultaneously recorded CA1 pyramidal cells were examined first with the cues in the standard configuration, then with the local and distal cues rotated by 90° in opposite directions. In response to this "double rotation," some place fields were reported to follow the local cues, others followed the distal cues, others stayed fixed in the framework of the room, and others "remapped" in an unpredictable way. Within simultaneously recorded ensembles, the level of discordance (i.e., different place fields changing differently) was below chance, but nevertheless all possible combinations were observed, including instances where one field followed the local cues while another at the same time followed the distal cues. The authors' interpretation of their results led them to conclude that hippocampal cells individually encode specific identifiable combinations of features in an environment.

There were two issues that motivated further investigation of this question. First, from the viewpoint of computational modeling, it would be useful to have data from larger ensembles of simultaneously recorded cells so that the levels of concordance versus discordance (that is, of groups of place fields all changing in the same way vs. different subgroups changing in different ways) could more precisely be quantified. Second, there was no systematic control for the possibility that random remapping would occasionally give rise to new place fields that appeared, just by chance, to be rotated versions of the original fields. The likelihood of this is

difficult to estimate because rotations were judged by eye, and when a unit had multiple fields (which occurs often in this task), each subfield was examined separately. It could not, therefore, entirely be ruled out that every case was "coincidental" in which two simultaneously recorded cells had fields that appeared to rotate with different sets of cues. Thus, the goals of the current study were to replicate the essential features of the Tanila et al. experiment, while recording from substantially larger ensembles of neurons and performing the data analysis in a way that controlled for the expected occasional incidence of spurious rotations. Preliminary results have been presented in abstract form (Brown and Skaggs, 1999).

2.2.Methods and Materials

2.2.1. Experimental subjects

All procedures were performed in accordance with National Institute of Health animal-care guidelines, following an Institutional Animal Care and Use Committee approved protocol. Four experimentally naive male Sprague-Dawley rats weighing 300–350 g at the time of surgery were used in this experiment. The animals were singly housed and maintained on a 12-h light/dark cycle. All training and recording was done during the light portion of the cycle. The rats were handled daily and food-deprived prior to training to decrease their weights to approximately 85% of baseline.

2.2.2. Surgical and recording methods

The type of chronically implanted microdrive array used in these experiments, and the techniques used for recording and analyzing spike activity from hippocampal CA1 pyramidal cells, have been described in detail previously (Skaggs et al., 1996). Briefly, we used custom-made microdrives that allowed us to independently drive 12 tetrodes and two reference electrodes into the hippocampus. For microdrive implantation, the animals were deeply anesthetized with Equithesin (a mixture of sodium pentobarbital and chloral hydrate). The microdrives were positioned stereotaxically at coordinates approximately 3.5 mm posterior to bregma and 2.2 mm lateral to the midline and cemented to skull-screws using dental acrylic. Immediately after surgery, the tetrodes and reference electrodes were lowered approximately 1 mm into the neocortex above the hippocampus. After the animal had recovered from surgery, the tetrodes were lowered gradually into the CA1 cell body layer, which was recognized by its

unique electrophysiological signature (unit activity and electroencephalography). Reference electrodes were placed nearby at locations where they were uncontaminated by unit activity.

For data acquisition, the rats were connected to a specially designed multi-channel headstage (Neuralynx, Tucson, AZ) containing operational amplifiers, for impedance reduction, and a ring of colored light-emitting diodes to permit position tracking. Two flexible cables ascended from the headstage to a port in the ceiling and continued to the recording system in an adjacent room. A pulley system was constructed to offset the weight of the cables so the animal could move freely around the maze with minimal drag. Recordings were made using a "Cheetah" parallel recording system (Neuralynx) consisting of eight 8-channel amplifiers with software-configurable high and low-pass filters feeding their output to a custom-made controller and A/D processor, which sampled the signals at 32 kHz per channel. This fed a formatted stream of data to a Sun Ultrasparc 2 workstation running custom-written acquisition and control software. Each time the signal on one of the tetrode channels crossed a specified threshold, a 1ms sample of data was saved from all four channels of the tetrode and written to disk. At the same time, position records containing information about the distribution of light across the video image were acquired at 60 Hz and written to disk.

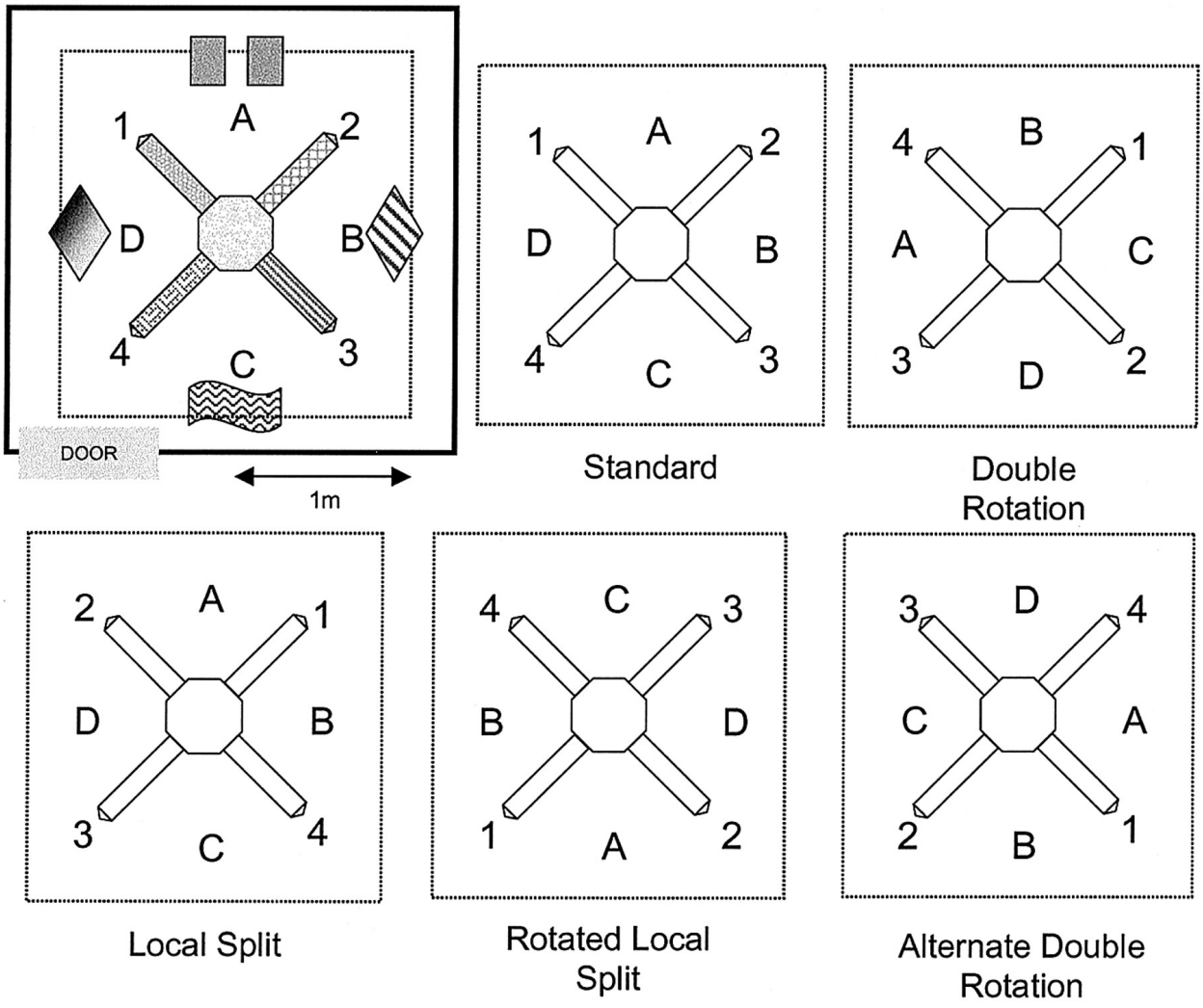
2.2.3. Behavioral task and training

The apparatus used in this experiment was intended to be similar to that used in a set of recent papers, including Shapiro et al. (1997) and Tanila et al. (1997a,b): an elevated, plus-shaped maze with a uniquely textured insert on each arm (Fig. 1, top left). The recording room contained a 2-m square region surrounded by dark blue ceiling-to-floor curtains. The maze was constructed of wood and consisted of an octagonal central platform (30 cm) and four arms (45 _ 10 cm). The ends of each arm tapered to a point and held a small aluminum food cup. The

central platform was covered with a fitted, removable sheet of aluminum, and the entire maze was painted flat gray. The maze arms were also covered by removable aluminum inserts to which texture cues were attached.

The experimental apparatus, and the entire environment visible from it, were designed to be symmetric with respect to rotations by any multiple of 90° with the exception of two sets of specific cues, designated "local" and "distal." The local cues consisted of four uniquely colored and textured inserts placed on the arms of the plus-maze (coarse plastic mesh, metal screen, ridged plastic, sandpaper). No specific olfactory cues were used. The distal cues consisted of four unique objects suspended from the center of each of the curtains surrounding the maze (aluminum foil, striped card, 2 paper bags, white towel). Room illumination was provided by four 25-W lights recessed symmetrically into the ceiling within the curtained enclosure. A video camera used to track the animals' movements was suspended from the ceiling directly above the center of the maze.

Figure 1. Top left: layout of the environment in which training and recording were performed. Rats were trained to run for food reward on a plus-shaped maze with distinctive inserts on each arm, surrounded by curtains with distinctive objects attached on each side (see text for details). Door at lower left leads to adjoining room containing recording equipment. *Remaining plots*: arrangement of local and distal cues in each of the 5 configurations used in the experiment. The standard configuration was used for training and for the 1st and last sessions on each day of recording; the double rotation configuration had the local cues rotated 90° clockwise while the distal cues were rotated 90° counterclockwise; the local split configuration had the distal cues unchanged while 2 of the local cues (1 and 3) were rotated clockwise and the other 2 rotated counterclockwise; the rotated local split was identical to the local split except that all controlled cues (local and distal) were rotated by 180° with respect to the room; and the alternate double rotation (used as a probe for *rat 1*) had the local cues rotated 180° while the distal cues were rotated 90° clockwise.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A
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D
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4
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3
DOOR
1m
Standard
Double Rotation
Local Split
Rotated Local Split
Alternate Double Rotation

Figure 1.

Training began prior to electrode implantation with a gradual adaptation to the recording environment. Initially, each rat was placed on the central platform of the maze once a day and allowed to explore freely for 30 min, after which it was returned to its home cage and fed. Pellets of rat chow (20 mg) were present at the ends of the arms and were replaced once the rat had eaten them and exited that arm. Once the rat was regularly running to the ends of the arms to obtain the food reward (3–4 days), a training paradigm was used to encourage the rat to quickly and completely sample the entire maze. At the start of a training session all four arms were baited with food and the rat was placed on the central platform. For each trial the rat was required to run down to the end of all of the four arms, to run down to the end of eight arms total, or to spend 8 min on the maze. Once any of these had occurred, the rat was confined to the central platform within a small (25 cm) opaque Plexiglas cylinder for a brief time, signaling the end of that trial. The arms were rebaited with food pellets during the confinement. The cylinder was then removed and the rat was allowed to begin a new trial. Initially the confinement period lasted 2 min; it was gradually shortened to about 30 s as the rats became more proficient at the task. Each rat ran several trials a day. Training continued daily until the animal would run for 30 min without entering a previously visited arm more than once per trial. This took between 7 and 10 days per rat. All training was done with the cues in the same "standard configuration."

After the rats recovered from surgery, daily sessions included a search for cells followed by additional training. Over the course of 2–10 days the tetrodes were slowly lowered into the CA1 layer of the hippocampus. As the tetrodes approached the cell layer they were moved less than 100 μm/day. Once all of the tetrodes had been moved for the day, the rats were subjected to additional training without being hooked up to the headstage and cables. In this case, the task was identical to the subsequent recording sessions: the confinement procedure was no longer

used and the arms were kept baited with food pellets at all times. The rat would find a food pellet at the end of an arm, and that arm would be rebaited immediately upon the animal's exit. After 2–3 days of running freely on the maze in this manner, each rat was hooked up to the headstage cables for the rest of the sessions. This was the final stage of training and generally coincided with the tetrodes' arrival at the CA1 layer. Recording sessions began once a rat was willing to run for at least 15 min on the maze and CA1 pyramidal cells had been identified on most of the tetrodes. Daily recording procedures began with a limited search for cells, an opportunity for fine adjustments to be made prior to recording. Tetrode movement prior to recording generally decreased stability during recording and was minimized.

2.2.4. Double rotation task

Each daily recording consisted of three sessions. In the first session, the rat ran on the maze for 5 minutes with the cues in the standard configuration. (All of the configurations used in the experiment are illustrated in Fig. 1.) The rat was then removed from the maze and placed in a high-walled cardboard cylindrical container so that it could not observe cue manipulations. (The rat was not disoriented or disconnected from the recording cable except in a few cases where the cable had become badly twisted during the session.) The local cues were rotated 90° clockwise, and the distal cues were rotated 90° counterclockwise. The rat was then returned to the maze for a second session with the cues arranged in the "double rotation" condition (Tanila et al. 1997a). After 5 min, the rat was removed and placed in the box again. The cues were returned to the standard configuration, and the rat was returned to the maze for a third and final 5-min recording session. The cue arrangements in the first and third sessions were identical. During recording, each arm was rebaited once the rat had exited that arm. Each animal

participated in one recording per day until the number of simultaneously recorded cells fell to less than 10 per ensemble for several consecutive days.

2.2.5. Local split task

A variation on the double rotation task was introduced if the unit responses to the double rotation configuration remained concordant over the first 7–10 days of recording (as happened for 2 of the 4 rats). For the remaining days, instead of the double rotation these rats were exposed to a "local split" cue arrangement in the middle recording session between the two standard sessions. In the local split session, the NW and SE local cues were rotated 90° clockwise, and the NE and SW local cues were rotated 90° counterclockwise. This manipulation was used to examine whether the control exerted by the local cues could be disrupted by perturbing their arrangement with respect to each other. (Note that the local split is formally equivalent to a double rotation of the local cues, leaving the distal cues fixed. The local split arrangement could also be described as a mirror-image flipping of the local cues.)

2.2.6. Probe trials

Two special cue-arrangements were used in small numbers of recording sessions to investigate questions that arose during the course of the study: an alternate double rotation in which the local cues were rotated 180° while the distal cues were rotated 90° clockwise. This was used for several days on one of the rats to test whether a small group of cells that appeared to be following the distal cues would continue to do so for a different cue arrangement. A rotated local split configuration, identical to the local split except that all controlled cues—both the local and distal cues—were rotated by 180° with respect to the room, was used on 1 day for

one of the rats to examine whether cells whose fields stayed fixed in the room/distal-cue frame would be controlled by room cues or by the distal cues if the two were dissociated.

2.2.7. Data analysis

Unit discrimination was performed offline based on the assumption that each recorded neuron would show a different pattern of spike waveform amplitudes across the four channels of a tetrode (McNaughton et al.,1983; O'Keefe and Recce, 1993). Units were discriminated using an interactive program (xclust, M. A. Wilson) that plots each spike as a point in two-dimensional (2D) space, according to its amplitudes on two selected tetrode channels. Spikes from an individual neuron tend to form clusters when plotted this way. The user draws polygons to define the edges of clusters. When the process is complete, a unit is defined by a conjunction of polygons drawn in several 2D projections.

The main data analyses for this study were based on correlation coefficients for spatial firing rate maps. The methods used to construct firing rate maps have been described in detail in earlier papers (Skaggs and McNaughton, 1998; Skaggs et al., 1996). Briefly, the position data were initially binned in a 64 x 64 grid, and the total occupancy time and total number of spikes fired were counted for each pixel in the grid. An "adaptive smoothing" algorithm was then used to calculate an estimated mean firing rate for each pixel by expanding a circle centered on the pixel until the total occupancy within the circle met a specific numerical criterion and then setting the estimated firing rate equal to the number of spikes divided by the occupancy time.

Correlation coefficients between firing rate maps for two sessions were calculated using all pixels having nonzero occupancy for both sessions. Correlations between firing rate maps for the first and last standard sessions on a given day were used as a measure of the quality and stability of spatial firing. For most purposes, a place field with a correlation coefficient greater

than 0.5 was considered to be a sufficiently reliable place field that could be used in the analysis of cue manipulations. Cells showing correlation coefficients less than zero were considered to have remapped. This is a rather conservative criterion but probably does not produce a large number of misclassifications. [See Skaggs and McNaughton (1998) for examples of the statistical distribution of correlations between unrelated place fields.] In any case, counts of remapping cells are only used in a comparative way in the current study and never assigned any absolute significance.

2.2.8. 180° rotation control

The most important question in this experiment was whether there were sessions in which some fields were controlled by the local cues while others were controlled by the distal cues. Place field rotations can be observed as high correlations between firing rate maps from session 1 and appropriately rotated firing rate maps from session 2. Operationally, the question was whether there were, within a single session, some cells showing high correlations for a rotation of 90° while others showed high correlations for -90°.

There is, however, a difficulty with this formulation, arising from the possibility of random remapping. If some fraction of fields remap unpredictably, then some subset of these will, just by chance, remap approximately to locations rotated 90° or -90° from their original locations. The expected incidence of this sort of "coincidental" rotation is not easy to calculate from first principles. There is a simple way to control for the incidence of coincidental rotations based on the structure of the apparatus. There were three sets of cues that a field might in principle be controlled by: the fixed room cues (producing a rotation of 0°); the distal cues (producing a rotation by 90°); and the local cues (producing a rotation by -90°). There were no cues that rotated by 180°. Therefore, if a place field appeared to rotate by 180°, onto the

opposite arm of the apparatus, it can be assumed that this must have been an coincidental rotation. Conversely, the number of fields rotating onto the fourth arm can serve as a control for the expected number of coincidental rotations onto each of the other arms.

Thus the main question in this experiment was approached operationally as follows: define the rotation (0º, 90º, or -90º) leading to the highest mean correlation as the "dominant" rotation for the session. This leaves two "non-dominant" rotations. The question was: did either of the two non-dominant rotations show significantly higher correlations than the 180° rotation? If so, then at least two subsets of cues (the dominant and 1 other) are deemed to have exerted significant control over place fields during the session. Conversely, if the non-dominant rotations did not differ significantly from the 180° rotation, there was considered to be no evidence in the data for anything more than the dominant rotation plus random remapping.

2.3.Results

2.3.1. General observations

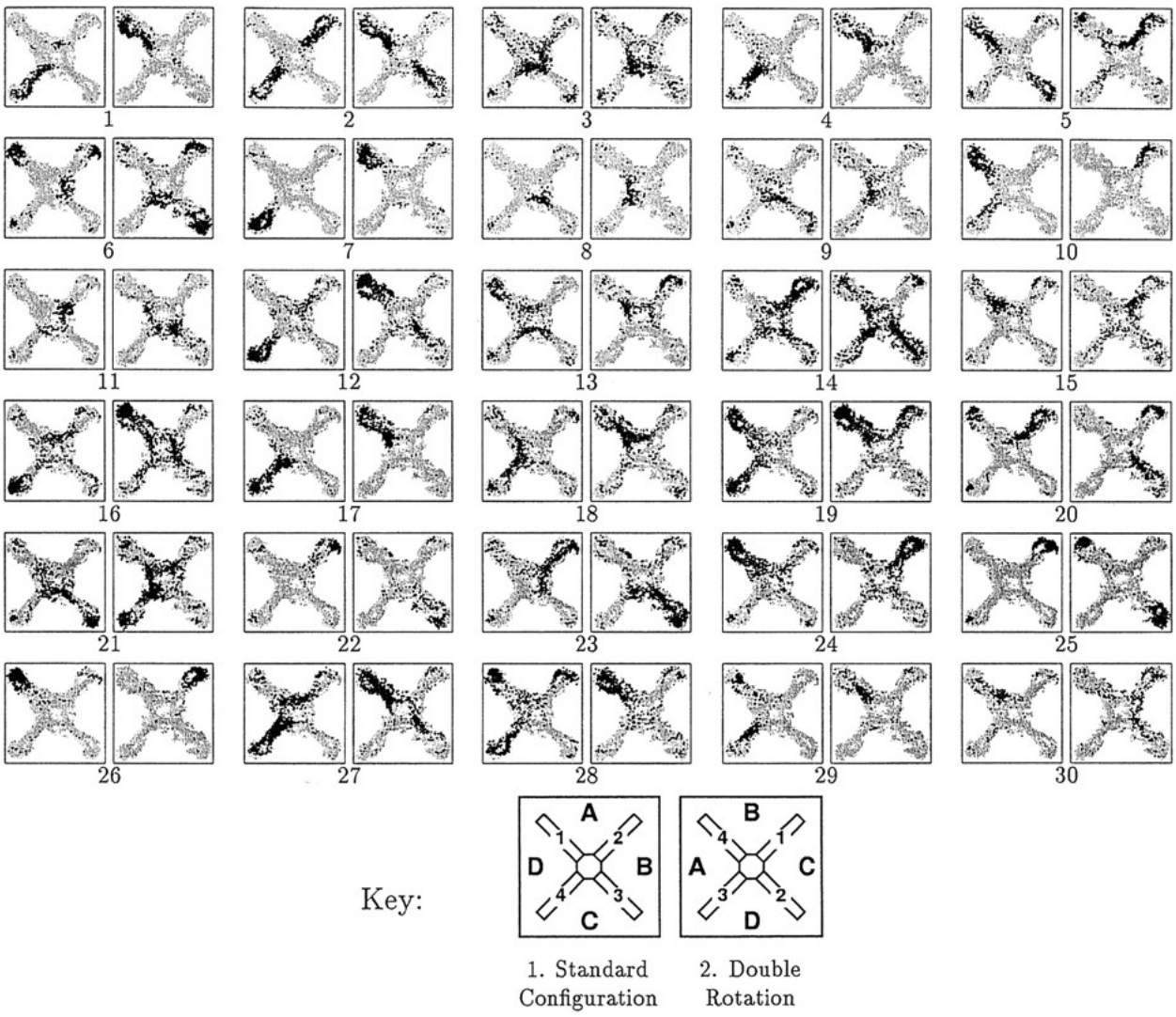
Data were obtained from four rats, which, for purposes of describing individual differences, will be designated rat 1 through rat 4. All rats learned to sample the entire maze equally well during training. Each rat developed a stereotypic pattern of movement on the maze (e.g., always running clockwise or counterclockwise), but there were no other obvious behavioral differences between rats during recordings. The number of recording days per rat ranged from 11 to 25, and the number of well-isolated pyramidal cells recorded ranged from 4 to 70 per ensemble. A typical session lasted 5 min and included 15–20 trials.

The cue manipulation performed for the first days of testing consisted of three 5-min sessions: standard configuration; double rotation configuration; and standard configuration. For all four rats, the result for the first day of testing was that all stable cells (i.e., cells that maintained the same spatial firing pattern in sessions 1 and 3) followed the local cues in the double rotation session (Fig. 2). For two of the rats (2 and 4), all cells continued to follow the local cues as long as the double rotation manipulation was continued (7 and 10 days, respectively). For rat 1, the second through fourth double rotation sessions were unusable due to a bad ground connection and/or electrode instability. From the fifth double rotation session onward, the place fields showed a combination of remapping and control by the distal cues. For rat 3, the first seven sessions showed complete control by the local cues, and all sessions thereafter showed a combination of distal cue control and remapping (Fig. 3).

The conclusion that all fields initially followed the local cues, for all four rats, is drawn on the basis of correlation plots of the sort illustrated in Fig. 4A, in which firing rate maps in the standard configuration are correlated with rotated firing rate maps derived from the double

rotation session. The x axis in these plots shows the correlation between firing rate maps from two standard configuration sessions and therefore represents a measure of the reproducibility of correlations. The y axis shows the correlation between the firing rate map from a standard session and the rate map from the double rotation session, rotated by 0, 90, -90, or 180°. In the illustrated example, all cells with large x coordinates also show large y coordinates for the 90° rotation, which corresponds to the local cues; therefore it is concluded that all reproducible fields rotated concordantly in this data set. Quantitatively, a field was deemed to remap if it produced an x coordinate greater than 0.5 and a y coordinate less than 0 (for the 90° rotation). By this definition, no fields remapped in any of the four rats in the initial sessions, and for rats 2 and 4, no fields ever remapped at all. It cannot be ruled out, however, that the properties of place fields may have changed in a more subtle way in response to cue manipulations.

Figure 2. Spatial firing plots for 30 simultaneously recorded pyramidal cells from *rat 1,* during the 2nd (double rotation) and 3rd (standard configuration) sessions. The 3rd session was used because some cells showed instability during the 1st session. The trajectory of the rat is shown in light gray, and a black dot is placed at the rat's location at the time of each action potential emitted by the cell. The key shows the arrangement of cues for each of the 2 sessions. In this data set, all place fields from well-isolated cells rotated with the local cues. This happened during the 1st day of recording for all 4 rats in the study.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30
Key:
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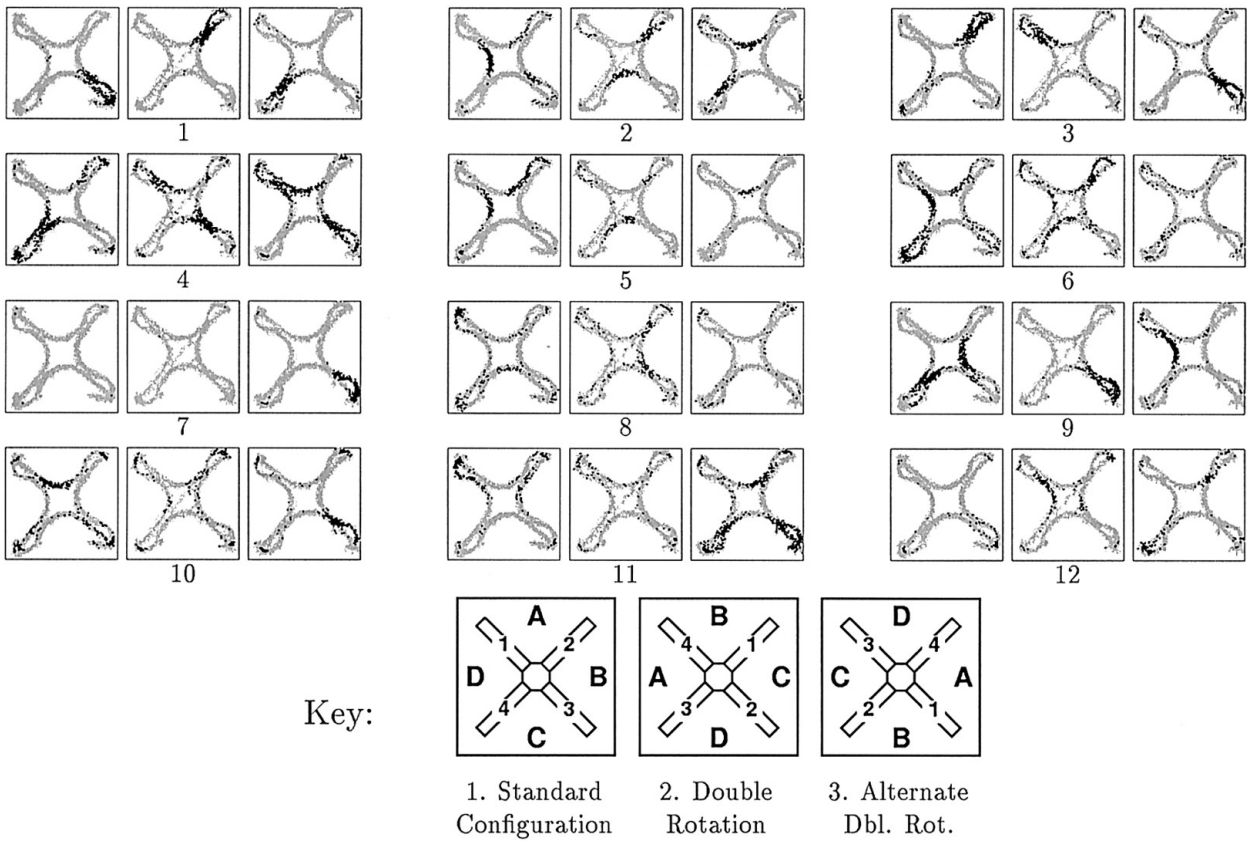
B
C
D
1. Standard Configuration
2. Double Rotation

Figure 2.

Figure 3. Spatial firing plots for 12 simultaneously recorded cells from *rat 1,* from a day on which some fields went with the distal cues while others remapped. Each group of 3 plots shows the spatial firing pattern for an individual cell in the 1st (standard configuration), 2nd (double rotation), and 3rd (alternate double rotation) sessions. *Cells 1-3* followed the distal cues throughout; the other cells remapped in each of the double rotation sessions.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Key:
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1. Standard Configuration
2. Double Rotation
3. Alternate Dbl. Rot.

Figure 3.

Figure 4. Examples of correlation plots for each of the 4 possible rotations, from 2 data sets from *rats 1* and *3.* Each point represents a single cell. The *x* axis, in all plots, shows the correlation of spatial firing patterns from *sessions 1* and *3,* which serves as a measure of the quality/reliability of the spatial firing pattern in the standard configuration. The *y* axis for each plot shows the correlation between the spatial firing pattern from *session 1* and the pattern from *session 2,* rotated to follow the set of cues specified by the label (rotation of 0° for ROOM, 90° for LOCAL, −90° for DISTAL, and 180° for OPPOSITE). *A*: in this data set, taken from the first double rotation session for *rat 1,* all high-quality place fields showed strong correlations for a rotation of 90° matching the local cues. This is taken to indicate that all place fields rotated with the local cues with no discordance. *B*: in this data set, taken from the 9th double rotation session for *rat 3,* the majority of place fields rotated in accordance with the distal cues, but several did not. Note that 3 of the exceptions showed high correlations for a rotation following the local cues. In this data set, there were no cells showing high correlations for a rotation of 180° (onto the opposite arm); but numerous examples of this were seen in other data sets.

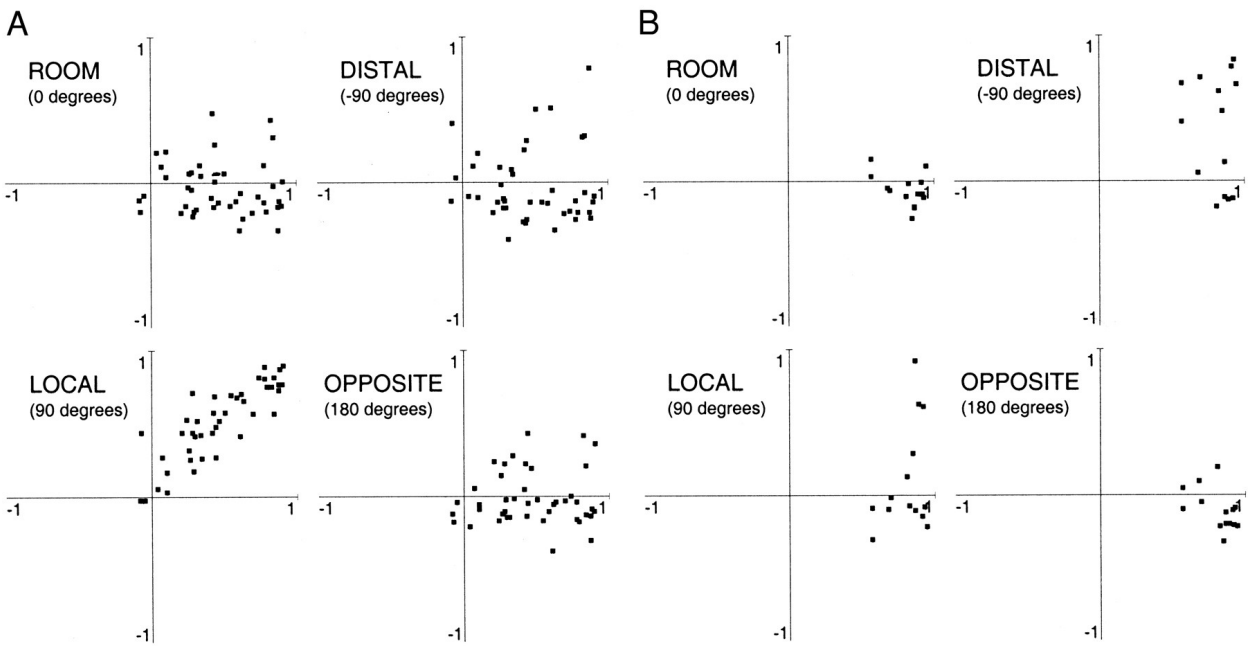

A
ROOM
(0 degrees)
DISTAL
(-90 degrees)
LOCAL
(90 degrees)
OPPOSITE
(180 degrees)
B
ROOM
(0 degrees)
DISTAL
(-90 degrees)
LOCAL
(90 degrees)
OPPOSITE
(180 degrees)

Figure 4.

As stated, the ensembles of two rats, 1 and 3, eventually began to both follow the distal cues and partially remap (Fig. 5). This accords with the observations by Shapiro et al. (1997) and Bostock et al. (1991) that the probability of remapping increases as a function of experience in a novel environment. It is not clear whether the level of remapping continued to increase gradually or switched abruptly at one time and remained constant thereafter. Figure 4B shows a plot of correlations for each of the possible rotations, from rat 1. The majority of cells showed high correlations for a rotation of _90°, corresponding to the distal cues. Several cells, though, did not. Note that three of the cells showed high correlations for a rotation of 90°, corresponding to the local cues. This exemplifies the observation, by Tanila et al. (1997), that a fraction of place fields may rotate in the opposite direction from the majority. In this particular data set, no place fields showed high correlations for a rotation of 180°. Fields that did so were, however, observed in other data sets. A statistical comparison of the numbers of fields rotating in discordance from the majority, versus the number rotating by 180°, is presented in the following text.

Figure 5. Incidence of remapping across days for *rats 1* and *3*. A cell was considered to have remapped if its correlation coefficient for the dominant rotation from the session was less than 0; only cells with correlation coefficients greater than 0.5 for the 1st and 2nd standard sessions were used for this comparison. *Rats 2* and *4* showed no remapping during any day of recording. (The gap in data from *rat 1* was caused by unusable recordings from days 3-6, due to a bad ground connection.) All data in this figure come from comparisons between standard configuration sessions and double rotation sessions.

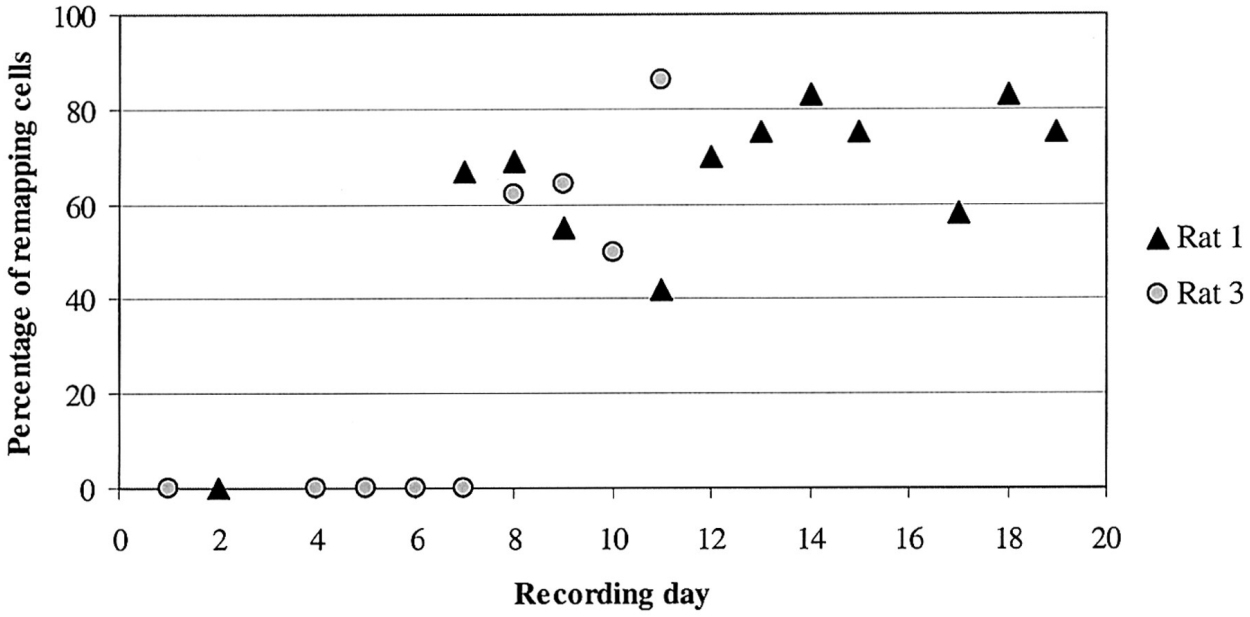

Percentage of remapping cells
100
80
60
40
20
0
0
2
4
6
8
10
12
14
16
18
20
Recording day
Rat 1
Rat 3

Figure 5.

For rat 1, there was a surprising aspect about the distribution of cue control among tetrodes. In this rat, following the switch from local cue control to distal control plus remapping, a number of cells recorded from tetrode 2 had place fields that rotated with the distal cues, while the remaining cells from tetrode 2 remapped unpredictably. None of the cells from the other eight tetrodes, though, showed anything except complete remapping. This discrepancy between tetrodes continued without change from days 7 through 19, which was the final day of recording. The number of usable cells on each tetrode varied considerably over this time. The largest ensemble for tetrode 2 contained seven usable cells of which four had fields that rotated with the distal cues (this is the session shown in Fig. 3). Distal cue control was confirmed by adding an additional 5-min session on days 15 and 17, in which the cues were configured in the alternate double rotation (see METHODS). Across days, there were a minimum of 59 distinct cells recorded from other tetrodes, all of which had fields that remapped. Strictly speaking, it is not legitimate to perform a statistical test on these numbers because of their post hoc nature, but a χ^2 test shows a significant difference with $P < 10\text{-}^{8}$, without any correction for multiple comparisons. For rat 3, there were no significant differences between tetrodes in the level of distal cue control.

The ensembles of the other two rats, 2 and 4, continued to follow the local cues as long as the double rotation was used. Once 2 and 4 had completed 7 and 10 recordings, respectively, it was deemed unproductive to continue this manipulation, and these rats were switched to the local split manipulation. For both of these rats, the local split caused all reliable place fields to stay fixed with respect to the distal/room cues, thus showing no influence of the local cues (which on the previous day had exerted 100% control; Fig. 6). Local split sessions were repeated

for 17 and 5 days (respectively), and throughout this time showed no recognizable remapping whatsoever.

Figure 6. Spatial firing plots for 9 cells from *rat 2* from the last day of double rotation and the immediately following first day of local split. (That is, on day 7 the middle session was a double rotation while on day 8 the middle session was a local split.) These were probably the same 9 cells on both days as judged by similarity of waveforms across the 4-tetrode channels and by similarity of spatial firing patterns in the standard configuration. In the double rotation, all 9 cells had place fields that rotated with the local cues; in the local split, all had place fields that stayed fixed in the room/distal-cue frame. These examples are representative of all other fields recorded on these days, as well.

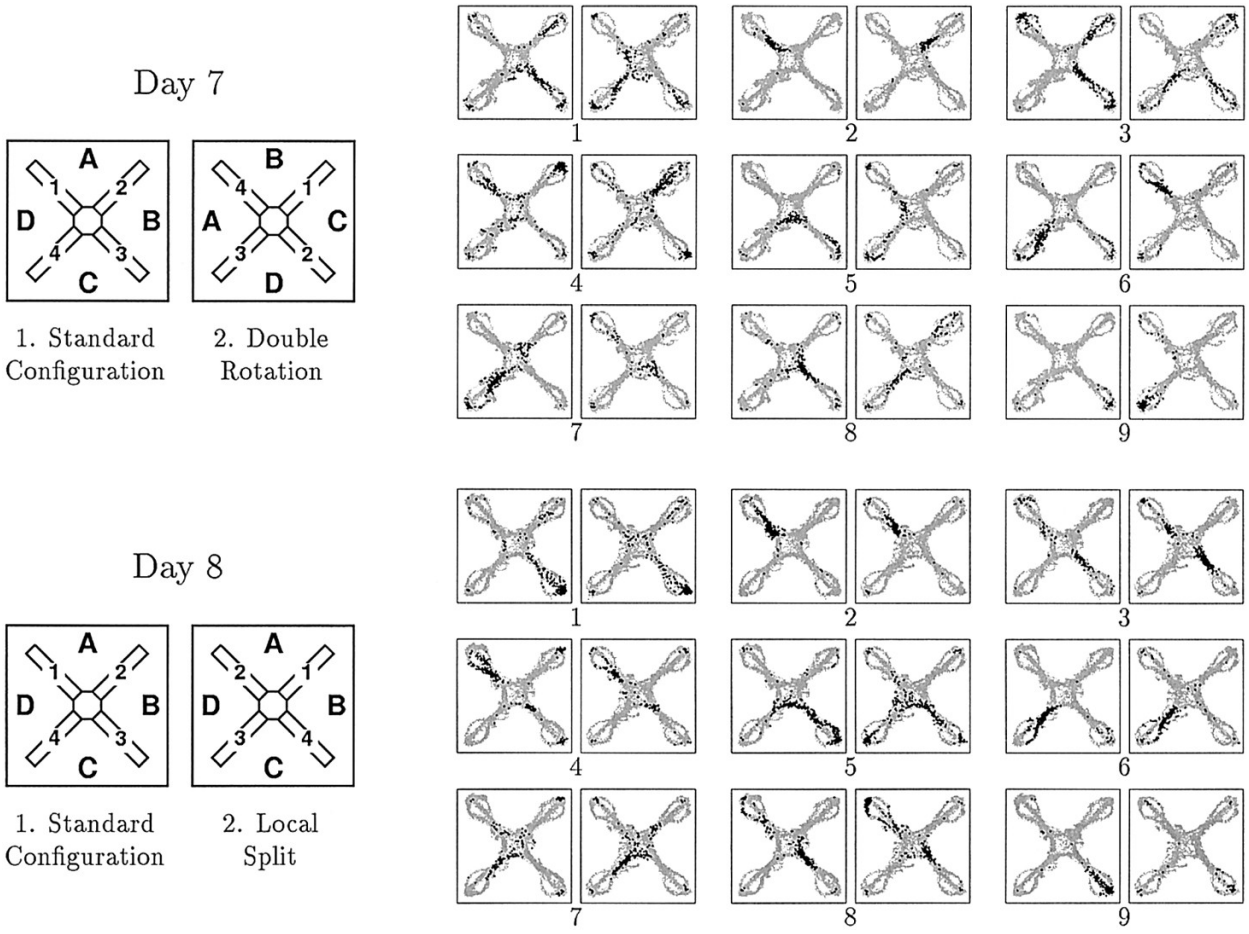

Day 7
A
D
B
C
1
2
3
4
1. Standard Configuration
B
A
C
D
1
2
3
4
2. Double Rotation
1
2
3
4
5
6
7
8
9
Day 8
A
D
B
C
1
2
3
4
1. Standard Configuration
A
D
B
C
2
1
3
4
2. Local Split
1
2
3
4
5
6
7
8
9

Figure 6.

For rat 2, to clarify whether control was being exerted by the distal cues or fixed room cues (whose relative locations did not change in the local split configuration), on the 14th day of recording (7th local split session), a probe was performed in which the controlled cues (local and distal) were arranged in the local split configuration but rotated 180° with respect to the room. In this case, all place fields rotated with the distal cues, none staying fixed in the room frame. This suggests that in the local split sessions, it was the distal cues, rather than the room cues, that exerted control over the place fields. For the same rat, after 14 local split sessions, the rat was subjected to another double rotation session to test whether the local cues had lost their influence in the interim. In this session, as in the earlier double rotation sessions, all reliable place fields rotated with the local cues.

2.3.2. Discordance analysis

For the sessions in which there was less than complete concordance (11 data sets from rat 1 and 4 data sets from rat 3, together constituting all of the data from these rats after they had switched from fully concordant to partially discordant responses), the data were examined to see whether there was any significant evidence of fields rotating discordantly from the dominant rotation for the session. (In all of these cases, the dominant rotation was -90°, following the distal cues.) This was done by correlating spatial firing rate maps for the standard configuration with rotated firing rate maps for the double rotation configuration. As a control, the distribution of correlations for the non-dominant rotation (90°) was compared with the distribution of correlations for a rotation of 180° (the rationale for this is explained in Data analysis). Figure 7 shows the correlations for the pooled data. There was no significant difference between the distributions; the mean correlation for the 180° rotation was actually larger than for the non-

dominant rotation (0.032 vs. _0.007), though neither was significantly different from zero. Comparing the shapes of the distributions using a Kolmogorov-Smirnov test yields a difference measure of $D = 0.142$, which is not significant though nearly so ($P = 0.067$). Thus, there is no real indication of above-chance numbers of fields following the non-dominant rotation in this experiment.

Figure 7. Statistical comparison of correlations for non-dominant rotations vs. 180° (control) rotations. *A*: each point represents a single cell on 1 day of recording—the data are pooled from 11 data sets. The vertical coordinate represents the correlation between the firing rate map for the standard configuration, and the map for the double rotation, rotated by 180°. (A high correlation indicates that the place field of the cell rotated by 180°.) The horizontal coordinate is the same thing except using a rotation of 90°, which corresponds to the local cues. Any tendency of cells to be controlled by the local cues would be reflected by an excess of cells with large *y* coordinates, compared to the number with large *x* coordinates. *B*: distribution of correlations, with firing rate maps from the double rotation session rotated by 90° (*top*) and 180° (*bottom*). The distributions were statistically indistinguishable, indicating that there was little evidence for any control over place field rotations by the non-dominant cue set.

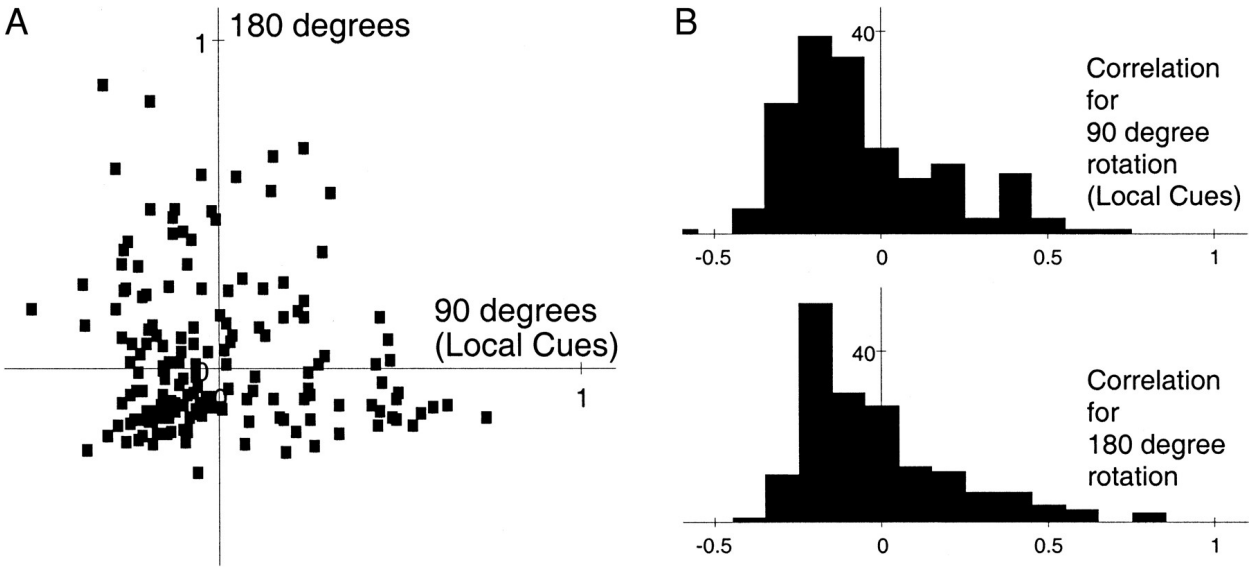

A
180 degrees
1
90 degrees
(Local Cues)
0
1
B
40
Correlation
for
90 degree
rotation
(Local Cues)
-0.5
0
0.5
1
40
Correlation
for
180 degree
rotation
-0.5
0
0.5
1

Figure 7.

2.4.Discussion

The present experiment was designed to replicate what were considered the essential features of the experiment of Tanila et al. (1997a) with two important additions: first, a control was added for the possibility that random remapping could give rise to the "coincidental" appearance of place fields rotating in synchrony with a set of controlled cues; second, larger populations of neurons were recorded from so that more quantitative information on the level of discordance could be obtained.

2.4.1. Concordant ensembles

One of the most striking results of the experiment was the very high level of concordance observed in the majority of data sets. All rats showed what appeared to be complete concordance in the initial double rotation sessions, with all high quality fields following the local cues; and two of the four rats showed complete concordance throughout the entire experiment even after the local split manipulation was introduced. This high level of concordance was clearly not a consequence of the rats "ignoring" the distal cues: two of the rats spontaneously switched to distal cue control (plus remapping) after several sessions, and the other two switched to distal cue control when placed in the local split configuration. Thus all four of the rats demonstrated some degree of distal cue control.

2.4.2. Lack of discordant ensembles

The second striking result is that no statistically significant evidence of discordant control by the local and distal cues could be shown. The only discordant responses were combinations of distal cue control and random remapping. This is in contrast to the conclusion reached in

Tanila et al. (1997a). It is important to emphasize, however, that the data from the current experiment were not in any important way inconsistent with the data reported by Tanila et al. Both studies observed simultaneously recorded cells responding in discordant ways. Both observed partial random remapping, in which some place fields followed one set of cues while other fields disappeared or shifted unpredictably; moreover, both found that the level of remapping tended to increase over time. Both observed instances in which some fields appeared to rotate with the distal cues while other fields appeared to rotate with the local cues. The essential difference is that the current experiment controlled for the possibility of "coincidental" rotations, whereas Tanila et al. considered all rotations to be meaningful.

2.4.3. Reconciling Tanila et al. (1997) with the present study

Certainly there were significant methodological differences between the current study and the experiment of Tanila et al.: one used brain-stimulation reward and the other used food reward; one used odor cues and the other did not; the relative sizes of the local and distal landmarks may have differed; training procedures may have differed; etc. There is no way to ascertain that these differences could not cause differences in the amount of discordance in the hippocampal representation. It is not, however, at this point necessary to postulate an explanation of this sort, because the data from the two experiments were generally consistent. Nevertheless, it must be clear that the inability to detect significant local-distal discordance in the current study does not imply that discordance could not be observed in other situations, or even in a replication of the same situation. A negative conclusion can only be established by a large body of evidence collected in a wide variety of different situations.

Even so, the data from this experiment are in several ways difficult to reconcile with the idea that individual hippocampal cells convey specific information about different subsets of

cues in an environment. In particular, for the two rats subjected to the local split manipulation, both showed the same pattern of results: while the double rotation was used, all place fields rotated with the local cues; the first day the local split was introduced, all fields stayed with the distal/room cues and seemed to completely ignore the local cues. It seems unlikely that all of the cells were "local cue cells" on 1 day and "distal cue cells" on the following day; rather, it seems more probable that the population as a whole was controlled by the global configuration of all of the landmarks in the environment. Indeed, one scenario that might account for the data would be to conjecture that the influence of the landmarks was mediated largely by the head-direction system, which is thought to respond to landmark information in a unified, "winner-takeall" manner (Knierim et al., 1995; Taube et al., 1990).

It is remarkable that two of the rats switched from local cue control to distal cue control after several days of experience, even though there were no apparent changes in behavior or in the contingencies of the task. A number of previous studies have found that in many situations distal cues exert a stronger influence than local cues over place fields and/or spatial navigation behavior (e.g., Cressant et al., 1997; Olton and Samuelson, 1976), but to our knowledge no such delayed shift in control has previously been reported, and the cause of it is unclear.

2.4.4. Theoretical significance of these results

What is the theoretical significance of these results? In light of the manner in which the issue was framed in the introduction to this chapter, they might be taken to support the cognitive map hypothesis over the relational declarative memory hypothesis. There is, however, a different way of looking at the matter. There are reasons for expecting unified, non-factorable representations in a cognitive mapping system, but there are reasons for expecting such representations in a memory system as well. In most theoretical models of one-trial memory

systems, such as the Hopfield network or various modifications of it (Amit 1989; Hertz et al. 1991), performance depends strongly on orthogonality between the patterns that are to be memorized. It is difficult to have orthogonality in a system where each unit's activity depends on a limited subset of features of the environment, because an environment containing a mixture of features from two other environments will then be represented by a hippocampal activity pattern partially overlapping with the representations of the other environments. In the most thoroughly studied models, this will cause interference between memorized patterns, interference that is undesirable. Thus it is not necessarily the case that the results of the current experiment go against either the memory theory or the spatial theory, but rather, that the opposite outcome would have presented difficulties for both theories.

It might be objected that partial remapping, which has clearly been demonstrated in several studies [e.g., Skaggs and McNaughton (1998)] as well as the current experiment and the Tanila et al. experiment that motivated it], presents the same difficulties. One possible response might be that partial remapping has thus far only been demonstrated in environments that have minimal differences from each other. It could, therefore, be a pathological behavior of the system, rarely seen in the ordinary life of an animal. This hypothesis suggests the prediction that, in a properly designed experiment, the incidence of partial remapping would be correlated with the level of interference between memories formed in two different, but similarly structured, environments.

3. EXPERIMENT 2: POLYSYNAPTIC PATHWAYS FROM THE VESTIBULAR NUCLEI TO THE LATERAL MAMMILLARY NUCLEUS OF THE RAT: SUBSTRATES FOR VESTIBULAR INPUT TO HEAD DIRECTION CELLS

3.1. Introduction

Neural activity in several areas of the rat brain corresponds with the animal's current head direction. "Head direction (HD) cells" have been studied primarily in the postsubiculum (PoS) (Taube et al. 1990), anterior dorsal thalamus (ADN) (Taube, 1995) and lateral mammillary nucleus (LMN) (Blair et al. 1998; Stackman and Taube, 1998). Neurons exhibiting direction-related activity have also been observed in the lateral dorsal thalamic nucleus (Mizumori and Williams, 1993), dorsal striatum (Weiner, 1993) and retrosplenial cortex (Chen et al. 1994). The activity of rat HD cells varies as a function of the animal's head direction in the horizontal plane, and is independent of the rat's location in the environment as well as ongoing behavior.

Extensive characterization of head direction cell activity has been performed (see Taube, 1998, and Sharp et al. 2001, for review) and evidence suggests that that these cells participate in navigation and spatial cognition (Muller et al. 1996). The circuitry responsible for the overall generation and maintenance of the HD signal has yet to be determined, although some relationships between areas containing HD cells have been characterized (see Taube, 1998, for review). For example, the HD signal in the LMN is thought to play a role in generating HD cell activity in the ADN. Blair and colleagues (1999) found that bilateral lesions of the LMN resulted in the loss of directional specificity of ADN HD cells. This result indicated that the LMN HD signal is necessary for ADN HD activity, and that LMN HD activity is a necessary precursor to ADN HD activity. Additionally, cells corresponding to the animal's angular head

velocity (AHV) have been found in the LMN (Blair et al., 1998; Stackman and Taube, 1998), and are likely to be involved in pathways supporting an internal representation of head direction.

Several sensory signals are thought to participate in shaping the firing patterns of HD cells, including vestibular, visual and proprioceptive information (Brown et al. 2002). A lesion study by Stackman and Taube (1997) demonstrated that elimination of labyrinthine inputs abolishes the directional sensitivity of HD cells in the ADN. Given that the vestibular complex is a first-order obligate synapse for labyrinthine input, these results suggest that the vestibular system plays an essential role in generating HD cell activity. Furthermore, a relatively direct polysynaptic pathway via the dorsal tegmental nucleus of Gudden (DTN) has been proposed that could relay vestibular signals to the LMN (Bassett and Taube, 2001; Sharp et al. 2001; Brown et al. 2002). This proposed pathway was developed from anatomical work using monosynaptic tracers showing that regions of the vestibular nuclei project to DTN, and that some DTN neurons project to LMN (Liu et al. 1984; Shibata 1987; Hayakawa and Zyo, 1985; Allen and Hopkins 1989). However, there currently is no direct evidence to show that vestibular nucleus neurons project to tegmental neurons that in turn project to areas containing a large number of HDC. Such evidence can only be gathered with the use of a transneuronal tracer that is transmitted sequentially across synaptically-linked cells that comprise a neural circuit.

The present study tested the hypothesis that a polysynaptic pathway connects the vestibular nuclei with the LMN via one or more relay nuclei. This hypothesis was tested using retrograde transneuronal viral replication and transport to define the synaptology of neural circuits impinging upon the LMN.

3.2.Methods and Materials

All procedures in this study conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Fifty-two adult male rats were used in these experiments. They were given food and water *ad libitum* and were kept in paired housing on a 12 hr/12 hr light/dark cycle. The characteristics of the two recombinants of the Bartha strain of pseudorabies virus (PRV) employed in this study, PRV-BaBlu and PRV-152, have been published elsewhere (Billig et al. 2000). Both viruses were the generous gift of Dr. Lynn Enquist (Princeton University, NJ). PRV-BaBlu expresses ß-galactosidase (ß-gal), and PRV-152 expresses enhanced green fluorescent protein (EGFP), under the gG and cytomegalovirus immediate early gene promoters, respectively. Both recombinants were grown in pig kidney (PK15) cells and were adjusted to a final concentration of 1 x 10^8 plaque-forming units/ml.

Two sets of experiments were performed. Initially, PRV-BaBlu was injected into the LMN of 12 rats in order to optimize the volume and placement of injections, and to establish the time course of the progression of infection produced by each recombinant. Post-injection survival times of 50, 60 and 72 hrs were found to result in the infection of first-order, and putative second- and third-order neurons, respectively. Once these data were gathered, the synaptic organization of circuits projecting to the LMN was characterized in 40 animals by injecting PRV-BaBlu into the left LMN, and PRV-152 into the right LMN, of each animal. Because PRV-BaBlu and PRV-152 express unique reporters, this paradigm allowed us to obtain two data sets from each case. This was possible because of the unique reporters that could be localized immunocytochemically in separate sets of sections (see below for more detail).

3.2.1. Surgical procedures

Animals were housed, injected with PRV, and euthanized within a biosafety level 2 (BSL-2) facility. Animals were acclimated to the facility for at least one week prior to surgery, which was performed using aseptic techniques. Rats were deeply anesthetized with 1-2% isoflurane, and fixed in a stereotaxic frame. The scalp was incised and retracted, and small craniotomies were made over the left and right LMN using a Dremel drill. An injection of PRV with a volume of 50 – 100 nl was made into the left and right LMN using a 1 µl Hamilton syringe equipped with a 32 gauge beveled-tip needle. Injections were placed at the following stereotaxic coordinates: AP – 4.65, ML +/- 1.10, DV – 9.45 (Paxinos and Watson, 1998), with the opening of the beveled needle oriented toward the LMN. The injections were made at the rate of 10 nl/min, and the syringe was left in place for 10 minutes following injection to ensure that virus would be less likely to move up the injection tract following removal of the needle. Following the removal of the needle, each craniotomy was plugged with bone wax, the scalp was sutured, and rats were returned to their home cages to recover from anesthesia. Analgesia was provided by 3 mg/kg intramuscular injections of Ketoprofen at 12-hour intervals after surgery. Following the designated survival times, rats were deeply anesthetized with 50 mg/kg intraperitoneal injections of sodium pentobarbital and perfused transcardially with 0.5 L of 9% saline followed by 1 L of 4% paraformaldehyde-lysine-periodate (PLP) fixative (McLean and Nakane, 1974). The brains were removed, postfixed for 2-4 hours in PLP, and cryoprotected for 2 days in 20% phosphate buffered sucrose. Postfixation and cryoprotection were done at 4°C.

3.2.2. Tissue processing and immunohistochemical procedures

Brains were sectioned at 35 µm in the coronal plane using a freezing microtome, and sections were collected sequentially in 6 wells of cryopreservant (Watson et al. 1986).

Cryoprotected sections were stored at –20°C until they were processed for immunohistochemical localization of infected neurons. Infected neurons were identified using a polyclonal antisera generated in rabbit against acetone-inactivated PRV (Card et al. 1990) that identifies all recombinants, or with antibodies specific for the unique protein reporters of the two recombinants. These included a mouse monoclonal antibody that recognized □-gal (1:1500; Sigma Chemical, St. Louis, MO) expressed by PRV-BaBlu and a rabbit polyclonal antibody that recognized EGFP (1:1,000; Molecular Probes, Eugene, OR) expressed by PRV-152. These antigens were localized in alternate sections using the avidin-biotin modification of the peroxidase-antiperoxidase procedure (Hsu et al. 1981), affinity purified secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA), and Vectastain reagents (Vector Laboratories, Burlingame, CA). Following immunohistochemical processing, sections were mounted on gelatin-coated slides, dehydrated, cleared and coverslipped using Cytoseal 60 (VWR Scientific, West Chester, PA).

3.2.3. Tissue analysis

Processed tissue sections were examined and photographed with a Zeiss Axioplan photomicroscope. Images were digitized using a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and a Simple-32 PCI image analysis system (Compix, Lake Oswego, OR). Mapping of the distribution of infected neurons in sections at a frequency of 210 μm through the rostrocaudal extent of the forebrain and brainstem was accomplished using Stereo Investigator software (MicroBrightField, Williston, VT). In order to test our hypothesis, this study focused primarily on brainstem projections to the mammillary complex.

There are no documented direct projections from the vestibular nuclei to LMN (Shibata, 1987; Matesz et al. 2002). We analyzed the patterns of infection throughout the entire brainstem,

but focused upon areas that receive vestibular input and have previously been shown to provide projections to the mammillary complex. We chose to focus on labeling in DTN, laterodorsal tegmental nucleus (LDTN) and ventral tegmental nucleus (VTN) because of their documented topographically organized projections to LMN, SUM and MMN, respectively (Shibata, 1987). DTN was considered as a potential relay nucleus for conveying vestibular information to LMN because it receives input from the vestibular nuclei (Liu et al. 1984). Additionally, two studies have demonstrated that some DTN neurons code for angular head velocity (Bassett and Taube, 2001; Sharp et al. 2001), raising the possibility that these neurons participate in the transformation of signals reflecting angular acceleration of the head to those reflecting head direction. These studies also found that that a small percentage of cells in the DTN were "classic" HD cells. Along with the anatomical connectivity of DTN and LMN, these findings imply a functional connection between the two areas.

The supragenual nucleus (SUG), nucleus prepositus hypoglossi (PH), and areas in the medullary reticular formation that receive vestibular input were also subjects of our attention. The SUG and PH project to DTN (Liu et al. 1984; Hayakawa and Zyo, 1985) and are thought to be a component of oculomotor pathways (Korp et al. 1989). Some areas of the medullary reticular formation receive vestibular input (Belknap and McCrea, 1988; Iwasaki et al. 1999) and project to DTN (Liu et al. 1984; Hayakawa and Zyo, 1985). Thus, they also represent potential relays for processed vestibular sensory information to contribute to HD cell activity. Finally, the vestibular nuclei were specifically analyzed because the goal of this study was to determine whether inputs from the vestibular nuclei are conveyed polysynaptically to LMN.

3.3.Results

This experiment was designed to test the hypothesis that a polysynaptic pathway connects the vestibular nuclei with the LMN via one or more relay nuclei. The results from 27 injections are presented in this analysis. Of the 11 injections that included the LMN, six were from the 60 hr survival time and five were from the 72 hr survival time. Sixteen cases served as controls. In these cases, the injection was principally in an area adjacent to LMN. Ten control cases were from the 60 hr survival time and six were from the 72 hr survival time. The injections not described here were unusable in the present analysis because they were located outside of the target areas or they failed to produce a productive infection. For example, any injection where the needle broke through the bottom of the brain was not used because virus spread into the subarachnoid space.

Several criteria were used to identify the zone of viral uptake that led to productive infection of neurons. Injection sites were first identified by locating the end of the cannula tract in the tissue. In all cases, at least two bins of tissue were processed to obtain an accurate localization of the cannula. Axon terminals have the highest affinity for alpha herpesviruses (Vahlne et al. 1980; Marchand and Schwab, 1986). Thus, virus is often taken up by terminals and transported retrogradely from the injection site. Because of this, the extent of infection around the cannula tip is not a reliable determinant of viral diffusion and uptake. Previous work has determined that the zone of virus uptake that leads to viral replication after an injection of 100 nl (1 x 10^8 pfu/ml) of PRV delivered at 20 nl/min is within an approximate 500 μm radius of the cannula tip (Jasmin et al. 1997; Card et al. 1999). Therefore, injection sites in this study were

defined by the end of the cannula tract plus a 500 μm radius surrounding that point. The zone of uptake was biased toward the LMN through the use and positioning of a needle with a beveled tip, so this is a conservative estimate of viral spread. Due to the small size and depth of the injection targets, most injections were not isolated to one nucleus (such as LMN), but usually extended to two or more (such as LMN plus SUM).

3.3.1. PRV transport following injection into LMN

Data were analyzed from 11 cases in which PRV was injected into the LMN. These animals were killed either 60 hours (n= 6) or 72 hours (n=5) post injection. The location and magnitude of infection in regions caudal to the injection sites in these cases are presented in Tables 1 and 2; identification of infected nuclei was based on standard atlases of the rat brain (Paxinos and Watson, 1998; Swanson, 1998). The estimated zone of PRV uptake in all of the cases was focused upon the LMN, but included adjacent nuclei to varying degrees. For example, the injection site in case 3 was centered in SUM but included LMN, medial mammillary nucleus (MMN) and ventral tuberomammillary nucleus (TMv). Conversely, the injection site in case 4 was centered in LMN but included TMv, and lateral MMN. In all cases, extensive retrograde infection of neurons that projected to the injection site was observed. The extent of infection at each injection site varied according to the nuclei involved, with the most extensive infection observed when the injection involved nuclei with local circuit connections.

3.3.1.1. 60 hr survival time.

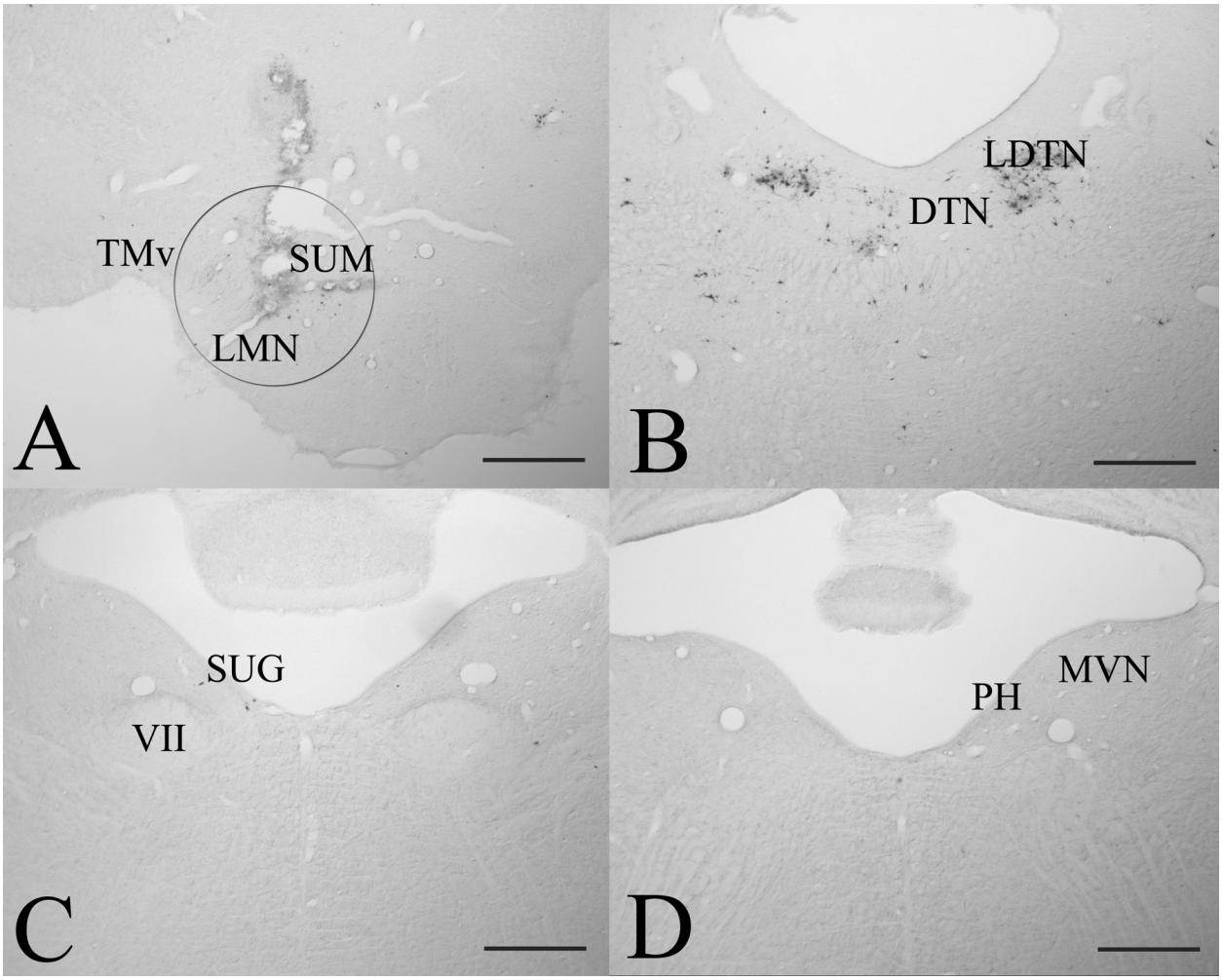
All six cases in the 60 hr survival time group exhibited infected cells in the DTN and laterodorsal tegmental nucleus (LDTN), which are known to have monosynaptic projections to LMN and SUM, respectively (Shibata, 1987; Hayakawa et al. 1993). DTN labeling was always more prominent ipsilateral to the injection site, while less extensive DTN labeling was observed contralaterally. Labeling in LDTN followed the same trend, except in cases 4 and 6 where the infected cells were concentrated on the contralateral side. Figure 1 illustrates the labeling patterns in an animal from the 60 hr survival time group (case 5). This injection site was centered in the LMN (Fig 1A) and light labeling was observed in LMN, SUM and TMv. The resultant infection in DTN (Fig 1B) was restricted to the ipsilateral side, whereas the infected cells in LDTN were observed bilaterally with a higher concentration on the ipsilateral side. Injection sites in cases 2, 3, and 4 included the MMN, and in all three cases labeled cells were observed in the ventral tegmental nucleus (VTN). The VTN projects monosynaptically to the MMN (Allen and Hopkins, 1989) and is one of the topographically organized projections from tegmentum to the mammillary complex.

Infected cells were observed in the SUG in four of the six cases in this group. These neurons were usually located bilaterally with a concentration on the ipsilateral side. Figure 1C illustrates light SUG labeling in case 5. The SUG is adjacent and directly anterior to the rostral extent of PrH. In cases where both nuclei were labeled, it was noted that there was a distinct space lacking infected cells between the caudal SUG and rostral PH. PH neurons confined to the rostral half of the nucleus were labeled in cases 2 and 3 only. No infected cells were found in the vestibular nuclei 60 hours subsequent to LMN injections. Figure 1D shows that PH and MVN in case 5 were devoid of infection.

Figure 1.

Photomicrographs of an injection site (A) and retrogradely infected brainstem nuclei (B-D) in a 60 hr survival time animal (case 5). This injection site included the lateral mammillary nucleus (LMN), supramammillary nucleus (SUM) and tuberomammillary nucleus pars ventralis (TMv). **A).** Light labeling in the left LMN and TMv; heavier labeling in SUM. **B).** Laterodorsal tegmental nucleus (LDTN) was heavily labeled bilaterally along with lighter labeling of the dorsal tegmental nucleus (DTN) ipsilateral to the injection site. **C.)** Light bilateral labeling in the supragenual nucleus (SUG) dorsal to the genu of the facial nerve (VII). **D.)** No labeling was observed in the prepositus hypoglossal nuclei (PH) or medial vestibular nuclei (MVN). Marker bars = 500 μm.

Figure 1.



In some animals, infected neurons were also located in the periaqueductal grey, interpeduncular nucleus, parabrachial nucleus, locus coeruleus and reticular formation. Regions of the reticular formation, such as the mesencephalic reticular nucleus, paragigantocellular reticular nucleus, gigantocellular reticular nucleus and pontine reticular nucleus, exhibited more prominent labeling when heavier infections were observed near the injection sites. For example, case 3 exhibited the most infected cells around the injection site, as well as the largest number of infected cells in other regions of the brain. Conversely, the lowest number of infected cells was observed near the injection site and elsewhere in the brain in cases 4 and 6.

3.3.1.2. 72 hr survival time.

The locations of infected neurons observed in the five 72 hr cases (Table 2) were similar to those found in the 60 hr survival time group, although labeling extended into more caudal nuclei in the longer survival time group. All five of these cases exhibited some labeling in the LMN as well as one or more surrounding nuclei. Figure 2 illustrates labeling in case 9, where an injection was placed deep in the mammillary complex between the LMN and the MMN pars lateralis (Fig 2A). Additional labeling near the injection site was located in TMv.

In all cases, the DTN and LDTN were found to contain infected cells bilaterally, with a concentration on the side ipsilateral to the injection site. Similar to the 60 hr group, the LDTN labeling was generally heavier than the DTN labeling, corresponding to the extent of labeling found in the SUM and LMN, respectively. Figure 2B provides an example of bilateral labeling in both the DTN and LDTN from case 9, as well as labeling in VTN that is notably heavier on the ipsilateral side. In this animal, infected cells in the MMN were confined to the side of the injection.

Labeling in the SUG was observed in four of the five cases. This labeling was always bilateral, although the relative number of infected SUG neurons ipsilateral and contralateral to the injection site varied between animals. Figure 2C shows SUG labeling that was much heavier on the ipsilateral side. Infected cells were found in the PH of all five cases, and an example of heavy PH labeling concentrated on the ipsilateral side is illustrated in Figure 2D. A higher magnification photomicrograph (Fig 2E) demonstrates the density of PH infection in this case. The PH infections in all five cases were limited to the rostral half of the nucleus. This pattern of labeling corresponds to the PH infections observed in the 60 hr survival time group, although the density of labeling was higher in the 72 hr survival time cases.

The MVN was found to contain cells that were infected by retrograde transneuronal transport of PRV in four of the five 72 hr cases where the injection site was localized largely to the LMN. This labeling was found to be bilateral in three of the four cases, with a slightly higher number of infected cells on the ipsilateral side. Figure 2D shows the MVN labeling found in case 9, which is shown at higher magnification in Figure 2F. The four cases containing infected MVN cells exhibited labeling throughout the rostrocaudal extent of MVN, although there was a concentration of infected cells in the rostral half of the nucleus. Labeled cells were not observed in the other vestibular nuclei in any of the cases. Case 11 did not exhibit any infected cells in the MVN, although the pattern of infection throughout the rest of the brain was consistent with the other 72 hr survival time cases with injections that included LMN. The number of infected cells in the MVN was higher in those cases where the LMN and DTN infections were most extensive (cases 7, 8, 9). Figure 3 illustrates the locations of infected cells at several levels of the brain from case 9, starting at the level of the injection site and continuing caudal to the MVN. Both

Figure 2.

Photomicrographs of an injection site (A) and retrogradely infected brainstem nuclei (B-F) in a 72 hr survival time animal (case 9). **A.)** Injection site included lateral mammillary nucleus (LMN), medial mammillary nucleus pars lateralis (MMNpl), supramammillary nucleus (SUM) and tuberomammillary nucleus pars ventralis (TMv). The cannula tract can be seen descending into the mammillary complex, and the tissue was cracked during processing. **B.)** Tegmental level labeling located bilaterally in both the DTN and LDTN, with primarily ipsilateral labeling in the VTN. **C.)** Heavy labeling of the contralateral SUG. **D.)** Extensive labeling of ipsilateral PH, light labeling of contralateral PH, and equivalent bilateral labeling of MVN. Higher power photomicrographs from panel D of the PH (E) and MVN **(F)** are illustrated. Marker bars in A-D = 500 μm, E = 125 μm, F = 62.5 μm .

Figure 2.

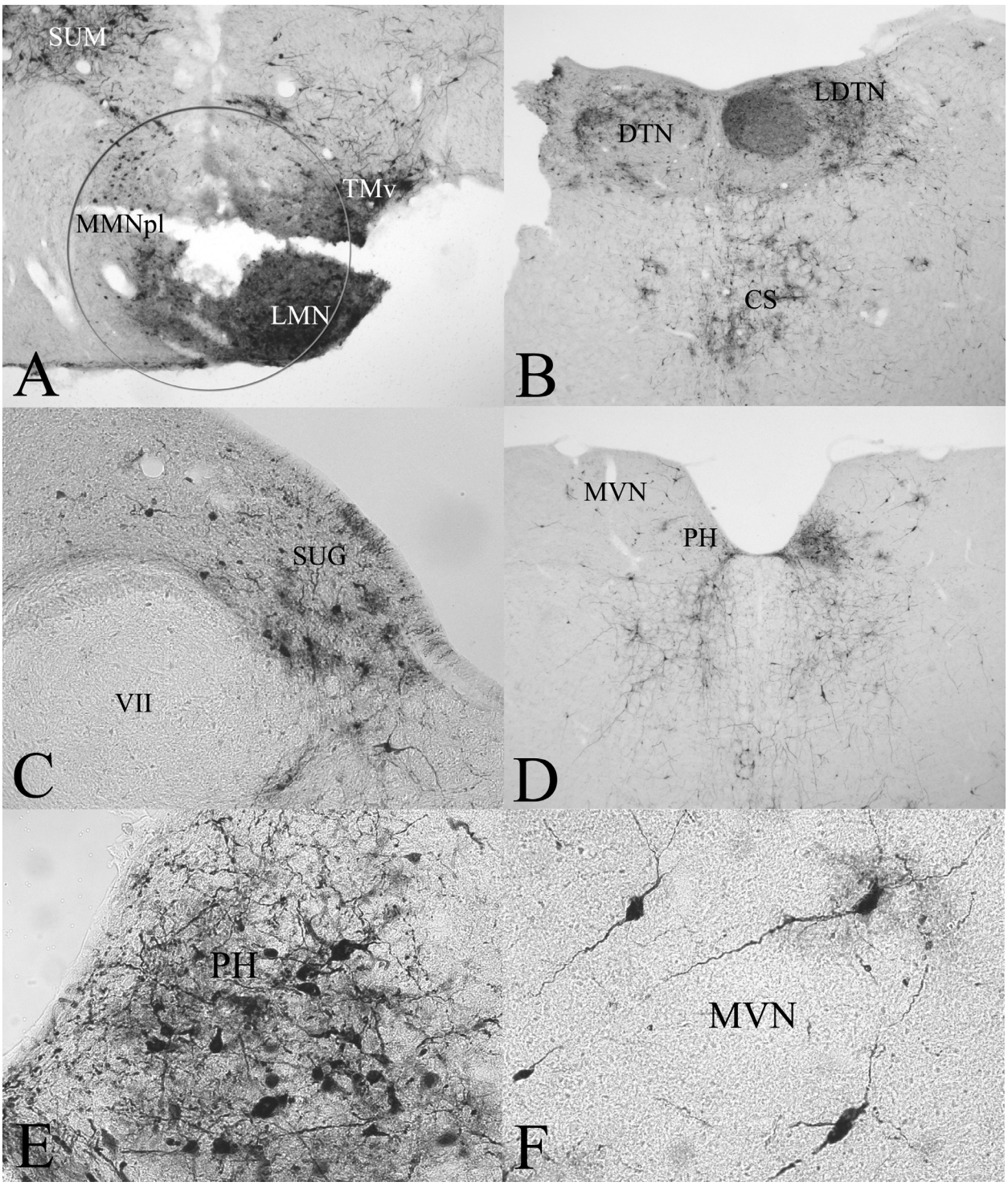


Figure 3.

The distribution of infected neurons from the case shown in Fig 2 (case 9) are illustrated. Each dot represents an individual neuron. Sections are arranged from rostral (A) to caudal (H), with the coronal planes relative to Bregma noted at the lower right of each section. The boxed areas in figures A, E, G, and H define regions illustrated in figures 2A, 2B, 2C and 2D, respectfully.

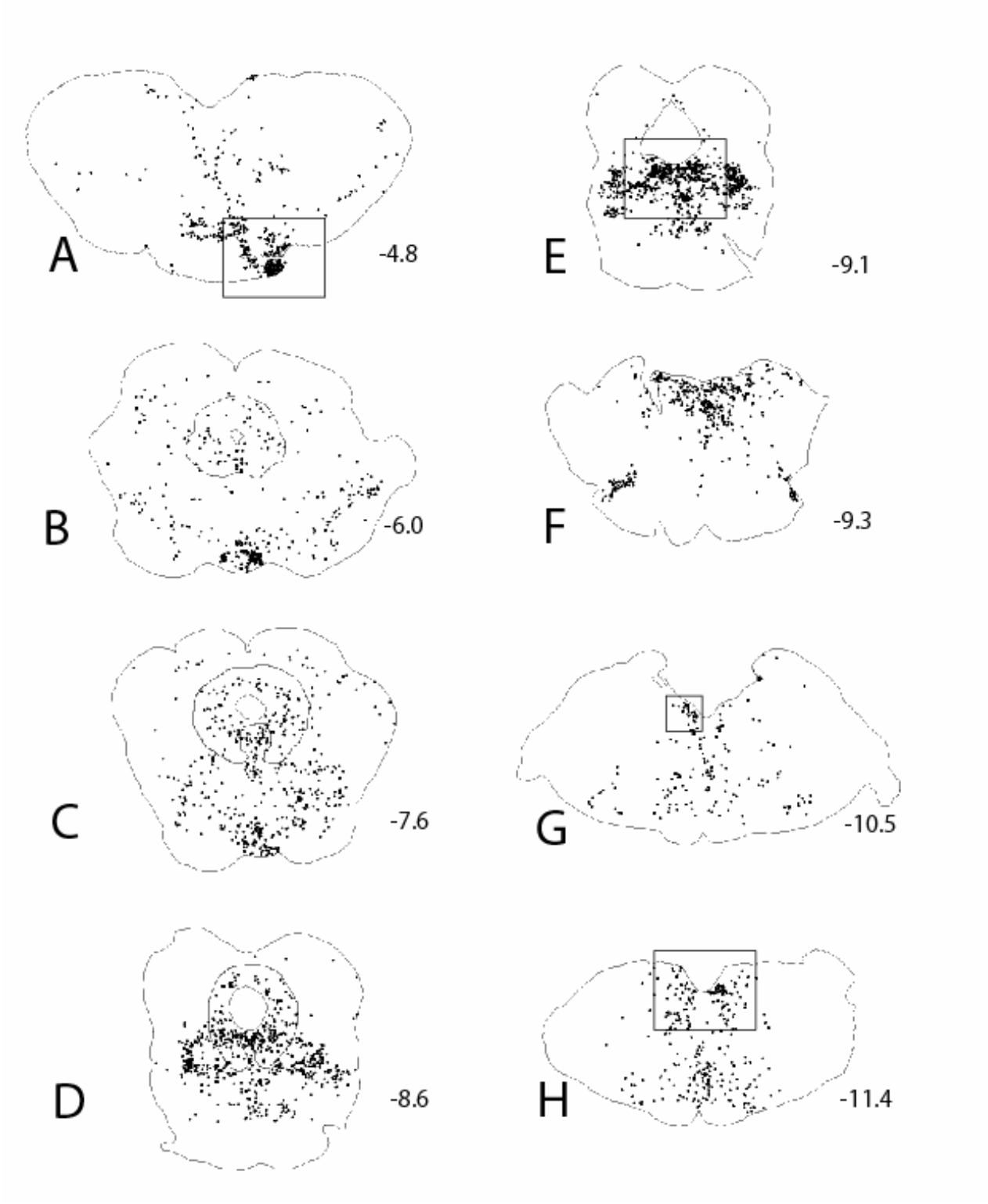


Figure 3.

the large number and wide distribution of infected neurons resulting from the retrograde transynaptic transport of PRV over 72 hrs are evident in this figure.

As also observed in the 60 hr survival time cases, infected neurons were noted in some animals in the periaqueductal grey, interpeduncular nucleus, parabrachial nucleus, locus coeruleus, reticular formation, and other areas (see Table 2). Labeling in areas of the reticular formation, such as the mesencephalic reticular nucleus, paragigantocellular reticular nucleus, gigantocellular reticular nucleus and pontine reticular nucleus, was more prominent when a large number of infected cells was present at the injection sites.

Forebrain labeling was also quantified in both the 60 hr and 72 hr cases, and was found to be consistent with the pattern that would be predicted based on relevant studies (Alonso and Kohler, 1984; Risold and Swanson, 1996, 1997). These studies demonstrated topographically organized bisynaptic pathways between the hippocampus and hypothalamus that are relayed through the lateral septum. The analysis of forebrain data were beyond the scope of the present study and will be presented in separate manuscript (Brown, 2004; Brown et al., 2004).

3.3.2. Control injections: PRV transport following injection into areas surrounding LMN

Control data were analyzed from 16 cases where PRV was placed into areas surrounding, but not including, the LMN. The goal of this analysis was to determine whether the brainstem labeling observed in the LMN injected animals was due to PRV transport from the LMN or from adjacent nuclei. These animals were killed 60 hours (n=10) or 72 hours (n=6) following injections. The locations of infected cells caudal to the injection sites in these brains are shown in Tables 3 (60 hr) and 4 (72 hr).

Figure 4.

Photomicrographs illustrating a control injection site (A) and the associated retrograde infection of brainstem nuclei (B-D) in case 12 with a 60 hr survival time. **A.)** This injection site was focused within the supramammillary nucleus (SUM) and tuberomammillary nucleus pars ventralis (TMv) and only slightly impinged upon the lateral mammillary nucleus (LMN). Labeling in TMv extends lateral and ventral to the LMN without including it. **B.)** Bilateral labeling was observed in LDTN and no infected neurons were present in the DTN. **C.)** Very light bilateral labeling is present in the SUG. **D.)** No labeling was found in PH or MVN. Marker bars = 500 μm.

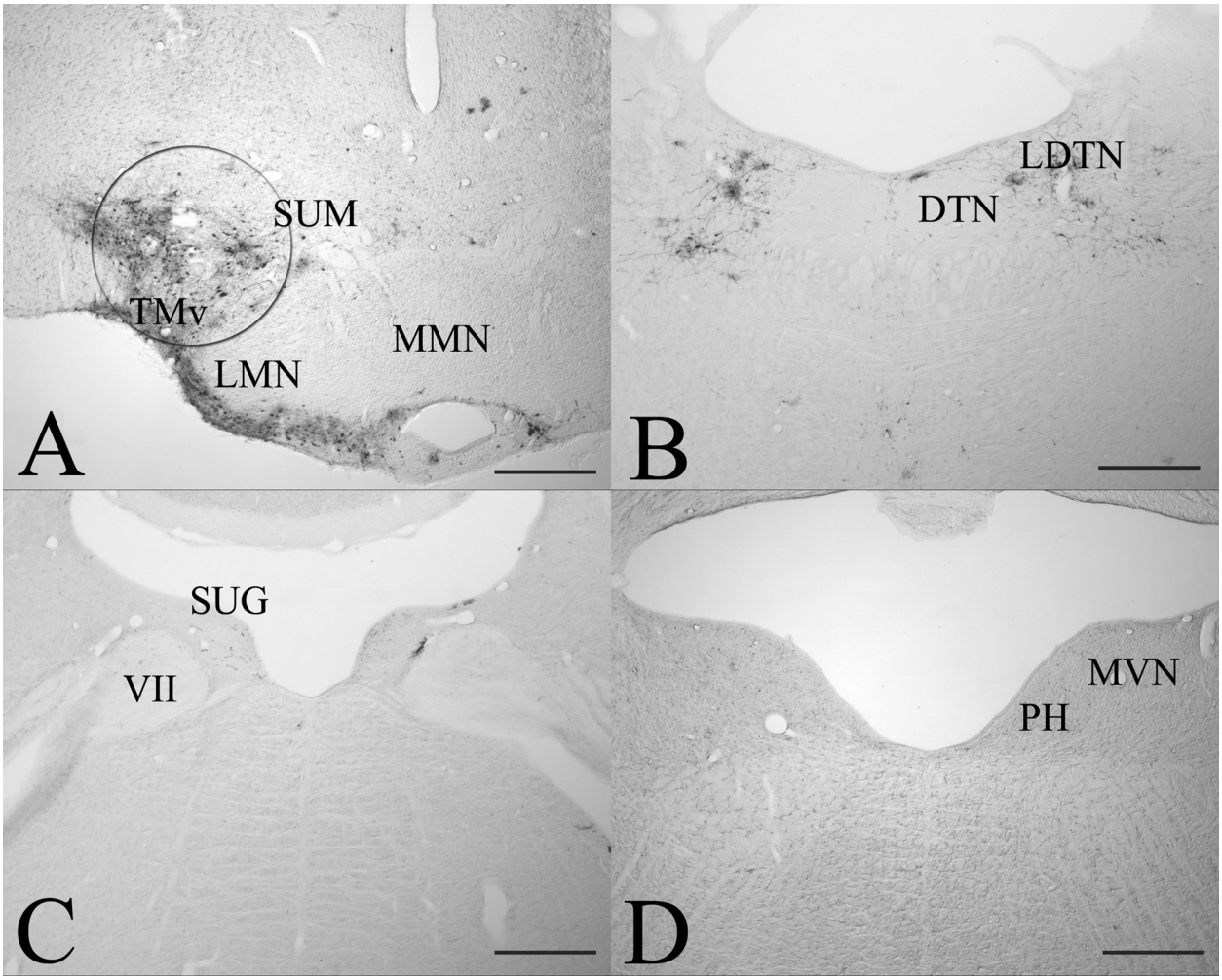


Figure 4.

Figure 4 illustrates representative 60 hr labeling from case 12, where the injection site was centered in TMv and included SUM and lateral hypothalamic area (Fig 4A). The TMv runs along the ventral boundary of the mammillary complex, as evidenced by the labeling shown in this case. No labeling was observed in the LMN in any sections of this brain, and the locations of infected neurons caudal to the injection site were different from those in cases where the injection site included LMN (compare Tables 1 and 3). Figure 5 illustrates a 72 hr control case (case 24), where labeling around the injection site was observed in the TMv, SUM, lateral hypothalamic area and substantia nigra pars reticulata, but not the LMN. The extensive infection in SNr lateral to the injection site presumably resulted from the interconnectivity within the nucleus and extensive axonal terminations near the injection site, similar to the TMv infection shown in Fig 4A.

In general, the labeling of DTN in the control cases differed dramatically from the DTN labeling observed in the LMN injected cases. Infected DTN cells in the control group were observed only in two of the ten control cases in the 60 hr survival time group (17 & 20) and in none of the cases in the 72 hr survival group. These two control cases exhibited very light, ipsilateral DTN infection. This differed from the LMN injected animals where DTN labeling was found in all of the cases, the number of infected cells was much higher, and the infected cells were found bilaterally in nine of the 11 rats. The LDTN was consistently infected in both the 60 hr and 72 hr control cases where the injection sites included SUM. This pattern was similar to the labeling observed in the LMN injection cases that additionally included SUM. LDTN labeling was usually bilateral with a greater number of cells found on the ipsilateral side. Figure 4B demonstrates the bilateral infection in LDTN from case 12 as well as the lack of

labeled cells in the DTN. Infected cells in the VTN were observed in two control cases (21 and 26); the injection sites in both animals included the MMN. Figure 5B illustrates the pattern of infection at the level of DTN and LDTN following a 72 hr survival time.

The SUG was labeled in two cases from the 60 hr control group (cases 12 and 14). Both displayed bilateral SUG labeling with higher numbers of infected cells on the ipsilateral side. There was no clear pattern of infection around the injection sites of these cases that set them apart from the rest of the control group. Figure 4C illustrates the light labeling in the SUG from case 12. Infected cells in the SUG of the 72 hr control group were observed in four of the six cases, also with the heaviest labeling found on the ipsilateral side. Figure 5C illustrates the labeling in SUG from case 24, which is representative of the SUG labeling found in the other three cases.

There were no infected cells in the PH, MVN, or other vestibular nuclei of any of the control cases. Figure 4D illustrates of the lack of labeling in rostral PH, as well as the absence of cells in rostral MVN in case 12. Figure 5D illustrates a similar the absence of infected cells in both the PH and MVN. Infected cells observed in the reticular formation of the control cases were limited to a sparse, ipsilateral distribution in the pontine reticular formation of cases 15 and 16, and the paragigantocellular reticular nucleus of case 22.

Figure 5.

Photomicrographs of a control injection site (A) and associated retrograde infection of brainstem (B-D) in an animal with a 72 hr survival time (case 24) are illustrated. **A.)** This injection included the SUM, TMv, lateral hypothalamic area and substantia nigra. **B.)** Bilateral labeling was apparent in LDTN but not DTN. **C.)** Light bilateral labeling in SUG, as well as locus coeruleus and nucleus raphe magnus are illustrated. **D.)** No labeled cells were found in PH or MVN. Marker bars = 500 μm.

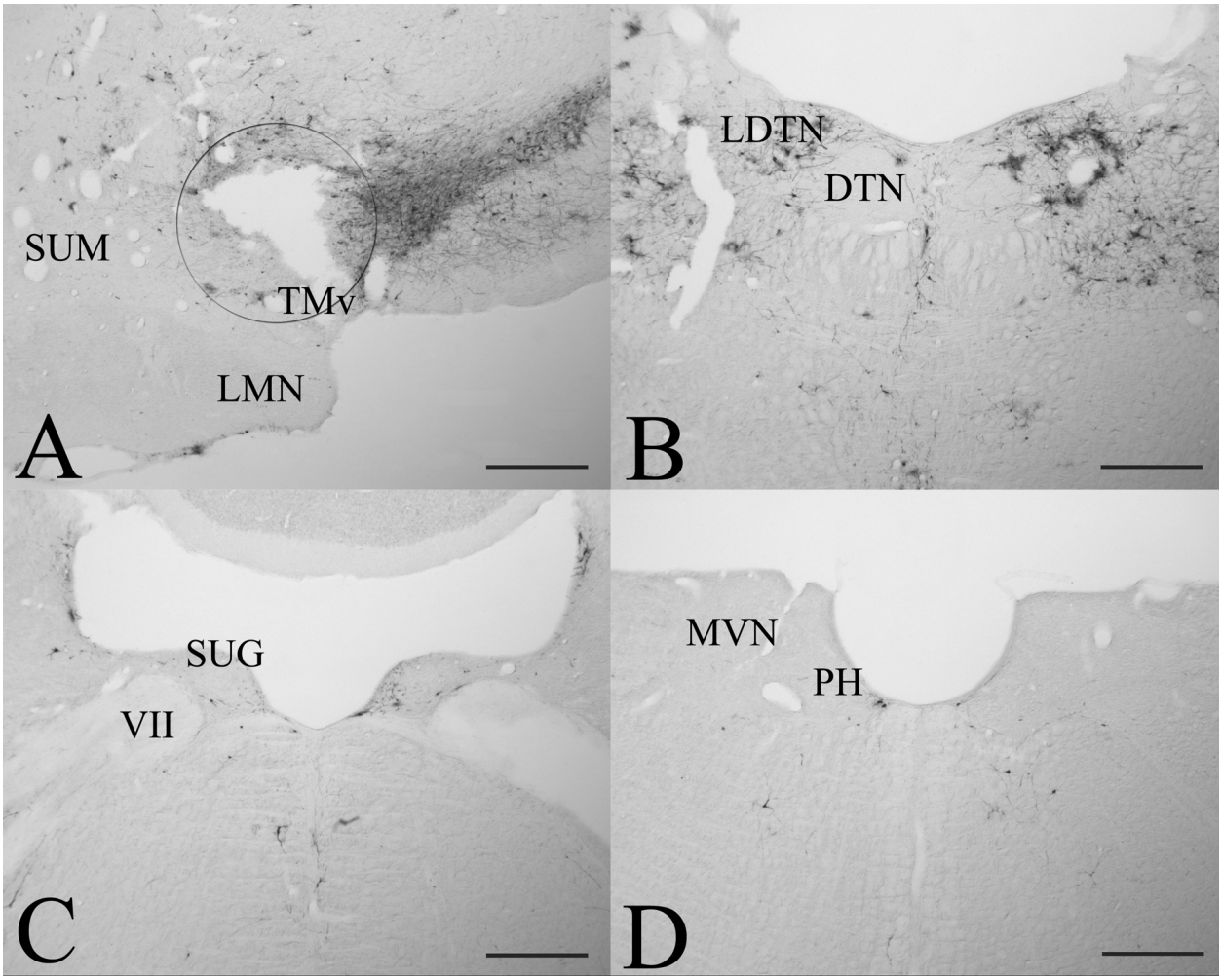


Figure 5.

Figure 6.

The distribution of infected neurons from the case shown in Fig 5 (case 24) are illustrated. Note that each dot represents an individual neuron. Sections are arranged from rostral (A) to caudal (H), with the coronal planes relative to Bregma noted at the lower right of each section. The boxed areas in figures A, E, G, and H define regions illustrated in figures 5A, 5B, 5C, 5D, respectively.

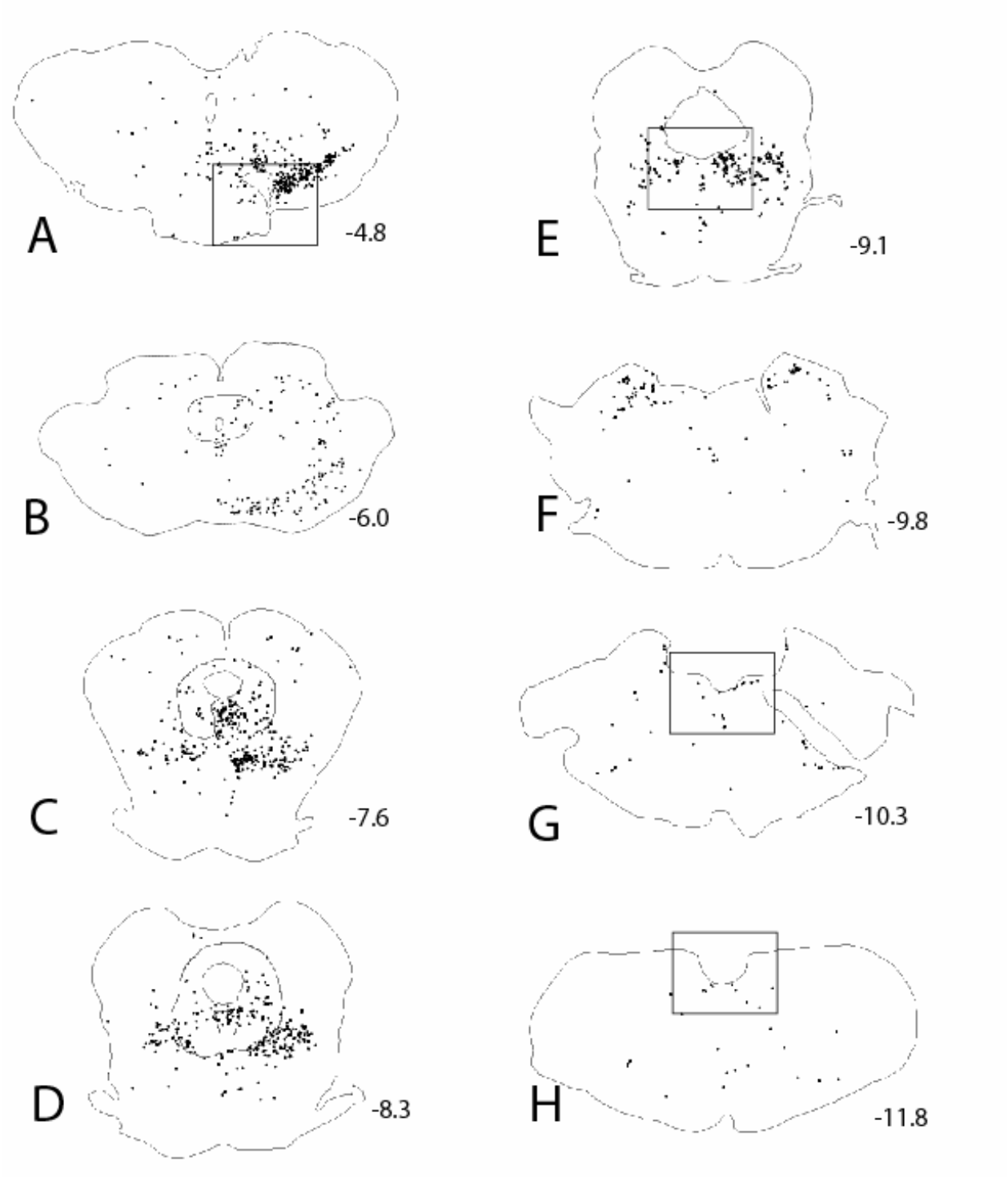


Figure 6.

3.4.Discussion

Previous electrophysiological observations have demonstrated that direction-sensitive firing of HD cells in the ADN is reduced or eliminated following chemical labyrinthectomy in rats (Stackman and Taube, 1997). Because ADN HD activity is dependent on an intact LMN (Blair et al. 1999), these observations raise the possibility that vestibular input to the LMN participates in shaping the directional activity of LMN HD cells or even LMN AHV cells. The absence of direct projections between the vestibular complex and the LMN indicates that these vestibular influences must be polysynaptic, but the identity and organization of projection pathways conveying labyrinthine information to LMN is not known. The present study used viral transneuronal tracing to provide insight into the pathways through which the vestibular system modulates the activity of cells in LMN that are related to the HD signal. Specifically, the data support the conclusion that vestibular information reaches the LMN via a polysynaptic circuitry that includes the DTN and other potential relays. Collectively, these data provide novel insights into the brainstem networks that contribute to spatial cognition and that may be relevant to an organism's ability to navigate effectively within its environment.

3.4.1. Technical considerations

The factors that influence the uptake and transport of PRV through neural circuitry following intracerebral injection are important considerations in evaluating the conclusions presented in this study. Prior work has demonstrated that the affinity of alpha herpesvirus envelope glycoproteins for neuronal profiles and extracellular matrix molecules restrict the

diffusion of PRV injected intraparenchymally (see Card et al. 1999, for review). As noted earlier, detailed studies indicate that the volume and concentration of PRV used in the present analysis is taken up within a 500 μm radius of injection (see Card, 2001 for review). Nevertheless, it is clear that the architecture of the region of injection exerts an important influence upon the zone of virus uptake. Important in this regard is the demonstration that axon terminals exhibit the highest affinity for PRV (Marchand and Schwab, 1986). Thus, the results of the present study must be viewed within the context of the architecture of the LMN and neighboring cell groups.

Previous studies report that the LMN and MMN contain few local circuit neurons and that the neuropil is composed principally of afferents from other regions (Allen and Hopkins, 1989; Hayakawa and Zyo, 1992; Takeuchi et al. 1985; Gonzalo Ruiz et al. 1993). In contrast, local circuit connections provide a more prominent component of the supramammillary nucleus neuropil (Hayakawa and Zyo, 1984). These observations are parsimonious with the sparse infection of LMN neurons observed in cases in which the injection site was centered within LMN and the prevalence of infected SUM neurons in cases where the injection involved this nucleus. The DTN provides a prominent afferent projection to the LMN (Shibata, 1987; Allen and Hopkins, 1989; Hayakawa and Zyo, 1992; Wirtshafter and Stratford, 1993) and this pathway is known to be reciprocal (Liu et al. 1984; Shibata, 1987; Hayakawa and Zyo, 1990). Therefore, it is possible that PRV injected into the LMN produced a retrograde infection of DTN neurons with subsequent transneuronal infection of LMN neurons projecting to the DTN. This interpretation is consistent with the more robust infection of LMN neurons in the 72 hour survival time.

The conservative approach employed in our analysis identified cases in which the injection was centered in the LMN and the orientation of the beveled needle biased the flow of virus into this subdivision of the mammillary complex. Nevertheless, we cannot exclude the possibility that PRV was taken up by neighboring cell groups in these cases. Comparative analysis of the retrograde infection produced by injection of virus into neighboring cell groups in control cases allowed us to address this issue. Such a comparison demonstrated that cases in which the injection was centered in the LMN always produced a retrograde infection of DTN whereas injections of the neighboring SUM rarely produced DTN infection but reliably infected the LDTN. Retrograde infection of neurons in the MVN was present in cases with a robust infection of the DTN and did not occur in cases in which there was robust infection of LDTN with little or no infection of DTN. These data demonstrate consistent patterns of infection that correlate with the location of the injection site in the caudal hypothalamus as well as with literature demonstrating differential projections of the DTN and LDTN to the LMN and SUM, respectively (Shibata, 1987; Allen and Hopkins, 1989; Hayakawa and Zyo, 1992). When considered with literature demonstrating the specificity of retrograde transport of PRV-Bartha through synaptically linked neurons (Pickard et al. 2002), these data strongly support the conclusion that the analytical approach employed in this analysis provides a reliable means of defining cell groups that contribute to the polysynaptic relay of vestibular information to the LMN.

3.4.2. The organization of polysynaptic pathways linking LMN and MVN.

Potential relay nuclei that carry vestibular information to the LMN can be identified by combining the patterns of labeling observed in the present study with current literature

describing the functional roles of areas containing infected cells. There are several tegmental nuclei that send ascending projections to the mammillary complex via topographically organized, parallel pathways, including the projection from the DTN to the LMN mentioned previously (Allen and Hopkins, 1989; Hayakawa and Zyo, 1992; Wirtshafter and Stratford, 1993). Our data are most consistent with the DTN serving as a relay of vestibular information to the LMN. The main LDTN projection to the mammillary complex terminates in the SUM (Shibata, 1987). However, Hayakawa and Zyo (1992) provided evidence for LDTN projections to LMN following injections of an anterograde tracer (WGA-HRP) into the LDTN. Also, the LMN has been found to exhibit very heavy ChAT labeling (Ruggiero et al., 1990), and the LDTN is also known to contain cholinergic neurons (Gonzalo-Ruiz et al., 1999). This suggests that the LDTN projects to the LMN, and therefore the LDTN labeling in the present study could partly be due to retrograde transport from the LMN. The present results, when combined with functional data in the literature regarding the AHV signals in the DTN (Bassett and Taube, 2001; Sharp et al., 2001) support the DTN as the probable relay in the polysynaptic circuit from MVN to LMN. The MMN receives input from the VTN of Gudden and the superior central nucleus (Hayakawa and Zyo, 1991). LDTN and VTN labeling was observed when the SUM and the MMN, but not the LMN, were included in the injection sites, and are therefore less likely to participate as relay nuclei in the polysynaptic circuit from MVN to LMN.

Electrophysiological recording data describing the functional activity of DTN neurons provides further strong evidence regarding their role as potential elements in the circuit relaying vestibular signals to the LMN. Studies by both Bassett and Taube (2001), and Sharp and colleagues (2001), found that DTN activity in freely behaving rats was related to their angular head velocity, which is a signal that could be used to generate HD cell activity. Furthermore, the

fact that a small percentage of HD cells were located in DTN (Basset and Taube, 2001; Sharp et al., 2001) indicates that the DTN could be performing an integrative function regarding vestibular data relevant to the HD system. Based on these data, in combination with the results of the present study, it is reasonable to conclude that the DTN participates in at least the relay of vestibular information to the LMN.

It is possible that relay nuclei besides the DTN participate in the circuit relaying vestibular information to HD cells in the LMN. In addition to the tegmental nuclei, the following regions exhibited relatively heavy labeling following PRV injections that included LMN: PH, SUG, reticular formation, interpeduncular nucleus (IPN), periaqueductal gray, parabrachial nucleus, and locus coeruleus. Whether or not these areas can be considered to be relay nuclei in the circuit of interest can be ascertained from the patterns of infection observed in the present study, plus their known functionality and/or connectivity with LMN, DTN and/or MVN.

Infected neurons were observed in the periaqueductal gray following both injections that included LMN and some control cases. The periaqueductal gray projects to the mammillary complex (Shibata, 1987), but projections to DTN appear to be limited (Liu et al. 1984; Hayakawa and Zyo, 1985). Furthermore, this region has not been documented to receive input from the MVN (Marchand and Hagino, 1983; Beitz et al., 1986). The parabrachial nuclei were heavily infected in several cases in this study where the injection site included LMN, but were also labeled in some control cases. Although the parabrachial nuclei receive vestibular inputs (Balaban et al. 2002), they do not project to the DTN (Liu et al. 1984; Hayakawa and Zyo, 1985) or LMN, and are therefore unlikely to be components of the circuit conveying vestibular signals to HD cells. The locus coeruleus is known to receive input from PH (Ennis and Aston-Jones,

1989) and the vestibular nuclei (Luppi et al. 1995), but does not project to DTN (Liu et al. 1984; Hayakawa and Zyo, 1985). The IPN projects to both the DTN and LDTN (Liu et al. 1984; Hayakawa and Zyo, 1985; Cornwall et al. 1990). In the present study, IPN neurons were labeled in both LMN-injected cases and control cases; IPN cells were also infected in animals where the LDTN, but not the DTN, was found to contain labeling. In addition, IPN has not been demonstrated to receive vestibular inputs (Marchand et al. 1980; Shibata et al. 1986). Thus, it seems unlikely that the periaqueductal gray, parabrachial nucleus, locus coeruleus play a primary role in relaying vestibular signals to DTN neurons that in turn project to LMN. The nucleus incertus contained infected neurons in some of our animals and is known to provide ascending projections to numerous areas including the LMN (Goto et al., 2001; Olucha-Bordonau et al., 2003). The nucleus incertus receives input from other areas labeled transynaptically in this study including the IPN, PAG and superior central nucleus. However, it is unlikely that the nucleus plays a role in carrying vestibular information as it does not receive input from the vestibular nuclei or dorsal tegmentum (Goto et al, 2001).

In contrast, PH both sends projections to the ipsilateral DTN (Liu et al. 1984; Hayakawa and Zyo 1985) and receives afferents from vestibular nuclei (Iwasaki et al, 1999), but does not project to LMN (Shibata, 1987). Furthermore, PH was heavily infected following injections of PRV into LMN, but not adjacent nuclei. It is therefore reasonable to consider PH as a potential relay for ascending vestibular signals to LMN neurons, as Bassett and Taube (2001) have predicted. Figure 7 illustrates the possible position of PH in the pathway, as it receives vestibular signals from MVN and also projects to DTN. However, PH receives both vestibular and optokinetic information (Lannou et al. 1984), as well as oculomotor signals (McCrea and Baker, 1985; Cannon and Robinson, 1987); the rostral portion of the nucleus, which contained

Figure 7.

Schematic representation of postulated connections between nuclei that contribute to the HD signal (in bold) based on the labeling in the present study. Other areas that may potentially relay vestibular information to the HD pathway include the reticular formation and supragenual nucleus. Connections between tegmental nuclei and mammillary nuclei are shown to provide an illustration of the topographical organization of projections between those regions.

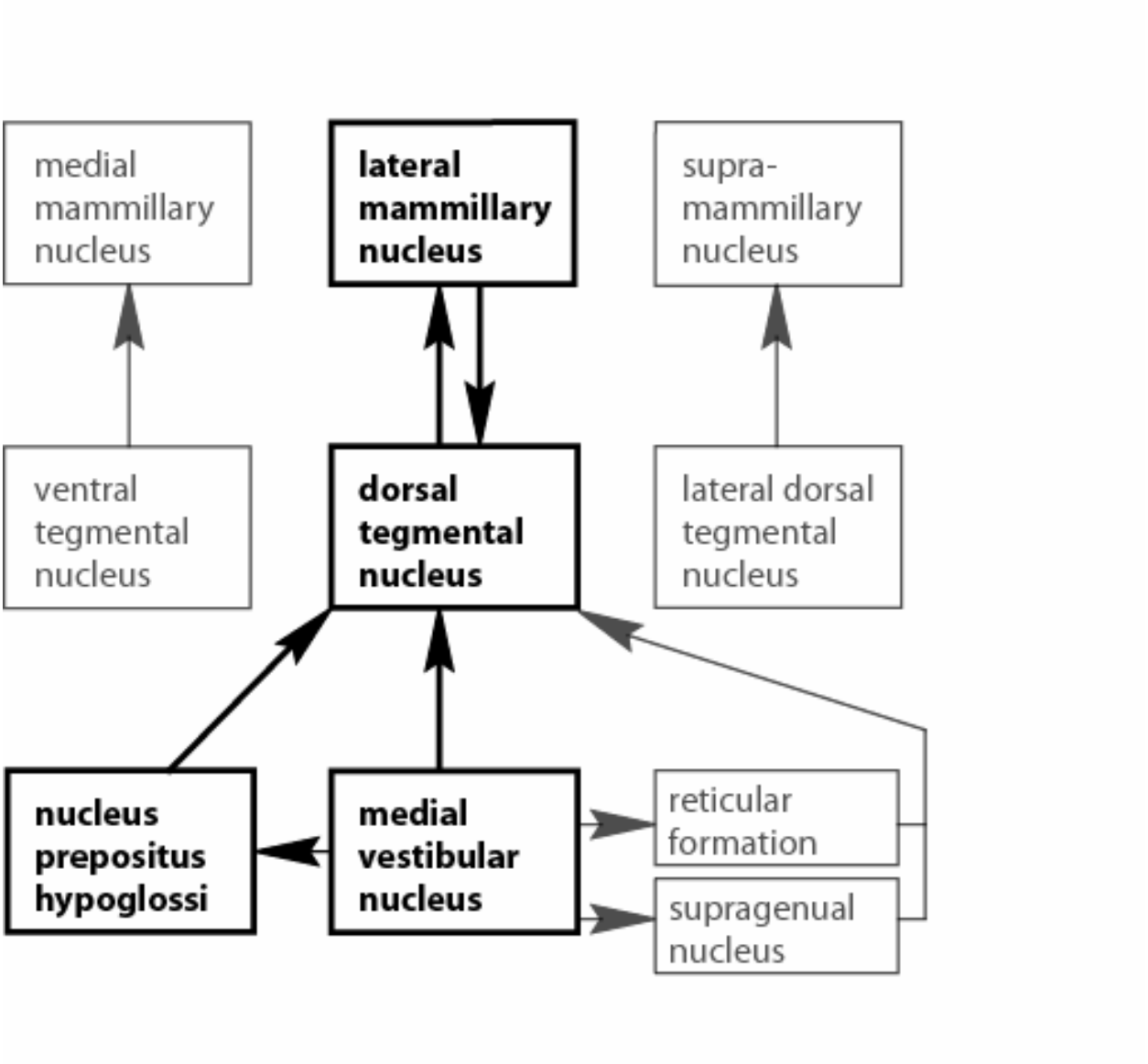

medial mammillary nucleus
lateral mammillary nucleus
supra-mammillary nucleus
ventral tegmental nucleus
dorsal tegmental nucleus
lateral dorsal tegmental nucleus
nucleus prepositus hypoglossi
medial vestibular nucleus
reticular formation
supragenual nucleus

Figure 7.

the neurons infected following injections of PRV into LMN in this study, is known to be a component of pathways that mediate horizontal eye movements (McCrea, 1988). Thus, it is alternatively possible that PH neurons that provide inputs to DTN integrate signals unrelated to the downstream generation of a head direction signal; the functional role of these neurons in the circuit is yet to be determined.

The mesencephalic reticular nucleus, pontine reticular formation and gigantocellular reticular nucleus were often heavily labeled in cases where the injection site was centered in the LMN. Portions of the medial reticular formation receive substantial vestibular inputs (Zelman et al. 1984; Matesz et al. 2002), and their previously reported connectivity with DTN (Liu et al. 1984) is similar to that found in the present study. However, there was no clear pattern of infection in the reticular formation that resulted from our injections. Thus, some parts of the reticular formation are potential components of the pathway relaying vestibular signals to LMN, although further experiments will be necessary to test this possibility.

The SUG has often been classified as one of the perihypoglossal nuclei or a rostral extension of PH (Brodal, 1983; Korp et al. 1989). As a result, its synaptology and functionality are often unclear in the literature. This nucleus is known to project to the contralateral DTN (Liu et al. 1984; Hayakawa and Zyo, 1985). Although a previous tracing study did not demonstrate the presence of efferents from the MVN to SUG in the rat (Matesz et al. 2002), further work is necessary to fully consider or eliminate SUG as a potential relay nucleus for ascending vestibular signals to HD cells.

The only vestibular nucleus that contained infected cells after PRV injection into LMN was the MVN, which was labeled in four of the five cases following a 72 hr survival time. It is possible that the temporal progression of PRV replication and transport from LMN in that case

was insufficient to generate infection of MVN neurons. Nonetheless, our results substantiate the hypothesis that the vestibular system projects to the LMN via a relatively direct polysynaptic pathway that, based on the current data, most likely includes the DTN as a primary relay nucleus. Previous studies have demonstrated direct projections from MVN to DTN (Liu et al. 1984; Hayakawa and Zyo, 1985). The present study also confirms that the LMN does not receive direct input from vestibular nuclei (Shibata, 1987). Therefore, our results are consistent with the scheme illustrated in Figure 7, where vestibular signals from the MVN are being relayed through the DTN to the LMN. It remains to be determined, however, whether direct projections from MVN to DTN play the predominant role in relaying vestibular signals to HD cells, or whether connections that involve PH, the reticular formation, or perhaps SUG are functionally important as well.

3.4.3. Functional implications of ascending vestibular projections to the LMN for the generation of the HD signal

One of the inputs to MVN is from the horizontal semicircular canals which generate angular head acceleration signals; many cells in MVN also receive convergent canal and otolith input (Schor et al. 1998). Although HD cells in the LMN code for head direction, there is evidence that AHV cells are also located in the LMN as well (Blair et al., 1998; Stackman and Taube, 1998). For vestibular information to be used in generating the HD signal, the angular head acceleration signals would have to be integrated twice over time to produce a position signal. Such neural integration could be accomplished over several steps that have been discussed previously, including routing the signal through cells that extract an AHV signal (Bassett and Taube, 2001; Sharp et al. 2001; Brown et al. 2002). Similar neural integration takes

place in computing eye position signals for the vestibulo-ocular (VOR) reflex (Baker, 1977). The relationship between LMN AHV and HD cells is unclear, and the results of this study can not indicate whether AHV or HD cells are receiving input from the polysynaptic pathway from the MVN. It is likely that this input influences both sets of cells in support of the HD signal.

Furthermore, it is probable that there is additional input or processing in the HD pathway because the HD signal in LMN is not a simple "head position" signal. HD cells code for head direction in the vertical plane, regardless of the relationship of the rat's head with its trunk (Taube, 1995). In addition, Stackman et al. (2000) and Kim et al. (2003) have provided evidence that, under some conditions, ADN HD cell activity remains stable in the vertical plane. Therefore, it is probable that other vestibular information, such as otolith signals, could play an important role in the maintenance of the HD signal. The nature of HD cell activity in a true three-dimensional environment is yet to be determined. Finally, vestibular neurons that respond to head rotation also respond to visual field rotation (Henn et al. 1974), and the role that optokinetic stimuli play in the generation of HD activity, if any, is unclear.

3.5.Summary

The present data indicate that the MVN projects to the LMN via a polysynaptic pathway that involves the DTN and perhaps other nuclei. The anatomical and functional relationship of the HD signal with vestibular, visual and optokinetic information appears to be quite complex. Additional work must be performed to differentiate the individual contributions of these types of information to the generation and maintenance of the HD signal.

Table 1. Locations of neurons infected with PRV injected into the LMN, and control injections excluding LMN, following survival times of 60 hrs and 72 hours.

	Nuclei included in injection site				
	LMN	**MMN**	**PMN**	**SUM**	**TMv**
60 hr LMN	ipsi contra	ipsi contra	ipsi contra	ipsi contra	ipsi contra
Case 1	x		xx xx		x x
Case 2	x	x	x x		xx xx
Case 3	x	xx	xx	xxx xxx	xx
Case 4	x	xx			x
Case 5	x		x	xx	x
Case 6	x			xx	x
	LMN	**MMN**	**PMN**	**SUM**	**TMv**
72 hr LMN	ipsi contra	ipsi contra	ipsi contra	ipsi contra	ipsi contra
Case 7	x	x	x	xx xxx	x xx
Case 8	x	x x	xx xx	xxx	xxx xxx
Case 9	xxx x	xxx		xxx xxx	xxx x
Case 10	x	x	x	x	x
Case 11	x			xx	x x
	LMN	**MMN**	**PMN**	**SUM**	**TMv**
60 hr control	ipsi contra	ipsi contra	ipsi contra	ipsi contra	ipsi contra
Case 12				xxx xxx	xxx x
Case 13					xxx
Case 14			xx	x x	x x
Case 15				x	
Case 16				x	
Case 17				xx	
Case 18					xx
Case 19				x	xx
Case 20			x		xx x
Case 21		x		x	x x
	LMN	**MMN**	**PMN**	**SUM**	**TMv**
72 hr control	ipsi contra	ipsi contra	ipsi contra	ipsi contra	ipsi contra
Case 22					xx xx
Case 23			xx	x	x x
Case 24			xx x	x x	x x
Case 25					x
Case 26		xx		x	x
Case 27				x	x

Table 1, cont. Locations of neurons infected with PRV injected into the LMN, and control injections excluding LMN, following survival times of 60 hrs and 72 hours.

	Hindbrain labeling													
	DTN		**LDTN**		**VTN**		**MRF**		**SUG**		**PrH**		**MVN**	
60 hr LMN	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
Case 1	x	x	x	x			xx	xx	x	x				
Case 2	x	x	xx	x	xx						x	x		
Case 3	x	x	xxx	xxx	x	x	xx	xx	xx	xx	x	x		
Case 4	x		x		x									
Case 5	x		xxx	xxx					x	x				
Case 6	x		x		x				x					
	DTN		**LDTN**		**VTN**		**MRF**		**SUG**		**PrH**		**MVN**	
72 hr LMN	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
Case 7	xxx	xxx	xx	xxx	x	x	xxx	xxx	x	x	x	x	x	x
Case 8	x	x	xxx	xxx			xxx	xxx	x	x	x	x	x	x
Case 9	xxx	xxx	xxx	xxx	xx	x	xxx	xxx	xx	x	xxx	xx	xx	x
Case 10	x	x	x	x	x		x	x			x		x	
Case 11	x	x	xx	xx			x	x	x	x	x			
	DTN		**LDTN**		**VTN**		**MRF**		**SUG**		**PrH**		**MVN**	
60 hr control	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
Case 12			xx	xx					x	x				
Case 13														
Case 14			x	x			x	x	xx	x				
Case 15			x	x			x							
Case 16			x				x							
Case 17	x		x	x										
Case 18			x				x							
Case 19			x											
Case 20	x													
Case 21			x	x	x		x							
	DTN		**LDTN**		**VTN**		**MRF**		**SUG**		**PrH**		**MVN**	
72 hr control	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra	ipsi	contra
Case 22			xx	x			x	x	x	x				
Case 23			x	x										
Case 24	x		xxx	x			xxx	xxx	x	x				
Case 25			x	x					x					
Case 26			x	x	x									
Case 27			x				x		x					

4. EXPERIMENT 3: POLYSYNAPTIC PATHWAYS FROM THE ENTORHINAL CORTEX AND HIPPOCAMPAL FORMATION TO THE LATERAL HYPOTHALAMUS VIA THE LATERAL SEPTUM

4.1.Introduction

The previous two chapters of this dissertation described experimental data related to internal representations of spatial location and orientation. Both of these signals arise from the processing of sensory information over polysynaptic neural pathways. However, the pathways through which these signals contribute to motivated behavior are unclear. This final data chapter describes an experiment that was designed to explore neural circuitry that may provide routes by which such information could be integrated and relayed to areas supporting motivated behavior.

Basic spatial navigation requires the integration of at least two types of information: spatial location and orientation (directional heading). In the rat, the neural processing of such information is thought to be supported by cells in the hippocampal formation whose activity is related to the location of the animal (place cells), and a more widely distributed set of nuclei throughout the limbic system that contain cells signaling the animal's head direction (head direction cells) (O'Keefe and Nadel, 1978; Taube, 1998; Redish, 1999). These internal representations of location and head direction have been studied extensively, although their relationship with motivated behavior has yet to be completely defined. In order to best approach the relationship of spatial and directional information with motivated behavior, it is useful to examine the polysynaptic circuitry that could support communication between neural regions involved in spatial information processing and motivated behavior.

There are two separate sets of neuroanatomical literature that shed light on this subject and provide the motivation for the work described in this chapter. This first set of data is the

primary focus of the present study, and it involves pathways though which hippocampal, and arguably spatial, information may be relayed to the hypothalamus (Fig 1A). The second set of data describes the organization of pathways between the parahippocampal region and hippocampal formation that process spatial information (Fig 1B). The integration of these two data sets is imperative to understanding the larger picture of spatial information processing and its relationship to motivated behavior.

Regions of hippocampus (CA3, CA1, subiculum) have been shown to project to the lateral septum (LS) in a topographically organized manner. The LS also sends projections to the hypothalamus in a topographically organized manner (Raisman, 1966; Swanson and Cowan, 1979; Risold and Swanson, 1996; Risold and Swanson 1997a). Additionally, Swanson and co-workers have proposed that hypothalamic nuclei can be functionally organized into longitudinal columns that support different classes of motivated behavior such as ingestive, social, and exploratory behaviors (Risold and Swanson, 1996; Risold and Swanson 1997a; Swanson 2000). Taken together, these data led Risold and Swanson (1996) to propose that the hippocampus is organized into functional domains that relay different types of information to these hypothalamic zones in a topographically organized fashion via the LS.

The second set of data describes the organization of circuitry that transports highly-processed sensory information from cortex into the hippocampal formation. Briefly, polymodal association cortices project to the parahippocampal region (postrhinal, perirhinal, and entorhinal cortices, plus presubiculum and parasubiculum). The parahippocampal region then projects in a topographically organized manner to the hippocampal formation (dentate gyrus, CA3, CA1 and subiculum) (Fig 1B). These connections have been studied extensively (for review, see Witter and Wouterlood, 2002). The organization of these pathways is extremely relevant to understand

the circuitry between the hippocampus and hypothalamus because 1) the entorhinal cortex is the gateway for cortical information to reach the hippocampus and 2) the entorhinal cortex also projects to the LS (Alonso and Kohler, 1984).

The present study aims to bring together these two sets of data by addressing the hypothesis that polysynaptic pathways from the hippocampal formation and parahippocampal region project to the hypothalamus via the LS in a topographically organized manner. Such an organization could provide a substrate for a topographically organized polysynaptic circuitry through which high level sensory information is processed, integrated, and transported from cortex to the hypothalamus, providing a spatial substrate for motivated behavior. The hypothalamic zone of primary interest is composed of lateral and caudal nuclei thought to be involved in exploratory and foraging behaviors, including the lateral hypothalamic area, lateral supramammillary nucleus and the mammillary complex (Swanson, 2000).

The circuits proposed in the current literature are based on studies in which monosynaptic neural tracers have been used to establish that neurons in a nucleus of interest project to, or receive projections from, neurons in another nucleus (Fig 2A). Injecting monosynaptic tracers in several different areas over the course of several experimental groups allows researches to hypothesize that the nuclei of interest are linked (Fig 2B). However, this type of analysis is not suited to establishing the synaptology of a polysynaptic circuit. As described in the previous chapter, the use of transneuronal tracers is a more appropriate tool for establishing the synaptology of neural circuits. While monosynaptic tracers can provide circumstantial evidence for polysynaptic circuits, the careful analysis of the transynaptic transport of viral tracers provides the key information that has heretofore been missing (Card, 2001).

In this study, injections of PRV were made into the lateral, caudal hypothalamus of adult male rats. The patterns of infected cells at different post-injection survival times was used to analyze the transynaptic transport of PRV through neural circuitry involving the LS, hippocampal formation and entorhinal cortex.

Figure 1.

The organization of proposed polysynaptic pathways based on monosynaptic tracing experiments. A) Hippocampal projections to the hypothalamus via the lateral septum. B) Cortical information funneled through the parahippocampal region and reaching the hippocampal formation.

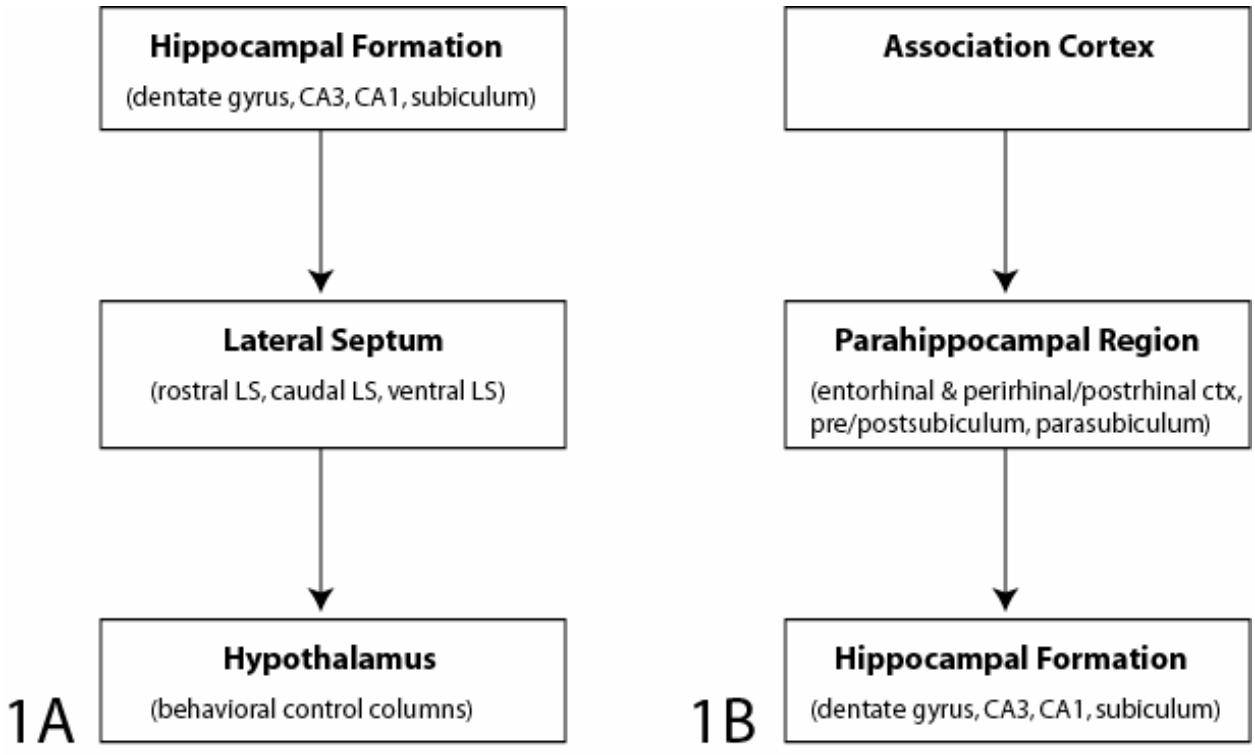

Hippocampal Formation
(dentate gyrus, CA3, CA1, subiculum)
Lateral Septum
(rostral LS, caudal LS, ventral LS)
Hypothalamus
(behavioral control columns)
1A
Association Cortex
Parahippocampal Region
(entorhinal & perirhinal/postrhinal ctx, pre/postsubiculum, parasubiculum)
Hippocampal Formation
(dentate gyrus, CA3, CA1, subiculum)
1B

Figure 1.

Figure 2.

Schematic diagram of neuroantomical tracing studies. A) Organization of Risold and Swanson (1996, 1997b) experiments in which bisynaptic pathways between the lateral septum and hypothalamus, as well as hippocampal projections to the lateral septum were examined. B) Organization of the hypothesis of the present study, indicating the proposed pathway through which PRV will travel following its injection into the hypothalamus.

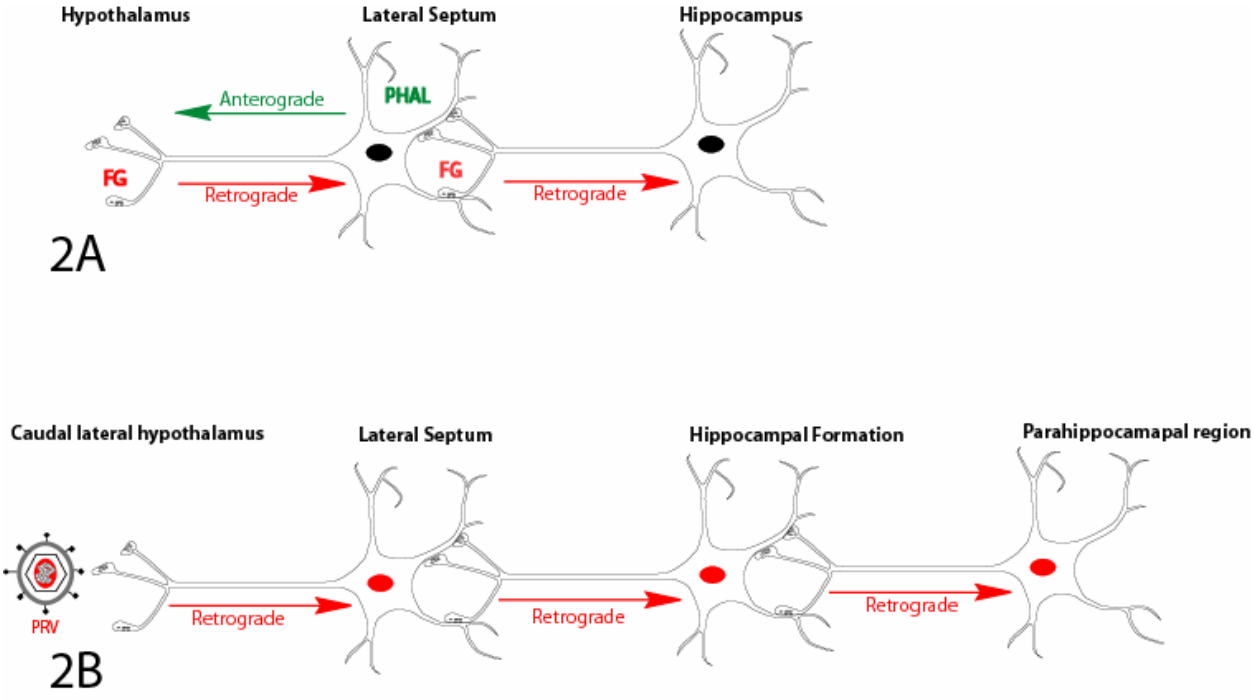

Hypothalamus
Lateral Septum
Hippocampus
Anterograde
PHAL
FG
Retrograde
FG
Retrograde
2A
Caudal lateral hypothalamus
Lateral Septum
Hippocampal Formation
Parahippocamapal region
PRV
Retrograde
Retrograde
Retrograde
2B

Figure 2.

4.2.Methods and Materials

The experimental methods used in this study were similar to those described in the previous chapter, especially because many of the animals contributed to both chapters. The two experimental groups were not identical, however, because there were numerous animals whose injection sites qualified them for only one of the two studies. The methods of data analysis used in the two studies also differed as described in detail below, as the brain regions of interest and the goals of the studies were completely different.

All procedures in this study conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee. Fifty-two adult male rats were used in these experiments. They were given food and water *ad libitum* and were kept in paired housing on a 12 hr/12 hr light/dark cycle. The characteristics of the two recombinants of the Bartha strain of pseudorabies virus (PRV) employed in this study, PRV-BaBlu and PRV-152, have been published elsewhere (Billig, et al., 2000). Both viruses were the generous gift of Dr. Lynn Enquist (Princeton University, NJ). PRV-BaBlu expresses β-galactosidase (β-gal), and PRV-152 expresses enhanced green fluorescent protein (EGFP), under the gG and cytomegalovirus immediate early gene promoters, respectively. Both recombinants were grown in pig kidney (PK15) cells and were adjusted to a final concentration of 1×10^8 plaque-forming units/ml.

Two sets of experiments were performed. First, 12 rats were used in order to optimize the volume and placement of injections, and to establish the time course of the progression of infection produced by the virus. Unilateral injections of PRV-BaBlu were made into the LMN of the 12 rats, which were killed following post-injection survival times of either 50, 60 or 72

hrs. Analysis of viral transport through synaptically linked neurons following these survival times (described below) revealed viral infections in first-order, and putative second- and third-order neurons, respectively. Once these data were gathered, the connectivity of synaptically-linked neurons projecting to the caudal lateral hypothalamus was characterized in 40 animals by injecting PRV-BaBlu into the left hypothalamus, and PRV-152 into the right hypothalamus, of each animal. Because PRV-BaBlu and PRV-152 express unique reporters, this paradigm allowed us to obtain two data sets from each case. This was possible because of the unique reporters that could be localized immunocytochemically in separate sets of sections. These rats were also killed following post-injection survival times of either 50, 60 and 72 hrs.

4.2.1. Surgical procedures

Animals were housed, injected with PRV, and euthanized within a biosafety level 2 (BSL-2) facility. Animals were acclimated to the facility for at least one week prior to surgery. Rats were deeply anesthetized with 1-2% isoflurane, and fixed in a stereotaxic frame. Using aseptic procedures, the scalp was incised and retracted, and small craniotomies were made over the left and right caudal lateral hypothalamus using a Dremel drill. An injection of PRV with a volume of 50 – 100 nl was made into the left and right target areas using a 1 µl Hamilton syringe equipped with a 32 gauge beveled-tip needle. Injections were placed around the following stereotaxic coordinates: AP – 4.65, ML +/- 1.10, DV – 9.45 (Paxinos and Watson, 1998), with the opening of the beveled needle oriented laterally. The range of injection sites can be seen in Figure 3.

Figure 3.

PRV injection sites mapped onto templates of the rat brain (Swanson, 1998). Each circle represents the location and spread of PRV virus in an animal analyzed in the present study. The circles are centered on the end of the syringe tract, and the radius of the circles represent the 500 μm distance that the virus may have spread following injection.

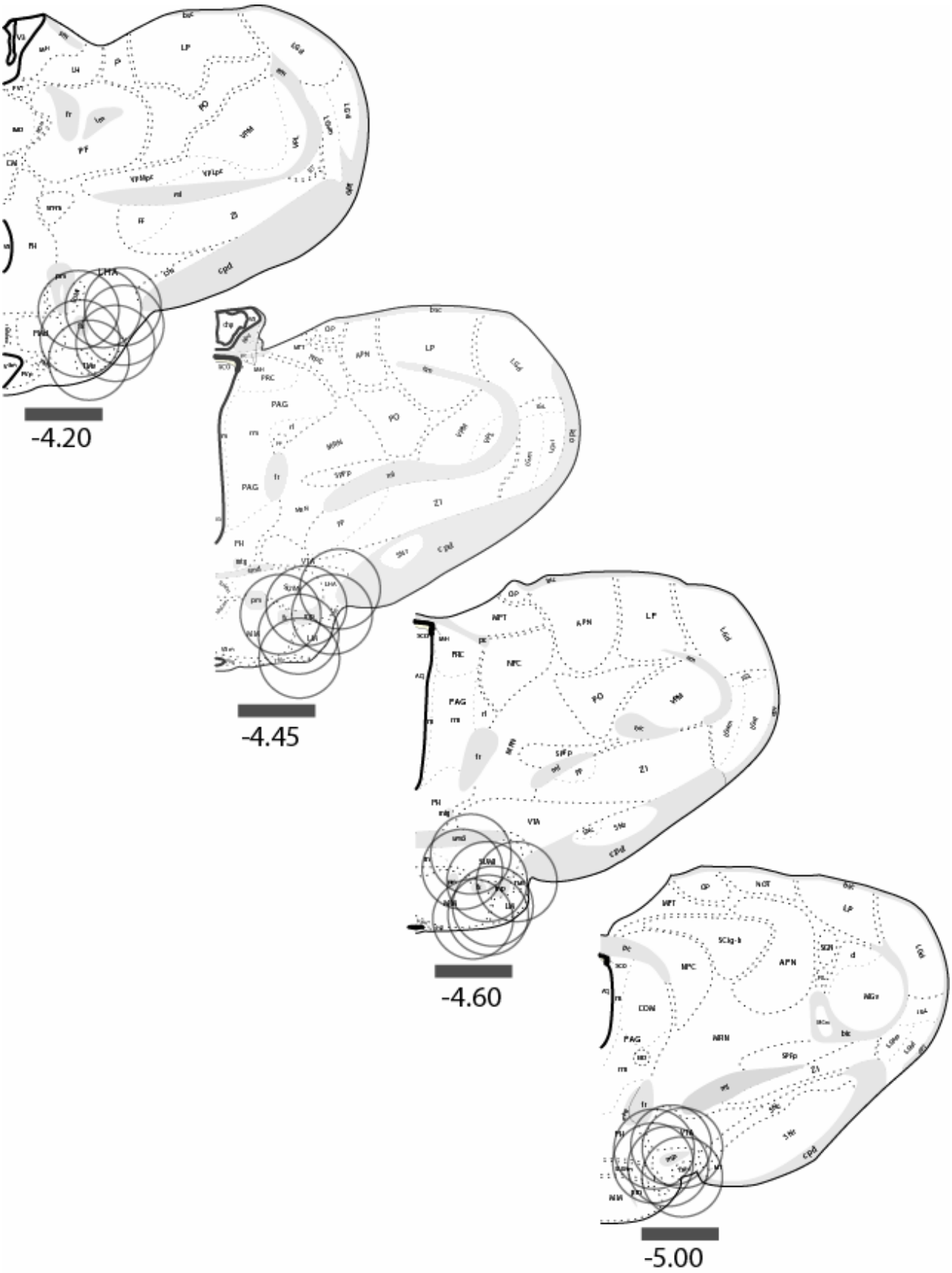


Figure 3.

The injections were made at the rate of 10 nl/min, and the syringe was left in place for 10 minutes following injection to ensure that virus would be less likely to move up the injection tract following removal of the needle. Following the removal of the needle, each craniotomy was plugged with bone wax, the scalp was sutured, and rats were returned to their home cages to recover from anesthesia. Analgesia was provided by 3 mg/kg intramuscular injections of Ketoprofen at 12-hour intervals after surgery. Following the designated survival times, rats were deeply anesthetized with 50 mg/kg intraperitoneal injections of sodium pentobarbital and perfused transcardially with 0.5 L of 9% saline followed by 1 L of 4% paraformaldehyde-lysine-periodate (PLP) fixative (McLean and Nakane, 1974). The brains were removed, postfixed for 2-4 hours in PLP, and cryoprotected for 2 days in 20% phosphate buffered sucrose. Postfixation and cryoprotection were done at 4°C.

4.2.2. Tissue processing and immunohistochemical procedures

Brains were sectioned at 35 μm in the coronal plane using a freezing microtome, and sections were collected sequentially in 6 wells of cryopreservant (Watson et al., 1986). Cryoprotected sections were stored at –20°C until they were processed for immunohistochemical localization of infected neurons. Infected neurons were identified using a polyclonal antisera generated in rabbit against acetone-inactivated PRV (Card et al., 1990) that identifies all recombinants, or with antibodies specific for the unique protein reporters of the two recombinants. These included a mouse monoclonal antibody that recognized □-gal (1:1500; Sigma Chemical, St. Louis, MO) expressed by PRV-BaBlu and a rabbit polyclonal antibody that recognized EGFP (1:1,000; Molecular Probes, Eugene, OR) expressed by PRV-152. These antigens were localized in alternate sections using the avidin-biotin modification of the

peroxidase-antiperoxidase procedure (Hsu et al., 1981), affinity purified secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA), and Vectastain reagents (Vector Laboratories, Burlingame, CA). Following immunohistochemical processing, sections were mounted on gelatin-coated slides, dehydrated, cleared and coverslipped using Cytoseal 60 (VWR Scientific, West Chester, PA).

4.2.3. Tissue analysis

Processed tissue sections were examined and photographed with a Zeiss Axioplan photomicroscope. Images were digitized using a Hamamatsu digital camera (Hamamatsu Photonics, Hamamatsu, Japan) and a Simple-32 PCI image analysis system (Compix, Lake Oswego, OR). Mapping of the distribution of infected neurons in sections at a frequency of 210 μm through the rostrocaudal extent of the forebrain and brainstem was accomplished using Stereo Investigator software (MicroBrightField, Williston, VT). In order to test our hypothesis, this study focused on the forebrain projections to the caudal lateral hypothalamus. We analyzed the patterns of infection throughout the entire forebrain, but focused primarily upon infected neurons observed in the lateral septum and extended hippocampal formation (hippocampal formation plus entorhinal cortex).

Historically, the LS had been divided cytoarchitectonically into three main areas: LSD (dorsal LS), LSI (intermediate (medial) LS) and LSV (ventral LS) (Swanson and Cowan, 1979; Paxinos and Watson, 1989; for review see Jakab and Leranth, 1995). However, this paper will use the more recent nomenclature based on Risold and Swanson (1997a), which identified subdivisions of the LS according to chemoarchitecture. For the most part, LSD can be considered LSc, LSI can be considered LSr, and LSV can be considered LSv. Data detailing the

subdivisions within those areas are presented in Table 3 of Risold and Swanson 1997a and provide the basis for the hypothalamic nomenclature in Swanson's altas of the rat brain (2004) that was used in the analysis of these data.

4.3.Results

This experiment was designed to test the hypothesis that a polysynaptic pathway links the hippocampal formation and the lateral, caudal hypothalamus via the lateral septum. This results section will describe in detail six cases (three 60 hr and three 72 hr survival times) that are representative of the range of the entire data set. Some cases not described here were unusable in the present analysis because they were located outside of the target areas or they failed to produce a productive infection.

Several criteria were used to identify the zone of viral uptake that lead to productive infection of neurons. Injection sites were first identified by locating the end of the cannula tract in the tissue. In all cases, at least two bins of tissue were processed to obtain an accurate localization of the cannula. Axon terminals have the highest affinity for alpha herpesviruses (Vahlne et al., 1980; Marchand and Schwab, 1986). Thus, virus is often taken up by terminals and transported retrogradely from the injection site. Because of this, the extent of infection around the cannula tip is not a reliable determinant of viral diffusion and uptake. Previous work has determined that the zone of virus uptake that leads viral replication after an injection of 100 nl (1 x 108 pfu/ml) of PRV delivered at 20 nl/min is within an approximate 500 μm radius of the cannula tip (Jasmin et al., 1997; Card et al., 1999). Therefore, injection sites in this study were defined by the end of the cannula tract plus a 500 μm radius surrounding that point. The zone of uptake was biased toward the lateral hypothalamus through the use and positioning of a needle with a beveled tip, so this is a conservative estimate of viral spread. Due to the small size and

depth of the nuclei in the target area, most injections were not isolated to one nucleus (such as SUM), but usually extended to two or more (such as SUM plus LHA).

4.3.1. PRV transport following 60 hr survival times

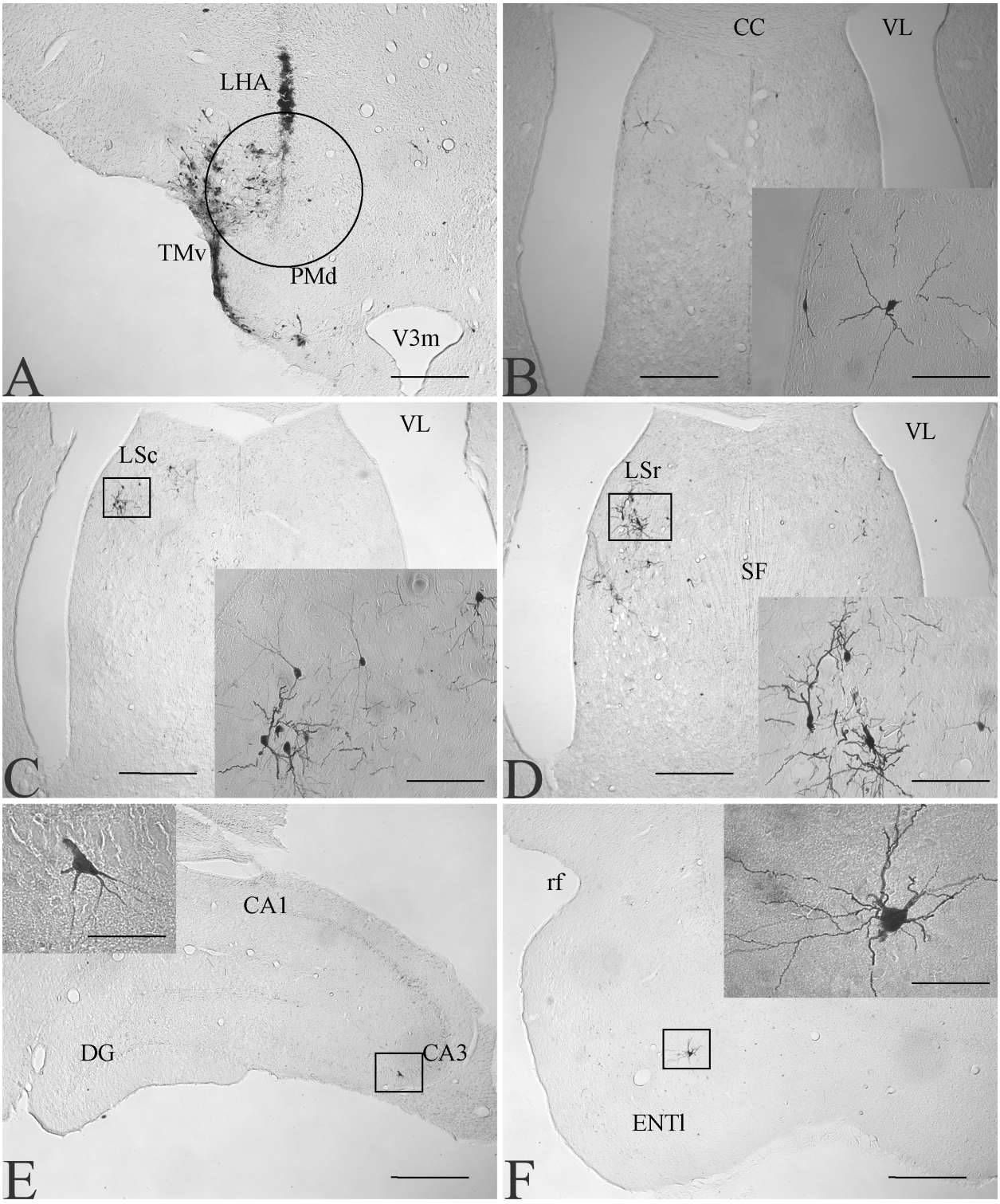
Data were analyzed from 23 cases in which PRV was injected into the caudal/lateral hypothalamus and the animals were killed 60 hours post injection. The location and magnitude of infected nuclei in these brains were identified based on standard atlases of the rat brain (Paxinos and Watson, 1998; Swanson, 1998; Swanson, 2004). The estimated zone of PRV uptake in all of the cases included several nuclei. For example, the injection site in Fig 4 was centered in LHA but included ventral tuberomammillary nucleus (TMv), dorsal premammillary nucleus (PMd), and SUM. Conversely, the injection site in Fig 5 was centered in MMN but included TMv, and SUM. In all cases, extensive retrograde infection of neurons that projected to the injection site was observed. The extent of infection at each injection site varied according to the nuclei involved, with the most extensive infection observed when the injection involved nuclei with local circuit connections. This concept is examined in greater detail in the Discussion.

The cases in the 60 hr survival time group exhibited infected cells in the LS, which is known to provide extensive monosynaptic projections to the hypothalamus (Risold and Swanson, 1997b). Specifically, the LS labeling was most often found in the dorsal LS in areas included in the caudal part of the LS (LSc). Some cases had infected cells in the LSr, septohippocampal nucleus (SH) and septofimbrial nucleus (SF). There were no cases in which the LSv contained infected cells at 60 hrs. For the most part, LS infections at 60 hr survival times were confined to the ipsilateral LS, although occasionally a few cells very found in the ipsilateral LS.

Figure 4.

Photomicrographs of an injection site (A) and retrogradely infected nuclei (B-F) in a 60 hr survival time animal. A) The circle represents the injection site centered at the end of the cannula tract, and its radius represents the theoretical 500μm spread of PRV. LHA and TMv contain infected cells. B-D) Rostral-caudal progression of lateral septal images. Boxed cells are shown at higher magnification in the insets. E) Dorsal hippocampal labeling limited to one cell in CA3. F) Infected cell in lateral entorhinal cortex.

Figure 4.



Hippocampal labeling was observed mostly in CA3 and there was minimal labeling in DG or CA1. The subiculum (SUB), including both dorsal (dSUB) and ventral (vSUB) subregions, was not observed to contain infected cells following 60 hrs. The parahippocampal region was found to contain very few (if any) infected cells, and these were found in lateral entorhinal cortex (ENTl) or the postpiriform transition area (TR) adjacent and medial to rostral ENTl. Infected cells in TR were only apparent in cases where the piriform cortex was also labeled. Entorhinal cells were found in both superficial (II and III) and deep (V and VI) layers. There were no labeled cells in medial entorhinal cortex, (ENTm), perirhinal cortex (PERI), or other caudal cortical areas. There was no labeling found in the presubiculum (PRE), postsubiculum (POST) or parasubiculum (PARA) or retrosplenial cortex (RS).

Each figure presented in this paper includes photomicrographs of the injection site, a series of LS sections, the dorsal hippocampal formation and rostral parahippocampal region. The figures showing 72 hr survival times also include additional photomicrographs of the hippocampal formation and the caudal parahippocampal region. There were infected cells found in areas outside of those shown in the figures. However, we feel that the labeling in the areas of interest (injection site, LS, hippocampal formation and parahippocampal region) are thoroughly represented in the figures. For example, several figures do not include photomicrographs of dSUB or ENTm, and the reader can therefore conclude that no infected cells were found in these areas of interest.

Figure 4 is an example of a case in which the injection site included the LHA, TMv and PMd (Fig 4A). Fig. 4B, C and D show the extent of LS labeling in a rostral to caudal series. Fig 4B and its inset show two infected cells in rostral LSc, Fig 4B shows several infected cells in

lateral LSc, and Fig 4C shows more infected cells in lateral, caudal LS in the LSr area. One infected cell is shown in dorsal/proximal CA3 (Fig 4E), and one infected cell in deep rostral ENTl is shown in 4F.

The injection site of the case shown in Figure 5 was located in the mammillary complex, and included the caudal medial mammillary nucleus (MM), SUM and TMv (5A). The extent of viral spread may have also included LMN and more medial subregions of the MM (outside of the plane of section. The entire LS contained infected cells in both LSc and LSr (5B, C and D). The hippocampal labeling was confined to dorsal/distal CA3 (5E) and did not included DG, but intermediate CA1 cell can be observed in 5F. Infected cells in ENTl were found in superficial layers (5F).

Figure 6A illustrates an injection site at which the syringe may have perforated the ventral surface of the brain. However, an adjacent section (6B) reveals that the ventral surface is intact. It is more likely that the syringe in this case got extremely close to the ventral surface without breaking it, and the section in 6A fell apart during processing. This section is also broken along the syringe tract, indicating that the plane of section included tissue divided by the path of the syringe. The injection site in this case is rostral and ventral to those in Fig 4 and 5, and it includes the TMv, LHA and possibly the LMN (rostral and outside of the plane of section). This brain had very few infected cells in the LS, none in the hippocampal formation, and only a few in the parahippocampal region. The only infected cells in the LS were located rostrally and confined to a very specific, medial region of LSr (6C). A higher magnification photomicrograph of these cells is shown in 6D. Alternatively, these cells could be located in SH and this subject is addressed in the Discussion. 6E illustrates the extent of parahippocampal labeling in both

superficial and deep ENTl, and 6F shows the lack of infected cells in the dorsal hippocampal formation in the same section as 6E.

Figure 5.

Photomicrographs of an injection site (A) and retrogradely infected nuclei (B-F) in a 60 hr survival time animal. A) Injection site centered in the caudal mammillary complex, with labeling in TMv and SUM. B-D) Lateral septal sections with labeled cells in LSc and LSr. E) Dorsal hippocampal labeling in CA3. F) Parahippocampal labeling in lateral entorhinal cortex and the post-piriform transition area.

Figure 5.

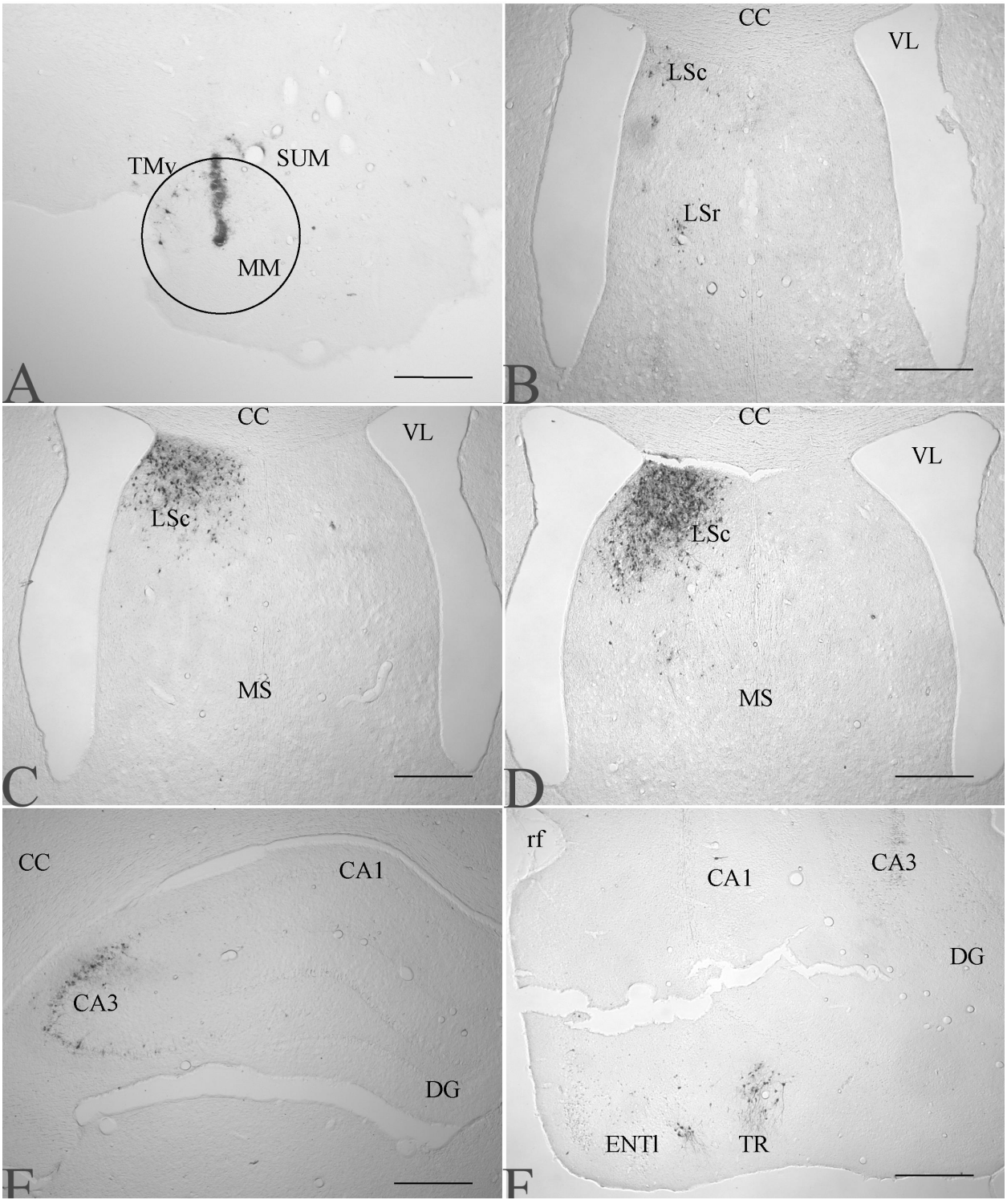
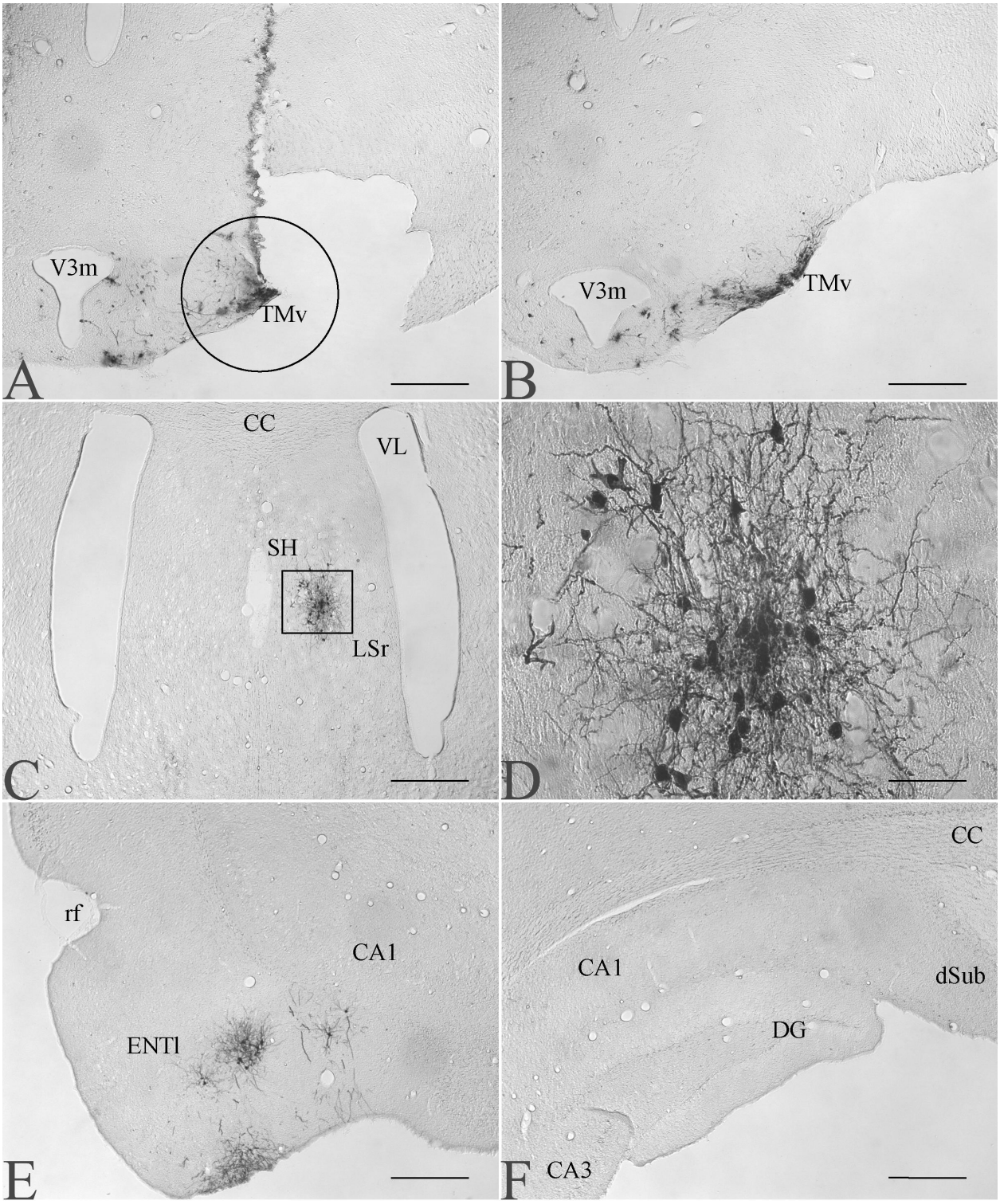

TMv
SUM
MM
A
CC
VL
LSc
LSr
B
CC
VL
LSc
MS
C
CC
VL
LSc
MS
D
CC
CA1
CA3
DG
E
rf
CA1
CA3
DG
ENTl
TR
F

Figure 6.

Photomicrographs of an injection site (A) and retrogradely infected nuclei (B-F) in a 60 hr survival time animal. A) Injection site centered in premammillary area and TMv. B) Section adjacent to injection site. C) Lateral septal labeling was confined to this septohippocampal/medial LSr area. D) Higher magnification view of cells in C. E) Labeling in lateral entorhinal cortex. F) No labeling was found in any areas of the hippocampal formation, including dorsal hippocampus shown here.

Figure 6.


V3m
TMv
A
V3m
TMv
B
CC
VL
SH
LSr
C
D
rf
CA1
ENTl
E
CC
CA1
dSub
DG
CA3
F

4.3.2. PRV transport following 72 hr survival times

Data were analyzed from 13 cases in which PRV was injected into the caudal/lateral hypothalamus and the animals were killed 72 hours post injection. These cases did not differ from those in the 60 hr survival group in terms of injection site location. Again, all of the injection sites were located in the caudal/lateral hypothalamus and included multiple nuclei. The extent and magnitude of infected cells observed in the 72 cases was much higher than that found in the 60 hr cases, as is expected following a longer survival time. In general, infected cells were found across a broad range of the LS, hippocampal formation and parahippocampal region. Other regions were also labeling, but will not be mentioned as they are outside the scope of this investigation.

Figure 7A illustrates a case in which the injection site is located in the LHA, and includes the SUM, TMv and PMd. Figure 7B shows LS labeling in LSc, SH and medial LSr. Labeling in LS extends caudally in both the LSc and LSr as shown in Figure 7C and 7D. There are also a few infected cells is the contralateral LS throughout its rostrocaudal extent. Infected cells in the dorsal hippocampal formation are shown in Figure 7E, where CA3 is labeled along its proximal/distal extent. There are also a few infected cells in DG but none inCA1. Figure 7F is a composite of several photomicrographs illustrating the hippocampal formation and parahippocampal region in one section. In the hippocampal formation, extensive labeling was found in CA3, while the labeling in CA1 and DG was less dense. Infected cells in CA1 are concentrated in the intermediate and ventral regions, but there are some scattered infected cells in dorsal CA1 as it continues into dSub. In the parahippocampal region, infected cells were

found in TR, ENTl and PERI. Lateral entorhinal cells in layer II can be seen throughout its medial lateral extent, and continue into PERI.

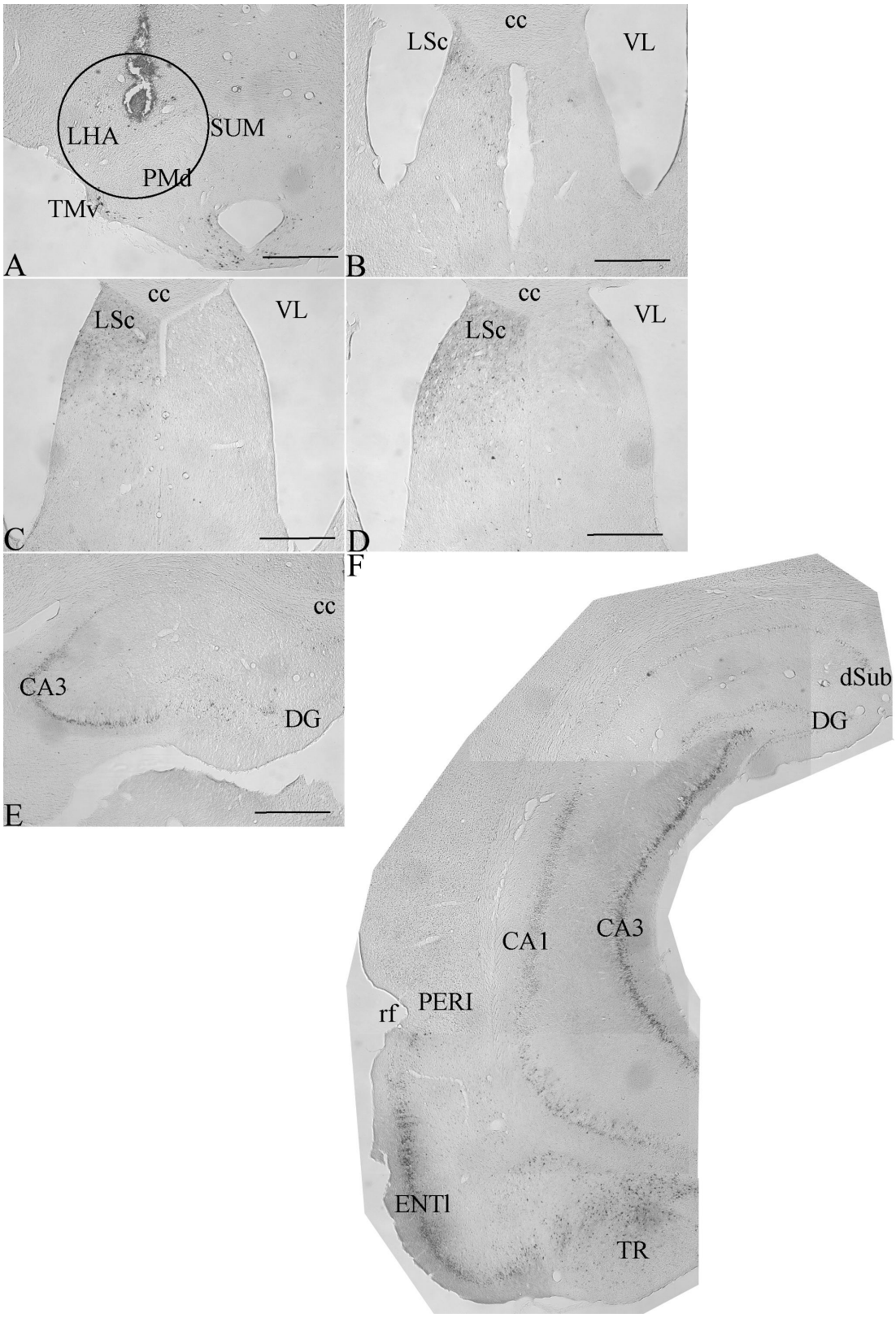
Figure 8 illustrates a case in which the injection was placed in the mammillary complex and included LMN, MMN and TMv. Labeling in Figure 8A can be observed in all of these areas as well as SUM. Infected cells were observed throughout all sections of the LS, and they were most heavily concentrated in the ipsilateral LSc and LSr (Fig 8B, C and D). There were also some infected cells in the medial septum (MS) and SF. In the dorsal hippocampal formation, CA3 was extensively infected (Fig 8E and F) along its proximal/distal extent, and DG was found to have several infected cells as well. Figure 8E illustrates heavy infection throughout the dorsal-ventral extent of the hippocampal formation, as well as the parahippocampal region in that slice. Similar to the case in figure 7, CA3 is densely infected while CA1 is most heavily labeled in its intermediate and ventral regions; dorsal CA1 and dSub also have some infected cells. Unlike the case in Figure 7, infected cells are also observed in vSub, ENTm. ENTl is heavily infected in both superficial and deep layers, which extend in to PERI. Additional, more caudal labeling in the parahippocampal region is illustrated in Figure 7H, where infected cells are located in POST, PARA, ENTm, ENTl and PERI. Layer V of entorhinal cortex is most heavily labeled in both ENTm and ENTl.

Finally, Figure 9 illustrates a case in which the injection site was located in the LHA, and included the SUM and TMv (Fig 9A). Similar to the case from Figure 8, LS labeling is extensive and heaviest on the ipsilateral side (Fig 9B, C and D). Dorsal CA3 contains many infected cells (Fig 9E), and dorsal CA1 and DG at this level were also infected but not shown. The rest of the hippocampal formation and parahippocampal region in Figure 9F are similar to 8G, although there is more extensive intermediate CA1 labeling in 9F. Figure 9G also illustrates

a pattern of infected cells that is similar to 8H. However, it is clear in 9G that POST is not labeled.

Figure 7.

Photomicrographs of an injection site (A) and retrogradely infected nuclei (B-F) in a 72 hr survival time animal. A) Injection site centered in LHA. B-D) Lateral septal images with labeling in LSc. E) Dorsal hippocampal labeling in CA3. F) More extensive labeling throughout intermediate and ventral hippocampal formation, as well as the lateral entorhinal cortex and perirhinal cortex.


LHA
SUM
PMd
TMv
A
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B
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LSc
VL
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LSc
VL
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F
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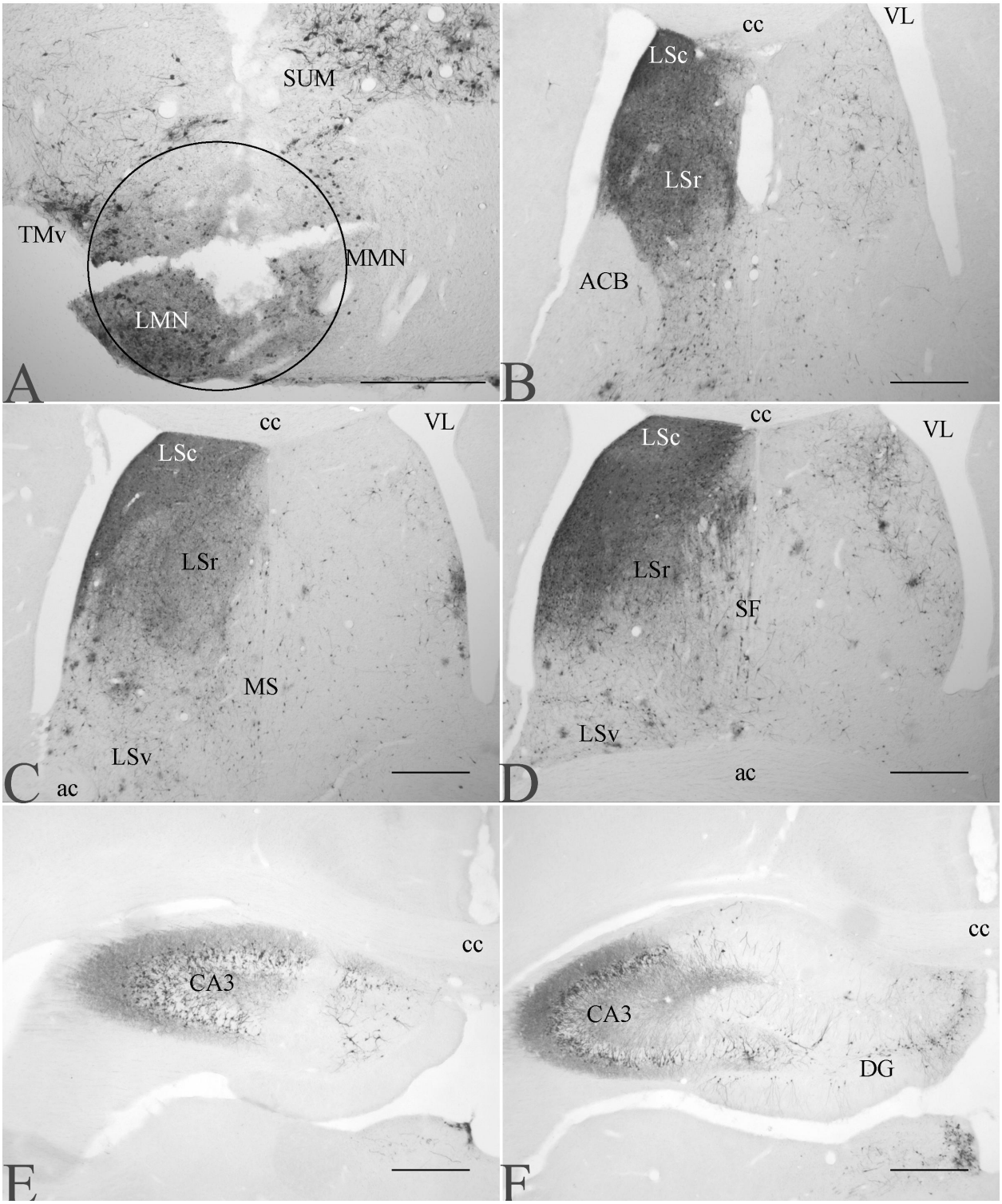
CA3
DG
E
dSub
DG
CA1
CA3
rf
PERI
ENTl
TR

Figure 7.

Figure 8.

Photomicrographs of an injection site (A) and retrogradely infected nuclei (B-H) in a 72 hr survival time animal. A) Injection site centered between LMN and MMN. B-D) Extensive labeling throughout ipsilateral lateral septum, with some more sparse labeling contralateral to the injection site. E-F) Infected cells in dorsal hippocampus in CA3 and DG. G) Parahippocampal region labeling was more extensive in 72 hr cases than in 60 hr cases. Labeling extends into dorsal and ventral subiculum as well as ventral medial entorhinal cortex. H) Caudal parahippocampal labeling extending into perirhinal cortex, parasubiculum and postsubiculum.

Figure 8.


SUM
TMv
MMN
LMN
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SF
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CA3
E
cc
CA3
DG
F

Figure 8 (cont).

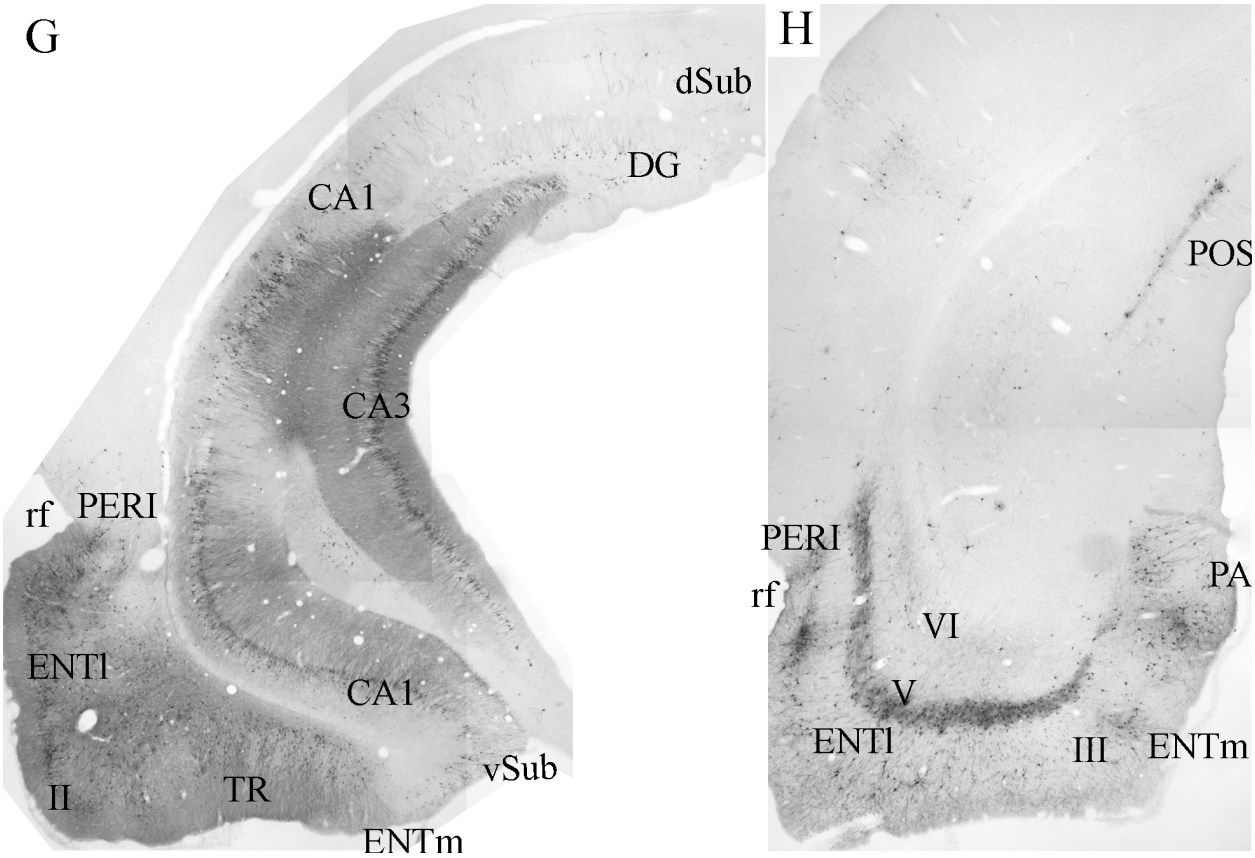
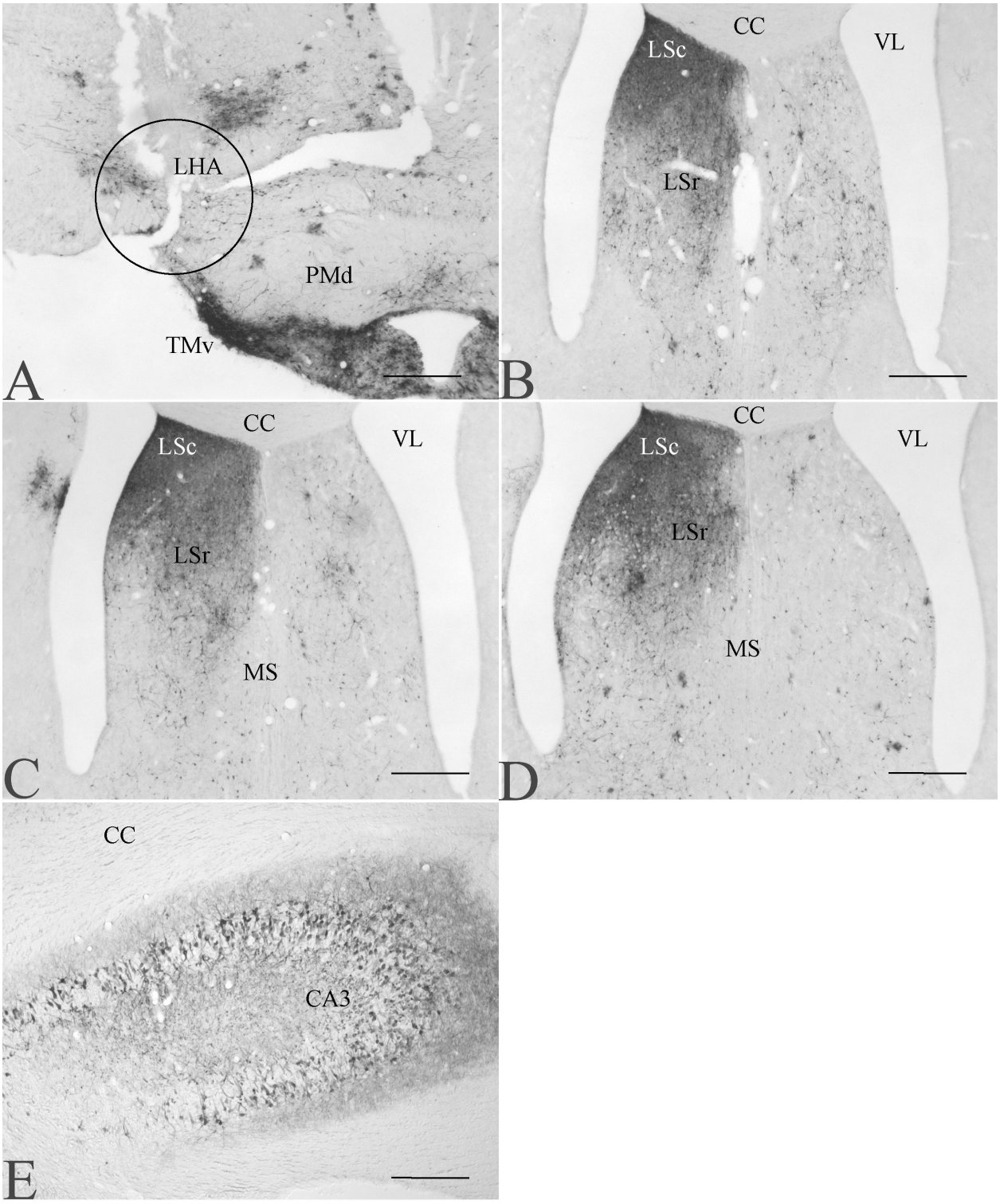


Figure 9.

Photomicrographs of an injection site (A) and retrogradely infected nuclei (B-G) in a 72 hr survival time animal. A) Injection site centered in LHA. B-D) Heavy labeling throughout LSc and LSr similar to Figure 8. E) Heavy dorsal CA3 infection. F) Additional labeling concentrated in intermediate hippocampal formation, with extensive parahippocampal labeling. G) Caudal parahippocampal labeling is again seen to include medial entorhinal cortex and parasubiculum.

Figure 9.


LHA
PMd
TMv
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LSc
VL
LSr
B
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VL
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MS
C
CC
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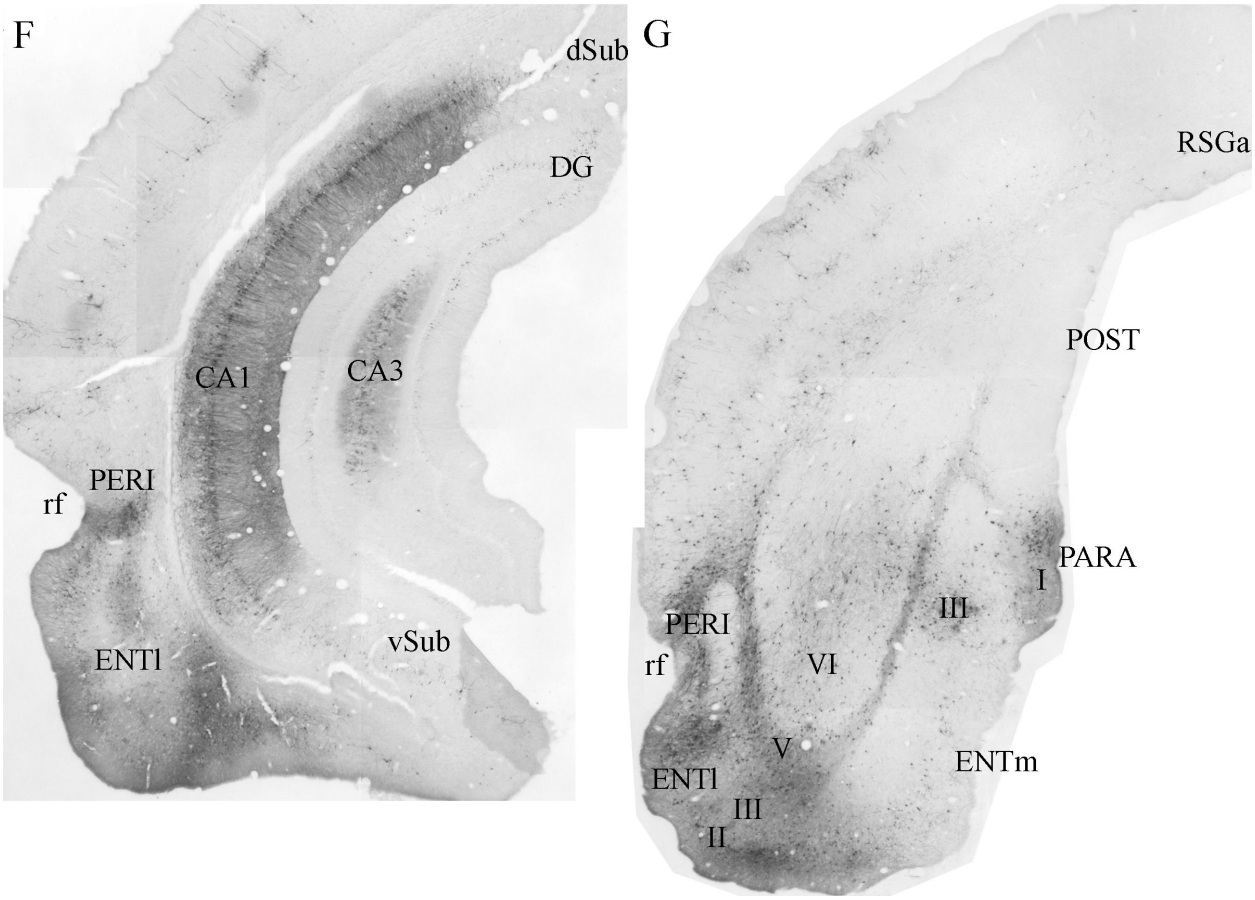
LSr
MS
D
CC
CA3
E

Figure 9 (cont).



4.4.Discussion

The present study both confirms and extends previous work examining monosynaptic projections between the hippocampal and lateral septum, and the lateral septum and hypothalamus (Risold and Swanson, 1996, 1997b). Our use of a polysynaptic retrograde viral tracer allows for the evaluation of the synaptology, or synaptic connectivity, of circuits predicted by monosynaptic tracing studies. These data support the hypothesis that the hippocampal formation projects to the hypothalamus via a polysynaptic pathway that includes the lateral septum, and extend it by providing insight into the microtopography of the circuitry. This conclusion will be supported by evidence and reasoning presented in this discussion section. Novel anatomical evidence suggesting that the entorhinal cortex participates in this circuit will be discussed, as predicted by Alonso and Kohler (1984). Finally, functional implications of the circuitry examined in this study will also be discussed.

4.4.1. Technical considerations

The factors that influence the uptake and transport of PRV through neural circuitry following intracerebral injection are important considerations in evaluating the conclusions presented in this study. Prior work has demonstrated that the affinity of alpha herpesvirus envelope glycoproteins for neuronal profiles and extracellular matrix molecules restrict the diffusion of PRV injected intraparenchymally (see Card et al., 1999, for review). As noted earlier, detailed studies indicate that the volume and concentration of PRV used in the present analysis is taken up within a 500 μm radius of injection (see Card, 2001 for review). Nevertheless, it is clear that the architecture of the region of injection exerts an important influence upon the zone of virus uptake. Important in this regard is the demonstration that axon

terminals exhibit the highest affinity for PRV (Marchand and Schwab, 1986). Thus, the results of the present study must be viewed within the context of the architecture of the cell groups located in our injection sites.

The extent of local circuitry within the nuclei in our injection sites must be taken into consideration when evaluating these data. Previous studies report that the mammillary complex contains few local circuit neurons and that the neuropil is composed principally of afferents from other regions (Allen and Hopkins, 1988; Hayakawa and Zyo, 1992; Takeuchi et al., 1985; Gonzalo Ruiz et al. 1993). In contrast, local circuit connections provide a more prominent component of the supramammillary nucleus neuropil (Hayakawa and Zyo, 1984). These observations are parsimonious with the sparse infection of LMN neurons observed in cases in which the injection site was centered within LMN and the prevalence of infected SUM neurons in cases where the injection involved this nucleus. There is no clear evidence suggesting that the TMv has a large amount of internal connectivity, although the extent of TMv infection in the present suggests that it may.

4.4.2. The organization of polysynaptic pathways linking the hippocampal formation and the hypothalamus.

4.4.2.1. Lateral septal projections to the hypothalamus.

Neurons in the lateral septum project to the hypothalamus in a topographically organized manner. This organization has been described by studies using monosynaptic tracers in both the rat (Swanson and Cowan, 1979; Risold and Swanson, 1996, 1997b) and guinea pig (Staiger and Nurnberger, 1991a). The general organization of these projections is such that progressively dorsal areas in the LS project to progressively lateral, longitudinal regions or "zones" of the hypothalamus (Swanson, 1987 book; Staiger and Nurnberger, 1991a; Risold and Swanson, 1996,

1997b). For example, the LSv projects to nuclei in the hypothalamus located in the periventricular region, while neurons in the LSr project to a medial zone of hypothalamic nuclei located lateral and adjacent to the periventricular zone. LSr targets include the anterior hypothalamic nucleus, dorsomedial hypothalamic nucleus, ventromedial hypothalamic nucleus and dorsal premammillary nucleus (Risold and Swanson, 1997b). These areas are thought to be involved in mediating social/defensive behaviors in rats (for extensive review of the literature supporting this hypothesis, see Risold and Swanson, 1997b.). The most lateral areas of the hypothalamus, including the lateral hypothalamic area, and lateral supramammillary area receive input from all areas of the LS.

Our data both confirm and extend these observations because the majority of our LS labeling, especially at the 60 hr survival time, was located in LSc. However, at the longer survival time, the number of cells infected in the LSr greatly increased with no remarkable differences in the injection sites between the 60 hr and 72 hr cases. This is most likely due to extensive intraseptal connectivity.

4.4.2.2. Intraseptal connectivity.

Areas of the lateral septum are heavily interconnected (Alonso and Frostcher, 1989; Jakab and Leranth, 1995; Phelan et al., 1989; Staiger and Nurnberger, 1991b), resulting in the likely transport of PRV between LSc, LSr and LSv at longer survival times. The organization of interconnectivity is such that LS axons collateralize within each subregion, and each subregion of LS also projects to the other subregions along rostrocaudal, dorsoventral and bilateral pathways (Jakab and Leranth, 1995). This provides an explanation for both of the following observations in our 72 hr cases in comparison to the 60 hr cases: greater density of infection

within LS subregions (especially LSc and LSc), and a greater range of infection (especially LSr). There is also evidence to suggest that LS neurons project to the MS (Swanson and Cowan, 1979; Leranth and Frotscher, 1989; Leranth, 1992; Witter et al., 1992, Risold and Swanon, 1997b), although the organization of this projection has been debated (Leranth et al., 1992; Witter et al., 1992). Although this projection was not examined in the present study, it provides insight into the functional role of the LS, discussed below.

4.4.2.3. Hippocampal projections to lateral septum.

The organization of hippocampal output to the LS has been studied extensively. CA3 has been shown to project to LSc, while CA1 and SUB project to LSr and LSv (Swanson and Cowan, 1977; Risold and Swanson, 1996, 1997b). Following 60 hr survival times, the CA3 subregion of the hippocampus was labeled in all of the cases in which LSc contained infected cells (see Fig. 4 and 5). Interestingly, CA3 did not contain infected cells when LSc was unlabeled, as shown in Fig. 6. Risold and Swanson (1997b) also suggest that more ventral regions of the LS are innervated by more dorsal regions of the hippocampal format. Our data do not speak to this issue, as most of the ventral labeling in the hippocampal formation was found only in the 72 hr cases. However, Fig 4B-E illustrate a case in which ventral LS corresponds with dorsal CA3 labeling.

Our cases from the 72 hr survival time exhibit extensive hippocampal labeling, especially compared to the 60 hr cases. For example, CA1 and SUB were rarely infected at 60 hrs, whereas they were sometimes filled with infected cells at 72 hrs (Fig 7F, 8G, 9F). CA1 and SUB labeling is interesting because, due to their projections to LS, they should only be labeled if LSr or LSv are infected. These extensive infections in CA1 and SUB are probably due to the internal

connectivity of the LS. For example, PRV may originally been transported retrogradely to LSc (as seen in the 60 hr cases), then to LSr, and then to CA1. As discussed in the previous section, this could explain both the increase in LSr and LSv labeling as well as CA1/SUB labeling.

The dentate gyrus is not known to have projections to LS, and none of our 60 hr cases had DG labeling. At the 72 hr survival time, DG was often observed to be sparsely infected (see Fig 8E, F and G; Fig 9F and G). These cells were most likely infected via their projections to CA3, which is why they were labeled only after the longer survival time.

It is interesting to note that dorsal CA1 was not infected to the extent predicted in the introduction. Apparently, retrograde transport of PRV from LSr to CA1 favored the intermedial and ventral CA1 subregions. There were a few infected cells in dorsal CA1 (Fig 8G and 9F) but not nearly to the extent of the rest of CA1. Dorsal CA1 is the area of the hippocampus which has been most widely studied in the place cell literature. Other areas in the hippocampal formation including CA3 (Barnes et al., 1990) and SUB (Sharp and Green, 1994; Sharp, 1997) have been shown to have place related activity.

4.4.2.4. Entorhinal projections to LS.

Alonso and Kohler (1984) used anterograde and retrograde monosynaptic tracing methods to show that deep ENTm, and both superficial and deep ENTl, send projections to the entire lateral septum in an overlapping manner. However, they found that the majority of the ENT projections terminate in the "intermediate" LS, which is now considered to be the medial LSr, or SH. Remarkably, Figure 6 provides compelling support for this projection. Fig 6C and D in our study illustrate PRV labeling in the identical LS area where Alonso and Kohler (1984) illustrate ENT projections to the LS using WGA-HRP injections into ENT(their Figure 10). In

Fig 6E of our study, there are infected cells in deep ENTl. Also, the hippocampal formation was devoid of infected neurons in this case. Taken together, both Alonso and Kohler (1984) and the present study provide evidence for entorhinal projections to the LS. In our study, however, the ENT infection was most likely due to transynaptic retrograde transport of PRV from the injection site to the LS, and from the LS to the ENT.

Risold and Swanson (1997b) did not observe any labeling in the entorhinal cortex following injections of retrograde tracers into LS. However, none of those injections were located in the medial LSr ("intermediate" LS) or SH. On the other hand, they did inject PHAL into the medial LSr, with resultant labeling in the caudal lateral hypothalamus (Risold and Swanson, 1997a Fig 16A and E). Their hypothalamic labeling corresponds to the injection site in the case of our Fig 6). Taken together, we believe that data from these three studies regarding the projection from ENT to LS to the lateral hypothalamus provides another layer of complexity regarding the functional implications of the circuit.

In the present study, the vast majority of infected cells in the ENT were located in the ENTl. This is interesting because it has been widely hypothesized that the ENTm is responsible for transporting spatial-related sensory information into the hippocampal formation, while the ENTl is more likely responsible for olfactory and auditory information (for review, see Aggleton et al., 2000; Witter and Wouterlood, 2002).

4.4.2.5. Head direction input to the circuit

The present study aimed to explore the potential pathways via which HD cells may contribute to the circuit of interest. Specifically, the POST contains HD cells (Taube et al., 1990), and projects monosynaptically to all areas of the hippocampal formation as well as the LMN (van Groen and Wyss, 1990). We expected POST to contain infected cells in each 72 hr

case whose injection site included the LMN (at least 7 cases). However, the only case in which the POST contained labeled cells is shown in Figure 8F, although several cases exhibited clear labeling of LMN neurons. It is therefore possible that POST terminals in LMN did not take up PRV, nor did the LMN neurons receiving synaptic input from POST. There is no specific evidence suggesting that HD cells in the LMN receive input from HD cells in POST, so perhaps this result is indicative of multiple subpopulations of cells within LMN. Regardless, a lack of labeling in POST is not necessarily indicative of a lack of involvement in LMN.

4.4.3. Functional implications of the proposed polysynaptic circuitry: Role of the lateral septum

These data can be used to augment the understanding of the role of the LS in information processing, the organization of entorhinal and hippocampal interactions with the LS, and the organization of spatial information processing.

The role of the LS has not been well defined, although the organization of its intrinsic connectivity suggests that it is involved in processing information (Staiger and Nurnberger 1991b). Each region of the LS seems to project ventrally to other regions of the LS. For example, (cite) showed that LSc neurons send axons ventrally that appear to terminate in LSr or LSv. Also, the LS arguably projects to the MS, which provides massive input to the entire hippocampal formation. The MS input to the hippocampus is thought to organize hippocampal activity by terminating on basket cells in the hippocampus, which provide inhibitory input to hippocampal pyramidal cells (Freund and Antal, 1988). One of the potential roles of the LS could therefore be integrate influence the MS input to the hippocampus.

The results from the present study also shed light on the role of the LS as the projections from the LS to the hypothalamus could be viewed in several ways. For example, the LS could

simply be relaying specific information from functional domains of entorhinal cortex and hippocampal formation to the hypothalamus. On the other hand, it could be integrating this information, along with additional entorhinal and hippocampal information as well as subcortical input, before relaying it to the hypothalamus. Specifically, it seems that as information flows through the entorhinal cortex and hippocampal formation, at each level there is a topographically organized projection to the LS. These areas in the LS in turn project in a topographically to the lateral/caudal hypothalamus. Perhaps the LS is integrating progressively processed information coming out of the entorhinal cortex and hippocampal formation along with subcortical input, before relaying it to the hypothalamus and MS.

Deeper insight into the organization of the LS and its subcortical inputs reveals another layer of complexity to the system. The principal cells in the LS, "somatospiny neurons" have been found to contain GABA (Jakab and Leranth, 1990) and are the cells that project outside of the septal area (Jakab and Leranth, 1995), presumably to the hypothalamic regions of interest in this study. The LS receives subcortical input from a wide variety of hypothalamic regions (Jakab and Leranth, 1995) to the extent that the projections from LS to hypothalamus are reciprocated. Taken together, this information adds another dimension of dynamic regulatory capacity to the LS. It is most likely an important integrative center for both hippocampal information and hypothalamic information.

These ideas are summarized by the connectivity in Figure 10, where different colored lines represent information originating in the entorhinal cortex and hippocampal formation as they progress through the polysynaptic circuit. However, it is important to realize that at every level of the model there is great potential for the integration of such information as it travels through the circuit.

Figure 10.

Schematic diagram illustrating the organization of information processing from cortex to the hypothalamus. "Multimodal association cortices" is a blanket term referring to the areas projecting into superficial entorhinal cortex, including perirhinal and postrhinal cortex, pre/postsubiculum and parasubiculum. The distributed superficial ENT projection to all of the LS nuclei is represented by the grey curved arrow at the left. Red and green lines represent projections from ENTm and ENTl, respectively. Hippocampal information is integrated internally as it proceeds through the hippocampus. More importantly, it is sent to LS following every "step" of the way. LS is also integrating information internally, and then projecting it to the hypothalamus. Subcortical input into these area is not shown for the sake of simplicity.

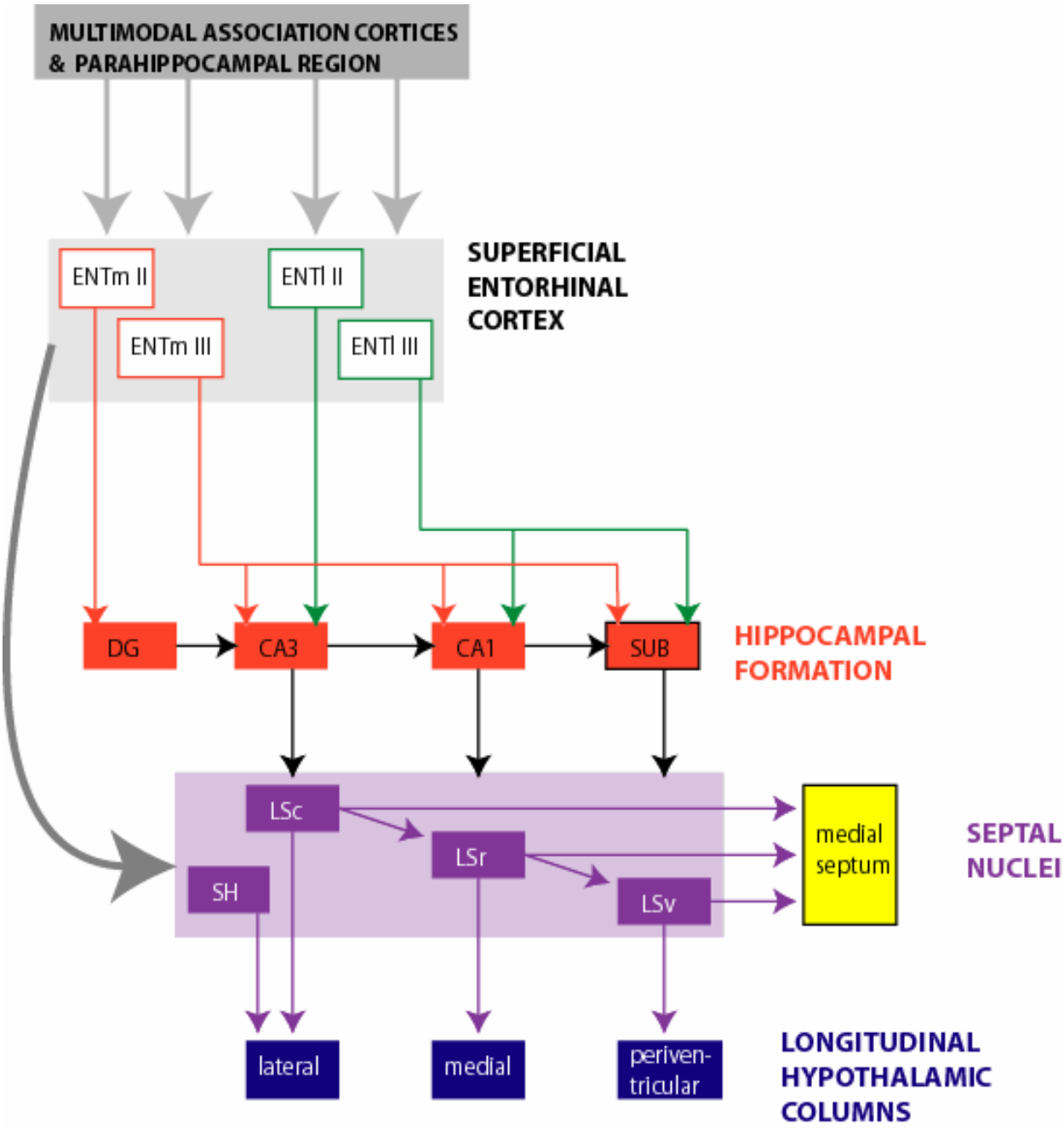

MULTIMODAL ASSOCIATION CORTICES
& PARAHIPPOCAMPAL REGION
ENTm II
ENTl II
ENTm III
ENTl III
SUPERFICIAL
ENTORHINAL
CORTEX
DG
CA3
CA1
SUB
HIPPOCAMPAL
FORMATION
LSc
LSr
LSv
SH
medial
septum
SEPTAL
NUCLEI
lateral
medial
periven-
tricular
LONGITUDINAL
HYPOTHALAMIC
COLUMNS

Figure 10.

4.5. Summary

In summary, the results described here represent merely the foundation for future work investigating the interactions between the extended hippocampal formation, lateral septum and hypothalamus. We have confirmed and extended previous work by providing evidence for a polysynaptic pathway linking the hippocampal formation with the hypothalamus via the lateral septum. As a result of this work, it is clear that the role of the lateral septum must be studied in greater detail in order to understand the relationship between spatial information and motivated behavior.

5. DISSERTATION DISCUSSION

In this general discussion section I would like to address three issues. First, in section 5.1 I will revisit the data presented in Chapter 2 in light of a more recently published experiment that used similar techniques but generated results that appear to be orthogonal to those from our study. Next, in section 5.2 I will extend the discussion of data from Chapter 3 in order to propose a somewhat novel view of the organization of the HD system. Finally, I will use section 5.3 to bring together the data from the two anatomical experiments and synthesize a model that describes how the HD model from section 5.2 fits into the data described in Chapter 4. I believe that addressing these issues in this manner will clarify the value of the dissertation relative to the current literature.

5.1. Extended Discussion of Chapter 2

What exactly does "place cell" activity code for? Scores of neurophysiological experiments over 30+ years have been designed and carried out in order to answer this one question. Most of these experiments examined the activity of hippocampal pyramidal cells under experimental conditions that differed slightly from one to the next, each one aiming to make its mark in the literature. As described in the introduction, the simplest conclusion that may be reached after all of this time is that whatever the hippocampus (or hippocampal pyramidal cells) is (are) doing, it sure is complicated! This is by no means meant to be a trite characterization. After all, one would imagine that if the answers were simple, it would not have taken 30 years of mostly intelligent scientific labor to reach the point where it is generally accepted that "place cells" code for more than just "place."

The experiment presented in Chapter 2 was conceived and conducted between 1998 and 2001, which makes it several years old at the time of this writing in 2004. It was designed to test the hypothesis that the hippocampus responds coherently to complex cue manipulations. Our results generally verified this hypothesis, indicating to us that hippocampal activity is better suited to the representation of spatial information than simple association between individual cues. However, we did not consider our results to be the death knell for relational theories of hippocampal function – just more supportive of the cognitive map theory.

I have had three years to think about this experiment. Over this time, I have come to more fully realize that our results indicated that the hippocampus is performing operations that are much more complicated than providing an internal representation of space to the rat. Therefore, it is important for to revisit the data in light of the current literature and the following four issues that have shaped my thoughts since 2001:

1) Data generated from another lab tested a similar hypotheses and led to conclusions relevant to those that we made (Knierim, 2002).

2) Some important questions remained unanswered by our published discussion (Brown and Skaggs, 2002).

3) Changes to the experimental paradigm that I would make in hindsight

4) There were two aspects of our experiment analysis that, in hindsight, were incredibly useful and should be applied to future experiments:
   a) The analysis of data from individual rats
   b) The analysis of changes in hippocampal activity over time

5.1.1. Reconciling Knierim (2002) with Brown and Skaggs (2002).

While our paper was under review, Knierim (2002) published a study whose organization was similar to others examining hippocampal pyramidal cell activity during cue conflict situations (Shapiro et al., 1997; Tanila et al., 1997; Knierim and McNaughton, 2001; Brown and

Skaggs, 2002). It was designed to test the effects of incremental changes in the relative positions of two cue sets on place cell activity. There were several major differences in the methods used by Knierim compared to our study. First, he used a circular track upon which the rats ran for food reward placed at arbitrary places along the track. Our study used a plus-shaped maze which forced the rats to sample an environment at predetermined intervals. The local cues used in his experiment were also textural cues placed on the circular track, and distal cues hung on curtains surrounding the track. Second, his testing sessions involved spreading apart the local and distal cue sets by intervals of 45º, 90º, 135º or 180º. Third, the training paradigms used in the two experiments were radically different. Otherwise, the two experiments were similar in most other areas.

Knierim (2002) found that when ensembles of simultaneously recorded hippocampal cells were recorded during cue conflict situations, some cells were controlled by the distal cue set while others were controlled by the local cue set. These results were unambiguous and provided evidence that the hippocampal activity can be discordant and controlled by multiple cue sets. How can this be reconciled with our data showing that discordant cue control never happened at a rate above that which was expected by chance?

The answer to this question is complicated. Most importantly, I believe that a major barrier to comparing the two studies is the fact that Knierim did not present an extensive analysis of data from individual rats. He did provide evidence that individual rats differed in the extent to which they were influenced by one cue set over another. However, his results would be more easily interpreted if specific differences between individual rats were discussed. I will cover the topic of the analysis of data from individual rats in a section below. Otherwise, I believe that the "discordance" between our experiment and Knierim's study is due to the differences in the

experimental design, especially the experimental apparatus and behavioral training. Additional reasons for the difference can be sorted out by looking at some minute details of our results.

5.1.1.1. Experimental apparatus.

First, our experimental apparatus was a plus-shaped track while Knierim's was an annulus (donut shape). The "plus" shape of our track forced the rats to run down maze arms that were set at 90º intervals. Had our maze allowed for a more thorough sampling of the available environment, such as an eight-arm radial maze, I believe that we may have seen more discordance in our results.

5.1.1.2. Training paradigm.

Second, the manner in which our rats were trained to run the maze was quite different from Knierim's training paradigm. His rats were allowed to run CW on the maze for food reward placed at one or two locations around the track. For our experiment, the rats had to be taught to sample all four maze arms before resampling an arm. This is a common task with radial maze experiments as it forces the rat to cover the entire available environment – something that is especially important in place cell experiments. In order to get our rats to achieve this behavior via training, I would initially confine them to the center of the maze after visiting four arms in order to get them to "reset" their strategy for sampling the maze. This effectively split the training into "trials" during which the rat could maximize its reward choosing previously unvisited arms during the four chances between confinements. I shorted the confinement times as the rats got better at sampling the entire maze. However, this procedure was not used during recording sessions. This led to a mismatch of sorts between training and recording, but more importantly I believe it led our rats to attend more closely to the local cue set. Over several

recording sessions without the confinement period, I believe that our rats became more aware of the entire environment and thereby were influenced by the other cue sets (distal cues and the reference frame of the recording room itself).

5.1.2. Important questions not fully addressed by the original paper's discussion.

The original published discussion was hampered by a lack of space necessary to answer several important questions regarding our data set. Plus, I have had three years to dwell on the results and I am therefore much better prepared to address them.

1. Why were some ensembles of cells controlled by the local cue set?

I propose that in our experiment the rats attended to the cue set that was closest (in space) to them until it another cue set became more useful. This proposal may at first seem to disagree with data sets that have shown that distal cues are more important than local cues in spatial tasks (Cressant et al., 1997, 1999; Gothard et al, 1996; Knierim et al., 1998; Muller and Kubie, 1987; O'Keefe and Nadel, 1978; Shapiro et al., 1997). It makes sense that distal cues would tend to exert more influence over spatial activity. As one moves though an environment, distal cues appear to move less than local cues relative to one's location. The most common example of this is how the moon seems to follow your car's path when you drive at night.

We therefore fully expected the distal cue set to exert all of the influence over the ensembles recorded in our experiment, and were surprised that they followed local cues during the initial recording sessions. Other studies have reported the importance of local cues to various extents, so it was not unprecedented that we came away with such results (Young et al., 1994; Gothard et al., 1996; Shapiro et al., 1997; Save et al., 2000, Knierim and McNaughton, 2001).

In fact, the place cell ensembles were always controlled by the local or distal cue sets until those two cues sets were scrambled in probe trials. In these sessions the room reference frame controlled ensemble activity. This leads me to believe that the rats were aware of the room reference frame, as well as consider the distal cue set as being perceived as spatially "in between" the local cue set and the room reference frame.

The reason for this is straightforward. The hippocampal activity did not correspond to the room reference frame until other cue sets, perhaps more "useful" cue sets, were found to be less stable. The rats were therefore aware of all of the sets of cues in the room, but only used those that were perceived to be the most stable. The apparent stability of a cue is a great predictor of its control over hippocampal place cell activity (Knierim et al., 1995). Knierim's group recorded hippocampal place cells from rats which were disoriented before and during recording sessions to the point where environmental cues were not longer a reliable predictor of spatial location within the recording chamber. Accordingly, their hippocampal place cell activity was not affected by rotations of those cues that were perceived to be unreliable. This makes good sense and fits well with our data.

It is important to note that we do not know which cue sets were controlling place cell activity at any time before we isolated cells and began recording. As previously stated I believe that the specific training that the rats received contributed to their attention to the local cue sets. The rats did not need to use a spatial strategy to obtain reward at the maximum rate, and therefore had no need to consider environmental cues other than those that were closest to the goal. The food reward was always placed at the end of an arm that contained a local cue, and in the processes of obtaining food the rat would likely notice the only differences between maze

arms, especially during training. The most obvious way to address this issue would be to record from the hippocampus during all phases of training and testing as described in a section below.

2. Our rats used a non-spatial strategy to run the plus maze. How did this affect the results?

The reward contingency in the task on which the rats were trained in our experiment was purposely non-spatial, except for during training when the rats were required to sample all four arms before any empty ones were rebaited. We created the experiment this way primarily to let the rats decide what type of strategy they would use to complete the task. Interestingly, the rats developed a non-spatial strategy to deal with this reward contingency: they simply sampled the arms in a clockwise or counterclockwise order. This turned out to be an excellent strategy because it allowed them to consistently find food in the next arm, and never enter an unbaited arm.

This type of strategy may at first seem like one that would lead the rats to ignore the environmental cues because, based on a strategy where the rats were sampling in a CW or CCW pattern, they were not predictors of reward. However, the hippocampal pyramidal cell activity clearly indicated that the environmental cues were relevant in terms of influencing place cell activity. The obvious "next step" in this case would be to perform a similar experiment where the reward contingencies were weighed and the rats would be forced to use a spatial strategy

3. What caused the switch from following local cues to following distal cues? And, why did the ensembles begin to remap in some rats?

I believe that these questions can be answered together. The more time the rats spent on the task, the less important the local cues became. After a certain amount of training and recording session they could reliably expect to obtain the food reward during their daily trip to

the recording room. At this point, the local cues became completely useless, and the rats became more aware of their surroundings. Distal cues then began to exert more control over place cell activity as they would in other task. Additionally, whatever cues corresponded to the room reference frame also became noticeable to the rat and exerted influence over place cell activity when the other cues became unstable in the probe trails. One way to test this hypothesis is to repeat the experiment with two changes that would allow us to access place cell activity during training and establish the timing of changes in cue set relevance.

5.1.3. Changes to the experimental design proposed in hindsight

Hindsight is a wonderful tool and I believe that the ideas presented here will undoubtedly influence any of my future work in this field.

1. Record hippocampal pyramidal cell activity from the beginning of training and continue throughout experimental testing.

Most place cell experiments reported in the literature are justifiably limited in scope because they are time and resource intensive projects. The actual recording of place cells doesn't begin until a rat has shown that it has learned the behavioral task. This is prudent because some rats simply never learn the task as a result of improper handling, training or stress. I believe that recoding place cells at the onset of training in every rat would be worth the resources committed to those rats which eventually are not useful for the main experiment. This is because we do not have a good idea about hippocampal place cell activity during the learning of spatial tasks.

Recording earlier in the experiment would allow us to determine whether the local cue set was the most important during the absolute beginning of the experiment. It would also allow us to determine the exact day upon which the distal cues because to exert more influence than the

local cues. One problem with this is that there are only so many productive recording days one can have when advancing tetrodes through the hippocampus. However, there are certain techniques too technical for this document that would hippocampal recordings to remain stable over longer periods of time.

2. Utilize the confinement paradigm throughout the recording sessions in one group of animals.

This would add a completely new and useful twist to the experiment because it would probably force that group of animals to use a more spatial strategy to gain the maximum reward. Differences in the control over place cell activity during spatial and non-spatial strategies could be assessed. I believe that this would lead to a more substantial understanding of the effects of different training paradigms on place cell activity during experiments.

3. Construct a reward contingency that would encourage a spatial strategy

This idea would allow us to, once and for all, determine whether the place cells rats using a spatial strategy in this experimental paradigm would follow cues according to their reward value and spatial location.

4. Add another group of animals to be given probe trials earlier in the recording process.

The ability to do probe trials in another group would alleviate the problem that probe trials may change the experience of the rats in such a way that would difficult to assess in the long run. Early probe trials involving the scrambling of local and distal cues would when the room reference frame became apparent or important to the rats. The probe trials discussed in CH 2 were more "reactionary" when we found out that some rats were reluctant remappers.

5. Record from larger CA1 ensembles.

The size of our place cell ensembles was highly variable across rats and recording sessions. Some of this can be blamed on "growing pains" and setting up a new lab. Regardless, it would be useful to have access to a larger number of place cells in each phase of the experiment.

6. Record from other areas in the hippocampal formation, especially the subiculum.

Finally, it goes without saying that recording ensembles in CA1 is merely a step in the right direction towards understanding hippocampal function and place cell activity. The discussion of CH 4, and the Synthesis section of this dissertation provide good evidence for exploring the entire hippocampal formation. I highlight the subiculum as a most interesting target because of elegant work describing how its place cells differ from CA (Sharp and Green, 1994; Sharp, 1996)

5.1.4. Additional comments on experimental design

As previously mentioned, there were two aspects of the experimental design that I have found to be of the utmost importance in the long-term analysis of the experiment. First, the individual analysis of data from each of the four rats in the experiment provided novel insight into differences in hippocampal activity between individual animals in a behavioral experiment. Second, the analysis of changes in hippocampal activity over time provided a novel view the differences between those individual rats. More importantly, it provided evidence that the hippocampus is a structure whose dynamic nature is in great need of further study.

5.1.4.1. Analysis of individual animals.

This aspect of our experimental design was determined mostly by the technique we used. Each rat was quite an investment of both time and energy. Training the animals was a lengthy process, as was the construction of the custom-made recording devices that held the 12 tetrode array. The entire experimental process built upon itself in such a way that any errors or failures along the way would result in suboptimal results.

Four rats were used in this experiment and it is unfortunate that we did not have time to use more animals. We considered the results from those four animals to be sufficient to answer our experimental question, although some reviewers correctly suggested that our case would be strengthened with additional data. Regardless, the use of only four animals allowed us to generate exceptional amounts of data from each animal. This led us to realize that the neural activity of each animal differed in important ways which we described in detail.

Most importantly, the four animals fell into two categories: those whose hippocampal activity remapped with no help from us, and those whose hippocampal activity remapped more reluctantly. It would be very interesting to observe more animals and determine whether this grouping would be maintained in larger experimental group. It is difficult to predict such a result, although it was such a clear different between the animals I assume that animals could fall into one group or another.

Why did some animals remap easily and some animals remap reluctantly? I think that this is a difficult question to answer, but it is an interesting one to consider in depth. I think that minor differences in training or other experiences in the rats' lives could have contributed to such differences. Additionally, the brains in those rats could have been wired slightly differently from one another: the "Nature" side of the Nature vs. Nurture puzzle. Regardless of the cause,

the perceptions that those four rats had of their training and recording sessions probably differed from one animal to the next.

Thinking about the changes in hippocampal activity over time sheds additional light on the subject. We observed that the hippocampal activity in some animals switched from correlating with local cues at the beginning of the experiment to correlating with distal cues further along in the experiment. Additionally, we observed that in some cases the amount of remapping changed as the experiment progressed. Given the physical constraints of the experimental design, it is likely that additional differences in hippocampal activity would have been apparent if we knew what to look for.

The rats in which we were recording were by no means operating according to our interests outside of the fact that we forced them to perform a certain behavioral task according to our schedule. Outside of that, their brains were arguably operating as such whether or not they had recording electrodes in them. We observed interesting and unpredicted changes in neural activity that was picked up by our recording electrodes. It is reasonable to conclude that numerous other interesting changes were occurring within the hippocampus of each rat that our electrodes were not privy to. Accordingly, the fact that we saw such apparent differences between animals (easy vs. reluctant remappers) indicates that there are probably myriad differences between individual animals that could be assessed if we had the appropriate tools –at least enough recording electrodes!!

As much as I tried to maintain a consistent training regimen between the rats, I am sure that their training experiences differed in ways that I couldn't measure. As much as I tried to keep the recording procedure and environment stable between animals, I am sure that the aspects of the procedure and environment differed in ways that were not apparent to my human senses.

To conclude, I believe that behavioral experiments in which neural activity is recorded are complicated because of so many experimental variables that can change between animals. Therefore, we would undoubtedly observe more complicated and interesting differences in neural activity between animals if only we had the means to do so – or the good fortune to stumble upon it. The more cells that we can simultaneously record in one animal, the more valuable the data from that single animal becomes. Gone is the time in our field when 3-4 cells are sufficient for a hippocampal pyramidal cell ensemble – and for good reason. There is a lot of information that we are missing by not observing the activity of the other million cells. There is a lot of information that we are missing simply by recording from only one part of the hippocampal formation at one time. Technology is catching up with desire, so such experiments will undoubtedly be the norm.

One caveat to this idea is that presenting unreasonably large data sets from single animals would be terribly exhausting to the reader, much like this section of the dissertation it to my committee. It is therefore the burden of the experimenters to present the data in a manner than is accessible to the reader. I'm not sure we accomplished that in our paper, but having three years to think about it has solidified my desired to present careful and understandable analysis of individual animals in any future experiments involving recording ensembles of cells.

5.1.4.2. Analysis of changes in hippocampal activity over time.

I am certain that we did not expect the ensembles recorded on any one rat on day 1 to be identical to that recorded on day 7. Not only did we expect to record different cells as we lowered our electrodes through the CA1 layer, but we were aware that there was the possibility of remapping. The phenomenon of remapping is difficult to analyze, because it is probably due

to uncontrolled variables within an experiment. The best way to approach this is to design experiments the capability to analyze changes in place field activity throughout the experimental process. I have discussed this idea in detail previously, so I won't expand upon it much further .

In a previous section I stated that it would benefit us to record from larger ensembles. The ability to maintain stable recordings of the same cells over long periods of time would be interesting as well – it would shed some light on learning process, in changes in perception of cues, etc. This is a real key to the understanding the place code. Had we been able to extend the recording sessions, we might have been able to address the questions regarding the *amount* and *characteristics* of the remapping that was taking place. For instance, if some rats completely remapped by day 10, would they remap again by day 20? Is remapping a singular experience, or is it more reflective of an ongoing dynamic process by which the hippocampus is encoding new information? I believe that remapping IS the recoding of new information, so I would expect that remapping would happen more than once.

It pleases me to no end that the experiment was conducted as such, because it opened a door in my mind for future work with hippocampal place cell recording. I imagine what it would be like to record simultaneously from input structures to the hippocampal formation, individual components of the hippocampal formation, and structures receiving first-order output from the hippocampal formation -- in an experiment that was designed to showcase the progression hippocampal activity during the learning of spatial tasks with non-spatial components – over a longer period of time, in order to best observe changes in the hippocampal code as a whole.

5.1.5. Conclusion

How does our data fit in with a modern theory of hippocampal function? At this point, the cognitive map theory and the relational theory can be combined into a theory that can explain most of the experimental results and is best described by Redish (1999). My understanding of this theory is that the hippocampus is not simply coding for the location of the rat in space. The relative location of the rat to objects possessing spatial information is being coded, but only in terms of a bigger picture – memory in general. The hippocampus is serving as a massive integrator of information after it is funneled through cortex, and it is binding the information together (spatial and non-spatial) to be used in memory (back to cortex), planning (prefrontal), behavior (prefrontal and hypothalamus), or to even be further bound in other integrative areas such as the lateral septum.

5.2. Extended Discussion of Chapter 3

5.2.1. How do these data contribute to the field?

The data presented in Chapter 3 suggest a pathway by which vestibular information can reach an area containing HD cells. Like all of the anatomical data presented in this dissertation, I believe that they contribute to the field in two ways. First, they can be used as a foundation upon which models of system function can be built. Second, they can be used to generate testable hypotheses that can outline future work in the field.

This section will use the data presented in Chapter 3 as a base to answer the following questions. What drives HD cell activity? What maintains the HD signal? How is error corrected in the HD signal? And, what is the neural circuitry responsibly for supporting all of these things?

This is only a sample of the wonderfully intriguing questions regarding head direction cells. One main reason why the HD system itself is so interesting to me is because it seems simple enough for us to figure it out within my lifetime. So far we have only scratched the surface of the subject, but I believe it has been with enough success in order to consider the subject's future to be bright. Regardless of when the system is "solved," continued investigations into the internal representation of orientation will increase our understanding of information processing in the brain, which can be applied to other neural systems. To what extent does the vestibular system affect HD cell activity? At present it remains unclear.

5.2.2. Interactions between brain regions involved in the HD system.

The truth is that no experiments categorically answering the questions proposed above have been performed. Are the data presented in Chapter 3 therefore less useful? I propose that, again, they can serve as the foundation for thinking about how the HD system might work on a larger scale.

In order to better understand the anatomical relationships between brain areas involved in spatial cognition, Pat Card and I created an extensive diagram (too large to include here) that represents the connectivity between nuclei of interest. This simple box-and-line diagram is based on neuroanatomical data currently available in the literature, although I have not cited the references supporting each line in the drawing. This diagram was intended to be a simple tool for organizing my thoughts regarding the processing of information within the hippocampal formation. As I added more and more connections to it, it grew into a tool that we could use to organize our thoughts regarding processing in the HD system as well.

The diagram is too complicated for general use so I have created a simplified version of one of the numerous concepts that fell out of the process of creating the diagram. It organizes

the HD system into four groups (Figure 1). These four groups of nuclei process the different types of information that I think are the critical for the HD system: the HD cells themselves, vestibular information, visual information, targets of HD cells in the hippocampal formation.

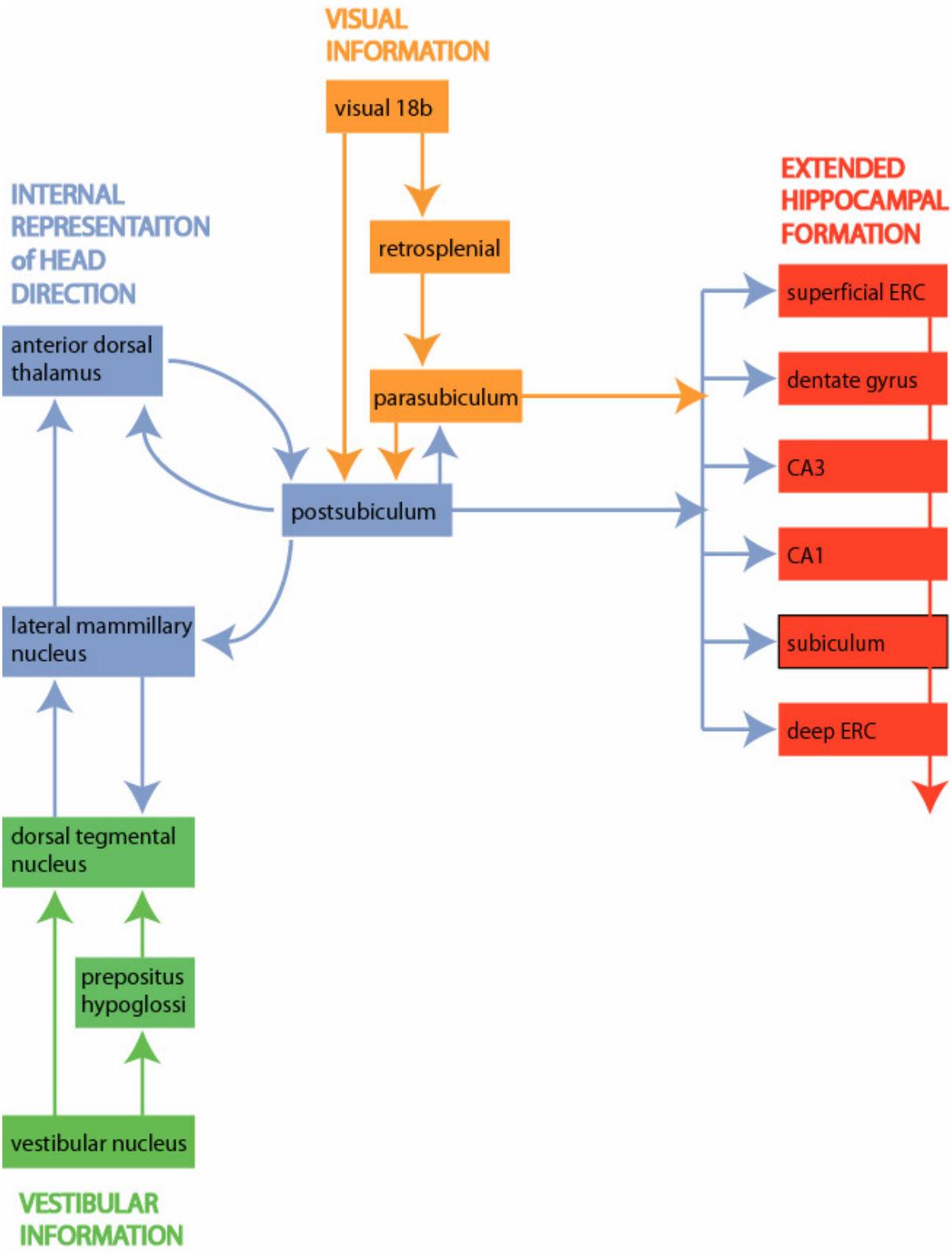
The first group in this model is blue, and is composed of the three main areas in which HD cells have been found: LMN, ATN and PoS. These areas in fact make a loop as LMN projects to ATN, ATN projects to PoS, and PoS projects back to LMN. PoS also projects back to ATN, but LMN does not project back to PoS. For purposes of simplification, HD cells found in other areas were left out of this model (for review, see Taube, 1998)

The second group, in green, is composed of the polysynaptic pathway that we established in Chapter 3. This is how vestibular information is carried into the HD system, and is targeted at the LMN.

Figure 1.

Proposed simplified schematic diagram of HD system. Blue areas contain HD cells and are responsible for the internal representation of head direction. Green areas represent the pathway through which vestibular information influences the HD cells. Orange areas represent visual pathways that can be used to calibrate the HD signal. Red areas represent the targets of HD output from POST.

Figure 1.


VISUAL INFORMATION
visual 18b
retrosplenial
parasubiculum
postsubiculum
INTERNAL REPRESENTAITON of HEAD DIRECTION
anterior dorsal thalamus
lateral mammillary nucleus
dorsal tegmental nucleus
prepositus hypoglossi
vestibular nucleus
VESTIBULAR INFORMATION
EXTENDED HIPPOCAMPAL FORMATION
superficial ERC
dentate gyrus
CA3
CA1
subiculum
deep ERC

The third group, orange, includes regions by which visual information is carried into the HD system. Visual information is thought to calibrate the HD signal. Knierim et at. (1995) performed an elegant study that established how HD cells work with and without visual input, at least in a lighted environment and a dark environment. In the dark, the HD signal tends to drift, while in the light the HD signal becomes "bound" to stable environmental cues over time.

The final group is composed of regions in the extended hippocampal formation that receive projections from the PoS. The organization makes clear the pathways through which HD information could be sent into the hippocampus for integration with other spatial information. PoS projects to each area in the hippocampal formation: DG, CA3, CA1 and Subiculum. This part of the model will be extended in the discussion of Chapter 4.

This model is obviously incomplete. There is so far no mention of how motor information reaches the system, or how the HD information may be useful in affecting motor output. The role of HD information in path integration is also not mentioned. These two related issues must certainly be added to the model in order for it to hold water. Additionally, the differences in place codes in the hippocampal formation, especially the place code in SUB, may be due to differences in the extent to which HD information is integrated with spatial information.

5.2.3. What exactly IS a head direction cell?

I think that this is the single most important question in the field. Although the anatomy of the circuitry must be better defined, the simple identification of HD cells outside of behavioral experiments is key to our understanding of the system. Individual HD cells have not been identified through means other than electrophysiological ones. Electron microscopy studies of areas known to contain HD cells provide us with information regarding the cell types in those

areas (in LMN: Allen and Hopkins, 1988), but it is unclear which of those cells are HD cells. Recently, cells that code for angular head velocity have been studied (Basset and Taube, 2001; Sharp et al., 2001). Their existence had been predicted in several models (Knierim et al. 1996; Skaggs et al., 1995; Zhang, 1996) and further investigation into their role is also very important. The fact that AHV cells and HD cells have been found in the same nuclei adds another layer of complication to the story. This layer will be a very useful one because responses of vestibular nucleus neurons to horizontal head rotations are considerably different from those of HD cells, requiring a complex transformation of signals by the circuitry that transmits signals between the two groups of neurons.

5.2.4. How could the role of the vestibular information in the HD system be isolated?

The strongest evidence suggesting that the vestibular system has a direct and profound influence on the firing of HD cells comes from the Stackman and Taube lesion study (1997). Consistent with that result, Stackman et al. (2002) more recently showed that inactivation of vestibular hair cells abolished directional sensitivity of HD cells in PoS. Taken at face value, these data suggest that the vestibular system has a fundamental influence on normal HD cell activity. When combined with our data, a strong case can be presented. However, these findings are not easily reconciled with current data regarding inputs to vestibular nucleus neurons and plastic changes that occur in the vestibular system following removal of labyrinthine inputs (see Brown et al,. 2002 for review).

We have proposed some recording experiments that would help determine the role played by the vestibular system in determining head direction (Brown et al., 2002). Physiological experiments could also be conducted to compare the firing and directional sensitivity of

individual HD cell during active head movements and passive rotations; such passive rotations should be conducted on head-restrained animals placed on a turntable capable of providing high-velocity, controlled movements. It is also important to differentiate the influence of visual from vestibular cues; if the vestibular system alone is sufficient for the generation and maintenance of normal HD cell activity, then HD cell should respond to rapid rotations of the animal conducted with the head fixed in both the light and dark. Findings from these experiments would be useful in determining the extent to which the vestibular system participates in determining HD cell activity, and may potentially open an important new research direction. Comparisons of the processing of vestibular signals by HD cells and neurons that mediate vestibulo-ocular, vestibulo-spinal, and vestibulo-autonomic responses will also likely reveal valuable new information regarding the physiology of the vestibular system.

5.3.Synthesis and Conclusion

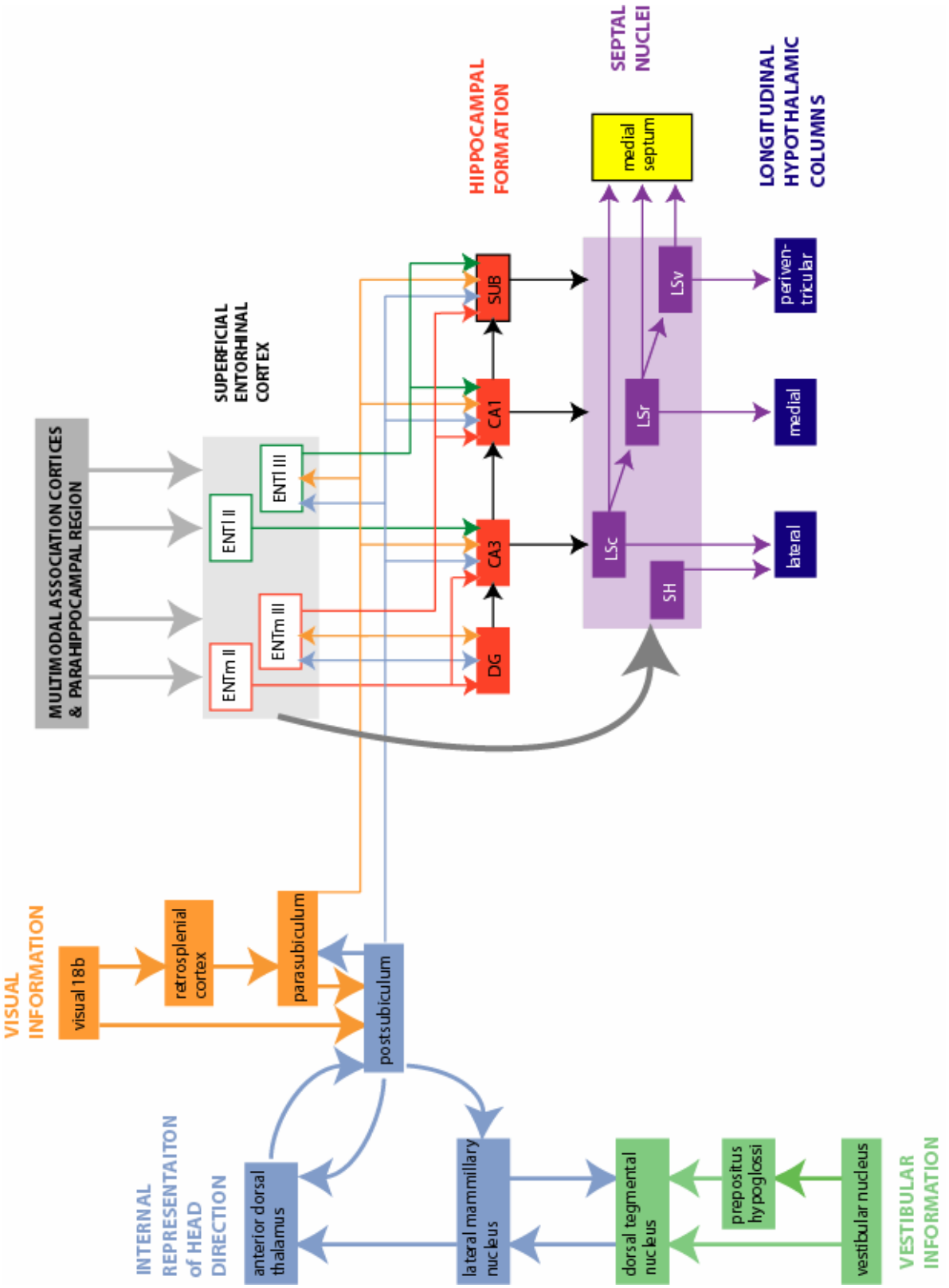
This section is based on a final figure which describe a combination of data from Chapter 3 with data from Chapter 4 in order to advance a more complete integration of the data collected for this dissertation. Figure 2 better represents the extent to which hippocampal information and HD information could be processed together in the overall scheme of the hippocampal-septo-hypothalamic pathways. Again, HD related information could be projected to all levels of the hippocampal formation. The parasubiculum, which is thought to process visual information, is also known to project throughout the hippocampal formation. Perhaps then the PoS input to the hippocampus is strictly a calibrated HD signal, while the ParaS is providing visual-based calibration information for the place code in the hippocampus.

Of course, this diagram is incomplete because it does not consider all of the other input that integration centers such as the hippocampal formation and lateral septum receive. However, as mentioned earlier, the work presented in this dissertation merely provides a foundation for future work. As a result of these studies, I hope to have the opportunity to focus on two major areas in future work. First, I am interested in better defining the polysynaptic input from multimodal association cortex into the parahippocampal region and especially the superficial entorhinal cortex. These pathways have been well described but like many other pathways in the literature, a great deal more could be gained by studying the synaptology of the circuitry. Second, I would like to achieve a better understanding of the organization of the lateral septum. It is clear to me that it is serving as an integrative center for hippocampal information and perhaps even subcortical information. I believe that additional anatomical and physiological studies would be useful to to better define the role of the lateral septum.

Figure 2.

Synthesis of HD system diagram with hippocampal-septo-hypothalamic pathways. This is a combination of Figure 10 from Chapter 4 and Figure 1 from Chapter 5, with a change in the layout of PoS and ParaS input into the extended hippocampal formation.

Figure 2.


VISUAL INFORMATION
visual 18b
retrosplenial cortex
parasubiculum
postsubiculum
INTERNAL REPRESENTAITON of HEAD DIRECTION
anterior dorsal thalamus
lateral mammillary nucleus
dorsal tegmental nucleus
prepositus hypoglossi
vestibular nucleus
VESTIBULAR INFORMATION
MULTIMODAL ASSOCIATION CORTICES & PARAHIPPOCAMPAL REGION
SUPERFICIAL ENTORHINAL CORTEX
ENTm II
ENTm III
ENTl II
ENTl III
HIPPOCAMPAL FORMATION
DG
CA3
CA1
SUB
SEPTAL NUCLEI
medial septum
LSc
LSr
LSv
SH
LONGITUDINAL HYPOTHALAMIC COLUMNS
lateral
medial
periventricular

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