CHOLINERGIC MODULATION OF THE SUPERFICIAL LAYERS OF THE PARASUBICULUM

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ABSTRACT

Cholinergic modulation of the superficial layers of the parasubiculum

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Recent evidence suggests that the parahippocampal area, including the entorhinal cortex and parasubiculum, may play a crucial role in spatial processing and memory formation. However, little is known about the basic cellular and network properties of the parasubiculum, an isocortical brain region that receives input from the hippocampus and other subcortical regions associated with spatial navigation, and projects exclusively to the superficial layers of the entorhinal cortex. Neurons in layer II of the parasubiculum demonstrate theta-frequency membrane potential oscillations at near-threshold voltages that are generated via an interplay between a persistent Na^+ current and the hyperpolarization-activated cationic current I_h, and these rhythmic fluctuations in membrane potential may contribute to the generation of oscillatory local field potentials. Further, the parasubiculum receives strong cholinergic projections from the medial septum. Acetylcholine has been linked to theta-frequency oscillations via regulation of cellular and network dynamics through membrane depolarization, while concurrently suppressing excitatory synaptic transmission, and it is likely that cholinergic receptor activation has similar effects in the parasubiculum. I found that activation of cholinergic receptors depolarizes layer II cells of the parasubiculum by exerting numerous effects on K^+ channels, including I_M and I_{Kir}, however also suppresses incoming excitatory synaptic transmission from the

CA1. These results indicate that increases in cholinergic tone during network-level theta-frequency oscillations in the parasubiculum may increase neuronal excitability by exerting strong effects on postsynaptic conductances, but may also regulate network dynamics by reducing the strength of incoming afferents.

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CONTRIBUTION OF AUTHORS

1. **Conductances mediating intrinsic theta-frequency membrane potential oscillations in layer II parasubicular neurons** by Stephen D. Glasgow and C. Andrew Chapman.
    - **Stephen Glasgow**: Designed the experiments, performed all electrophysiological recordings, analyzed and plotted the data, and wrote the manuscript for publication.
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3. **Muscarinic depolarization of layer II neurons of the parasubiculum** by Stephen D. Glasgow and C. Andrew Chapman.

- **Stephen Glasgow**: Designed the experiments, performed all electrophysiological recordings, analyzed and plotted the data, and wrote the manuscript for publication.
- **Andrew Chapman**: Designed the experiments, and assisted with the writing of the manuscript.

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AHP	afterhyperpolarization
ANOVA	analysis of variance
APV	2-amino-5-phosphonovaleric acid
$ATSO_4$	atropine sulfate
Ba^{2+}	barium sulfate
CA1-4	*Cornu Ammonis* fields of the hippocampus
Ca^{2+}	calcium
CNQX	7-nitro-2,3-dioxo-1,4-dihydroquinoxaline-6-carbonitrile
Cs^{+}	cesium
EEG	electroencephalographic
E_K	equilibrium potential for potassium
EPSP	excitatory postsynaptic potential
fEPSP	field excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
HD	head-direction
I_{Kir}	inward rectifying potassium current
I_{Kv}	voltage-gated potassium current
I_h	hyperpolarization-activated nonspecific cation current
I_M	muscarinically-activated potassium current
I_{NaP}	persistent sodium current
I_{NCM}	nonselective mixed cationic current
K^{+}	potassium
LTD	long-term depression
LTP	long-term potentiation
LY	Lucifer Yellow
Na^{+}	sodium
N-K	Newman-Keuls
NMDA	N-methyl-D-aspartate
PaS	parasubiculum
PHA-L	Phaseolus vulgaris leucoagglutinin
PKC	protein kinase C
Rin	input resistance
Rs	series resistance
SEM	standard error of the mean
TEA	tetraethylammonium
TTX	tetrodotoxin
XE-991	10,10-*bis*(4-Pyridinylmethyl)-9(10*H*) -anthracenone dihydrochloride
ZD7288	4-Ethylphenylamino-1,2-dimethyl-6-m ethylaminopyrimidinium chloride

CHAPTER 1

GENERAL INTRODUCTION

PART 1

THE PARASUBICULUM AND ACETYLCHOLINE: OVERVIEW

The parasubiculum is a key structure within the hippocampal formation that is thought to contribute to major cognitive functions associated with the medial temporal lobe including declarative memory, spatial navigation, and sensorimotor integration. It receives major synaptic inputs from the CA1 region of the hippocampus, the medial septum, and the anterior thalamus, and its major output pathway terminates within layer II of the entorhinal cortex (Caballero-Bleda & Witter, 1993, 1994; Kohler, 1985; van Groen & Wyss, 1990a). Layer II neurons of the entorhinal cortex form the origin of the perforant path, which carries the majority of the highly processed sensory input to the hippocampus, and the parasubiculum therefore occupies a central position to strongly influence the function of the hippocampal formation (Amaral & Witter, 1995a; Andersen, Holmqvist, & Voorhoeve, 1966b; Burwell, 2000; Kohler, 1985, 1986; Swanson & Cowan, 1977). Further, the parasubiculum is known to be able to either inhibit or facilitate the synaptic response of the entorhinal cortex to inputs from sensory cortex in a time-dependent manner (Caruana & Chapman, 2004). This suggests that the relative timing of inputs to the entorhinal cortex from sensory cortices and from the parasubiculum play an important role in sensory and mnemonic functions of the region, and that synchronized population neuronal activity that affects the timing of synaptic events may play an important role in interactions between these structures. Both the parasubiculum and entorhinal cortex express theta-frequency (4-12 Hz) electroencephalographic (EEG) activity (Alonso &

Garcia-Austt, 1987a; Glasgow & Chapman, 2007). This activity is mediated, in part, by theta-frequency oscillations in membrane potential (Alonso & Llinas, 1989; Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Glasgow & Chapman, 2007) by cholinergic inputs from the medial septum (Alonso & Kohler, 1984; Gaykema, Luiten, Nyakas, & Traber, 1990; Swanson & Cowan, 1979). In both hippocampal and entorhinal neurons, acetylcholine results in the depolarization of membrane potential to voltages near the threshold for action potentials (Benardo & Prince, 1982a; Benson, Blitzer, & Landau, 1988; Klink & Alonso, 1997b), and this is thought to be a major mechanism that can contribute to theta-frequency EEG activity because the membrane depolarization leads to the emergence of theta-frequency membrane potential oscillations in layer II parasubicular neurons that are driven by voltage-dependent conductances (Chapman & Lacaille, 1999a, 1999b; Dickson & Alonso, 1997; Dickson, Magistretti, Shalinsky, Hamam, & Alonso, 2000; Glasgow & Chapman, 2007; Hasselmo & Brandon, 2008; Klink & Alonso, 1993; Schmitz, Gloveli, Behr, Dugladze, & Heinemann, 1998; Shalinsky, Magistretti, Ma, & Alonso, 2002). Two chapters of this thesis describe experiments that have investigated the muscarinic-receptor-dependent conductances that mediate the depolarization of parasubicular neurons, and have also investigated the conductances that interact to generate theta-frequency membrane potential oscillations after parasubicular neurons have been depolarized. These experiments are a major contribution to our understanding of the mechanisms that generate theta activity within the parasubiculum. This thesis has also examined how cholinergic inputs modulate

synaptic responses within the parasubiculum; cholinergic receptor activation has been linked to suppression of glutamate-mediated synaptic transmission in numerous brain areas (Hamam, Sinai, Poirier, & Chapman, 2007; Hasselmo, 2006; Hasselmo & Bower, 1991; Yun et al., 2000), but there has been no prior investigation of how acetylcholine modulates synaptic responses within layer II parasubicular neurons. These cholinergic effects on the functioning of parasubicular neurons are likely to have an important impact on how the parasubiculum contributes to hippocampal-dependent processes, such as spatial navigation and memory consolidation.

Neuronal processes that support declarative memory formation function are mediated in part by the medial temporal lobe, and the parasubiculum contributes to temporal lobe function. Memory formation and consolidation are essential processes that enable an animal to interact with their environment, as an animal must be able to form complex associations between objects, events, and other aspects of their environment to thrive. Relationships between stimuli can be complex and may necessitate the integration of sensory information from multiple sensory modalities for encoding and consolidation of a cohesive mnemonic representation. Moreover, the content of memory is often updated, and this requires that representations be malleable and modifiable. Research into brain mechanisms of memory has shown that learning and memory depend on a remarkably intricate network composed of multiple brain structures that support associations between stimuli and the integration of stimuli within memory representations (Amaral, 1993; Bird & Burgess, 2008; Burgess, 2008b; Buzsaki

& Draguhn, 2004; Harris, 2005). These representations are continuously shaped through neuronal systems that attach emotional and motivational significance to these representations (Squire & Zola-Morgan, 1991). The medial temporal lobe mediates many of these processes, and therefore it is likely that the parasubiculum contributes to the mnemonic processes associated with the medial temporal lobe function.

Early neuropsychological studies of patients with bilateral lesions of the hippocampus and related medial temporal lobe structures revealed that the loss of these structures led to severe deficits in long-term memory formation without noticeable intellectual impairments or changes in personality (Scoville & Milner, 1957). Most notable of these patients was patient H. M., who displayed both anterograde and retrograde amnesia following surgical lesioning of the hippocampus. Moreover, H. M. displayed severe anterograde amnesia, characterized by the completely inability to form any new memories. This finding indicates that the hippocampus is essential to the acquisition and consolidation of new memories. In contrast, patients with damage to the hippocampal formation were able to learn new motor skills, suggesting that nondeclarative memory functions do not depend on the hippocampus and associated structures. Anatomically, the observed deficits in memory consolidation were initially thought to be related specifically to damage to the hippocampus, as patients that underwent resections of extrahippocampal regions, including the uncus, the amygdaloid, and periamygdaloid areas, failed to show noticeable impairments on memory function (Scoville & Milner, 1957).

However, subsequent case studies of patients with bilateral damage confined to the CA1 region of the hippocampus following an ischemic episode revealed only moderate deficits in anterograde memory, whereas more extensive damage to hippocampal areas including the CA1 and subicular complex resulted in severe memory impairments (Rempel-Clower, Zola, Squire, & Amaral, 1996; Zola-Morgan, Squire, & Amaral, 1986). The parasubiculum is a component of the subicular complex, which was damaged in H. M. (Corkin, Amaral, Gonzalez, Johnson, & Hyman, 1997; Scoville & Milner, 1957) and other patients with severe memory deficits (Ploner et al., 2000; Zola-Morgan, Squire, Amaral, & Suzuki, 1989), suggesting that damage to other areas of the hippocampal formation, including the parasubiculum, may contribute to some aspects of their impaired memory function. A substantial body of work examining performance on memory tests in monkeys has also suggested that the memory impairments may be due to damage to adjoining areas in the parahippocampal cortices which include the pre- and parasubiculum, as well as perirhinal, postrhinal, and entorhinal cortices (Corkin et al., 1997; Ploner et al., 2000; Scoville & Milner, 1957; Squire & Zola-Morgan, 1991; Zola-Morgan et al., 1989). Studies in monkeys using delayed nonmatching-to-sample tests have assessed the contribution of the parahippocampal region to object recognition memory performance (Zola-Morgan et al., 1989). In this task, the animal is shown an object, and after a delay from 8 s to 10 min, is shown the same object alongside another novel object. In order to receive a food reward, the monkey must remember the sample object for the duration of the delay and choose the novel object. Animals

with damage to the parahippocampal region performed significantly worse than controls and took over ten times longer to learn the task compared to animals with no damage. Interestingly, animals with combined lesions of both the hippocampus and amygdala, but sparing the parahippocampal region, performed significantly better on both memory tests and acquisition of the task compared to animals with lesions to the parahippocampal region, but worse compared to controls. These results indicate that the parahippocampal cortical region including the perirhinal cortex makes a strong contribution to normal object recognition memory function, but that the parasubiculum, that is typically lesioned along with the hippocampal formation may not be required for object recognition memory (Murray & Mishkin, 1986; Zola-Morgan et al., 1989).

The hippocampal formation, however, has also been implicated in spatial navigation, and the parasubiculum is thought to play an important role in spatial processing. Foundational studies using rat models of spatial memory found that lesions to the hippocampus disrupted navigational memory performance on a water maze task (Morris, Garrud, Rawlins, & O'Keefe, 1982). In this task, the rat was placed in a circular pool filled with water that had been rendered opaque and, from a range of starting points, the animal was required to locate a platform that was submerged below the surface of the water. Rats with bilateral hippocampal lesions performed poorly on this task, while control animals and animals with superficial cortical lesions were able to use spatial cues surrounding the maze to locate the platform. In contrast, if the platform protruded slightly from the water, both lesion and control animals performed equally well, indicating

that the deficit observed in the hippocampal lesion group was not due to impairments in perception of spatial cues or motor behavior, but rather that it was a deficit in the ability to recall the relationships between extramaze cues and the location of the platform (Morris et al., 1982). However, subsequent studies demonstrated that animals with lesions that were focused on parahippocampal areas, including the subicular complex, also show profound deficits in performance on the water maze task (Schenk & Morris, 1985), suggesting that parahippocampal areas such as the parasubiculum are important in spatial memory processes.

The parahippocampal region is richly interconnected with the hippocampus proper (Amaral & Witter, 1989, 1995a; Blackstad, 1956), and based on anatomical and physiological evidence, it is likely that many processes associated with temporal lobe function are mediated by interactions with the entorhinal cortex. The entorhinal cortex receives a massive amount of sensory inputs from sensory and associational cortices, and it also provides the main sensory input to the dentate gyrus and CA1 regions of the hippocampal formation (Witter, Groenewegen, Lopes da Silva, & Lohman, 1989). While superficial layer entorhinal cortex neurons receive a number of sensory inputs both directly and indirectly from the neocortex, they also receive direct monosynaptic inputs from adjacent structures, the presubiculum and parasubiculum, that terminate on neurons in the entorhinal cortex that project to the hippocampus (Caballero-Bleda & Witter, 1993, 1994; Kohler, 1985; van Groen & Wyss, 1990a). Recent evidence suggests that the entorhinal cortex is not simply a passive relay for

sensory inputs to the hippocampus, but plays a more active role the integration of sensory information that underlies the mnemonic and computational functions of the dentate gyrus and hippocampus proper (Fyhn, Molden, Witter, Moser, & Moser, 2004; Hafting, Fyhn, Molden, Moser, & Moser, 2005; Witter & Moser, 2006). Consequently, modulation of synaptic properties and firing patterns of entorhinal neurons may influence upstream processes dependent on hippocampal functioning, including spatial navigation and plastic changes associated with memory consolidation (Hasselmo, Hay, Ilyn, & Gorchetchnikov, 2002; Molter & Yamaguchi, 2008). The parasubiculum exerts powerful control over the responsiveness of these entorhinal neurons, and can shape how they respond to sensory information in a timing-dependent manner (Caruana & Chapman, 2004). These findings suggest a crucial role for the parasubiculum in the modulation of entorhinal function, however very little is known about the basic intrinsic properties of parasubicular neurons, and how synchronous network activity within the parasubiculum may interact with other regions of the hippocampal formation and parahippocampal region.

Structures within the hippocampus and parahippocampal regions display prominent theta-frequency (4-12 Hz) EEG activity, and this activity is thought to be a major mechanism that contributes to the computational functions of the medial temporal lobe by synchronizing the activity of large populations of neurons (Alonso & Garcia-Austt, 1987a, 1987b; Alonso & Llinas, 1989; Glasgow & Chapman, 2007; L. W. Leung & Yim, 1991; Mitchell & Ranck, 1980). The hippocampal theta rhythm has been linked to active exploration (Vanderwolf,

1969), sensorimotor integration (Bland, 1986; Bland & Oddie, 2001), and memory consolidation (Buzsaki, 2002; Huerta & Lisman, 1995; Hyman, Wyble, Goyal, Rossi, & Hasselmo, 2003). Moreover, theta activity has been implicated in spatial memory and spatial navigation, and likely contributes to the firing of specialized “place cells” within the hippocampus, which fire in relation to specific locations in the environment (O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978). Hippocampal place cells fire in relation to the phase of theta activity as the animal moves through the environment (O'Keefe & Recce, 1993; Skaggs, McNaughton, Wilson, & Barnes, 1996), and the parasubiculum also contains place cells that fire in relation to local theta activity (Boccara et al., 2010; Cacucci, Lever, Wills, Burgess, & O'Keefe, 2004; Taube, 1995b). Thus, the firing of many cells within the hippocampal formation is locked to specific phases of local theta-frequency field activity and this is thought to contribute to mechanisms that support spatial navigation. These phase relationships are also thought to contribute to mechanisms of memory formation. Coordinated cell firing can support synaptic integration and the induction of long-term synaptic changes, and this can lead to the formation of cell-assemblies, a large-scale network of interconnected neurons in a given brain region that interact in a temporally-coordinated manner and may form the basis of declarative memory (Harris, 2005; Hebb, 1949; Holscher, Anwyl, & Rowan, 1997; Hyman et al., 2003). These findings provide evidence for an important role of theta activity in contributing to functions of the parasubiculum and hippocampal formation related to spatial navigation and memory processing.

Cholinergic inputs to the hippocampal formation from the medial septum are thought to play a major role in the generation of theta activity (Buzsaki, 2002; Stewart & Fox, 1990). Hippocampal theta activity is thought to be generated primarily via a combination of tonic cholinergic excitation and rhythmic γ-amino-butyric acid (GABA)-mediated inhibitory inputs from the medial septum (Bland, Konopacki, & Dyck, 2002; Buzsaki, 2002; Chapman & Lacaille, 1999b; Cobb, Buhl, Halasy, Paulsen, & Somogyi, 1995; Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Klink & Alonso, 1993; L. W. Leung & Yim, 1991). Tonic cholinergic input to the hippocampus can lead to depolarization of membrane potential in principal cells of the hippocampal formation, and this can contribute to rhythmic population activity because hippocampal neurons display intrinsic theta-frequency oscillations in membrane potential at depolarized membrane potentials (Bland et al., 2002; L. W. Leung & Yim, 1991). In addition to these cholinergic effects that promote theta activity, the rhythmic activity of local inhibitory neurons, and septal inhibitory cells that project to the hippocampal formation, can synchronize the activity of principal cells (Chapman & Lacaille, 1999a, 1999b; Goutagny, Manseau, Jackson, Danik, & Williams, 2008; Manseau, Goutagny, Danik, & Williams, 2008; Sotty et al., 2003). Although the parasubiculum also receives a heavy septal cholinergic input (Alonso & Kohler, 1984; Gaykema et al., 1990; Swanson & Cowan, 1979), little is known of the modulatory effects of acetylcholine on neuronal and synaptic processing within the parasubiculum.

Similar to other areas of the hippocampal formation (Alonso & Garcia-Austt, 1987a; Buzsaki, 2002), cholinergically-dependent theta activity is generated locally in the superficial layers of the parasubiculum, and it is likely that cholinergic receptor activation depolarizes parasubicular cells to near-threshold voltages where these cells express theta-frequency oscillations in membrane potential that may contribute to the generation of local EEG activity (Glasgow & Chapman, 2007). The superficial layers of the parasubiculum receive strong projections from the medial septum, and show high levels of acetylcholinesterase, an enzyme that degrades acetylcholine, indicating that these septal projections likely carry cholinergic fibers (Alonso & Kohler, 1984). Acetylcholine-containing cells of the medial septum are known to be active during exploratory behaviors (King, Recce, & O'Keefe, 1998), and cholinergic receptor activation can exert a wide range of excitatory and inhibitory effects on principal cells in the hippocampal formation. During theta activity, septal inputs to the parasubiculum are therefore likely to contribute to the generation of parasubicular theta activity by depolarizing principal cells. In addition, although cholinergic receptor activation is known to modulate the strength of excitatory synaptic responses within the neocortex (Gulledge, Bucci, Zhang, Matsui, & Yeh, 2009; Hasselmo & Bower, 1991) and hippocampal formation (Cheong et al., 2001; Hamam et al., 2007; Hasselmo & Schnell, 1994; Yun et al., 2000), it also remains unclear how acetylcholine modulates theta activity and synaptic responses within parasubicular neurons.

This thesis investigated the mechanisms by which parasubicular neurons express membrane potential oscillations, and how increases in cholinergic tone during theta activity may modulate the synaptic and intrinsic properties of parasubicular neurons. In the following chapters, I describe experiments using in vitro intracellular recordings that have shown how parasubicular neurons can contribute to synchronized theta activity within the parasubiculum, and how increases in cholinergic tone during theta activity may influence synaptic transmission to the superficial layers of the parasubiculum. These experiments provide insight into the role of the parasubiculum during large-scale network synchronization, and have demonstrated the mechanisms by which acetylcholine can modulate the intrinsic cellular and synaptic properties of layer II parasubicular neurons. The following sections of the *General Introduction* provide an overview of the anatomy and physiology of the parasubiculum and surrounding regions, review the role of the parasubiculum in spatial navigation, and propose a possible role for acetylcholine in the modulation of intrinsic excitability and synaptic function in the superficial layers of the parasubiculum.

PART 2: CONNECTIONS OF THE PARASUBICULUM AND ASSOCIATED REGIONS

General anatomy and physiology

Situated directly posterior to the hippocampus and ventral to the subiculum, the parahippocampal region, including the entorhinal cortex, perirhinal cortex, and pre- and parasubiculum, provides the main input and output

pathways of the hippocampal formation (Blackstad, 1956; Kerr, Agster, Furtak, & Burwell, 2007; Witter et al., 1989). The parahippocampal region lines the ventral and caudal area of the mammalian brain, and is dominated by the entorhinal cortex, which is demarcated medially at the border of the parasubiculum and laterally by the perirhinal fissure. The entorhinal cortex is thought to contribute to hippocampal-dependent processes including memory formation and spatial navigation; it receives major sensory inputs from the perirhinal, postrhinal and piriform cortices, and it also provides major output pathways that terminate in the dentate gyrus, and CA3 and CA1 regions of the hippocampus (Amaral & Witter, 1995a; Andersen, Holmqvist, & Voorhoeve, 1966a; Kohler, 1986; Witter et al., 1989). The parasubiculum is a wedge-like multi-layered cortical structure that projects to layer II of the entorhinal cortex, as well as to the dentate gyrus and CA3 region of the hippocampus (Amaral & Witter, 1989, 1995a; Caballero-Bleda & Witter, 1993, 1994; van Groen & Wyss, 1990a; Witter et al., 1989), and this suggests that activity in the parasubiculum can influence hippocampal function both directly and by acting on the entorhinal cortex. The following sections detail the intrinsic and extrinsic connections of the hippocampal formation and parahippocampal region (Figure 1.1), including the parasubiculum and its relation to the entorhinal cortex, and review empirical data relating to the electrophysiological properties of parasubicular neurons.

Cytoarchitectonics of the hippocampal formation

In order to understand the role of the parasubiculum, it is necessary to review the nomenclature related to structural grouping of the hippocampal formation. Comprised of six cytoarchitecturally-distinct regions, the hippocampal formation includes the dentate gyrus, hippocampal gyrus (which includes subfields CA1, CA2, and CA3), subiculum, presubiculum, parasubiculum, and entorhinal cortex (Amaral, 1993; Amaral, Dolorfo, & Alvarez-Royo, 1991; Amaral & Witter, 1995a; Blackstad, 1956). The umbrella term of "hippocampal formation" underlines the extensive interconnectivity of these areas. Although comprised of cytoarchitecturally-distinct cortical fields, all of the structures within the hippocampal formation, including the parasubiculum, are strongly interconnected with one another.

On the basis of cytoarchitecture, the areas of the hippocampal formation can be divided into two major categories. Three-layered *allocortical* structures, which include the dentate gyrus, the hippocampus proper, and the subiculum, are characterized by a single neuronal cell layer with plexiform layers above and below the cell layer, whereas the pre- and parasubiculum, as well as entorhinal cortex show a distinct six-layered lamination reminiscent of the cortical profile in neocortical regions. As such, entorhinal cortex and the pre- and parasubiculum have been termed *isocortical*.

The term "hippocampal complex", which refers to the dentate gyrus, hippocampus (including CA1, CA2, and CA3), and subiculum, is composed exclusively of the allocortical areas in the hippocampal formation (Amaral & Witter, 1989, 1995a). The structure of the hippocampal complex resembles a

seahorse (hence the name of the structure) or alternately described as two interlocking “C”s, and characterized by a single principal cell layer with extensive dendritic branching in basal and apical orientations. In contrast to the organization of connections between adjacent portions of the neocortex, which show a high degree of reciprocity, the areas of the hippocampal formation are strongly linked to one another through extensive unidirectional excitatory projections. Within the basic circuitry of the hippocampal formation (Andersen, Bliss, & Skrede, 1971), neurons in layer II of the entorhinal cortex project to granule cells of the dentate gyrus which give rise to excitatory fibers, termed the mossy fibers that innervate principal neurons in the CA3 region. The CA3 pyramidal neurons provide extensive excitation within the CA3 through recurrent axonal connections, and also send substantial excitatory projections to the pyramidal neurons of the CA1 region via the Schaffer collaterals (Andersen, Bliss, Lomo, Olsen, & Skrede, 1969; Andersen et al., 1971; Andresen, Bliss, & Skrede, 1971). Principal cells of the CA1 region project extensively to the pyramidal neurons in the subiculum, as well as to the parasubiculum and deep layers of the entorhinal cortex (Cenquizca & Swanson, 2007). The subiculum serves as a major output region for the hippocampus, and neurons in this area project to the pre- and parasubiculum, as well as to the deep layers of the entorhinal cortex (Kohler, 1985; S. M. O'Mara, Commins, Anderson, & Gigg, 2001).

The *subicular complex* is a set of cytoarchitecturally-distinct and relatively small cortical fields that were originally grouped together based on anatomical

proximity, and which includes the subiculum, postsubiculum, prosubiculum, presubiculum, and parasubiculum. First described by Lorente de Nó (1934), the term “subicular complex” is somewhat controversial, given the differences in structure and cytoarchitecture of its sub-regions, and many authors now emphasize that the components of the subicular complex are quite separate and distinct cortical entities (Amaral & Witter, 1995a; Kohler, 1985; S. M. O'Mara et al., 2001). While disagreement exists regarding the precise definition of the subicular complex, it can be generally described as a transitional zone located between the CA1 region of the hippocampus and the entorhinal cortex, and is bordered dorsally by the retrospenial cortex (Lopes da Silva, Witter, Boeijinga, & Lohman, 1990; S. M. O'Mara et al., 2001). Cytoarchitecturally, the subicular complex is marked by the gradual transition from the three-layered subiculum into the presubiculum, which is characterized by presence of a densely-packed cell layer superficial to a cell-free layer (lamina dissecans). These characteristics suggest that the subicular complex is a transitionary area between the allocortex and isocortex, with each subregion showing distinct laminar profiles (Lorente de Nó, 1934). Additionally, the connections of the caudal regions of the subicular complex, including the pre- and parasubiculum, are hodologically-distinct from the subiculum proper, with marked differences in their output pathways. As mentioned previously, in contrast to the three-layered hippocampal complex, the entorhinal cortex and pre- and parasubiculum are more similar to the six-layered neocortex, which can be grouped together and referred to as either *retrohippocampal* (behind the hippocampus) or *parahippocampal* (alongside the

hippocampus). Therefore, the subicular complex includes regions that can be viewed as lying within both the hippocampal complex and the parahippocampal cortex. For the purpose of this thesis, the parasubiculum will be defined as a component of the subicular complex, while concurrently serving as a central component of the parahippocampal region.

Connections of the hippocampal formation

Although much of the evidence describing the lamination and connections of the entorhinal cortex and adjacent parahippocampal cortices, including the parasubiculum, has been obtained from studies in the rat, data from cats, guinea pigs, mice, and monkeys show a remarkable similarity, suggesting that the topographical organization of this region is conserved across species (for review, see (Insausti, 1993).

The superficial layers of the entorhinal cortex were first described by Ramón y Cajal (1911), and further refined by his student Lorente de Nó (1934). Layer I of the entorhinal cortex is primarily cell-free, however layer II is populated with stellate and pyramidal neurons that form the origin of the perforant path. These projection neurons are characterized by extensive dendritic branching within the cell layer and, to a lesser extent, layer I, and the axons of these neurons show few collaterals within the entorhinal cortex and instead project almost exclusively to the dentate gyrus and CA3. Similarly, layer III contains pyramidal neurons with apical dendrites that extend through layer II to form a terminal tuft in layer I. These cells are differentiated from layer II cells in their

projections, with layer III neurons preferentially innervating the stratum lacunosum-moleculare area of the CA1 region of the hippocampus and the molecular layer of the subiculum. The organization of the superficial layers of the entorhinal cortex implies that inputs from other brain regions to layers II and III can differentially modulate activity in distinct hippocampal subfields (Caballero-Bleda & Witter, 1994; Lopes da Silva et al., 1990). For example, inputs that terminate in layer I are likely to innervate layer II and III neurons with equal chance. Afferents that terminate on layer II neurons can influence activity in perforant path projections to the dentate gyrus and CA3 region, whereas afferents that innervate layer III neurons can modulate the temporoammonic input to the CA1 region (Witter et al., 1989; Witter & Moser, 2006).

The parahippocampal region has dense reciprocal connections with major sensory and associational cortical areas, and provides the hippocampus with the majority of its cortical sensory input through projection neurons located in the superficial layers of the entorhinal cortex (Amaral & Witter, 1989; Burwell, 2000; Naber, Witter, & Lopes Silva, 2000; Witter, Wouterlood, Naber, & Van Haeften, 2000). The superficial layers of the entorhinal cortex receive widespread inputs from a number of cortical sensory areas via connections with the perirhinal cortex, which is situated directly adjacent the lateral entorhinal cortex. All layers of the entorhinal cortex, in turn, reciprocate these projections (Insausti, Herrero, & Witter, 1997; Van Hoesen & Pandya, 1975; Witter et al., 1989). In addition to inputs from the perirhinal cortex, the superficial layers of the entorhinal cortex also receive strong direct projections from other cortical and subcortical regions.

Olfactory areas including the olfactory bulb, anterior olfactory nucleus, and piriform cortex, send substantial projections to layer I and to the superficial portion of layer II, and make contact on dendrites of layer II and layer III cells (Boeijinga & Van Groen, 1984; Gnatkovsky, Uva, & de Curtis, 2004; Krettek & Price, 1977; Room, Groenewegen, & Lohman, 1984; Scalia & Winans, 1975). The entorhinal cortex also receives direct inputs from the insular and postrhinal cortices, and minor projections from the retrosplenial, posterior parietal, infralimbic, prelimbic, visual association, and anterior cingulate cortices (Burwell & Amaral, 1998; B. F. Jones & Witter, 2007; van Groen & Wyss, 1992; Witter et al., 1989). The entorhinal cortex also receives inputs from subcortical structures including the basolateral amygdala, hypothalamus, supramammillary nucleus, raphe nucleus, and nucleus reuniens of the thalamus. An additional prominent projection from the medial septum terminates densely in lamina dissecans and, to a lesser extent, layer II (Alonso & Kohler, 1984). Therefore, inputs from a number of neocortical and subcortical areas terminate on the dendrites of superficial neurons in the entorhinal cortex. These anatomical connections indicate that the superficial layers of the entorhinal cortex receive a great deal of sensory information, and that modulation of activity in neurons located in the superficial layers may influence upstream processes in the hippocampus.

Entorhinal cortex as an interface for the hippocampal formation

Initial theories on the function of the hippocampal formation suggested that the entorhinal cortex was simply a passive relay for cortical information

destined for hippocampal processing, and the output of the hippocampus was often believed to be directed towards subcortical regions, such as the anterior thalamus and the mammillary complex (Douglas, 1967; Raisman, Cowan, & Powell, 1966). In turn, these putative termination sites were thought to project the majority of hippocampally-processed information back to the neocortex through associational areas. However, in their seminal study using autoradiographic tracers injected directly into various fields of the hippocampus, Swanson and Cowan (1977) were unable to observe any evidence supporting direct hippocampal projections to the mammillary complex or anterior thalamus. Rather, they reported strong topographically-organized projections to retrohippocampal areas, including a substantial projection to the deep layers of the entorhinal cortex, and additional studies revealed that these direct projections emanated from the CA1 and subiculum, which form the primary output region of the hippocampus (Andersen, Bland, & Dudar, 1973; Burwell, 2000; Cenquizca & Swanson, 2007; Kohler, 1985, 1986; Sorensen & Shipley, 1979; Swanson & Cowan, 1977; Witter et al., 1989). Interestingly, deep layer entorhinal neurons have extensive connections with extrahippocampal associational cortices (Witter, 1993), which suggests that the entorhinal cortex serves not as simply an input relay for the hippocampus, but rather may govern *interactions* between the hippocampus and the neocortex (Witter, Naber, et al., 2000). Given the reciprocal connections of the entorhinal cortex with brain areas involved in sensory processing, and the key role it plays in serving as the input-output center for hippocampal processing, it is clear that extrinsic modulation of both the

superficial and deep layers of the entorhinal cortex may play a crucial role in mediating processes carried out by the hippocampus and the neocortex.

The superficial and deep layers of the entorhinal cortex form both the entry and exit points, respectively, for information in the hippocampal circuit. Therefore, the entorhinal cortex serves as a convergence point for information from the neocortex, but also plays a vital role in the dissemination of hippocampally-processed information back to neocortical structures. Interactions between the neocortex and the hippocampus are thought to be critical for hippocampal-dependent sensory and mnemonic processing (Squire & Zola-Morgan, 1991). Neurons in the deep layers of the entorhinal cortex also send excitatory synaptic connections to the superficial layers of the entorhinal cortex, and form synaptic connections on both projection neurons as well as local inhibitory interneurons (Kloosterman, Van Haeften, Witter, & Lopes Da Silva, 2003; van Haeften, Baks-te-Bulte, Goede, Wouterlood, & Witter, 2003). The projection from the deep layers to the superficial layers has two major implications. First, excitatory projections from layer V neurons to the superficial layers of the entorhinal cortex suggest that information processed by the hippocampus that is carried to layer V may be able to influence how entorhinal projection neurons in the superficial layers respond to incoming sensory information. Second, innervation of local inhibitory interneurons in the superficial layers by layer V neurons may also serve to gate excitation and consequently inhibit the transfer of information from the entorhinal cortex to the hippocampal formation. Such a mechanism could serve to continuously modulate incoming

sensory information to the hippocampal formation, and promote coordination between the input and output layers of the entorhinal cortex in a manner that is dependent on hippocampal input to layer V neurons. Therefore, inputs to both the superficial and deep layers of the entorhinal cortex are likely to play an important role in modulating the activity of entorhinal projection neurons to the hippocampal formation.

The superficial layers of the entorhinal cortex receive inputs, not only from a number of different sensory and associational cortical areas, but also from portions of the parahippocampal region. In addition to inputs from the deep layers of the entorhinal cortex and perirhinal cortex, the superficial layers of the entorhinal cortex also receive a substantial input from the adjacent pre- and parasubiculum, and activity in these areas may also play a substantial role in influencing entorhinal function, and consequently hippocampal function (Caballero-Bleda & Witter, 1993, 1994; Kohler, 1985; van Groen & Wyss, 1990a; Witter et al., 1989). Although a great deal is known about the physiology of the connections of the entorhinal cortex and hippocampus, relatively little is known about the basic physiology of the connections of the parasubiculum.

Anatomy and physiology of the parasubiculum

As mentioned previously, traditional terminology includes the parasubiculum within the subicular complex, but each structure within the subicular complex has a unique pattern of connectivity with the hippocampus and parahippocampal cortices. While the parasubiculum more closely resembles the

six-layered neocortex rather than the allocortical subiculum, the parasubiculum differs from neocortex in several fundamental ways (Amaral & Witter, 1995a). The most apparent cytoarchitectonic differentiation between the parasubiculum and neocortex is the presence of the cell-free lamina dissecans, which separates the superficial and deep layers. Further, in the neocortex, large neurons located in the deep layers of the cortex (layers V-VI) typically serve as the main output neurons, whereas in the parasubiculum, the primary output neurons are located in the superficial layers.

The laminar profile of the parasubiculum has received relatively little attention. Layer I of the parasubiculum consists mostly of fibers *en passant* and contains few cells. In contrast to the entorhinal cortex and presubiculum, which demonstrate tight clustering of superficial principal cells in a transverse layer, and an easily distinguishable border between layers II and III, the superficial layers of the parasubiculum are diffuse and display no obvious border between layers II and III (Funahashi & Stewart, 1997a, 1997b; Glasgow & Chapman, 2007; Kohler, 1985). As such, layer II and III are generally labeled together as the superficial layers. Layers II/III of the parasubiculum contain primarily a broad clustering of medium- to large-sized cells with predominant pyramidal morphology, but it also contains cells with stellate morphology (Funahashi & Stewart, 1997a; Kohler, 1985). The dendritic arborization of these superficial neurons is relatively well confined to within the same cell layer with some ramifications in layer I. Below layers II/III is the lamina dissecans, a cell-free zone that is believed to be a remnant of phylogenetically conserved cell layer that may have been present in

the past. Layer IV is relatively thin, and contains few cells. Layers V and VI, which are the deepest layers of the parasubiculum, contain pyramidal and polymorphic cells, respectively, that project primarily to other deep layer neurons in the adjacent presubiculum and medial entorhinal cortex via horizontal collaterals, as well as to superficial parasubicular neurons via ascending fibers (Funahashi & Stewart, 1997a, 1997b, 1998; Kohler, 1985).

Efferent intrahippocampal connections of the parasubiculum

Only a handful of studies have provided conclusive evidence of the connections of the pre- and parasubiculum. Work by Kohler (1985) and van Groen and Wyss (1990) provided much of the anatomical evidence suggesting strong roles for pre- and parasubiculum in modulating the activity of superficial entorhinal neurons. Using small confined injections of the anterograde tracer Phaseolus vulgaris leucoagglutinin (PHA-L) into either the pre- or parasubiculum, Kohler (1985) demonstrated a marked separation of terminal sites within the entorhinal cortex. Specifically, the superficial layers of the presubiculum send axon collaterals that course ventrally through the deep layers of the presubiculum, and terminate at sites in layers I and III of the medial portion of the entorhinal cortex, with increased density of termination in layer III. In contrast to the projections from the presubiculum, injections of PHA-L within the superficial layers of the parasubiculum resulted in evidence of a strong projection to layer II of both the medial and lateral entorhinal cortex (Kohler, 1985). Extending out from the superficial layers, parasubicular fibers course through layers III and IV of

the entorhinal cortex. Similar to presubicular fibers, axon collaterals from parasubicular neurons project ventrally, and follow an organized topographical distribution such that parasubicular neurons always project to somewhat more ventral locations in the entorhinal cortex. Further, parasubicular projections follow distinct proximodistal topography; cells in the proximal parasubiculum (i.e. abutting the border of the presubiculum) project to the most medial portions of the medial entorhinal cortex, whereas more distal portions of the parasubiculum (i.e. close to the border with the medial entorhinal cortex) tend to project to lateral portions of the medial entorhinal cortex. Additionally, distal portions of the parasubiculum send substantial projections to the entirety of the lateral entorhinal cortex and terminate primarily on deep layer II neurons, although parasubicular fibers also do contact superficial layer III lateral entorhinal cells near the border of the perirhinal cortex (Caballero-Bleda & Witter, 1993).

Further anatomical work has shown that parasubicular axons terminate on dendrites and cell bodies of neurons that form the origins of the perforant path. Caballero-Bleda and Witter (1994) injected small quantities of the anterograde tracer PHA-L into the parasubiculum, as well as a retrograde tracer into the hippocampus, and individual projection neurons of the entorhinal cortex were then filled with Lucifer Yellow (LY). This methodology was particularly powerful, as it allowed for morphological identification of entorhinal projection neurons, and provided strong evidence regarding the location and number of appositions that parasubicular fibers make on entorhinal projection neurons. Axons arising from superficial parasubicular neurons formed two to three times more synapses on

distal and perisomatic dendrites of identified layer II medial entorhinal projection neurons compared to processes of layer III cells. This suggests that the activity of superficial layer parasubicular neurons can directly affect the activity of cells of origin of the perforant path and temporoammonic inputs to the hippocampus, and are likely to modulate hippocampal-dependent functions that are dependent on the responsiveness of the superficial layers of the entorhinal cortex to extrahippocampal sensory inputs.

In addition to heavy projections to the medial and lateral entorhinal cortex, the parasubiculum also has much less pronounced connections with all areas of the hippocampal formation. Injections of [^{3}H]amino acids into the parasubiculum result in labeling of the ipsilateral CA1 region, subiculum, postsubiculum, and presubiculum, as well as labeling in the contralateral pre- and parasubiculum and entorhinal cortex (van Groen & Wyss, 1990a). Additional experiments using PHA-L demonstrated that the outputs of the superficial layers of the parasubiculum are organized into three main pathways in addition to the projections to the medial and lateral entorhinal cortex (van Groen & Wyss, 1990a). The first projection courses through layer I of the parasubiculum, forms a terminal plexus in layers I and III of the presubiculum, and also courses dorsally to send additional collaterals that terminate in the deep layers of the postsubiculum. A second bundle of fibers from the parasubiculum travels via the fimbria/fornix and provides weak inputs to stratum moleculare of the temporal third of the CA1 region and subiculum. The remaining fibers of this projection then cross the hippocampal commissure and terminate in the contralateral

entorhinal cortex and pre- and parasubiculum (van Groen & Wyss, 1990a). Finally, the superficial layers of the parasubiculum also give rise to a fairly substantial projection to the outer two-thirds of the molecular layer of the dentate gyrus, and a minor projection to the hilar region of the dentate gyrus (Amaral & Witter, 1995a; Kohler, 1985; Witter et al., 1989; Witter, Holtrop, & van de Loosdrecht, 1988). Therefore, although the major output of the parasubiculum is to layer II of the medial and lateral entorhinal cortex, there are also extensive, although weaker, projections within the hippocampal formation through which the parasubiculum can modulate hippocampal-dependent processes (Witter et al., 1989).

Afferent intrahippocampal connections of the parasubiculum

Many of the output connections of the parasubiculum are reciprocated, and the parasubiculum receives several major synaptic inputs. Initial studies found that injections of retrograde tracers into the parasubiculum resulted in the labeling of neurons throughout the hippocampal formation and surrounding areas, including the entorhinal and perirhinal cortices, all CA fields of the hippocampus, the subiculum, presubiculum, and postsubiculum (van Groen & Wyss, 1990a). Injections of anterograde tracers into these areas demonstrated that the majority of inputs to the parasubiculum terminate exclusively on layer II neurons. Layer II neurons of the entorhinal cortex send a relatively light projection back to the parasubiculum, and the superficial layers of the pre- and postsubiculum also send modest projections to layer II of the parasubiculum

(Kohler, 1985; Room & Groenewegen, 1986b; van Groen & Wyss, 1990a; Witter, Room, Groenewegen, & Lohman, 1986). The subiculum sends a modest projection that traverses the deep layers of the pre- and parasubiculum, making a number of contacts with deep layer neurons, before terminating on neurons in the superficial layers of the parasubiculum (Kloosterman, Witter, & Van Haeften, 2003; Kohler, 1985; Swanson & Cowan, 1977). Additionally, recent studies have demonstrated that all dorsoventral levels of the CA1 project to the parasubiculum, terminating primarily in layer I, with additional light terminal densities in layers IV and V (Cenquizca & Swanson, 2007; van Groen & Wyss, 1990b). These findings indicate that the parasubiculum receives information from the CA1 region and the subiculum, which are the major output structures of the hippocampal complex. Because these hippocampal inputs terminate on the dendrites of layer II of the parasubiculum, these connections may serve to modulate the activity of layer II parasubicular neurons that project to layer II of the entorhinal cortex.

Extrahippocampal connections of the parasubiculum

Interestingly, the parasubiculum projects to very few extrahippocampal areas. In contrast to the massive innervation of the entorhinal cortex that arises from layer II of the parasubiculum, the majority of the modest extrahippocampal parasubicular projection to areas 17 and 18b of the visual cortex originates in the deep layers of the parasubiculum (Donovan & Wyss, 1983). The deep layers of the parasubiculum (layers V-VI) also send a dense plexus of fibers that

terminates in the anterodorsal thalamus (Amaral & Witter, 1995a; van Groen & Wyss, 1990a). The parasubiculum and presubiculum also give rise to prominent bilateral projections to the mammillary complex, with heavier ipsilateral projections (Donovan & Wyss, 1983; Meibach & Siegel, 1977; Swanson & Cowan, 1977). A weak projection from the deep layers of the parasubiculum also terminates in the ventral claustrum (Witter, Room, Groenewegen, & Lohman, 1988).

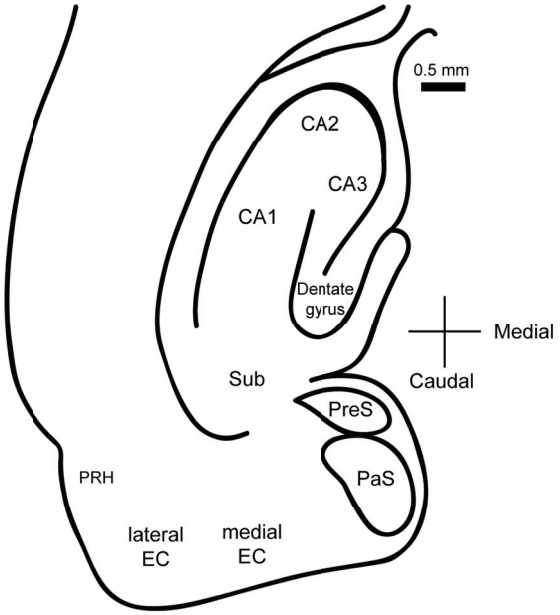
The parasubiculum receives inputs from a number of extrahippocampal cortical and subcortical regions. All layers receive light projections from the deep layers of the retrosplenial cortex, and neurons in layer II/III receive afferents from occipital visual areas (Shibata, 1994; van Groen & Wyss, 1992; Vogt & Miller, 1983). Recently, additional projections from the anterior cingulate, infralimbic and prelimbic cortices have been described, and are believed to terminate primarily in layers I and V of the parasubiculum (B. F. Jones & Witter, 2007). Layers I and II also receive substantial inputs from the claustrum, basolateral amygdala, and endopiriform nucleus (Krettek & Price, 1977; Pikkarainen, Ronkko, Savander, Insausti, & Pitkanen, 1999; van Groen & Wyss, 1990a; Witter, Room, et al., 1988). In addition to these cortical inputs, the parasubiculum also receives inputs from a number of subcortical brain regions. Most prominently, the superficial layers of the parasubiculum receive a dense projection from the rostral anterodorsal thalamus, as well as substantial inputs from the rostral portion of the laterodorsal thalamus and nucleus reuniens (Herkenham, 1978; van Groen & Wyss, 1990a). Superficial layers of the

parasubiculum also receive direct projections from areas that are associated with neuromodulation, including large inputs from the ventral tegmental area, locus coeruleus, and the midbrain raphe nuclei, areas that house dopaminergic, noradrenergic, and serotonergic cells, respectively (Kohler, Chan-Palay, & Steinbusch, 1981; Room & Groenewegen, 1986a; Swanson, 1982; van Groen & Wyss, 1990a; Witter et al., 1989). Moreover, the parasubiculum receives an extremely dense projection of fibers from the medial septum and diagonal band of Broca, which terminate across all layers (Alonso & Kohler, 1984; Gaykema et al., 1990; Swanson & Cowan, 1979). Although the neurochemical identity of all of the components of this pathway have not been identified conclusively, there is solid evidence that many of these fibers employ the neurotransmitter acetylcholine (Alonso & Kohler, 1984; Amaral & Kurz, 1985; Gaykema et al., 1990; van Groen & Wyss, 1990a).

Given the strong connections between the parasubiculum and areas that have been implicated in spatial navigation (i.e., CA1 of the hippocampus, medial septum, anterior thalamus, retrosplenial cortex), it is likely that the input and output pathways of the parasubiculum may contribute to spatial processing, and that it may do so via its output to layer II of the entorhinal cortex. This output pathway may affect how the hippocampal formation responds to incoming information from extrahippocampal areas (Caruana & Chapman, 2004), and it may also contribute to the spatial functions of the entorhinal cortex (Boccara et al., 2010; Hafting et al., 2005; Sargolini et al., 2006; Witter & Moser, 2006). Thus, the connectivity of the parasubiculum is consistent with a role of the

parasubiculum in the spatial and mnemonic functions of the hippocampal formation.

A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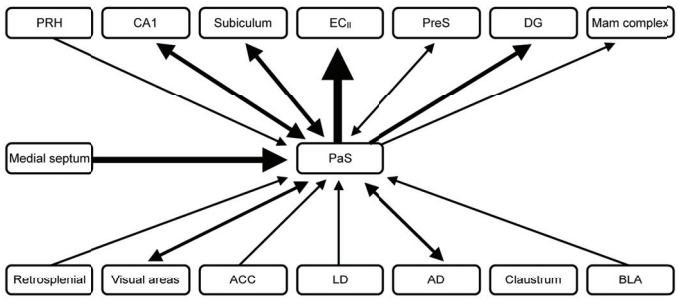
PRH
CA1
Subiculum
ECII
PreS
DG
Mam complex
Medial septum
PaS
Retrosplenial
Visual areas
ACC
LD
AD
Claustrum
BLA

Figure 1.1. The anatomical location and connections of the parasubiculum. **A**. The location of the parasubiculum (PaS) is shown on a tracing of a representative horizontal section. **B**. A summary diagram detailing the extrinsic cortical and subcortical connections with the parasubiculum. Bold lines indicate major projections. The CA1 region of the hippocampus, subiculum, anterodorsal thalamus, and the medial septum send major projections to the superficial layers of the parasubiculum, which in turn sends a major projection to the superficial layers of the entorhinal cortex (adapted from Amaral & Witter, 1995). PRH: perirhinal cortex; CA1: Cornu Ammonis region 1; EC: entorhinal cortex; PreS: presubiculum; DG: dentate gyrus; Mam complex: mammillary complex; PaS: parasubiculum; ACC: anterior cingulate cortex; LD: laterodorsal thalamus; AD: anterodorsal thalamus; BLA: basolateral amygdala.

PART 3: THE PARASUBICULUM AND SPATIAL PROCESSING

A variety of lines of evidence suggest that the parasubiculum may contribute to spatial memory and navigation. Early studies of maze-learning indicated that animals are able to form internalized maps of their environment and, therefore, that processes within the brain must necessarily mediate the formation of internalized representation of space (Tolman, 1948). Initial studies examining the role of the brain in spatial navigation suggested that the hippocampal formation is involved in spatial behavior; rats with damage to part or all of the hippocampus demonstrated persistent hyperactivity in novel environments compared to control animals that show rapid reductions in exploratory behavior with time that were thought to be due to increased familiarity with the environment (Teitelbaum & Milner, 1963). Similarly, both rats and humans with hippocampal damage perform poorly on spatial memory tasks such as the radial arm task, Morris water maze, and the Hebb-Williams maze task (Bartsch et al., 2010; Douglas, 1967; Gross, Chorover, & Cohen, 1965; Hock & Bunsey, 1998; R. L. Isaacson, Schmaltz, & Douglas, 1966; Morris et al., 1982; M. B. Moser, Moser, Forrest, Andersen, & Morris, 1995; Smith, 1988; Smith & Milner, 1981). In addition, hippocampal lesions impair spatial context-dependent discrimination (Frankland, Cestari, Filipkowski, McDonald, & Silva, 1998; Save, Poucet, Foreman, & Buhot, 1992) and place learning (for review, see (Suzuki, 2006) suggesting that the hippocampus and related structures contribute to spatial cognition. It is now known that many cortical areas are involved in the representation of the external spatial environment (Ekstrom et al., 2003; Witter &

Moser, 2006), and recent evidence suggests that parahippocampal areas including the entorhinal cortex and the parasubiculum are important for spatial processing (Boccara et al., 2010; Hafting et al., 2005; Hargreaves, Rao, Lee, & Knierim, 2005; P. Liu, Jarrard, & Bilkey, 2001, 2004; Ploner et al., 2000; Sargolini et al., 2006; Solstad, Boccara, Kropff, Moser, & Moser, 2008). The following sections will discuss the role of the parasubiculum in spatial navigation and processing.

Effects of parasubicular damage on spatial behaviour

Evidence supporting a role for the parasubiculum in spatial memory is sparse. However, the parasubiculum receives strong inputs from areas associated with spatial navigation, including the hippocampus, retrosplenial cortex, and anterior thalamus and it also sends its major output to layer II of the entorhinal cortex (Amaral & Witter, 1995a; Witter et al., 1989). This suggests that the parasubiculum may play an important role in spatial processing associated with these structures. The superficial layers of the entorhinal cortex provide the majority of neocortical input to the hippocampal formation, and the entorhinal cortex is likely to carry much of the sensory information that the hippocampal formation requires for its spatial functions. Accordingly, early experiments examining the role of the parahippocampal region in spatial memory processes demonstrated that combined lesions that included the entorhinal cortex and the pre- and parasubiculum often resulted in severe memory deficits on a myriad of spatial memory tasks, including the Morris water maze (Galani,

Jarrard, Will, & Kelche, 1997; Hardman et al., 1997; Schenk & Morris, 1985) and radial arm maze (Y. H. Cho & Kesner, 1996; Olton, Walker, & Gage, 1978), as well as substantial impairments on memory tasks that require object-place associations (P. Liu et al., 2001; Malkova & Mishkin, 2003). Additional studies indicated that lesions to the parahippocampal region, including the entorhinal and perirhinal cortices, as well as the pre- and parasubiculum, resulted in deficits on tests that assess spatial discrimination, while failing to affect simple black-white discrimination (Rothblat, Vnek, Gleason, & Kromer, 1993). Taken together, these findings suggest that the parahippocampal region is important in spatial processes, including navigation and spatial object associations, but is not required for simple perceptual abilities (Burgess, 2008b; Witter & Moser, 2006).

Since cortical inputs reach the hippocampus via the entorhinal cortex, spatial and memory deficits resulting from lesions of the retrohippocampal region may be due to 1) a disruption of processes associated with the entorhinal cortex function, 2) a loss of cortical innervation of the hippocampus through its connections with the entorhinal cortex, or 3) a disruption of processes associated with other regions of the parahippocampal area. Animals with lesions of the entorhinal cortex using ibotenic acid, which spares fibers of passage, do not show impaired performance on spatial maze tasks (Bannerman et al., 2001), and show only minor deficits on tasks such as the radial arm maze, which assess forms of spatial memory (Galani, Obis, Coutureau, Jarrard, & Cassel, 2002; Jarrard, Davidson, & Bowring, 2004). These studies suggest that other regions of the parahippocampal cortex, including the pre- and parasubiculum, may play

important roles in spatial navigation and spatial memory, and that the spatial functions of the medial entorhinal cortex are likely to be related to its anatomical and functional links to adjacent parahippocampal structures including the parasubiculum.

Lesion studies have demonstrated that the pre- and parasubiculum contributes to some forms of spatial memory. Specifically, Kesner and Giles (1998) found that electrolytic lesions of the pre- and parasubiculum, but not of the entorhinal and perirhinal cortices, resulted in deficits in continuous spatial recognition memory. In this study, rats were placed in a 12-arm radial maze, and were trained to enter each of the arms to obtain a food reward. The rats were presented with access to each arm sequentially, and through the 12 presentations, access was given to a small number of previously accessed arms. Successful completion of each trial was dependent on the rat's ability to "remember" which arms had been visited previously using extramaze spatial cues; access to novel arms was reinforced with a food reward, whereas previously encountered arms were not. After a period of training, all rats showed increased latencies to enter and traverse previously entered arms, suggesting that the animals were able to retain a spatial representation of arms that had already been visited. Upon reaching criterion performance levels, various regions of the parahippocampal region were lesioned, including the medial and lateral entorhinal cortex, as well as the pre- and parasubiculum. Animals with lesions confined to the lateral or medial entorhinal cortex failed to show any impairment, and demonstrated long latencies to enter and traverse arms that had

been presented repeatedly, indicating that lesions to the entorhinal cortex do not impair short-term memory for spatial cues. In contrast, animals with lesions to the pre- and parasubiculum demonstrated consistently short latencies to enter previously accessed arms. This finding was interpreted to suggest that destruction of the pre- and parasubiculum resulted in an inability to remember prior spatial locations (Kesner & Giles, 1998), and since this task requires that the animal remember which arms have been previously visited in order to successfully complete the trial, the impairments associated with damage to the pre- and parasubiculum suggest that these areas are required for a form of short-term spatial "working memory" (Olton & Papas, 1979).

A similar study aimed at assessing the contribution of the pre- and parasubiculum to working memory have confirmed and extended these findings. Jarrard et al. (2004) used a novel radial arm maze paradigm to examine the role of the pre- and parasubiculum in working memory. In the spatial cue version of the task, the rat was placed in the center of a radial arm maze that allowed for free access to all arms, and was trained to search for sucrose pellets at the end of all eight arms. After four arms were visited, the rat was removed for a period of one minute, and then returned to the center of the maze. In order to successfully complete a trial, the animal must recall relationships between extramaze cues to determine which arms were previously accessed, and seek the unvisited arms. Compared to control animals, rats with lesions to the pre- and parasubiculum commit significantly more errors in arm entries when only using spatial cues, suggesting that the pre- and parasubiculum are involved in

forms of spatial working memory. However, in a variation of this task in which the floor of each arm was covered in a distinct material (ie. wire mesh, carpet, sandpaper, etc.), animals with damage to the pre- and parasubiculum showed *enhanced* performance, and committed fewer errors in access to arms compared to control animals, as well as animals with lesions to either the entorhinal cortex or subiculum (Jarrard et al., 2004). The perirhinal cortex is important in object recognition (Murray & Richmond, 2001) and sends heavy projections to layer II entorhinal neurons (Amaral & Witter, 1995a; Witter et al., 1989). Lesions of the pre- and parasubiculum may impair the ability of the animal to use a spatial strategy and may simultaneously promote the use of a more efficient object-recognition cue-based strategy, and this interpretation is consistent with a spatial role of the parasubiculum.

Additional work using excitotoxic lesions of the pre- and parasubiculum have demonstrated that these areas contribute to spatial memory processes and object recognition (P. Liu et al., 2001). Liu and colleagues (2001) demonstrated that animals with ibotenic acid lesions of the pre- and parasubiculum show major performance deficits on tasks that require spatial memory, such as the Morris water maze (Morris et al., 1982). In this study, animals were placed in a water maze pool, and trained to use extramaze cues to locate a submerged platform. Five days after the final training session, animals were assessed on a probe trial, in which the platform was removed, and the percentage of time spent swimming in the quadrant of the pool that previously housed the platform was assessed. Compared to control animals, animals with lesions to the pre- and parasubiculum

took significantly longer to locate a submerged platform during the training phase of the experiment, suggesting impairments in the acquisition of the task. Similarly, during the probe phase in which the platform was removed, these animals spent significantly less time in the quadrant that previously held the platform, suggesting a deficit in the ability of the animal to maintain a spatial representation of the platform's location. Interestingly, some animals were able to find the previous location of the platform after repeated exposures to a visible platform, despite extensive damage to the pre- and parasubiculum (P. Liu et al., 2001). These findings demonstrate that, while the parasubiculum contributes to the integration of spatial cues, it is not *required* for spatial navigation. Similarly, animals with lesions to the pre- and parasubiculum also performed poorly on object recognition tests (P. Liu et al., 2001). After a series of training sessions with familiar objects in an open field, animals were presented with novel objects. Upon introduction of a novel object to the test arena, control animals spent significantly more time investigating the novel object, while animals with lesions to the pre- and parasubiculum show only moderate increases in time spent investigating the new object, suggesting an impairment in the ability to recall and associate familiarity and novelty. Taken together, these studies suggest a role of the parasubiculum in spatial processing, but that it may also contribute to object recognition in some contexts.

Place-dependent cell firing in the hippocampal formation

Work by John O'Keefe and his colleagues demonstrated the presence of cells in the rat hippocampus that fire selectively in relation to the animal's position in space, independent of other behaviour-dependent correlates (O'Keefe, 1976; O'Keefe & Dostrovsky, 1971; O'Keefe & Nadel, 1978). The firing rate of these "place cells" is modulated as a function of the animal's location in space, and demonstrates distinct characteristic firing properties consisting of a rapid succession of complex spikes at frequencies of 5 to 20 Hz when the animal moves into a specific area of their environment referred to as "place fields" (*X* and *Y*; (O'Keefe, 1979; Ranck, 1973). Remarkably, the firing rate of a place cell becomes virtually silent when the animal is outside of the respective place field (Best & Ranck, 1982; Miller & Best, 1980; O'Keefe & Dostrovsky, 1971; Olton, Branch, & Best, 1978). The actual dimensions of place fields vary considerably between cells, ranging from very narrow to relatively large, but they typically represent approximately 10% to 20% of the rat's environment (Best, White, & Minai, 2001; O'Keefe, 1979; O'Keefe & Nadel, 1978). Recent evidence suggests that place cell activity may even vary as a function of the vertical plane in three-dimensional space (Knierim & McNaughton, 2001; Knierim, McNaughton, & Poe, 2000). While place cells are distributed throughout the hippocampal formation, cells in the dorsal hippocampus display the most robust and spatial information-rich place fields. Interestingly, these place fields are persistent representations, enduring for weeks to months (Thompson & Best, 1990), and cannot be disrupted unless large-scale alterations are made to environmental cues (Muller & Kubie, 1987). Additionally, place cells are phylogenetically conserved across

species, and have been reported in mice (McHugh, Blum, Tsien, Tonegawa, & Wilson, 1996), homing pigeons (Hough & Bingman, 2004), monkeys (Hori et al., 2005; Ono, Nakamura, Nishijo, & Eifuku, 1993), and humans (Ekstrom et al., 2003), with some recent work suggesting that insects such as honey bees also form spatial maps (Menzel et al., 2005). O'Keefe and Nadel (1978) suggested that animals may form internalized cognitive representations of their environment via the activity of place cells.

The activity of place cells is strongly determined by location, and is independent of other aspects of behaviour, such as head-direction and running speed (Muller, Bostock, Taube, & Kubie, 1994). Accurate spatial navigation requires both the representation of location, as well as direction of travel, and requires both types of information to formulate trajectories through the environment (Taube, 1998). Since the majority of place cells in the hippocampus do not demonstrate directional tuning, directional information is likely coded by neural systems outside of the hippocampus. The presence of a neural directional navigation system was first reported in the subicular complex on the basis of evidence of cells that fire in relation to head direction (Taube, Muller, & Ranck, 1990), but it is now known that cells in other areas of the brain also show similar directional tuning, including cells in the anterodorsal thalamus (Blair & Sharp, 1995; Taube, 1998), laterodorsal thalamus (Mizumori & Williams, 1993), retrosplenial cortex (J. Cho & Sharp, 2001), and the entorhinal cortex (Sargolini et al., 2006). These so-called 'head direction' (HD) cells discharge preferentially when the animal is oriented in a particular arbitrary direction relative to the frame

of the surrounding environment (Taube, 1998). The activity of these cells is independent of the animal's location in the environment, insensitive to changes in pitch or rolls of the head, and not correlated with any observable ongoing behaviour (Taube, 1998), suggesting that these cells are involved solely in the processing of directional information. The relationship of the entorhinal cortex with areas that show location- and direction-specific cell firing suggest that the entorhinal cortex may also contribute to navigational processes.

Early electrophysiological investigations by a number of investigators tracking the firing rate of individual cells during spatial exploration found that neurons in the ventromedial entorhinal cortex displayed only modest spatial selectivity and, compared to CA1 place cells, transmitted very low levels of spatial information per spike (Barnes, McNaughton, Mizumori, Leonard, & Lin, 1990; Frank, Brown, & Wilson, 2000; Mizumori, Ward, & Lavoie, 1992; Quirk, Muller, Kubie, & Ranck, 1992). These findings suggested that the entorhinal cortex is not a central component of the computational processes underlying the formation of allocentric spatial maps. However, while the ventral portions of the entorhinal cortex project primarily to the ventral areas of the hippocampus, the dorsolateral band of the medial entorhinal cortex sends strong inputs to dorsal areas of the hippocampus which contain the most robust place cell activity and have been demonstrated to play an important role in spatial memory (Fyhn et al., 2004; E. Moser, Moser, & Andersen, 1993; Witter et al., 1989). In a series of groundbreaking studies, Edvard Moser and colleagues investigated the activity of cells in the dorsolateral band of the medial entorhinal cortex during spatial

exploration (Fyhn et al., 2004; Hafting et al., 2005; Witter & Moser, 2006). They found that a subset of cells in the dorsolateral band of the medial entorhinal cortex fire in a regular, tessellating distribution across a two-dimensional area as an animal moves through an environment. Because the multiple place fields observed for each of these cells were located at locations that could be covered by the vertices of a grid of equilateral triangles, the cells were termed “grid cells,” and may form the basis of an internal allocentric metric system (Fyhn et al., 2004; Hafting et al., 2005). Comparisons between these dorsolateral medial entorhinal cells and hippocampal place cells revealed similar information bit rates, suggesting that the entorhinal cells process large amounts of spatial information and thus contribute significantly to spatial representations (Fyhn et al., 2004; Hafting et al., 2005; Hargreaves et al., 2005). Although grid cells have been reported across all layers of the medial entorhinal cortex, layer II contains the highest proportion of cells that fire in a grid-like manner, whereas cells in deep layers show far fewer grid properties (Hafting et al., 2005; Witter & Moser, 2006). Grid cell activity is driven by extra-hippocampal sensory inputs, as ablation of the hippocampus fails to degrade the entorhinal place fields, suggesting that highly-refined spatial processing occurs outside of, and independent of, the hippocampus (Hafting, Fyhn, Bonnevie, Moser, & Moser, 2008). Furthermore, lesioning of the medial entorhinal cortex disrupts the activity of CA1 place cells, suggesting that grid cells are necessary for place cell representations within the hippocampus (Brun et al., 2008). These data suggest that grid cells in the entorhinal cortex, combined with head-directional and

locational information from the presubiculum and CA1, respectively, may serve as the basis of an allocentric spatial map within the hippocampal formation, and also suggest that the entorhinal cortex makes a central contribution to the representation of space in other areas of the hippocampal formation.

Following the discovery of grid cells in the entorhinal cortex, two other spatially-tuned cell populations have been reported in the entorhinal cortex. A subset of layer III-VI cells show tuning to both direction and location, and have a firing rate that is modulated by the speed of the animal within the environment; these are termed velocity-sensitive conjunctive grid by direction cells (Sargolini et al., 2006). An additional group of cells respond solely when an animal is traveling along the physical geometric border of their environment, and are termed 'border' cells (Solstad et al., 2008). Taken together, this suggests that cells of the medial entorhinal cortex code for several forms of information relevant to spatial navigation, and that this information may contribute to the activity of place cells within the hippocampal formation.

The firing activity of parasubicular neurons also show location- and directional specificity (Cacucci et al., 2004; Hargreaves et al., 2005; Hargreaves, Yoganarasimha, & Knierim, 2007; Taube, 1995b). Initial studies of parasubicular cells showed that their firing is similar to hippocampal place cells but with substantially larger place fields (Taube, 1995b). Similar to place cells, the parasubicular cells fired in a specific area of the testing chamber, irrespective of the animal's direction and ongoing behavior, but, subsequent recordings in the pre- and parasubiculum demonstrated the presence of "place-by-direction" cells,

which encode spatial information about both location and direction in allocentric space (Cacucci et al., 2004). Place-by-direction cells demonstrate properties similar to location-specific place cells of the hippocampus as well as the directionally-tuned cells in the dorsal presubiculum (Sargolini et al., 2006). Although the first studies of place-dependent firing in the parasubiculum used small testing enclosures which may have prevented the detection of the multiple firing fields characteristic of grid cells (Fyhn et al., 2004; Hafting et al., 2005), recent work from Edvard Moser's lab using larger testing environments has demonstrated the presence of grid cells, as well as, HD, border, and conjunctive cells within all layers of the parasubiculum (Boccara et al., 2010). Approximately 20% of all parasubicular neurons fire in a location-specific manner, and form grids of firing fields across local environments (Boccara et al., 2010). Grid cells in the parasubiculum are similar to entorhinal grid cells, and, unlike neurons in layer II of the medial entorhinal cortex that typically fail to respond to direction and location, the superficial layers of the parasubiculum contain a significant number of conjunctive cells. It is therefore likely that the place and place-by-direction cells reported previously using small testing enclosures (Cacucci et al., 2004; Taube, 1995b) may reflect the activity of grid and conjunctive grid cells, respectively (Boccara et al., 2010). These findings indicate that grid cells are not exclusive to the entorhinal cortex, and are expressed across all layers of the parasubiculum. The diversity of the parasubicular cell types that are sensitive to different aspects of spatial location and navigation suggests that the parasubiculum, through its output to the entorhinal cortex and other portions of

the hippocampal formation, may play a rich role in spatial processing and navigation.

The parasubiculum contains cells that fire in relation to external space. However, because the parasubiculum sends projections to the superficial layers of the entorhinal cortex, it is possible that the parasubiculum may also be involved in governing location-specific cell firing in other regions of the hippocampal formation. Consistent with this idea, lesions of the pre- and parasubiculum disrupt the place field specificity, and reduce the spatial information content, of hippocampal place cell firing (P. Liu et al., 2004). These data indicate that the pre- and parasubiculum contribute to the maintenance and specificity of hippocampal place cell firing fields, and may contribute to spatial representations within the hippocampus, either directly via connections with the hippocampus proper, or through indirect connections with the entorhinal cortex (Kohler, 1985; P. Liu et al., 2004; van Groen & Wyss, 1990a; Witter et al., 1989). However, it remains unclear whether the parasubiculum is necessary for the maintenance of grid cells in the medial entorhinal cortex. Taken together, however, these data indicate that the pre- and parasubiculum are likely to contribute to spatial navigational processes mediated by place cells of the hippocampal formation.

Cholinergic modulation of theta-frequency activity in the parasubiculum

The firing activity of many of the cells throughout the hippocampal formation, including the parasubiculum, are modulated by local

electroencephalographic (EEG) activity (Buzsaki, 2002; Buzsaki & Draguhn, 2004; O'Keefe, 1993; Skaggs et al., 1996). Synchronized neuronal population activity is associated with coordinated synaptic activity that is reflected in EEG recordings of the local extracellular field potential, and this synchronization may serve as a temporal structure to coordinate interactions both within cortical structures, and between synaptically connected brain regions. In mammals, EEG activity in the hippocampal formation is dominated by the theta rhythm, a 4-12 Hz sinusoidal-like waveform that has been linked to computational processes that mediate active exploration, sensorimotor integration, and memory consolidation (Bland, 1986; Buzsaki, 2002; Hasselmo, 2005; Vanderwolf, 1969). Although theta activity can be observed most robustly in stratum lacunosum-moleculare of the CA1 region of hippocampus, it has also been recorded in the dentate gyrus and CA3 region (Bland & Whishaw, 1976; Kramis, Vanderwolf, & Bland, 1975). In addition to the hippocampus, theta activity has been recorded in adjacent structures including the entorhinal, perirhinal, and cingulate cortices, the amygdala, and more recently, in the superficial layers of the parasubiculum (Alonso & Garcia-Austt, 1987a; Buzsaki, 2002; Glasgow & Chapman, 2007; L. W. Leung & Borst, 1987; Mitchell & Ranck, 1980; Pare & Collins, 2000). Hippocampal theta oscillations can be distinguished into two main classes based on pharmacological and behavioral evidence (Kramis et al., 1975). Type I theta is resistant to systemic administration of the cholinergic antagonist atropine, and is typically observed during active exploration of an environment or manipulation of objects. In contrast, type II theta is predominantly observed during alert

immobility or during urethane anesthesia, and is completely abolished by administration of atropine, and so is considered to be atropine-sensitive (Kramis et al., 1975; Vanderwolf, 1969). The functional differentiation between atropine-sensitive and –insensitive theta is not well-understood, but it appears to underlie two distinct forms of network synchronization.

Despite over half a century of intensive research, the precise function of the theta rhythm remains unclear. It has been postulated that theta activity may contribute to mechanisms underlying memory formation through the binding of "cell assemblies" (Harris, Csicsvari, Hirase, Dragoi, & Buzsaki, 2003; Kudrimoti, Barnes, & McNaughton, 1999; Maurer, Cowen, Burke, Barnes, & McNaughton, 2006a; O'Neill, Senior, Allen, Huxter, & Csicsvari, 2008). Hebbian cell assemblies are large cortical networks of neurons that are proposed to represent a percept or engram based on a specific spatiotemporal sequence of activation of synaptic inputs (Hebb, 1949). Hebb proposed that, by strengthening the synaptic connections between neurons that are activated by a perceptual stimulus, an assembly of cells could be formed that represent the stimulus or percept. The addition of multiple assemblies could form representations of increasingly complex stimuli. Within this framework, therefore, Hebb proposed that learning is the result of the activity-dependent strengthening of synaptic connections that link the component neurons of the cell assembly. Moreover, he suggested that the synaptic connections within the cell assembly could be strengthened based on repeated activation of the pre- and post-synaptic neuron, such that if a presynaptic neuron consistently contributed to the firing of the

postsynaptic neuron, then repeated activation of the synapse would lead to the lasting strengthening of the connection (Hebb, 1949).

In the first physiological study to provide support for this hypothesis, Timothy Bliss and Terje Lømo demonstrated that repeated activation of entorhinal inputs to the dentate gyrus using high frequency trains of stimulation in both the anesthetized and freely-moving rabbit can lead to a potentiation of the amplitude of synaptic responses (Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973). Responses to stimulation were enhanced for up to 3 days, leading to the idea that this long-term potentiation (LTP) of responses could serve as a neural mechanism underlying long-term memory storage. It is now known that many different brain areas show LTP, and LTP has been observed in both neocortical and limbic structures (Bliss & Collingridge, 1993). Further, LTP can be induced using physiologically realistic stimulation parameters that are likely to be active during learning, including theta-patterned stimulation protocols (Larson, Wong, & Lynch, 1986). At the network level, the theta rhythm may serve to promote the temporal summation of excitatory synaptic responses, and this feature may contribute to the formation of cell assemblies by enhancing postsynaptic activation. Additionally, theta activity may serve to rhythmically synchronize cells so that inputs to certain cell groups arrive at particular phases of the oscillation. For example, weak inputs that arrive at the trough of the field oscillation are not likely to result in a postsynaptic spike, whereas relatively weak inputs arriving at the peak of the oscillation may result in neural activation (Hyman et al., 2003; Judge & Hasselmo, 2004). In other words, the phase of the local theta cycle may

serve as a temporal filter, with synaptic inputs that arrive out of phase being discarded, and appropriately timed inputs resulting in long-term synaptic enhancement (Hyman et al., 2003; Orr, Rao, Houston, McNaughton, & Barnes, 2001; Pavlides, Greenstein, Grudman, & Winson, 1988). Therefore, a major function of theta activity in the hippocampal formation is likely to be the synchronization of local synaptic activity to enhance the postsynaptic depolarization required for activity-dependent strengthening of synaptic connections. This may serve as one of the primary neurophysiological mechanisms for the development of cell assemblies that underlie memory formation.

The parasubiculum sends its major output projection to layer II EC cells, and parasubicular theta activity may therefore play an important role in shaping computational processes within the entorhinal cortex (Amaral & Witter, 1989, 1995b; K. Wu & Leung, 1998). In anesthetized animals *in vivo*, Caruana and Chapman (2004) demonstrated that the parasubiculum can alter how the entorhinal cortex responds to incoming sensory information carried by inputs from the piriform cortex. A strong conditioning stimulation pulse delivered to the parasubiculum 5 ms prior to a test pulse delivered to the piriform cortex resulted in an inhibition of the resulting synaptic response in the entorhinal cortex. In contrast, entorhinal responses were enhanced when the conditioning pulse delivered to the parasubiculum was delivered 20-150 ms prior to the test pulse in the piriform cortex. Because parasubicular neurons receive synaptic inputs from the CA1 and subiculum, the parasubiculum may represent a mechanism by

which ongoing output from these hippocampal structures can modify the responsivity of entorhinal projection neurons to inputs from sensory and associational cortices. Moreover, the time-dependent nature of the facilitation of the responsiveness of the entorhinal cortex by the parasubiculum suggests that endogenous rhythmic activity may be important in facilitating synaptic responses (Caruana & Chapman, 2004). Parasubicular stimulation promotes entorhinal responses at the period of both gamma- and theta-frequencies, and inhibits excitatory transmission at short intervals that match the period of high frequency sharp waves. Therefore, the parasubiculum is well-poised to affect the integration of new information within the entorhinal cortex, and thus, it is necessary to understand the basic cellular and network mechanisms that govern the output of the parasubiculum during rhythmic oscillations.

Studies of cellular discharge patterns in parahippocampal cortices in relation to local field activity have revealed marked laminar differences in activity associated with characteristic EEG patterns (Chrobak & Buzsaki, 1994). Deep layer cells increase their activity during sharp waves which are large amplitude (1-3 mV) aperiodic field events that are behaviorally correlated with awake immobility and are thought to contribute to memory consolidation (Buzsaki, 1986; Foster & Wilson, 2006; A. K. Lee & Wilson, 2002; O'Neill et al., 2008). In contrast, the discharge patterns of superficial layer cells in retrohippocampal cortices are typically phase-related to local theta activity, and do not respond during sharp waves (Chrobak & Buzsaki, 1994). Consistent with this finding, a number of studies have found that parasubicular cells fire in close relation to

local theta field activity (Boccara et al., 2010; Cacucci et al., 2004; Chrobak & Buzsaki, 1994; Taube, 1995b), although the proportion of theta-related cells is lower than found in the adjacent entorhinal cortex (Boccara et al., 2010; Taube, 1995b).

Theta-frequency EEG activity in the hippocampal formation and entorhinal cortex is thought to rely on tonic cholinergic depolarization and phasic GABAergic inhibition from the medial septum (Alonso & Garcia-Austt, 1987a, 1987b; Bland, 2000; Buzsaki, 2002; Sotty et al., 2003; Sun, Zhao, Nelson, & Alkon, 2001; Swanson & Cowan, 1979), and similar mechanisms may also be at play in the parasubiculum. The superficial layers of the parasubiculum receive a strong cholinergic projection from the medial septum (Alonso & Kohler, 1984; Swanson & Cowan, 1979; van Groen & Wyss, 1990a), and also receives inhibitory GABAergic innervation from septal projections and local inhibitory interneurons (Kohler, Wu, & Chan-Palay, 1985). Furthermore, systemic administration of the cholinergic antagonist atropine abolishes local theta-frequency EEG activity in the parasubiculum (Glasgow & Chapman, 2007), indicating that theta activity in the parasubiculum is dependent on cholinergic mechanisms and may be supported by both septal inputs and intrinsic networks. Hippocampal theta activity can be observed without cholinergic activation in vitro (Goutagny, Jackson, & Williams, 2009), but bath application of the muscarinic agonist carbachol leads to theta-like activity in conventional *in vitro* hippocampal and entorhinal slices (Dickson & Alonso, 1997; Williams & Kauer, 1997) suggesting that cholinergic mechanisms that contribute to theta-frequency

activity *in vivo* are also intact in slice preparations (Bland, Colom, Konopacki, & Roth, 1988; Konopacki, Bland, MacIver, & Roth, 1987; Konopacki, Bland, & Roth, 1988a, 1988b).

Acetylcholine may enhance theta activity by depolarizing neurons to result in oscillations of membrane potential at theta-frequency. Cholinergic agonism depolarizes hippocampal interneurons and entorhinal stellate cells to near-threshold and threshold voltages, resulting in the generation of subthreshold membrane potential oscillations and repetitive spike cluster firing (Alonso & Llinas, 1989; Chapman & Lacaille, 1999a; Dickson & Alonso, 1997; Klink & Alonso, 1997b). This suggests that cholinergic receptor activation may also depolarize layer II parasubicular neurons to promote the genesis of network level oscillations (Buzsaki & Draguhn, 2004).

Intrinsic membrane potential oscillations have been reported in several brain regions within the hippocampal formation where they can be generated by the combination of several different intrinsic voltage-dependent conductances (Burgess, Barry, & O'Keefe, 2007; Buzsaki, 2002). Field potential oscillations are associated with active currents near the soma and proximal dendrites of oscillating cells (Bland et al., 2002), and these synaptic inputs are likely driven in part by synchronized membrane potential oscillations among principal neurons. In addition to synaptic currents, depolarization of a diverse range of cell types using positive steady current injection to near-threshold voltages results in theta-frequency oscillations in membrane potential, suggesting that voltage-dependent conductances are responsible for the generation of this activity. Neurons of the

olivary nucleus demonstrate theta-frequency oscillations that are dependent on the activation of voltage-gated Ca^{2+} currents (Llinas & Yarom, 1986). In contrast, theta-frequency membrane potential oscillations in lacunosum-moleculare interneurons of the hippocampal field CA1 are due to an interaction between the persistent sodium current (I_{NaP}) and a voltage-gated A-type potassium current ($I_{Kv4.3}$) (Bourdeau, Morin, Laurent, Azzi, & Lacaille, 2007; Chapman & Lacaille, 1999b). Similarly, oscillations in both CA1 pyramidal cells and layer II entorhinal stellate cells were initially linked to persistent Na^+ currents and TEA-sensitive K^+ conductances (Alonso & Llinas, 1989; Klink & Alonso, 1993), however further studies suggest that oscillations in stellate cells of layer II medial entorhinal cortex rely on an interplay between I_{NaP} and a mixed cationic hyperpolarization-activated current I_h (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000). Specifically, the depolarizing phase of the oscillation is due to the non-inactivating persistent sodium current I_{NaP}, and the hyperpolarizing phase is due to the time-delayed deactivation of I_h. The delayed activation and deactivation kinetics of I_h are believed to account for the timing of the oscillations, and function as a temporal regulator in a “push-pull” fashion (Dickson et al., 2000). That is, hyperpolarization-dependent activation of I_h pushes the membrane potential to depolarized voltages during the up-swing of the oscillation, and depolarization-dependent deactivation of I_h pulls the membrane back to more hyperpolarized voltages. Frequency resonance studies, that use frequency-modulated intracellular current injections to determine the optimal frequencies at which the membrane potential of neurons entrains to current injection (see Hu,

Vervaeke, & Storm, 2002), have also suggested that an interaction between I_{NaP} and the muscarinically-activated M-current (I_M) may be responsible for peak resonance in the theta-frequency range in CA1 pyramidal cells (Hu, Vervaeke, & Storm, 2002, 2007). Similar findings have been reported in deep layer entorhinal cortex neurons (Hamam, Kennedy, Alonso, & Amaral, 2000). This suggests that different populations of cells in the parahippocampal cortex may use different ionic mechanisms to generate local theta network activity. Similar to cells of the medial entorhinal cortex, both principal cells and putative interneurons in the superficial layers of the parasubiculum demonstrate intrinsically-generated voltage-dependent membrane potential oscillations (Glasgow & Chapman, 2007), but the ionic conductances underlying the generation of these oscillations are not clear. Further, although cholinergic septal inputs are thought to result in depolarization of the membrane potential in hippocampal and entorhinal neurons to voltages at which oscillations are observed (Alonso & Garcia-Austt, 1987a; Bland, 1986; Buzsaki, 2002; Chapman & Lacaille, 1999a; Goutagny et al., 2008; Jeffery, Donnett, & O'Keefe, 1995; Konopacki et al., 1988b; Mizumori, Perez, Alvarado, Barnes, & McNaughton, 1990), it is not clear whether septal inputs to the parasubiculum serve a similar role.

Cholinergic inputs from the medial septum are likely to play an important role in the generation of theta-frequency membrane potential oscillations by depolarizing parasubicular neurons to near-threshold and threshold voltages where these oscillations are expressed (Alonso & Kohler, 1984; Chapman & Lacaille, 1999a; Klink & Alonso, 1997b), however it remains unclear how

cholinergic receptor activation can modulate the membrane potential of parasubicular neurons. Cholinergic agonism is known to result in significant depolarization of the resting membrane potential in hippocampal, entorhinal, and neocortical neurons through activation of M_1 receptors (Benson et al., 1988; Dutar & Nicoll, 1988; Guerineau, Bossu, Gahwiler, & Gerber, 1995; Haj-Dahmane & Andrade, 1998; Klink & Alonso, 1997a; Uchimura & North, 1990), however the possible mechanisms in parasubicular neurons is unclear. In the hippocampus and some other cortical regions, activation of muscarinic receptors has been reported to result in suppression of a myriad of K^+ channels, and the development of a slow depolarization in membrane potential (Brown & Adams, 1980; Cole & Nicoll, 1984; Hu et al., 2007; McCormick & Prince, 1986). In addition to the potassium current I_M, which is inhibited by muscarinic receptor activation (Brown & Adams, 1980; Brown & Passmore, 2009; Delmas & Brown, 2005), muscarinic receptor activation is known to modulate additional potassium conductances. Acetylcholine depolarizes striatal neurons via inhibition of I_M, a leak K^+ conductance, and the inward-rectifying K^+ channel Kir2.3 (Hsu, Yang, Huang, & Gean, 1996; Shen, Hamilton, Nathanson, & Surmeier, 2005; Shen et al., 2007), and similar findings have been reported in the amygdala (Womble & Moises, 1992), nucleus accumbens (Uchimura & North, 1990), reticular formation (Gerber, Stevens, McCarley, & Greene, 1991), and in sympathetic neurons (H. S. Wang & McKinnon, 1996). In contrast, M_1 muscarinic receptor activation in layer II stellate neurons of the medial entorhinal cortex leads to depolarization due to the activation of a Ca^{2+}-modulated non-selective cationic current, I_{NCM}, that is

primarily carried by Na^+ ions (Klink & Alonso, 1997a; Shalinsky et al., 2002). Cholinergic depolarization of layer V medial prefrontal neurons share a similar mechanism, and results in the activation of a mixed cationic current that primarily carries Na^+ ions (Haj-Dahmane & Andrade, 1998). Through activation of a cationic current, or suppression of a K^+ conductance, cholinergic receptor activation can serve as a mechanism to depolarize layer II parasubicular neurons to near-threshold voltages, and lead to the emergence of voltage-dependent theta-frequency membrane potential oscillations.

While muscarinic receptor activation is well-known to result in depolarization of principal neurons, cholinergic agonists have also been found to *suppress* excitatory glutamate-mediated synaptic transmission in the hippocampus (Hasselmo & Schnell, 1994; Hounsgaard, 1978; Sheridan & Sutor, 1990; Valentino & Dingledine, 1981) and entorhinal cortex (Cheong et al., 2001; Hamam et al., 2007; Yun et al., 2000). Muscarinically-mediated depression of synaptic transmission in these areas has been attributed to a reduction in presynaptic glutamate release, however the exact mechanism underlying this suppression is not clear (Auerbach & Segal, 1996; Hasselmo & Bower, 1992). Cholinergic receptor activation is known to modulate Ca^{2+} currents involved in presynaptic transmitter release through an adenosine-like pathway (Qian & Saggau, 1997). Therefore, while the majority of parasubicular neurons are likely depolarized to threshold voltages during theta activity via activation of muscarinic receptors, suppression of excitatory inputs may serve to concurrently limit recurrent excitation during theta activity (Hasselmo, 2006).

PART 4: SUMMARY OF EXPERIMENTAL CHAPTERS

There are three experimental chapters in this thesis. The first chapter used whole-cell patch clamp experiments to determine the mechanisms underlying the generation of voltage-dependent theta-frequency membrane potential oscillations in layer II parasubicular neurons. Results showed that oscillations in these cells are due to an interaction between a persistent sodium current, likely I_{NaP}, and a hyperpolarization-activity mixed cationic current I_h. The conductances that mediate the generation of membrane potential oscillations in layer II parasubicular neurons are similar to those found in layer II stellate cells in medial entorhinal cortex, as well as subicular, perirhinal, and sensorimotor cortical neurons, but the mechanisms do differ from those found in hippocampal principal cells (Garcia-Munoz, Barrio, & Buno, 1993; Hu et al., 2002; L. W. Leung & Yim, 1991), hippocampal interneurons (Bourdeau et al., 2007; Chapman & Lacaille, 1999b) as well as neurons in thalamus, inferior olivary nucleus, and supramammillary complex (Alonso & Llinas, 1992; Jahnsen & Llinas, 1984a; Llinas & Yarom, 1986). Rhythmic oscillation of membrane potential may contribute to the generation of local field potentials, promote rhythmic variations in synaptic responsiveness of principal cells, and may promote network synchronization through the output of inhibitory interneurons that can synchronize the firing of large numbers of principal cells (Burgess, 2008a; Burgess, Barry, & O'Keefe, 2007; Buzsaki, 2002; Buzsaki & Draguhn, 2004; Chapman & Lacaille, 1999a; Giocomo, Zilli, Fransen, & Hasselmo, 2007). Moreover, these oscillations may play a major role in coordinating neural activity

in the parasubiculum that may contribute to spatial processes, and are likely to be important in coordinating the output of parasubicular neurons to layer II cells of the entorhinal cortex.

The second experimental chapter investigated the ionic mechanisms underlying cholinergic depolarization of layer II parasubicular neurons. Acetylcholine is a neurotransmitter that is known to lead to membrane depolarization and overall increases in neuronal excitability in cells throughout the hippocampal formation. The parasubiculum receives massive cholinergic innervation from the medial septum, and it is likely that these fibers are active during theta-related behaviors, such as active exploration. As in the hippocampus, increases in cholinergic tone are likely to contribute to increases in excitability in the parasubiculum, and this may play a central role in parasubicular network activity. Moreover, cholinergic inputs from the medial septum are likely to play an important role depolarizing parasubicular neurons to membrane voltages at which theta-frequency oscillations are expressed. Using whole-cell patch clamp recordings from morphologically-identified layer II parasubicular neurons, I found that high concentrations of carbachol resulted in strong depolarization of membrane potential to near-threshold voltages in most layer II parasubicular neurons, and in some cases, led to repeated spiking. Cholinergic receptor activation also led to changes in action potential waveform including a reduction in action potential amplitude, and reductions in fast and medium afterhyperpolarizations. Pharmacological tests were used to determine that cholinergic receptor activation promotes overall excitability in a concentration-

dependent manner through activation of M_1 receptors and the resulting inhibition of two potassium currents including the muscarinically-inhibited K^+ current I_M and an inward rectifying K^+ current. These findings demonstrate that increases in cholinergic input during theta-related behaviors likely results in depolarization of parasubiculum neurons that can contribute to the genesis of theta activity in the superficial layers by leading to intrinsic voltage-dependent oscillatory activity.

The third experimental chapter of this thesis examined the effects of cholinergic receptor activation on synaptic transmission within the superficial layers of the parasubiculum. I found that synaptic responses were greatly suppressed during cholinergic receptor activation with the agonist carbachol, and that this suppression occurs in a concentration-dependent manner. The receptors that mediate the effect were assessed by application of carbachol in the presence of muscarinic receptor blockers. Both low and high doses of carbachol resulted in the cholinergic suppression of excitatory synaptic transmission in the superficial layers of the parasubiculum, and this suppression was mediated via activation of M_1 receptors. Further experiments demonstrated that cholinergic suppression of synaptic transmission in the parasubiculum is not due to increases in local inhibitory circuits. Finally, to assess whether cholinergic suppression is mediated by pre- or post-synaptic mechanisms, I used pairs of stimulation pulses to examine changes in paired-pulse facilitation induced by cholinergic agonism. I found that cholinergic receptor activation increased the paired-pulse ratio, suggesting that the cholinergic suppression of synaptic responses is mediated via a presynaptic reduction in transmitter release.

These findings indicate that, during theta-related behaviors, increases in cholinergic tone are likely to exert a wide variety of effects on parasubicular neurons. Membrane depolarization associated with cholinergic receptor activation is likely to result in substantial increases in overall excitability and may promote network synchronization by contributing to theta-frequency EEG activity. However, cholinergic receptor activation is also associated with a suppression of excitatory synaptic transmission, resulting in reduction in the relative impact of synaptic inputs to the superficial layers of the parasubiculum. Although the functional implications of these findings are not yet clear, it is likely that acetylcholine plays a central role in modulating spatial and mnemonic processes associated with the parasubiculum. Further, since cholinergic neurons are active during theta-frequency EEG activity, the cholinergic system is well poised to make substantial contributions to the temporal coordination of synaptic activity between the parasubiculum and the structures it projects to, such as the entorhinal cortex. Because the parasubiculum sends its major output to the projection neurons of the superficial layers of the entorhinal cortex, cholinergic regulation of synaptic interactions between the parasubiculum and entorhinal cortex is likely to play a major role in parasubicular modulation of processes associated with hippocampal function.

CHAPTER 2

CONDUCTANCES MEDIATING INTRINSIC THETA-FREQUENCY MEMBRANE POTENTIAL OSCILLATIONS IN LAYER II PARASUBICULAR NEURONS

Stephen D. Glasgow and C. Andrew Chapman

ABSTRACT

Ionic conductances that generate membrane potential oscillations in neurons of layer II of the parasubiculum were investigated using whole-cell current clamp recordings in horizontal slices from the rat brain. Blockade of ionotropic glutamate and GABA synaptic transmission did not reduce the power of the oscillations, indicating that oscillations are not dependent on synaptic inputs. Oscillations were eliminated when cells were hyperpolarized 6 to 10 mV below spike threshold, indicating that they are mediated by voltage-dependent conductances. Application of tetrodotoxin (TTX) completely eliminated oscillations, suggesting that Na^+ currents are required for the generation of the oscillations. Oscillations were not reduced by blocking Ca^{2+} currents with Cd^{2+} or Ca^{2+}-free ACSF, or by blocking K^+ conductances with either 50 μM or 5 mM 4-aminopyridine (4-AP), 30 mM tetraethylammonium (TEA), or Ba^{2+} (1-2 mM). Oscillations also persisted during blockade of the muscarinic-dependent K^+ current, I_M, using the selective antagonist XE-991 (10 μM). However, oscillations were significantly attenuated by blocking the hyperpolarization-activated cationic current I_h with Cs^+ and were almost completely blocked by the more potent I_h blocker ZD7288 (100 μM). Intrinsic membrane potential oscillations in neurons of layer II of the parasubiculum are therefore likely driven by an interaction between an inward persistent Na^+ current and time-dependent deactivation of I_h. These voltage-dependent conductances provide a mechanism for the generation of membrane potential oscillations that can help support rhythmic network activity within the parasubiculum during theta-related behaviors.

The subicular complex includes the subiculum, presubiculum and parasubiculum, and has recently attracted interest due to the roles that it may play in the modulation of activity in both the hippocampus and entorhinal cortex (Craig & Commins, 2006; Hargreaves et al., 2005; P. Liu et al., 2004; S. O'Mara, 2005). The parasubiculum receives inputs from the CA1 region, medial septum, and anterior thalamus, and has a single major output projection to layer II of the medial and lateral entorhinal cortex (Amaral & Witter, 1989; Caballero-Bleda & Witter, 1993, 1994; Funahashi & Stewart, 1997a; Shibata, 1993; Swanson & Cowan, 1979; Wouterlood, Saldana, & Witter, 1990). It is therefore well-positioned to make a substantial contribution to the computational processes of the hippocampal formation. The parasubiculum contains place cells that fire in relation to head-direction, and this suggests that the parasubicular input to the entorhinal cortex may contribute to the specialized place cell representations carried by grid cells in the dorsolateral entorhinal cortex (Cacucci et al., 2004; Hafting et al., 2005; Hargreaves et al., 2005; Taube, 1995b). Further, layer II of the entorhinal cortex is the major target for cortical sensory inputs to the hippocampal formation, and layer II cells provide much of the processed sensory input received by the dentate gyrus and CA3 regions (Amaral & Witter, 1989; Kerr et al., 2007). Stimulation of parasubicular inputs to the entorhinal cortex can facilitate entorhinal cortex responses to subsequent inputs from the piriform cortex (Caruana & Chapman, 2004), and the parasubiculum may therefore also modulate transmission of highly processed sensory input to the hippocampal formation.

Neuronal synchronization during theta-frequency (4 to 12 Hz) EEG activity is thought to contribute to the computational functions of the entorhinal cortex and hippocampus (Bland, 1986; Bland, Oddie, & Colom, 1999; Buzsaki, 2002, 2005; Buzsaki, Leung, & Vanderwolf, 1983; Hasselmo, 2005; Vertes, 2005), and it is also now clear that the activity of parasubicular neurons is modulated by local theta activity. Low-amplitude theta activity was recorded near the parasubiculum in early mapping studies (Bland & Whishaw, 1976), but because this activity might have been volume-conducted from adjacent structures we recently used depth profiles with moving bipolar electrodes in urethane-anaesthetized rats to verify that theta-frequency EEG activity is generated locally within the superficial layers of the parasubiculum (Glasgow and Chapman 2007). The presence of place cells in the parasubiculum suggests that the region contributes to spatial navigation (Hargreaves et al., 2005; Hargreaves et al., 2007; Taube, 1995b), and a substantial proportion of these cells fire with a consistent phase-relation to theta activity (Cacucci et al., 2004; Taube, 1995b). This indicates that theta oscillations modulate the firing of parasubicular neurons during theta-related behaviors such as active exploration.

Using whole-cell current clamp recordings in acute brain slices, we previously found that depolarization to near-threshold voltages resulted in theta-frequency membrane potential oscillations in approximately 80% of layer II parasubicular neurons (Glasgow and Chapman 2007). The oscillations persisted in the presence of synaptic blockers and were blocked by hyperpolarization, indicating that they are driven by intrinsic, voltage-dependent conductances.

Theta-frequency membrane potential oscillations have been observed in CA1 pyramidal cells *in vivo* (Bland et al., 2002; Ylinen et al., 1995) and *in vitro* (Hu et al., 2002; L. W. Leung & Yim, 1991), as well as in interneurons in stratum lacunosum-moleculare (L-M) of the CA1 region (Bourdeau et al., 2007; Chapman & Lacaille, 1999b) and in layer II and V entorhinal cortex neurons (Alonso & Llinas, 1989; Hamam et al., 2000; Klink & Alonso, 1993; Schmitz et al., 1998). However, the ionic conductances that combine to produce subthreshold membrane potential oscillations differ. In L-M interneurons, oscillations result from an interaction between a persistent sodium current (I_{NaP}), and a 4-AP-sensitive A-type K^+-current mediated by Kv4.3 channels (Bourdeau et al., 2007; Chapman & Lacaille, 1999b). Oscillations in CA1 pyramidal cells and in entorhinal cortex neurons are also mediated by sodium currents (Klink & Alonso, 1993; L. W. Leung & Yim, 1991; Schmitz et al., 1998) but have been linked to TEA sensitive K^+ currents (Leung and Yim 1991), the muscarinic-sensitive K^+ current I_M (Hu et al., 2002, 2007; Yoshida & Alonso, 2007), and the hyperpolarization-activated cationic current I_h (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Fransen, Alonso, Dickson, Magistretti, & Hasselmo, 2004; Hu et al., 2002).

The current study investigated ionic conductances responsible for the generation of voltage-dependent membrane potential oscillations in layer II cells of the parasubiculum using whole-cell current clamp recordings. Results indicate that oscillations are generated by mechanisms similar to those that drive oscillations in principal neurons of the entorhinal cortex (Dickson, Magistretti,

Shalinsky, Fransen, et al., 2000), and likely rely on an interaction between I_{NaP} and I_h.

METHODS

Slice preparation

Methods used for in vitro recordings were similar to those in previous reports (Chapman & Lacaille, 1999b; Glasgow & Chapman, 2007) and were conducted in accordance with guidelines of the Canadian Council on Animal Care. Acute brain slices were obtained from 4 to 6 week old male Long Evans rats (Charles River, Montreal, QC). The rat was deeply anesthetized with halothane and decapitated. The brain was quickly removed and submerged in cold ACSF (4 ºC) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose saturated with 95% O_2 and 5% CO_2 (pH ~7.3; 300-310 mOsm). Horizontal brain slices (300 µm) containing the parasubiculum were cut with a vibratome (WPI,Vibroslice NVSL), and allowed to recover at room temperature for ~1 h. Individual slices were then transferred to a recording chamber, and superfused with oxygenated ACSF at room temperature (22-24º C) at a rate of 1.5-2.0 ml/min. Cells of the superficial layers of the parasubiculum were visualized using an upright microscope (Leica, DM-LFS) equipped with a long-range water immersion objective (40x), differential interference contrast optics, and a near-infrared camera (COHU). The borders of the superficial layers of the parasubiculum were delineated using criteria described previously (Funahashi & Stewart, 1997b; Glasgow & Chapman, 2007). Layer II of the parasubiculum contains relatively large principal neurons, and is diffuse and disorganized compared to the relatively compact superficial layers of the medial entorhinal cortex and presubiculum (Amaral & Witter, 1989;

Funahashi & Stewart, 1997a, 1997b). Layer II parasubicular cells can also be distinguished from deep layer cells by lack of burst firing in response to current injection (Funahashi & Stewart, 1997a; R. S. Jones & Heinemann, 1988).

Whole cell recordings

Intracellular patch pipettes were pulled using a horizontal puller (Sutter Instruments, P-97) and contained (in mM) 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.5 ethylene glyco-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2 ATP-Tris, and 0.4 GTP-Tris (pH calibrated to 7.20 - 7.26 using KOH; 270-280 mOsm). The patch pipette (4-8 MΩ) was lowered under visual guidance to contact with the soma of the target parasubicular cell, and gentle suction was applied. After achieving a tight seal (>1 GΩ) under voltage-clamp, strong suction was applied to obtain whole-cell configuration. The cells were allowed to recover for approximately five minutes before recordings proceeded. Whole-cell current clamp recordings of membrane potential (DC-10 kHz) were amplified using an Axoclamp 200B amplifier (Axon Instr.), monitored using a digital oscilloscope, and digitized at 20 kHz (Axon Instr., Digidata 1322A) for storage on hard disk using the software package Clampex 8.2 (Axon Instr.). Recordings were accepted if the series resistance was below 30 MΩ (mean, 11.10 ± 0.75).

The majority of layer II parasubicular neurons demonstrated membrane potential oscillations when depolarized to near-threshold voltage levels using steady current injection (Glasgow and Chapman 2007). Ten-sec duration

recordings were obtained at a range of voltages relative to action potential threshold by varying the level of constant current injection. Oscillation frequency is temperature dependent (Glasgow and Chapman 2007), but the amplitude is similar at both room temperature and 32° C, and the recordings reported here were therefore obtained at room temperature to reduce the metabolic demands on the cells. After initial baseline tests, recordings were repeated at the same voltages in the presence of pharmacological agents. The effects of drugs on action potentials and voltage responses to hyperpolarizing and depolarizing current steps were monitored regularly throughout the experiment.

Pharmacological manipulations

All drugs were stored in frozen stock solutions and were added to ACSF just prior to recordings. Sodium currents were blocked by tetrodotoxin (TTX; 0.5 μM). Calcium- and Ca^{2+}-dependent currents were blocked by application of $CdCl_2$ (50 μM) or by perfusion of Ca^{2+}-free ACSF in which Ca^{2+} was replaced with Mg^{2+}. Potassium channels were blocked using 4-amino-pyridine (4-AP; 50 μM and 5 mM), tetraethylammonium (TEA; 30 mM) or Ba^{2+} (2 mM) in the presence of the ionotropic glutamate receptor antagonist kynurenic acid (KYNA; 1 mM) and the $GABA_A$ receptor antagonist bicuculline methiodide (BIC; 25 μM). The $GABA_B$ receptor antagonist CGP 55845 (1 μM) was added to the bath when TEA and high doses of 4-AP were used. Normal osmolarity was maintained in control ACSF when testing high doses of 4-AP and TEA by reducing Na^+, and replaced it with equimolar choline. When assessing the effects of Ba^{2+} and Cd^{2+},

PO_4 and SO_4 were removed. The muscarinic-sensitive K^+ current I_M was assessed using the selective Kv7.2/3 channel blocker XE-991 (10 μM). The hyperpolarization-activated inward-rectifying current I_h was blocked using 1 mM CsCl, 2 mM CsCl in the presence of synaptic blockers kynurenic acid (1 mM) and bicuculline (25 μM), or the I_h blocker ZD7288 (100 μM; Dickson et al. 2000, see also(Chevaleyre & Castillo, 2002)). All drugs were purchased from Sigma (St. Louis, MO), except for ZD7288 (Tocris, Bristol, UK) and XE-991 (Ascent Scientific, Weston, UK).

Analysis

Ten-sec samples of membrane potential at near-threshold voltages were prepared for spectral analysis by reducing the effective sampling rate to 1 kHz. A 2.048-s segment that contained no action potentials was chosen and passed through a Blackman window prior to computing the power spectral density. The power spectrum was calculated as the squared magnitude of the fast Fourier transform (Clampfit 8.2, Axon Instr.), and was averaged across three samples of membrane potential. The power of oscillations between 1.5 and 5.9 Hz was expressed as a percent of total power. Paired *t*-tests and repeated measures ANOVA were used to analyze alterations in peak frequency and theta-band power.

Electrophysiological characteristics of layer II parasubicular neurons were analyzed using the Clampfit 8.2 software package (Axon Instr.). Action potential height was measured from resting membrane potential, and action potential

width and fast and medium afterhyperpolarizations (fAHP and mAHP) were measured from action potential threshold. Input resistance was calculated from the peak voltage response to a 500 ms, –200 pA current step, and inward rectification was quantified by expressing the peak voltage response as a proportion of the steady-state response (rectification ratio; Chapman and Lacaille, 1999a).

RESULTS

Membrane potential oscillations and electrophysiological characteristics of layer II parasubicular neurons were similar to those observed previously using whole cell recordings (Funahashi & Stewart, 1997a, 1997b; Glasgow & Chapman, 2007). Mean resting membrane potential in oscillatory cells was –60.1 ± 0.5 mV and a peak input resistance was 120.1 ± 4.0 MΩ, with moderate inward rectification during hyperpolarizing current pulses observed in all cells (rectification ratio: 1.15 ± 0.01). Action potentials (amplitude, 120.2 ± 1.0 mV; duration, 3.7 ± 0.1 ms) were typically followed by fast and medium duration afterhyperpolarizations (fAHP, amplitude: 8.7 ± 0.3 mV; mAHP, amplitude: 6.2 ± 0.3 mV). Oscillatory cells displayed relatively high action potential thresholds (-42.8 ± 0.5 mV; Glasgow and Chapman 2007), and holding the cell near spike threshold using positive current injection resulted in 2 to 5 Hz membrane potential oscillations (mean peak frequency: 2.65 ±0.06 Hz) and intermittent repetitive spiking in almost all PaS neurons recorded (*Fig*. 1; 75 of 83 cells, or 90.3%). The average theta-band power was 0.42 ± 0.02 mV^2/Hz between 1.5 and 5.9 Hz, and accounted for 53.8 ± 1.3% of total power. Non-oscillatory cells

displayed comparable electrophysiological properties, except for larger fAHPs (10.6 ± 0.9 mV, t_{81}= 2.17, $p < 0.05$) and mAHPs (8.1 ± 0.7 mV, t_{81}= 2.10, $p < 0.05$).

Blockade of glutamate- and $GABA_A$-mediated synaptic transmission with kynurenic acid (1 mM), and bicuculline (25 μM) was used to verify that oscillations were intrinsic and did not require ionotropic glutamate or GABA synaptic input from other cells (Glasgow and Chapman 2007). Bath application of the synaptic antagonists (n= 15) failed to disrupt oscillations in membrane potential (percent of total power: 50.0 ± 3.2 % in blockers vs. 45.5 ± 3.4 % in control ACSF, $p > 0.05$; frequency: 2.6 ± 0.2 in blockers vs. 2.4 ± 0.1 Hz in control ACSF, $p > 0.05$), indicating that synaptic inputs are not necessary for oscillations (Figure 2.1). However, oscillations were eliminated by membrane hyperpolarization to 6 to 10 mV below threshold, suggesting that they are generated by intrinsic voltage-dependent conductances.

Oscillations are Dependent on Sodium Currents. The role of Na^+ currents in generating membrane potential oscillations was tested using the Na^+ channel blocker tetrodotoxin (TTX). Bath application of TTX (0.5 μM) both eliminated Na^+-dependent action potentials evoked by depolarizing current pulses, and also entirely blocked membrane potential oscillations (n= 7). Application of TTX reduced power in the theta band from 55.7 ± 4.2% to 33.8 ± 5.8% of total power (t_6 =3.79, $p < 0.01$; Figure 2.2), and raw power values were reduced from 0.58 ± 0.07 to 0.04 ± 0.01 mV^2/Hz (t_6 =7.80, $p < 0.001$). Membrane potential oscillations in layer II parasubicular neurons are therefore dependent on inward Na^+ currents.

Oscillations are observed at subthreshold membrane potentials in the absence of sustained repetitive spiking in control ACSF, suggesting that oscillations do not require Na^+ currents that drive action potentials, but rather may depend on a persistent non-inactivating Na^+ current (Klink & Alonso, 1993; Magistretti, Ragsdale, & Alonso, 1999).

Oscillations do not Require Ca^{2+} Currents. The potential role of Ca^{2+} and Ca^{2+}-dependent K^+ conductances in the generation of oscillations was tested through bath application of the Ca^{2+} channel blocker Cd^{2+} (50 μM; *n*= 5) and Ca^{2+}-free ACSF (*n*= 5) (*Fig*. 3). Amplitudes of afterhyperpolarizations, which are dependent on Ca^{2+}-activated K^+ conductances, were reduced by Cd^{2+} (fAHP, 4.1 ± 1.9 mV in Cd^{2+} vs. 7.8 ± 1.8 mV in control ACSF, t_4 =3.03, $p < 0.05$; mAHP, 2.0 ± 1.3 mV vs. 5.0 ± 1.6 mV, t_4 = 2.44, $p < 0.05$) and by Ca^{2+}-free ACSF (fAHP, 3.6 ± 1.3 mV in Ca^{2+}-free ACSF vs. 7.8 ± 1.3 mV in control ACSF, t_4 = 3.92, $p < 0.01$; mAHP, 1.7 ± 0.9 vs. 5.8 ± 0.9 mV, t_4 = 2.88, $p < 0.05$) (*Fig*. 3B). The reduced fAHP was also associated with a moderate increase in action potential duration in both Cd^{2+} (5.7 ± 0.9 ms vs. 3.6 ± 0.4 ms in control ACSF, t_4 = 2.83, $p < 0.05$) and Ca^{2+}-free ACSF (6.4 ± 0.5 ms vs. 3.9 ± 0.3 ms in control ACSF, t_4 = 4.04, $p < 0.01$). The frequency and power of oscillations, however, were unaffected by either Cd^{2+} (frequency: 3.1 ± 0.3 Hz in Cd^{2+} vs. 3.2 ± 0.3 Hz in control ACSF; power: 53.9 ± 6.3 % vs. 54.9 ± 4.2 %) or Ca^{2+}-free ACSF (frequency: 3.0 ± 0.2 Hz in Ca^{2+}-free ACSF vs. 3.0 ± 0.2 Hz in control ACSF; power: 65.8 ± 4.7 % vs. 68.2 ± 4.6 %) (Figure 2.3C), indicating that Ca^{2+} and Ca^{2+}-mediated K^+ currents

are not necessary for the generation of subthreshold membrane potential oscillations in layer II parasubicular neurons.

Potassium Currents. The potential role of K^+ conductances in oscillations was tested using several K^+ channel blockers. Parasubicular neurons have relatively high action potential thresholds which suggests that the voltage-gated outward-rectifying current I_D and the voltage-dependent potassium conductance I_A, which activate close to –40 and –50 mV respectively, may play roles in the repolarizing phase of oscillations (R. L. Wu & Barish, 1992). The slowly inactivating K^+ current I_D is sensitive to low doses of 4-AP, whereas high doses of 4-AP block the transient K^+ current I_A (Storm, 1990). Because theta-frequency oscillations in hippocampal L-M interneurons require the voltage-dependent potassium conductance I_A mediated by Kv4.3 channels sensitive to high doses of 4-AP, we tested the effects of both low (50 μM, n= 6) and high doses (5 mM, n= 5) of 4-AP on oscillations (Bourdeau et al. 2007; Chapman and Lacaille 1999b). Both low and high doses of 4-AP had strong effects on electrophysiological properties of parasubicular cells, but did not have a significant effect on oscillations. Bath application of 4-AP significantly increased spike duration (50 μM, 6.7 ± 0.8 ms in 4-AP vs. 4.0 ± 0.1 in control ACSF ms, t_5= 2.52, $p < 0.05$; 5 mM, 11.5 ± 4.0 vs. 4.0 ± 0.4 ms, t_4= 2.12, $p = 0.05$). Fast and medium AHPs were also significantly reduced by 50 μm 4-AP (fAHP: 2.2 ± 1.2 mV in 4-AP vs. 8.2 ± 1.4 mV in control ACSF, t_5= 5.48, $p < 0.01$; mAHP: 5.9 ± 1.7 to 1.4 ± 0.6 mV, t_5= 3.06, $p < 0.05$; Figure 2.4B), and were completely abolished by 5 mM 4-AP (Figure 2.4F). However, there were no significant changes in either the

frequency (50 μM, 2.9 ± 0.1 Hz in 4-AP vs. 2.4 ± 0.1 Hz in control ACSF; 5 mM, 2.8 ± 0.3 vs. 2.6 ± 0.2 Hz) or power of oscillations (50 μM, 50.0 ± 1.7 % in 4-AP vs. 51.5 ± 6.4 % in control ACSF; 5 mM, 50.3 ± 6.8 % in 4-AP vs. 42.4 ± 5.6 % in blockers; *Fig*. 4), suggesting that neither I_A nor I_D are necessary for the generation of subthreshold membrane potential oscillations in parasubicular neurons.

Tetraethylammonium (TEA) blocks delayed rectifier potassium channels (Beck, Ficker, & Heinemann, 1992; Chikwendu & McBain, 1996) that could contribute to oscillations (Klink & Alonso, 1993; L. W. Leung & Yim, 1991) and the effect of high doses of TEA on oscillations was therefore tested in parasubicular neurons. Bath application of TEA (30 mM) in the presence of synaptic blockers (n= 4; 1 mM kynurenic acid, 25 μM bicuculline, and 1 μM CGP-55845) resulted in spike broadening, long repolarization periods, and completely abolished fast and medium AHPs (Figure 2.5B). However, membrane potential oscillations were not significantly affected in either frequency (2.2 ± 0.1 Hz in TEA vs. 2.3 ± 0.1 Hz in control ACSF) or power (57.7 ± 3.6 % in TEA vs. 54.0 ± 6.3 % in blockers; Figures 2.5A and C). Oscillations in layer II parasubicular neurons therefore do not require K^+ currents sensitive to TEA.

The potential contribution of K^+ channels to oscillations was further tested by application of the widely acting K^+ channel blocker Ba^{2+} in the presence of kynurenic acid, bicuculline, and CGP-55845 (2 mM; n= 6; *Fig*. 2.5). Application of Ba^{2+} resulted in greatly increased action potential duration (10.7 ± 2.3 ms in Ba^{2+} vs. 3.7 ± 0.3 ms in blockers, t_5= 3.32, $p < 0.05$), decreased action potential

amplitude (107.6 ± 5.4 vs. 120.1 ± 4.2 mV, t_5= 8.11, $p < 0.001$), and abolished both the fAHP and mAHP. Application of Ba^{2+} also increased peak and steady state input resistance (249.1 ± 67.1 MΩ in Ba^{2+} vs. 98.3 ± 21.2 MΩ in blockers, t_5= 3.65, $p < 0.05$, and 159.6 ± 44.0 vs. 85.7 ± 15.9, t_5= 2.81, $p < 0.05$, respectively). However, barium did not significantly reduce oscillation power (48.0 ± 4.5 % in Ba^{2+} vs. 55.4 ± 4.5 % in blockers) or frequency (3.0 ± 0.4 Hz in Ba^{2+} vs. 2.8 ± 0.3 Hz in blockers). The hyperpolarizing phase of oscillations therefore appears not to require activation of Ba^{2+}-sensitive K^+ channels.

Recent evidence has indicated that the muscarinic-sensitive outward K^+ current I_M modulates intrinsic neuronal excitability and may play a significant role in the generation of subthreshold theta-frequency membrane potential oscillations in both CA1 pyramidal neurons and in layer V entorhinal neurons (Hu et al., 2002, 2007; Shalinsky et al., 2002; Yoshida & Alonso, 2007). Bath application of the selective Kv7.2/3 channel antagonist, XE-991 (10 μM), resulted in a moderate decrease in fast AHP (5.8 ± 1.2 mV in XE-991 vs. 7.9 ± 0.7 mV in control ACSF, t_4 = 2.09, p = 0.052) (Yoshida & Alonso, 2007), but failed to disrupt oscillations (62.7 ± 2.1 % in XE-991 vs. 60.8 ± 3.4 % in control ACSF; Figure 2.6). This indicates that I_M is not required for oscillations in superficial parasubicular neurons.

Hyperpolarization-activated current I_h. The hyperpolarization-activated cationic current I_h contributes to theta-frequency oscillations in CA1 neurons and stellate cells of the entorhinal cortex through time-dependent activation and deactivation (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Hu et al.,

2002). The parasubiculum shows high levels of HCN1 protein expression (Notomi & Shigemoto, 2004), suggesting that I_h may play a substantial role in regulating the excitability of parasubicular neurons, and contribute to the generation of subthreshold oscillations. Therefore, its contribution to oscillations in parasubicular neurons was tested using the I_h blockers Cs^+ (1 mM, n= 4; 2 mM, n= 5) and ZD7288 (100 μM, n=10). The inward rectifying sag in voltage responses to hyperpolarizing current steps was eliminated by bath application of Cs^+ (rectification ratio, 1 mM: 0.98 ± 0.02 in Cs^+ vs. 1.14 ± 0.04 in control ACSF; 2 mM: 1.02 ± 0.01 vs. 1.15 ± 0.02), and this increased steady-state input resistance (1 mM: 226.3 ± 103.5 MΩ in Cs^+ vs. 115.8 ± 17.6 MΩ in control ACSF; 2 mM: 136.8 ± 20.3 MΩ vs. 99.2 ± 3.1 MΩ). In addition, Cs^+ also significantly attenuated the power of membrane potential oscillations.

Theta-band power was reduced from 57.1 ± 5.0% to 34.5 ± 2.3% in 1 mM Cs^+ (t_3= 3.24, $p < 0.05$; 0.28 ± 0.05 mV^2/Hz in Cs^+ vs. 0.37 ± 0.06 mV^2/Hz in control ACSF, t_3= 3.93, $p < 0.05$;), and was reduced from 52.3 ± 5.5% to 31.5 ± 2.2% in 2 mM Cs^+ (t_4= 3.06, $p < 0.05$; 0.20 ± 0.05 mV^2/Hz in Cs^+ vs. 0.44 ± 0.04 mV^2/Hz in blockers, t_4= 3.23, $p < 0.05$; Figure 2.7). The peak frequency of oscillations was not significantly affected by Cs^+ (1 mM: 3.3 ± 0.3 Hz in Cs^+ vs. 3.2 ± 0.4 Hz in control ACSF; 2 mM: 3.7 ± 0.4 vs. 2.9 ± 0.3 Hz).

A residual I_h current can sometimes be observed in the presence of Cs^+ (Dickson et al. 2000), and the role of I_h in generating oscillations was therefore tested further using the more potent I_h blocker, ZD7288 (100 μM). Bath application of ZD7288 eliminated inward rectification in response to –200 pA

current pulses (n= 10; rectification ratio: 1.00 ± 0.01 in ZD7288 vs. 1.11 ± 0.03 in control ACSF; t_9= 4.17, p < 0.01) and also eliminated inward rectification in response to larger, –400 pA, pulses (n= 4; rectification ratio: 0.99 ± 0.01 in ZD7288 vs. 1.17 ± 0.04 in control ACSF). In addition, ZD7288 also almost completely blocked oscillations, and oscillation power was reduced from 55.8 ± 2.8% in control ACSF to 30.6 ± 2.6% in ZD7288 (t_9= 6.92, p < 0.001; 0.14 ± 0.02 mV^2/Hz in ZD7288 vs. 0.57 ± 0.06 mV^2/Hz in control ACSF, t_9= 7.25, p < 0.001; Figure 2.7). Application of ZD7288 also resulted in a reduction in the fast afterhyperpolarization (from 6.9 ± 0.3 to 2.2 ± 0.8 mV; t_9= 7.14, p < 0.001) and an increase in action potential duration (from 3.9 ± 0.2 to 5.6 ± 0.5 ms; t_9= 3.55, p < 0.01), but the block of oscillations by ZD7288 was highly effective, even in cells that showed smaller changes in spike repolarization. The block of oscillations by Cs^+ and by the more potent I_h blocker ZD7288 indicate that oscillations in parasubicular neurons are likely mediated by the hyperpolarization activated cationic current I_h.

DISCUSSION

The present study has identified intrinsic voltage-dependent conductances that drive theta-frequency membrane potential oscillations in putative pyramidal and stellate neurons in layer II of the parasubiculum. Oscillations persisted during blockade of ionotropic glutamate and GABA transmission and are therefore not dependent on synaptic inputs from other neurons (Glasgow and Chapman 2007). Further, parasubicular neurons express membrane potential

oscillations at near-threshold voltages and are eliminated by hyperpolarization, suggesting that the oscillations are mediated by voltage-dependent conductances. Here, we have found that membrane potential oscillations in layer II cells of the parasubiculum are primarily generated by an interplay between a sodium conductance and the hyperpolarization-activated mixed cationic current I_h (Figures 2.2 and 2.7). These currents have also been shown to underlie the generation of theta-frequency membrane potential oscillations in layer II stellate neurons of the entorhinal cortex (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Fransen et al., 2004; Hamam et al., 2000) and mediate membrane potential resonance to sinusoidal current injection at hyperpolarized potentials in CA1 pyramidal neurons (Hu et al., 2002). This mechanism contrasts with oscillations in hippocampal inhibitory interneurons that are dependent on Na^+ and a transient A-type K^+ current (Chapman and Lacaille 1999a; Bordeau et al. 2007).

Membrane potential oscillations provide a major mechanism that can contribute to the local genesis of theta activity in the parasubiculum, and the oscillations likely modulate neuronal firing in relation to ongoing theta activity within the hippocampal formation (Bland et al., 2002; Glasgow & Chapman, 2007; Taube, 1995b). Many place cells in the parasubiculum fire in relation to the theta rhythm, indicating that theta activity helps determine the firing behavior of parasubicular neurons involved in spatial processing (Cacucci et al., 2004; Hargreaves et al., 2005; Hargreaves et al., 2007; Taube, 1995b). In addition, the single major output of the parasubiculum is to layer II of the entorhinal cortex,

which receive converging inputs from many cortical regions and provides the hippocampus with much of its highly processed sensory input (Caballero-Bleda & Witter, 1993, 1994; van Groen & Wyss, 1990a). Time-dependent stimulation of the parasubiculum can either enhance or suppress entorhinal cortex responses to sensory inputs from other cortical regions, suggesting that coordinated activity in this pathway plays an important role in the modulation of how the entorhinal cortex processes other inputs (Caruana & Chapman, 2004). Theta activity, which is prominent during active exploration, is therefore likely to help regulate the manner in which parasubicular efferents combine with extrinsic cortical sensory inputs to the hippocampal formation (Caruana & Chapman, 2004; Sejnowski & Paulsen, 2006).

Conductances Generating Oscillations. Oscillations were completely eliminated by the Na^+ channel blocker TTX, and were also strongly reduced by application of Cs^+ and the potent I_h blocker ZD7288. This suggests that oscillations in parasubicular neurons are generated by mechanisms analogous to those in stellate neurons of the medial entorhinal cortex and subicular pyramidal neurons (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Fransen et al., 2004; Klink & Alonso, 1993; W. T. Wang et al., 2006). Similar to oscillations in these areas, oscillations in parasubicular cells likely result from sodium-dependent depolarization to near-threshold voltage levels within the activation range of the persistent sodium current I_{NaP}, and a regulation of the frequency of oscillations by the time-dependent activation and deactivation kinetics of I_h (Dickson et al. 2000; Fransen et al. 2004). Coactivation of I_{NaP} and I_h leads to the

depolarizing phase of the oscillations that results in the voltage- and time-dependent deactivation of I_h. Subsequent hyperpolarization then leads to the slow reactivation of I_h and promotion of the depolarizing phase of the oscillations (Dickson et al. 2000; Fransen et al. 2004). This interaction between I_{NaP} and I_h is also thought to contribute to membrane potential resonance in sensorimotor cortex neurons (Hutcheon, Miura, & Puil, 1996a, 1996b) and in CA1 neurons at hyperpolarized potentials (Hu et al. 2002). Both I_{NaP} and I_h currents may also mediate oscillations in pyramidal cells of the subiculum and in layers II/V of perirhinal cortex where neurons show inward rectification during hyperpolarizing current steps (Bilkey & Heinemann, 1999; Hamam et al., 2000; W. T. Wang et al., 2006). In addition to I_{NaP}, TTX also blocks the transient sodium current, and it is therefore possible that the depolarizing phase of the oscillations may be mediated in part by the activation of transient sodium channels responsible for window currents (Ketelaars, Gorter, van Vliet, Lopes da Silva, & Wadman, 2001).

Cesium blocks I_h currents only partially and, because residual I_h currents can maintain oscillations in the presence of cesium (Dickson et al. 2000), the persistence of some oscillations in Cs^+ cannot be taken to rule-out the involvement of I_h (Klink and Alonso 1993; Dickson et al. 2000). It was initially concluded that I_h does not contribute to oscillations in entorhinal neurons because oscillations persisted during Cs^+ application (Klink and Alonso 1993), but it was later found that Cs^+ reduces I_h by only about 60-75%, and that while Cs^+ can disrupt oscillations, periods of clear oscillations are still observed (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; R. S. Jones, 1994).

Similarly, bath application of Cs^+ resulted in a significant attenuation of theta-band membrane potential oscillations in layer II parasubicular neurons, but some periods of oscillatory activity were observed intermittently. Periods of oscillatory activity are also observed in entorhinal stellate cells from HCN1 knockout mice in which the I_h current is greatly reduced (Nolan et al., 2007), and this suggests that the HCN1-mediated component of I_h may not be required for oscillations in these cells. However, as in stellate cells of the entorhinal cortex (Dickson et al. 2000), complete block of I_h with ZD7288 eliminated oscillations in parasubicular neurons, indicating that they are likely dependent on I_h (Figure 2.7). ZD7288 has been shown to result in a non-specific, slowly developing suppression of synaptic transmission at mossy fiber synapses (Chevaleyre and Castillo 2002) but we have found that oscillations in parasubicular neurons are not dependent on synaptic transmission and are rapidly blocked by ZD7288 (Figures 2.1 and 2.7). In addition, although ZD7288 can result in a partial block of inward rectifying K^+ channels (Wilson 2005), the block of oscillations is not easily attributable to effects of ZD7288 on I_{Kir} because oscillations persisted in the presence of Ba^{2+} (Figure 2.5). The cells tested here showed a reduction in the fast afterhyperpolarization following ZD7288. However, oscillations were blocked effectively in all cells exposed to ZD7288, and the reduction in oscillations was not related to between-cell variability in the effect of ZD7288 on spike repolarization. Thus, although non-specific effects of Cs^+ and ZD7288 cannot be ruled out entirely, the effects of Cs^+ and ZD7288 on oscillations is likely to be mainly attributable to their effects on I_h.

Voltage-gated potassium channels can contribute to oscillations and rhythmic firing activity in a variety of cell types. Oscillations in hippocampal L-M interneurons are generated by an interaction between I_{NaP} and a A-type potassium current mediated by Kv4.3 channels that is sensitive to 4-AP (Chapman and Lacaille 1999a; Bordeau et al. 2007). In CA1 pyramidal cells, oscillations are maintained in 200 μM 4-AP, but are disrupted by moderate doses of TEA, suggesting that oscillations in these cells do not depend on activation of I_A but rather on TEA-sensitive delayed rectifying potassium currents (Garcia-Munoz et al., 1993; L. W. Leung & Yim, 1991). The conclusion that TEA-sensitive currents could contribute to oscillations was made tentatively, however, because of the strong bursting behavior induced by TEA. In the present study, oscillations were not significantly affected either by low or high doses of 4-AP or by TEA, indicating that I_A and delayed rectifier K^+ channels do not play a central role in the oscillations of parasubicular neurons.

Muscarinic receptor activation closes Kv7/KCNQ channels, and can affect neuronal excitability by depolarizing membrane potential, altering spike frequency adaptation and suppressing spike afterpotentials (Gu, Vervaeke, Hu, & Storm, 2005; Hu et al., 2002, 2007; Lawrence, Saraga, et al., 2006; Womble & Moises, 1992, 1993; Yoshida & Alonso, 2007; Yue & Yaari, 2004, 2006). The muscarinic-sensitive inward rectifying potassium current (I_M) is active at near-threshold potentials, and has been linked to membrane potential oscillations in hippocampal neurons (Gutfreund, yarom, & Segev, 1995; Hu et al., 2002). Block of I_M suppresses oscillations in layer V entorhinal cells (Yoshida & Alonso, 2007),

and I_M is also required for theta-frequency resonance responses in CA1 pyramidal neurons at potentials near threshold (Hu et al. 2002). Here, we have used both Ba^{2+}, a wide-acting K^+ channel blocker which blocks leak conductances as well as inward rectifying potassium channels including I_M, and the selective I_M antagonist XE-991 to determine whether oscillations in parasubicular neurons are dependent on I_M (Benson et al., 1988; Hu et al., 2007; Yoshida & Alonso, 2007). It was initially found that oscillations in entorhinal cortex neurons were disrupted by Ba^{2+} (Klink and Alonso 1993), but this was later attributed to greatly increased synaptic inputs (Dickson et al. 2000). When synaptic blockers are present, membrane potential oscillations in entorhinal cortex actually increase in amplitude in the presence of Ba^{2+} due to an increase in membrane resistance (Dickson et al. 2000). Oscillations in CA1 neurons are not disrupted by Ba^{2+} (Leung and Yim 1991), and oscillations in L-M interneurons also persist during blockade of I_M using the selective antagonist XE-991 (Bordeau et al. 2007). Similarly, we have found that oscillations in parasubicular neurons persist in the presence of Ba^{2+} so that oscillations are not dependent on I_{Kir} conductances such as I_M. Further, we have also recently observed that bath application of the cholinergic agonist carbachol which acts in part by inhibiting I_M (Womble & Moises, 1992, 1993), does not disrupt oscillations in layer II cells of the parasubiculum (S.D. Glasgow and C.A. Chapman, Chapter 3). Finally, our present results show that selective blockade of I_M with XE-991 fails to disrupt oscillations in layer II parasubicular neurons, indicating that I_M is not required for the generation of the oscillations.

Calcium currents and Ca^{2+}-dependent K^{+} currents (Sah, 1996) contribute to oscillations in the thalamus, inferior olivary nucleus and mammillary complex (Alonso & Llinas, 1992; Jahnsen & Llinas, 1984b; Llinas & Yarom, 1986). However, the present study demonstrated that oscillations in parasubicular neurons were not significantly affected by Ca^{2+}-free ACSF or by the Ca^{2+} channel blocker Cd^{2+}, suggesting that Ca^{2+} conductances are not required for the generation of this activity.

Extrinsic Mechanisms Contributing to Theta Activity. In addition to the ionic conductances described here that drive oscillations in individual neurons, other extrinsic mechanisms are required to synchronize theta-frequency population activity and lead to the associated field potential. Parasubicular neurons recorded here usually required positive constant current injection to depolarize neurons to the subthreshold range where they expressed oscillations, and we found previously that parasubicular theta activity *in vivo* is dependent on cholinergic mechanisms (Glasgow and Chapman 2007). As is the case in the entorhinal cortex (Klink & Alonso, 1997b) and CA1 region (Chapman & Lacaille, 1999a), it is likely that septal cholinergic projections to the parasubiculum results in muscarinic depolarization to near-threshold voltages during theta activity (Alonso & Kohler, 1984; Benson et al., 1988; Hu et al., 2002, 2007; Klink & Alonso, 1997a). Cholinergic theta activity likely serves as a mechanism contributing to timing-dependent changes in synaptic responsivity in the parasubiculum, and cholinergic effects on neuronal excitability, spike timing, and synaptic integration need to be assessed further.

In the CA1 in the intact brain, field activity associated with theta activity is generated by rhythmic perisomatic inhibition and excitatory inputs to distal dendrites (Buzsaki, 2002). We have found previously that putative inhibitory interneurons in the parasubiculum also display membrane potential oscillations (Glasgow and Chapman 2007), and the parasubiculum contains large numbers of glutamic acid decarboxylase (GAD) and GABA-immunoreactive cells (Kohler et al., 1985). Inhibitory interneurons contact many parasubicular neurons, and the rhythmic inhibition of large numbers of principal cells can contribute synchronization of theta activity by "rebound depolarizations" via synchronous activation of I_h (Chapman & Lacaille, 1999a; Cobb et al., 1995). Rhythmic excitatory synaptic input to the parasubiculum from the CA1 region and other glutamate inputs from theta-related structures such as the subiculum, the anterior thalamus, and the deep layers of the entorhinal cortex (Kohler, 1985, 1986; Shibata, 1993; van Groen & Wyss, 1990a; Vertes, Albo, & Viana Di Prisco, 2001) might also contribute to neuronal synchronization in the parasubiculum, and to the membrane currents that generate associated field activity. Thus, membrane potential oscillations are likely to combine with extrinsic synaptic and neuromodulatory inputs in the generation and synchronization of theta activity within the parasubiculum.

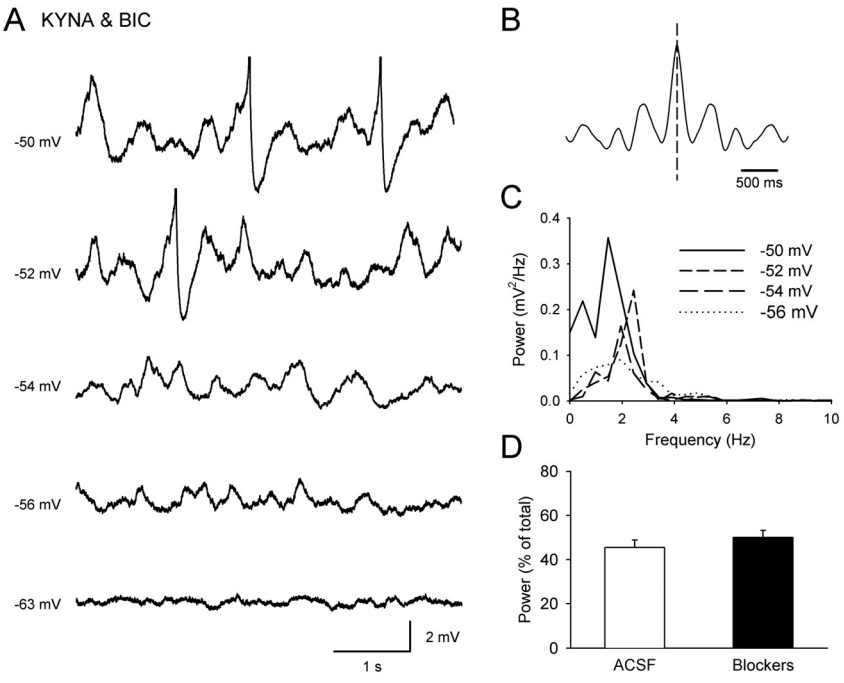
A
KYNA & BIC
-50 mV
-52 mV
-54 mV
-56 mV
-63 mV
2 mV
1 s
B
500 ms
C
Power (mV²/Hz)
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
Frequency (Hz)
-50 mV
-52 mV
-54 mV
-56 mV
D
Power (% of total)
80
60
40
20
0
ACSF
Blockers

Figure 2.1. Membrane potential oscillations in layer II cells of the parasubiculum are voltage-dependent, and persist during blockade of fast ionotropic glutamatergic and GABAergic synaptic transmission. **A**: Whole-cell current clamp recordings were taken at a range of membrane potentials relative to action potential threshold during bath application of synaptic antagonists kynurenic acid (KYNA; 1 mM) and bicuculline (BIC; 25 μM). Depolarization of cells to near-threshold voltages using steady positive current injection resulted in membrane potential oscillations at 2 to 5 Hz. Oscillations are abolished by hyperpolarization. Note that action potentials are truncated in this and subsequent figures. **B**: An autocorrelogram reflects the rhythmicity of membrane potential oscillations in the same cell as in panel A at a membrane potential of -52 mV. **C**: Power spectra for recordings at a range of voltages are shown for the same cell as in A. The power of oscillations increased as membrane potential was raised to threshold, with no significant change in frequency. **D**: Group data show that the power of oscillations is not affected by blockade of synaptic transmission, indicating that membrane potential oscillations in layer II parasubicular neurons do not require extrinsic synaptic inputs.

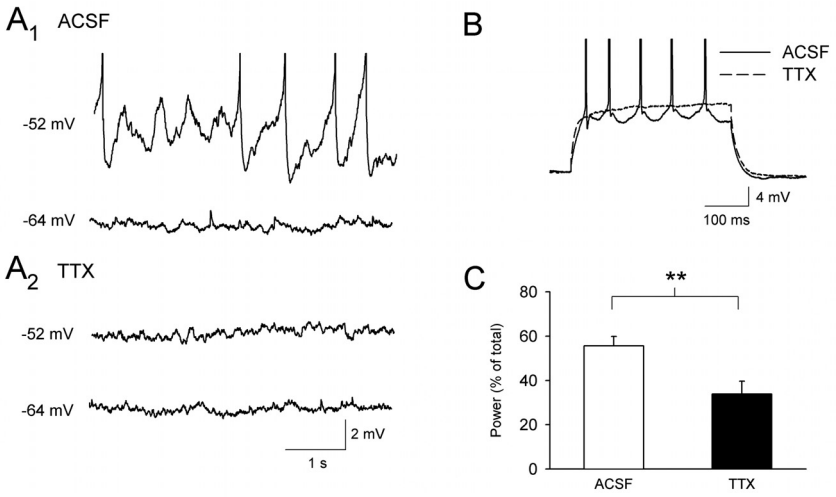
A1 ACSF
-52 mV
-64 mV
A2 TTX
-52 mV
-64 mV
2 mV
1 s
B
ACSF
TTX
4 mV
100 ms
C
**
Power (% of total)
80
60
40
20
0
ACSF
TTX

Figure 2.2. Oscillations in parasubicular neurons are dependent on voltage-gated sodium channels. **A**: Voltage-dependent oscillations were eliminated by constant bath application of the Na^+ channel blocker tetrodotoxin (TTX; 0.5 μM; *n*= 7). Recordings were obtained at the membrane potentials indicated at left. **B**: Responses of the same cell to positive current pulses at resting membrane potential in normal ACSF and in the presence of TTX show that TTX blocked Na^+-dependent action potentials. **C**: Group data show a significant reduction in percent of total power in the theta-band during bath application of TTX (**: $p < 0.01$). This was associated with a large reduction in raw power values within the theta band (0.04 ± 0.01 vs. 0.58 ± 0.07 to mV^2/Hz, $p < 0.001$). The block of oscillations by TTX suggests that inward Na^+ currents contribute to the depolarizing phase of oscillations.

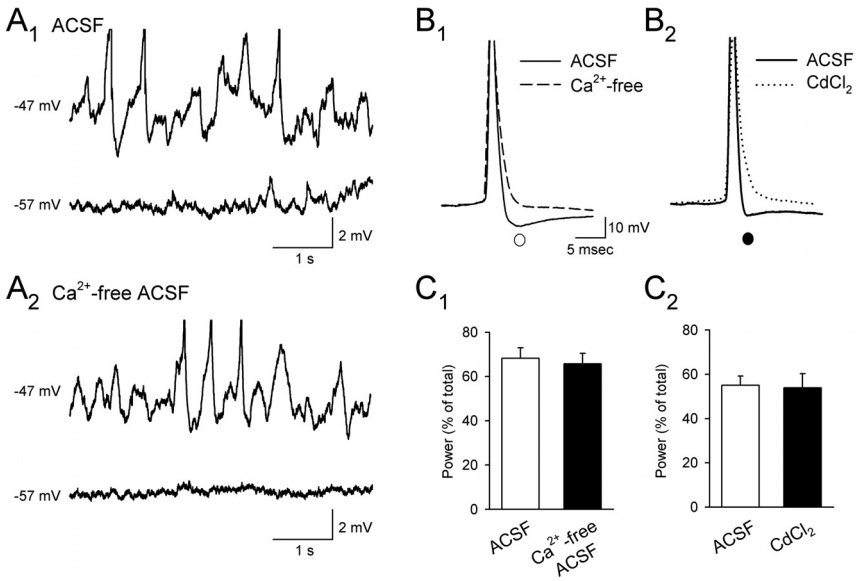
A1 ACSF
-47 mV
-57 mV
2 mV
1 s
A2 Ca2+-free ACSF
-47 mV
-57 mV
2 mV
1 s
B1
ACSF
Ca2+-free
10 mV
5 msec
B2
ACSF
CdCl2
C1
Power (% of total)
80
60
40
20
0
ACSF
Ca2+-free ACSF
C2
Power (% of total)
80
60
40
20
0
ACSF
CdCl2

Figure 2.3. Oscillations are not dependent on calcium currents. **A**: Voltage-dependent oscillations were not reduced in tests in which Ca^{2+} currents were reduced using Ca^{2+}-free ACSF (*n*= 5). Similar results were obtained using Cd^{2+} (n= 5; 50 μM). **B**: Superimposed action potentials show reductions in the amplitude of fast afterhyperpolarizations during application of Ca^{2+}-free ACSF (B_1) and Cd^{2+} (B_2) at the latency indicated by circles**.** **C**: The mean power of oscillations was unaffected by application of either Ca^{2+}-free ACSF (C_1) or Cd^{2+} (C_2). Inward Ca^{2+} currents and Ca^{2+}-dependent K^+ conductances are therefore not required for membrane potential oscillations in parasubicular neurons.

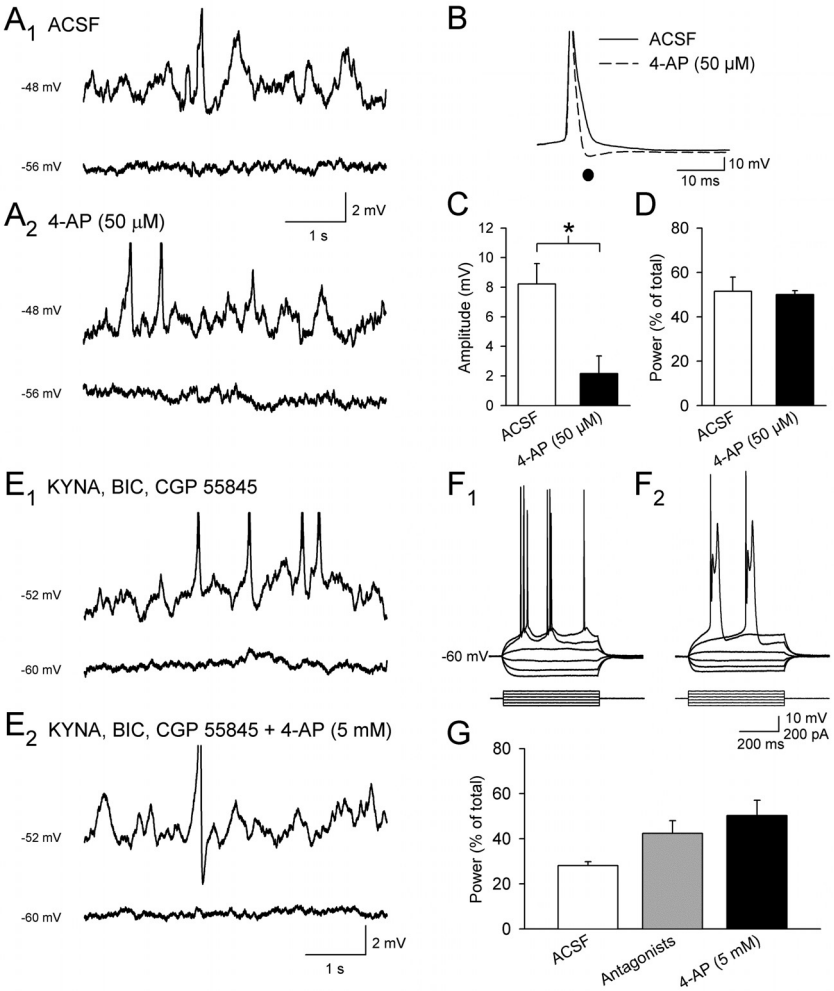
A1 ACSF
-48 mV
-56 mV
A2 4-AP (50 μM)
2 mV
1 s
-48 mV
-56 mV
E1 KYNA, BIC, CGP 55845
-52 mV
-60 mV
E2 KYNA, BIC, CGP 55845 + 4-AP (5 mM)
-52 mV
-60 mV
2 mV
1 s
B
ACSF
4-AP (50 μM)
10 mV
10 ms
C
Amplitude (mV)
12
10
8
6
4
2
0
*
ACSF
4-AP (50 μM)
D
Power (% of total)
80
60
40
20
0
ACSF
4-AP (50 μM)
F1
F2
-60 mV
10 mV
200 pA
200 ms
G
Power (% of total)
80
60
40
20
0
ACSF
Antagonists
4-AP (5 mM)

Figure 2.4. Oscillations are not dependent on voltage-dependent potassium currents sensitive to either low (50 μM) or high (5 mM) doses of 4-AP. **A**: Oscillations were not significantly affected by a low dose of 4-AP (50 μM). **B**: Superimposed action potentials from the same cell as in A show a reduction in the fast afterhyperpolarization in 50 μM 4-AP. **C and D**: The reduction in the fast afterhyperpolarization was significant for the group of cells tested (n=6; *: $p < 0.05$), but there was no significant change in the mean power of subthreshold oscillations in the presence of 50 μM 4-AP. **E**: Oscillations recorded in the presence of antagonists for ionotropic glutamate and $GABA_A$ and $GABA_B$ receptors were not significantly reduced during bath application of a high dose of 5 mM 4-AP. **F**: Membrane potential responses to hyperpolarizing and depolarizing current pulses in are shown for the same cell as in E during bath application of synaptic antagonists (F_1) and 5 mM 4-AP (F_2). Note the multiple spikes and delayed repolarization. **G**: Mean power of oscillations at near-threshold voltages was not significantly affected by the high dose of 4-AP (5 mM; *n*= 5).

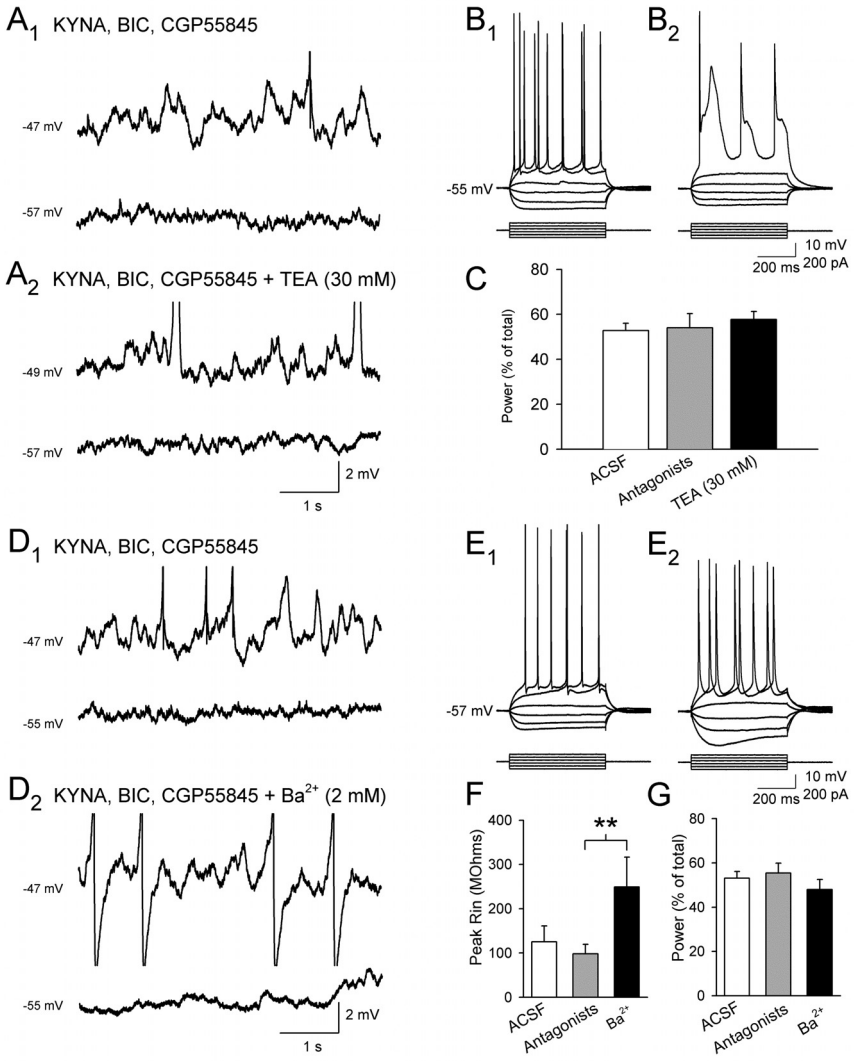
A1 KYNA, BIC, CGP55845
-47 mV
-57 mV
A2 KYNA, BIC, CGP55845 + TEA (30 mM)
-49 mV
-57 mV
2 mV
1 s
D1 KYNA, BIC, CGP55845
-47 mV
-55 mV
D2 KYNA, BIC, CGP55845 + Ba2+ (2 mM)
-47 mV
-55 mV
2 mV
1 s
B1
B2
-55 mV
10 mV
200 ms 200 pA
C
Power (% of total)
ACSF
Antagonists
TEA (30 mM)
E1
E2
-57 mV
10 mV
200 ms 200 pA
F
Peak Rin (MOhms)
**
ACSF
Antagonists
Ba2+
G
Power (% of total)
ACSF
Antagonists
Ba2+

Figure 2.5. Blockade of potassium channels with either TEA or Ba^{2+} does not block oscillations. **A**: Voltage-dependent oscillations recorded in the presence of antagonists of glutamatergic, $GABA_A$ and $GABA_B$ receptors were not reduced by 30 mM TEA (n= 4). **B**: Membrane voltage responses to hyperpolarizing and depolarizing current pulses in ACSF containing the synaptic antagonists (B_1) and with the addition of 30 mM TEA (B_2). Note the significant spike broadening, and the elimination of fast and medium afterhyperpolarizations by TEA. **C**: The mean power of oscillations was not significantly affected by TEA. **D**: Voltage-dependent oscillations recorded in the presence of glutamate and $GABA_A$ and $GABA_B$ receptor antagonists were not reduced by the broad-acting K^+ channel blocker Ba^{2+} (2 mM; n= 6). **E**: Membrane potential responses to hyperpolarizing and depolarizing current pulses in ACSF containing synaptic antagonists (E_1) and with the addition of 2 mM Ba^{2+} (E_2). Note the increased spike duration, reduced fAHPs, and increased peak and steady-state input resistance. **F and G**: Peak input resistance was increased during bath application of Ba^{2+} (**: $p < 0.01$), but the mean power of oscillations was not affected, indicating that outward K^+ currents sensitive to Ba^{2+} are not required for oscillations in parasubicular neurons.

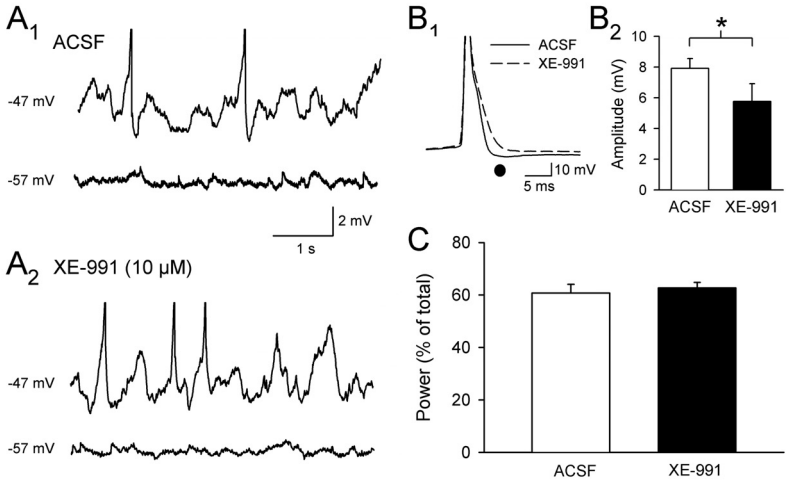
A1
ACSF
-47 mV
-57 mV
2 mV
1 s
A2
XE-991 (10 μM)
-47 mV
-57 mV
B1
ACSF
XE-991
10 mV
5 ms
B2
*
Amplitude (mV)
10
8
6
4
2
0
ACSF
XE-991
C
Power (% of total)
80
60
40
20
0
ACSF
XE-991

Figure 2.6. The muscarine-sensitive potassium current I_M is not required for the generation of membrane potential oscillations in parasubicular neurons. **A**: The power and frequency of oscillations were not significantly affected by the selective I_M antagonist XE-991 (10 μM). **B**: Superimposed action potentials reflect a reduction in the fAHP following bath application of XE-991. **C**: Group data show that the power of oscillations in parasubicular cells was not significantly affected by XE-991 (*n*= 5).

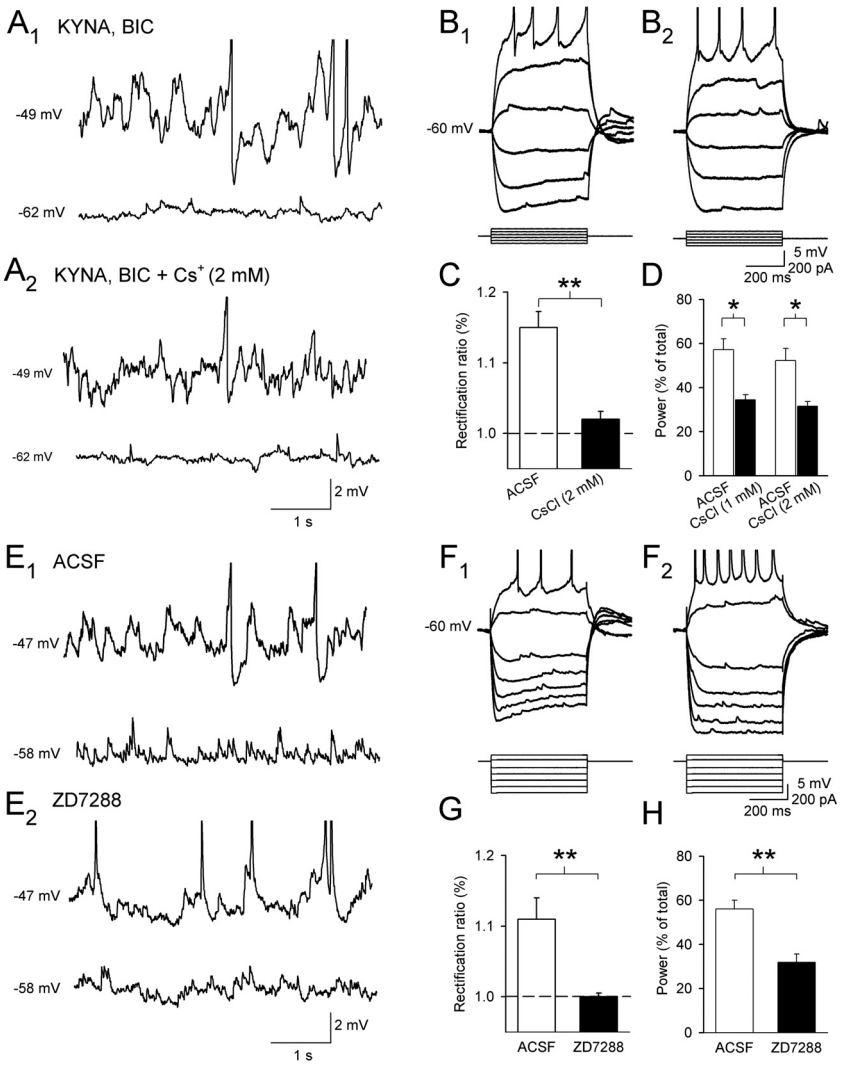
A1 KYNA, BIC
-49 mV
-62 mV
A2 KYNA, BIC + Cs+ (2 mM)
-49 mV
-62 mV
2 mV
1 s
E1 ACSF
-47 mV
-58 mV
E2 ZD7288
-47 mV
-58 mV
2 mV
1 s
B1
B2
-60 mV
5 mV
200 pA
200 ms
C
**
Rectification ratio (%)
1.2
1.1
1.0
ACSF
CsCl (2 mM)
D
*
*
Power (% of total)
80
60
40
20
0
ACSF
CsCl (1 mM)
ACSF
CsCl (2 mM)
F1
F2
-60 mV
5 mV
200 pA
200 ms
G
**
Rectification ratio (%)
1.2
1.1
1.0
ACSF
ZD7288
H
**
Power (% of total)
80
60
40
20
0
ACSF
ZD7288

Figure 2.7. Blocking the hyperpolarization-activated cationic current I_h disrupts voltage-dependent oscillations in parasubicular neurons. **A**: Voltage-dependent oscillations recorded from a parasubicular neuron in the presence of ionotropic glutamate and GABA receptor antagonists were markedly reduced by Cs^+ (2 mM). **B**: Responses to hyperpolarizing and depolarizing current pulses are shown before (B_1) and during bath application of Cs^+ (B_2) for the same cell as in A. Action potentials have been truncated here and in panel F. The voltage- and time-dependent inward rectification typical of parasubicular cells was not observed in the presence of Cs^+, and rebound potential responses were abolished. **C**: There was a significant reduction in the rectification ratio during bath application of 2 mM Cs^+ (*: $p < 0.05$). **D**: Both 1 mM (n= 4) and 2 mM (n= 5) Cs^+ significantly reduced the power of oscillations (*: $p < 0.05$). **E**: Voltage-dependent oscillations were also blocked by the potent I_h blocker ZD7288 (100 μM). **F**: Membrane potential responses to current steps in control ACSF (F_1) and ZD 7288 (F_2) show the complete block of inward rectification. Also note the spike broadening and reduction of the fast afterhyperpolarization. **G**: Changes in mean rectification ratio reflect the elimination of inward rectification in cells treated with ZD7288 (n=10; **: $p < 0.01$). **H**: The block of oscillations by ZD7288 was also reflected in a significant reduction of theta-band power (**: $p < 0.001$).

CHAPTER 3

MUSCARINIC DEPOLARIZATION OF LAYER II NEURONS OF THE PARASUBICULUM.

Stephen D. Glasgow and C. Andrew Chapman

ABSTRACT

The parasubiculum sends its major output to layer II of the entorhinal cortex, and is therefore well-positioned to modulate sensory and mnemonic functions associated with the medial temporal lobe. The parasubiculum also receives strong cholinergic innervation from the basal forebrain, and similar to effects on neurons through the hippocampal formation, cholinergic receptor activation is likely to result in membrane potential depolarization through activation or occlusion of a variety of ionic conductances in layer II parasubicular neurons.. The present study used whole cell current- and voltage-clamp recordings to determine how the activity of layer II parasubicular neurons is modulated by activation of muscarinic cholinergic receptors. Bath application of carbachol (10-50 μM) typically resulted in a postsynaptically-mediated, dose-dependent depolarization of morphologically-identified layer II stellate and pyramidal cells. Bath application of the selective M_1 receptor antagonist pirenzepine (1 μM), but not the M_2-preferring antagonist methoctramine (1 μM), blocked the depolarization, indicating that the depolarization is dependent on M_1 receptors. In voltage-clamp recordings, carbachol also resulted in the gradual development of an inward current that was not completely blocked by the selective Kv7.2/3 channel antagonist XE-991. The remaining current reversed near E_K and was inhibited by the K^+ channel blocker Ba^{2+}, suggesting that M_1 receptor activation results in the reduction of a current that uses K^+ as its primary ion. Moreover, this K^+ current showed rectification at depolarized voltages, similar to K^+ conductances mediated by Kir 2.3 channels. Therefore, cholinergic

innervation of the parasubiculum depolarizes layer II parasubicular neurons through effects on I_M as well as an additional K^+ conductance.

Recent evidence suggests that the subicular complex, including the pre- and parasubiculum, occupies an important node within the brain navigational system (Boccara et al., 2010; Hargreaves et al., 2005; Solstad et al., 2008). The parasubiculum receives numerous cortical and subcortical inputs, including substantial projections from the CA1 region of the hippocampus, the anterior thalamus, and medial septum (Alonso & Kohler, 1984; Boeijinga & Van Groen, 1984; Cenquizca & Swanson, 2007; Shibata, 1993; Swanson & Cowan, 1977; van Groen & Wyss, 1990a). In turn, layer II parasubicular cells terminate on projection neurons in layer II of the entorhinal cortex that form the origin of the perforant path (Caballero-Bleda & Witter, 1993, 1994; van Groen & Wyss, 1990a). Layer II of the entorhinal cortex serves as an interface between multimodal sensory associational cortices and the hippocampus, and this suggests that activity in the parasubiculum may modulate how the entorhinal cortex transfers information into the hippocampus (Amaral & Witter, 1989; Caballero-Bleda & Witter, 1993, 1994). Consistent with anatomical data, stimulation of the parasubiculum can facilitate the response of entorhinal cortex cells to inputs from the piriform cortex, demonstrating that the parasubiculum can modulate how the entorhinal cortex responds to incoming sensory information (Caruana & Chapman, 2004).

The parasubiculum receives highly-processed spatial information from head direction cells in the anterior thalamus, and from place cells of the proximal CA1 region of the hippocampus (Cenquizca & Swanson, 2007; Henriksen et al., 2010; Jung, Wiener, & McNaughton, 1994; O'Keefe & Dostrovsky, 1971; Shibata,

1993; Taube, 1995a; van Groen & Wyss, 1990a, 1990b). The convergent spatial information received by the parasubiculum may allow parasubicular neurons to contribute to the activity of grid cells in the entorhinal cortex. *In vivo* studies have also demonstrated that the parasubiculum contains cells that fire in response to head direction and location (Taube, 1995b), and more recently, subsets of parasubicular neurons have been reported that resemble grid, conjunctive head by direction, and border cells of the dorsolateral band of the medial entorhinal cortex (Boccara et al., 2010; Cacucci et al., 2004; Hargreaves et al., 2005; Solstad et al., 2008). Further, lesions of the parasubiculum result in impairments in object-recognition and spatial working memory tasks that are thought to result from disrupted place field specificity in CA1 neurons (P. Liu et al., 2001, 2004), suggesting an important role for the parasubiculum in the formation and maintenance of spatial representations within the hippocampal region.

The parasubiculum receives dense cholinergic and GABAergic projections from the medial septum (Alonso & Kohler, 1984; Swanson & Cowan, 1977), and the superficial layers of the parasubiculum generate cholinergically-dependent theta frequency field activity (Boccara et al., 2010; Cacucci et al., 2004; Glasgow & Chapman, 2007). Neuronal synchronization associated with theta-frequency EEG activity is thought to contribute to the formation and maintenance of spatial representations within the entorhinal cortex (Alonso & Garcia-Austt, 1987b; Chrobak & Buzsaki, 1994; Stewart, Quirk, Barry, & Fox, 1992) and CA1 region (Henriksen et al., 2010; O'Keefe & Recce, 1993), and approximately half of the spatially tuned cells in the parasubiculum are strongly modulated by local theta-

frequency field activity (Boccara et al., 2010; Cacucci et al., 2004; Hargreaves et al., 2005; Hargreaves et al., 2007; Taube, 1995b). Theta frequency (4-12 Hz) activity is a sinusoidal-like rhythmic EEG waveform observed throughout the hippocampal formation, and has been linked to cognitive and behavioral processes, including sensorimotor integration, memory consolidation and spatial navigation (Alonso & Garcia-Austt, 1987a; Bland, 1986; Buzsaki, 2002; Glasgow & Chapman, 2007; Hasselmo, 2005; Hasselmo & Brandon, 2008). Behavioral states associated with the generation of theta EEG activity, such as active exploration, are correlated with increases in acetylcholine as measured by microdialysis (Marrosu et al., 1995; Zhang, Lin, & Nicolelis, 2010). Although the mechanisms underlying the generation of theta activity have not been fully determined, it is thought that cholinergic projections from the medial septum to the hippocampal formation provide tonic depolarization which can result in the emergence of intrinsic voltage-dependent membrane potential oscillations at theta-frequency and, when combined with phasic septal and local inhibitory inputs, lead to the generation and synchronization of theta-frequency population activity (Bland, 1986; Buzsaki, 2002; Goutagny et al., 2009; J. D. Green & Arduini, 1954; M. G. Lee, Chrobak, Sik, Wiley, & Buzsaki, 1994; Stewart et al., 1992; Stumpf, Petsche, & Gogolak, 1962). I have recently demonstrated that layer II neurons of the parasubiculum display theta-frequency oscillations in membrane potential at near-threshold voltages (Glasgow & Chapman, 2007, 2008). Cholinergic inputs from the medial septum may therefore serve to depolarize cells to near-threshold voltages to result in the induction of theta-

frequency membrane potential oscillations that can contribute to the generation of theta-frequency EEG activity.

The cholinergic system plays a critical role in the modulation of neural processes in both cortical and subcortical structures, and has been implicated in declarative memory function (Hasselmo, 2006). Acetylcholine induces prominent changes in the overall neuronal excitability, including alterations in spike properties, increases in input resistances, and sustained membrane potential depolarization via alterations in various conductances (Chapman & Lacaille, 1999a; Dutar & Nicoll, 1988; Klink & Alonso, 1997a). Early work in hippocampal neurons demonstrated that stimulation of muscarinic receptors leads to inhibition of K^+ conductances, including the muscarine-sensitive K^+ current I_M and a leak K^+ current (Benson et al., 1988; Madison, Lancaster, & Nicoll, 1987). In contrast, muscarinic depolarization in prefrontal and entorhinal cortex principal neurons has been attributed primarily to activation of a Ca^{2+}-modulated nonselective cationic current (Egorov, Angelova, Heinemann, & Muller, 2003; Haj-Dahmane & Andrade, 1996; Shalinsky et al., 2002). However, it remains unclear how parasubicular neurons respond to cholinergic receptor activation, and whether muscarinic receptor stimulation may contribute to the generation of theta-frequency membrane potential oscillations through modulation of K^+ and cationic conductances.

We have recently demonstrated that the parasubiculum expresses theta-frequency EEG activity that is dependent on cholinergic inputs, and that parasubicular neurons also generate intrinsic theta-frequency membrane

potential oscillations (Glasgow & Chapman, 2007, 2008). Cholinergic inputs to the parasubiculum are therefore likely to contribute to the local generation of theta activity by depolarizing principal neurons to near-threshold voltages leading to the generation of intrinsic membrane potential oscillations. Here, I demonstrate that cholinergic receptor activation in morphologically-identified layer II neurons of the parasubiculum modulates neuronal excitability via M_1 receptors, and that the depolarization of parasubicular neurons is dependent on suppression of I_M- and an additional K^+ conductance likely mediated by Kir2 channels.

METHODS

Slice preparation

The methods used for slice preparation have been described in previous reports (Glasgow & Chapman, 2007, 2008), and were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Concordia University Research Ethics committee. Acute brain slices were obtained from 4 to 6 week old Long-Evans male rats (Charles River Laboratories, Montreal). The rat was deeply anesthetized using halothane and decapitated. The brain was quickly extracted and submerged in ACSF (4 ºC) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose saturated with 95% O_2 and 5% CO_2 (pH ~7.3; 300-310 mOsm). Horizontal hippocampal slices (300 µM thick) containing the parasubiculum were cut using a vibratome (WPI, Vibroslice NVSL), and allowed to recover for ~1.5 h at room temperature.

Individual slices were transferred to a recording chamber, and superfused with oxygenated ACSF (~22-24º C) at a rate of 1.5 - 2.0 ml/min. Slice landmarks, such as the characteristic broadening of layer II/III of the parasubiculum compared to the relatively compact superficial layers of the adjacent presubiculum and medial entorhinal cortex, as well as the location of the angular bundle, were used to identify the location of the parasubiculum (Funahashi & Stewart, 1997b; Glasgow & Chapman, 2007, 2008). Individual parasubicular neurons were visualized using an upright microscope (Leica, DM-LFS) equipped with a long-range (40x) water immersion objective using differential interference contrast optics, and a near-infrared camera (COHU).

Electrophysiological recordings and data analysis

Somatic whole-cell recordings were obtained using patch recording pipettes (4-8 MΩ) containing (in mM): 140 K-gluconate, 5 NaCl, 2 $MgCl_2$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 0.5 ethylene glyco-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2 ATP-Tris, 0.4 GTP-Tris (pH calibrated to 7.20 - 7.26 using KOH; 270-280 mOsm). Biocytin (0.1%) was added in some experiments to allow morphological identification of neurons. Pipettes were prepared from borosilicate glass capillaries (1.0 mm OD, 0.5 mm ID) using a horizontal puller (Sutter Instr., P-97). Recorded cells were located in layer II near the border with layer I, and none of the neurons sampled showed burst firing in response to a 500 ms positive current injection that is characteristic of layer V cells (Funahashi & Stewart, 1997a). The patch pipette was slowly

lowered to contact the soma of the target cell, and gentle suction was applied in voltage-clamp mode to obtain a tight seal (>1 GΩ) after which strong negative pressure was applied to obtain whole-cell configuration. Cells were allowed to recover for ~5 min before recordings were taken to allow for equilibration of the intracellular patch solution. Membrane voltage was amplified in current-clamp mode, and membrane currents were measured in voltage-clamp mode using an Axoclamp 200B amplifier (Axon Instr.). Signals were monitored on a digital oscilloscope, digitized (Axon Instr., Digidata 1322A), and sampled at 20 KHz for storage on hard-disk using pClamp 8.2 software package (Axon Instr.). Current-clamp recordings were filtered at 10 kHz, and voltage-clamp data were filtered at 2 kHz. Continuous recordings of membrane potential and current were stored onto VHS tape by PCL coding using a Neurocorder converter (DR-886, Neurodata) for offline analysis.

Recordings were accepted if the resting membrane potential was ≤ -50 mV. For current clamp experiments, series resistance was estimated by compensating for the discontinuity in the voltage response to –50 pA intracellular current pulses, and recordings were accepted if series resistance was < 35 MΩ (mean: 13 ± 1 MΩ). Changes in input resistance were monitored regularly using 500 ms hyperpolarizing current pulses (–100 pA) applied at a frequency of 0.1 Hz. For voltage clamp recordings, series resistance was estimated by cancellation of the fast component of whole-cell capacitive transients using a -2 mV voltage step, and was typically compensated ~40-60% (range: 10-15 MΩ; mean: 14 ± 1 MΩ). Series resistance was monitored throughout the duration of

the experiment, and the experiment was discontinued if Rs changed by ≥ 15%. Further, all membrane potentials reported here were not corrected for the liquid junction potential, which was estimated using the method described by Neher (Neher, 1992) to be ~8 mV.

The conductances underlying carbachol-induced depolarization were assessed in voltage clamp experiments by use of slow voltage-ramps at 2 min intervals throughout the experiment (Ma, Shalinsky, Alonso, & Dickson, 2007; Shalinsky et al., 2002). The holding potential for voltage-clamp experiments was -60 mV. Ramp voltage protocols consisted of 4-s linear depolarizations from -120 to -40 mV, and were preceeded by a 1-s fixed step at -120 mV. The sampling frequency of voltage ramps was reduced to 2 KHz for offline analysis. Currents elicited by carbachol were computed by subtraction of ramp-evoked current traces during drug application from control traces, and linear regression fittings between -120 mV and -70 mV were used to calculate reversal potentials of the subtracted current.

The electrophysiological characteristics of parasubicular neurons were analyzed using the Clampfit 8.2 software package (Axon Instr.). Action potential amplitude was measured from resting membrane potential. Action potential duration and afterhyperpolarizations were measured from action potential threshold. Input resistance was measured as the peak voltage response to a 500 ms, –100 pA current pulse from –60 mV, and inward rectification was quantified as the ratio between peak and steady-state input resistance in response to a -200 pA hyperpolarizing current pulse (Chapman & Lacaille,

1999a). Similarly, the anodal break potential was measured as the peak depolarization following the offset of a -200 pA pulse relative to baseline voltages.

To analyze subthreshold membrane potential oscillations, cells were depolarized to near-threshold voltages using positive constant current injection. Cells were typically maintained at this depolarized voltage for ≥ 30s. Samples of membrane potential at near-threshold voltages were prepared for spectral analysis by reducing the effective sampling rate to 1 kHz. Samples of 2.048-s duration that contained no action potentials were passed through a Blackman window, and the power spectrum was then computed as average squared magnitude of the fast Fourier transformation across three non-overlapping samples. Membrane potential oscillations in parasubicular neurons at room temperature increase in frequency without alterations in power at higher temperatures (Glasgow & Chapman, 2007). The recordings reported here were obtained at room temperature, and the power of membrane potential oscillations was therefore calculated for the frequency band of 1.5-5.9 Hz, and expressed as a percentage of the total power of the signal.

Statistical analyses assessed alterations in electrophysiological properties after pharmacological manipulations using one-way repeated measures ANOVA, paired t-tests, and pairwise multiple comparisons using Student-Newman-Keuls method for parametric data, and Mann-Whitney *U* tests for nonparametric data. The significance level was set at an alpha of 0.05. Data are presented as means ± SE.

Immunohistochemistry

Morphological identification of biocytin-filled neurons in the intact slice has been reported previously in detail elsewhere (Hamam & Kennedy, 2003; Mueller, Chapman, & Stewart, 2006). Immediately following completion of electrophysiological recordings, individual slices were fixed in 4% paraformaldehyde in 0.1 M sodium-phosphate buffer (NaPB, ph: 7.5) for ~24h at 4º C, and stored in 0.1 M NaPB for 2-4 weeks. Slices were rinsed 3x (5 min per wash) in 0.1 NaPB, and incubated in 1% H_20_2 for 30 min to block endogenous peroxidases. To block non-specific binding, slices were then rinsed and incubated in PHT (1% heat-inactivated normal goat serum and 0.3% Triton X-100 in 0.1 NaPB, pH: 7.5) for 2h. The slices were subsequently transferred to an avidin-biotin-horseradish-peroxidase complex (ABC kit, Vector, Burlington, ON) in 0.1 NaPB overnight at room temperature on an oscillating table. After six successive 1h rinses in PHT, the slices were incubated in a Tris-buffered saline solution containing 0.01% H_2O_2, 0.5% 3,3′-diaminobenzidine (DAB), and 0.02% $NiSO_4$ for 10-15 min. Staining intensity was monitored, and quenched by rinsing the slices. Slices were then dehydrated in progressive concentrations of glycerol (25%, 50%, 75%, 100%), and stored at 4º C in 100% glycerol, and mounted on glass slides.

Following successful histological processing, biocytin-labeled neurons were identified using a low-magnification light microscope, and reconstructed using the Neurolucida neuron tracing system (Microbrightfield Inc., Colchester,

VT) using an upright microscope (Leica DM-5000B) mounted with a digital camera (Hamamatsu ORCA-ER, Hamamatsu Photonics Deutschland, Germany) connected to a workstation computer. Quantitative data regarding neuronal cell bodies and dendrites were obtained using NeuroExplorer software package (Microbrightfield Inc.). Photomicrographs of successfully labeled neurons were taken at various stages on the Z-axis of the intact slice using an upright microscope (Leica DFC480) affixed with a high-resolution digital camera, captured and stored on a hard disk, and two-dimensional composites of each successfully labeled cell were digitally-reconstructed using Adobe Photoshop CS3 software (Adobe Systems Inc).

Pharmacological manipulations

Drugs were typically stored in frozen stock solution, and diluted to proper concentrations in ACSF prior to recordings. The effects of cholinergic receptor activation on layer II parasubicular neurons were routinely assessed using bath application carbamylcholine chloride (carbachol, CCh, 5-50 μM), a cholinergic agonist that is poorly metabolized by acetylcholinesterase (Dutar & Nicoll, 1988). To determine whether cholinergic depolarization of layer II parasubicular neurons was dependent on increases in local network synaptic transmission, fast ionotropic synaptic transmission was blocked using 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM), DL-(±)-2-amino-5-phosphonopentanoic acid (AP5, 50 μM), and bicuculline methiodide (10 μM). Cholinergic effects on muscarinic receptors were assessed using the nonselective antagonist atropine sulfate

($ATSO_4$, 1 μM), M_1-preferring blocker pirenzepine dihydrochloride (1 μM), and the M_2 antagonist methoctramine tetrahydrochloride (1 μM). To assess the contribution of Na^+ conductances to cholinergic depolarization, Na^+ channels were blocked using tetrodotoxin (TTX, 0.5 μM). The muscarinic-sensitive KCNQ Kv7.2/3 channel I_M was blocked using 10,10-bis(4-pyridinylmethyl)-9(10H)-antracenone (XE-991, 10 μM). In voltage-clamp experiments, the potent I_h channel blocker 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium chloride (ZD7288, 50 μM) and TTX (0.5 μM) were routinely added to the bath. Potassium conductances were blocked with the wide-acting K^+ blocker barium sulfate (200 μM). When barium was added to the bath, PO_4 and SO_4 were removed from ACSF. Most drugs were obtained from Sigma-Aldrich (St. Louis, MO, USA) except for pirenzepine, XE-991, ZD7288, and bicuculline methiodide, which were purchased from Ascent Scientific (Princeton, NJ, USA).

RESULTS

Stable whole-cell recordings were obtained from 122 layer II parasubicular cells. Electrophysiological properties and firing patterns were similar to those reported previously (Funahashi & Stewart, 1997a; Glasgow & Chapman, 2007). The superficial layers of the parasubiculum are under considerable inhibitory tone (Funahashi & Stewart, 1998), and layer II cells were typically quiescent at resting membrane potentials (mean: -59.9 ± 1.0 mV). Layer II parasubicular neurons demonstrate time-dependent inward rectification in response to hyperpolarizing current pulses (mean rectification ratio: 1.21 ± 0.02) with

moderate peak input resistance (mean: 127.5 ± 4.4 MΩ). Neurons reported here exhibited high action potential thresholds (mean: -39.6 ± 0.4 mV), and overshooting action potentials (mean amplitude: 125.0 ± 0.8 mV; duration: 3.5 ± 0.1 ms) were typically followed by fast (fAHP; mean: 8.6 ± 0.3 mV) and medium (mAHP; mean: 6.1 ± 0.2 mV) afterhyperpolarizations. Further, the -200 pA pulse offset was typically followed by an anodal break potential (mean: 4.5 ± 0.3 mV). In contrast to subicular and deep layer parasubicular neurons (Funahashi & Stewart, 1997b), superficial layer neurons typically responded to 500-ms positive current injection with repetitive regular spiking and little to no spike frequency adaptation (Figure 3.1A).

Properties of morphologically identified stellate and pyramidal neurons.

Layer II of the parasubiculum contains both pyramidal-like and stellate-like neurons. Morphological identification was obtained for 18 biocytin-filled neurons, and the morphological characteristics of the layer II parasubicular neurons observed here were similar to previous reports (Funahashi & Stewart, 1997a, 1997b). All identified neurons were located near the superficial edge of layer II of the parasubiculum. Five of 18 cells (28% of stained cells) displayed stellate-like neuronal morphology, and these cells typically had 3 to 5 basal dendrites emanating from the soma with each primary dendrite bifurcating several times (Figure 3.1A_1). Similar to previous reports, stellate cells typically had spiny dendrites throughout all processes. Thirteen of 18 cells (72%) showed pyramidal morphology (Figure 3.1A_2). In some cases, axons were traced out of the parasubiculum via layer I, and terminated in layer II of the entorhinal cortex. In

the present study, no axons were observed descending towards the deep layers. Pyramidal cells had multiple basal dendrites as well as one or two apical dendrites that terminated in layer I. In addition, pyramidal-like neurons tended to have more profuse basal dendrites that occasionally extended to the border between layer III and layer IV, while stellate-like neurons showed a more clustered dendritic arbor confined to the superficial layers, and rarely displayed prominent basal dendrites projecting into deep parasubicular layers. However, there were no detectable differences between pyramidal and stellate neurons in somatic diameter (16.6 ± 1.4 μm for pyramidals vs. 14.0 ± 1.3 μm for stellates; n.s., $p = 0.19$) or overall length of dendritic arbor (2046 ± 409 μm for pyramidals vs. 2437 ± 296 μm for stellates; n.s., $p = 0.45$). Taken together, these findings suggest that the differences in dendritic arbour between the stellate and pyramidal neurons are not reflected in quantitative measures of cell morphology.

Morphologically identified pyramidal and stellate neurons exhibited very similar electrophysiological characteristics. Both cell types fired repetitive spikes in response to a depolarizing current injection (+100 pA), and no cells responded in a burst-like manner (Figure 3.1A). Input resistances were similar in both cell types (108.0 ± 4.3 MΩ in pyramidals vs. 109.7 ± 7.1 MΩ in stellates; n.s., $p = 0.84$) with moderate rectification in response to hyperpolarizing current pulses (1.19 ± 0.02 in pyramidal cells vs. 1.15 ± 0.02 in stellate cells; n.s., $p = 0.28$). Although layer V parasubicular stellate neurons display a large anodal break potential (Funahashi & Stewart, 1997a), there was only a small depolarizing response at the offset of -200 pA pulses in layer II neurons (4.0 ± 0.4 mV in

pyramidals vs. 3.4 ± 0.2 mV in stellates; n.s., p = 0.37) that typically did not reach action potential threshold. In summary, both the pyramidal- and stellate-like neurons reported here display similar electrophysiological properties, suggesting a homogeneity in basic electroresponsiveness across these cell types in layer II of the parasubiculum (Funahashi & Stewart, 1997a).

Many parasubicular neurons display theta-frequency oscillations in membrane potential at near-threshold voltages that are dependent on the interplay between I_{NaP} and I_h (Glasgow & Chapman, 2007, 2008). Consistent with our previous findings, the majority of layer II parasubicular neurons tested in current clamp experiments also showed membrane potential oscillations at near-threshold voltages (62 of 74 cells tested; 83.7%) that accounted for 52.4 ± 0.2% of total power (0.74 ± 0.06 mV^2/Hz between 1.5 and 5.9 Hz). To investigate whether cell type was associated with the expression of theta-frequency membrane potential oscillations, biocytin-filled cells were grouped based on cell morphology (Figure 3.1B). Layer II parasubicular stellate neurons (Figure 3.1B_1) and pyramidal neurons (Figure 3.1B_2) both showed prominent theta-frequency membrane potential oscillations. Interestingly, identified pyramidal neurons displayed significantly higher levels of theta band power (1.31 ± 0.25 mV^2/Hz) compared to stellate cells (0.42 ± 0.07 mV^2/Hz; t_{13}=2.48, p <0.05) (Figure 3.1C), and theta-band frequencies in pyramidal cells accounted for a larger portion of the total signal compared to stellate cells (61.7 ± 2.0% vs. 47.9 ± 0.05%; t_{13}=3.09, p <0.01). Therefore, both layer II pyramidal and stellate neurons of the

parasubiculum demonstrate theta-frequency subthreshold membrane potential oscillations, however pyramidal neurons show larger amplitude oscillations.

Cholinergic modulation of layer II parasubicular neurons. Current-clamp experiments were performed to assess the voltage responses of layer II cells of the parasubiculum to cholinergic agonism using a stable analog of acetylcholine, carbachol (CCh, 5-50 μM). To determine the effects of cholinergic receptor activation on the resting membrane potential and firing properties of parasubicular neurons, carbachol was bath applied for 2 - 5 min (Figure 3.2). Low doses of CCh (5 μM) failed to result in significant changes in the resting membrane potential of layer II parasubicular neurons (0.9 ± 1.7 mV, n = 3), however higher doses of 10 to 50 μM CCh resulted in the slow depolarization of resting membrane potential. As illustrated in Figure 3.2, bath application of 25 μM CCh resulted in significant sustained depolarization, and led to the emergence of repetitive spike activity in some cells. Of the 78 cells sampled in current clamp experiments, 63 layer II parasubicular neurons (80.7%) responded to 10 to 50 μM carbachol with depolarization of membrane potential, and mean depolarization increased as a function of concentration (10 μM: 4.8 ± 1.1 mV, n = 4; 25 μM: 8.1 ± 1.1 mV, n = 14; 50 μM: 7.0 ± 1.6 mV, n = 14; main effect of CCh: $F_{1, 31}$ = 26.55, p < 0.001; Figure 3.2, inset). The effects of CCh were maximal between 25-50 μM, and subsequent experiments therefore investigated the mechanisms underlying cholinergic depolarization of layer II neurons using these higher concentrations.

Interestingly, carbachol-sensitive and insensitive neurons did not differ significantly in electrophysiological or morphological properties. Carbachol-insensitive neurons displayed trends toward slightly smaller fast AHPs (-8.8 ± 0.3 mV in responders vs. -7.5 ± 0.7 mV in nonresponders; Mann-Whitney *U*, $p = 0.09$), and slightly smaller anodal break potentials at the offset of -200 pA current pulses (4.7 ± 0.4 mV in responders vs. 3.4 ± 0.2 mV in nonresponders; Mann-Whitney *U*, $p = 0.07$), but these effects were not significant. Further, the presence and magnitude of subthreshold membrane potential oscillations induced by constant current injection did not vary between cells that were sensitive or insensitive to carbachol (data not shown). Thus, the electrophysiological profiles of layer II parasubicular do not predict their sensitivity to carbachol.

Previous studies in the hippocampus, subiculum and layer II of the entorhinal cortex have reported that cholinergic receptor activation results in increases in apparent input resistances (Benardo & Prince, 1982a; Kawasaki, Palmieri, & Avoli, 1999; Madison et al., 1987). Because 10-50 μM carbachol typically resulted in a depolarization to near-threshold and threshold voltages that could alter activation of voltage-dependent conductances, input resistance and passive electrical properties were routinely measured from -60 mV by injecting negative constant current. Bath application of CCh (25 μM) was associated with no appreciable change in peak input resistance, and a minor increase in steady-state input resistance in 13 of 14 cells (from 108.5 ± 7.3 MΩ in ACSF to 122.9 ± 8.9 MΩ in CCh; $p = 0.06$), and reduced the input resistance in one cell (39.4%)

(Figure 3.3A, 3.3B$_1$). The slight increase in input resistance observed in some cells is consistent with inhibition of K^+ conductances (Brown & Adams, 1980; Halliwell & Adams, 1982; Madison et al., 1987).

The hyperpolarization-activated mixed cationic current I_h is important in the generation of time-dependent depolarizing "sag" responses during negative current pulses in both subicular pyramidal neurons and stellate cells of the entorhinal cortex (Klink & Alonso, 1993; van Welie, Remme, van Hooft, & Wadman, 2006), and plays a crucial role in the generation and maintenance of membrane potential oscillations in parahippocampal neurons (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000). Further, recent work has suggested that CCh may modulate theta frequency resonance profiles by affecting I_h (Heys, Giocomo, & Hasselmo, 2010). While CCh (25 μM) did not significantly affect rectification ratio in parasubicular neurons (from 1.26 ± 0.05 in ACSF to 1.24 ± 0.07 in 25 μM CCh; $n = 14$; $p = 0.41$), a group of cells tested with 50 μM CCh ($n = 7$) demonstrated a significant reduction in rectification ratio (from 1.17 ± 0.03 in ACSF to 1.10 ± 0.04 in CCh; $t_6 = 3.50$, $p < 0.05$; data not shown). These data indicate that cholinergic receptor activation does not affect the amplitude of I_h at hyperpolarized voltages during bath application of CCh at concentrations lower than 50 μM.

The amplitudes of anodal break potentials were consistently reduced in both 25 μM CCh (5.4 ± 0.7 mV in ACSF vs. 2.7 ± 0.5 mV in CCh; $t_{12} = 4.34$, $p < 0.01$; Figure 3.3B$_2$), and 50 μM CCh (3.8 ± 0.7 mV in ACSF vs. 1.6 ± 0.4 mV in CCh; $t_6 = 3.63$, $p < 0.05$). Although I_h can contribute to anodal break excitation

(van Welie et al., 2006), break potentials in layer II entorhinal neurons are mediated primarily via the activation of a voltage-dependent Na^+ conductance (Klink & Alonso, 1993, 1997b). To assess the contribution of Na^+ currents to cholinergically-mediated reductions in anodal break excitation, the potent I_{Na} channel blocker TTX (0.5 μM) was added to the bath. Application of TTX reduced the amplitude of the anodal break potential (from 4.8 ± 1.2 mV in ACSF to 0.7 ± 0.4 mV in TTX) without significantly altering hyperpolarization-induced rectification. Subsequent application of CCh did not lead to further reductions in anodal break potentials (n = 3, data not shown), indicating that CCh-induced reductions anodal break potentials in layer II parasubicular neurons are likely due to an attenuation of voltage-dependent Na^+ conductances, rather than a suppression of I_h.

Previous reports have shown that carbachol can modulate the ionic conductances responsible for action potential generation and repolarization in hippocampal pyramidal neurons, as well as stellate and non-stellate entorhinal cells (Figenschou, Hu, & Storm, 1996; Klink & Alonso, 1997b). Hippocampal pyramidal neurons show carbachol-sensitive fast and medium AHPs that are mediated via Ca^{2+}-dependent and independent K^+ channels, including I_C and I_M (Storm, 1987, 1989). In parasubicular neurons, cholinergic receptor activation resulted in significant reductions of both fast (from 8.5 ± 0.5 mV in ACSF to 5.3 ± 0.9 mV in CCh; t_{12} = 4.65, p < 0.01) and medium afterhyperpolarizations (from 5.4 ± 0.6 mV in ACSF to 3.8 ± 0.8 mV in CCh; t_{12} = 2.36, p < 0.05) (Figure 3.3C). The suppression of the fast AHP was associated with a slight increase in the

duration of the action potential (3.7 ± 0.2 ms in ACSF to 4.2 ± 0.2 ms in CCh; t_{12} = 2.11, p = 0.06). Additionally, carbachol shifted the action potential threshold by ~1.5 mV, and reduced the spike amplitude (from 122.3 ± 2.0 mV in ACSF to 107.9 ± 4.3 mV in CCh; t_{12} = 4.09, p < 0.01) (Figure 3.3D). In a subset of cells assessed (n = 6), the effects of CCh on electrophysiological properties returned to baseline values after a 15 min washout period in normal ACSF. These results indicate that CCh affects conductances associated with spike repolarization and afterhyperpolarizations, and may include modulation of K^+, Ca^{2+}-dependent K^+, and Na^+ currents.

Cholinergic depolarization is not mediated by changes in synaptic inputs. Cholinergic agonism is known to result in strong depolarization of neurons in other hippocampal structures that project to the parasubiculum, and enhanced synaptic input could contribute to the depolarization of individual parasubicular neurons (Buhl, Tamas, & Fisahn, 1998; Dickson & Alonso, 1997; Fisahn, Pike, Buhl, & Paulsen, 1998; Williams & Kauer, 1997). To assess the contribution of changes in local synaptic activity to the cholinergic depolarization, and to assess whether CCh acts via intrinsic conductances in layer II parasubicular neurons, CCh was coapplied with CNQX (20 μM), AP-5 (50 μM), and bicuculline methiodide (25 μM) to block AMPA and kainate, NMDA, and $GABA_A$-mediated synaptic transmission, respectively (n = 5; data not shown). Blockade of fast glutamatergic and GABAergic receptors failed to block the CCh-induced depolarization, and bath application of CCh (50 μM) resulted in significant depolarization from -56.2 ± 0.7 mV to -48.4 ± 1.8 mV (n = 5, $F_{2,8}$ = 15.15, p <

0.01; N-K, $p < 0.01$) (data not shown). Resting membrane potential returned to baseline voltages (mean: -57.2 ± 1.1 mV) after drug washout (N-K, $p < 0.01$). This demonstrates that alterations in fast excitatory and inhibitory synaptic transmission are not required for CCh-induced depolarization of layer II parasubicular neurons, and suggest that CCh exerts direct actions on layer II cells of the parasubiculum.

Carbachol-induced depolarization is dependent on M_1 receptors. To assess the dependence of the CCh-induced depolarization on muscarinic receptor activation, the potent muscarinic receptor antagonist atropine ($ATSO_4$; 1 μM) was added to the bath for 15-20 min prior to CCh (50 μM) application. The CCh-induced depolarization was completely blocked in the presence of $ATSO_4$ (from -58.2 ± 1.9 mV in $ATSO_4$ to -57.7 ± 1.8 mV in CCh; $n = 7$, $p = 0.67$; Figure 3.4), indicating that muscarinic receptors are required. Further, bath application of $ATSO_4$ also blocked effects of CCh on action potential amplitude and width, as well as fast afterhyperpolarizations, but did not block reductions in anodal break potentials (data not shown).

Additional experiments were aimed at determining the specific muscarinic receptor subtype involved in the CCh-induced depolarization. First, the responsiveness of layer II parasubicular neurons was assessed using high concentrations of CCh which elicited strong depolarization layer II cells from -61.4 ± 1.5 mV in ACSF to -56.2 ± 1.8 mV in CCh ($n = 6$; $F_{3, 15} = 12.17$, $p < 0.05$; N-K, $p < 0.001$) that was reversed following wash in normal ACSF. Subsequent antagonism of M_1-like receptors using the M_1-preferring muscarinic antagonist

pirenzepine (1 μM) for a period of 15 min completely blocked CCh-induced membrane depolarization (mean RMP: -60.1 ± 2.0 mV in pirenzepine vs. -60.2 ± 1.8 mV in pirenzepine and CCh; N-K, $p = 0.73$; Figure 3.5A,B). Pirenzepine also blocked the effects of CCh on the action potential and anodal break potential, suggesting that these effects are mediated by an M_1-dependent pathway. In contrast, the M_2-preferring antagonist, methoctramine (1 μm) failed to block depolarization associated with CCh application (mean RMP: -58.4 ± 2.5 mV in methoctramine vs. -54.5 ± 1.7 mV in methoctramine and CCh; $n = 3$; $F_{2,4} = 17.93$, $p < 0.01$; N-K, $p < 0.05$; Figure 3.5B). Taken together, these results indicate that CCh-induced depolarization is dependent on actions on M_1-like receptors.

Cholinergic depolarization is mediated by I_M and an additional K^+ conductance. Cholinergic depolarization in hippocampal neurons has been primarily attributed to inhibition of the voltage-dependent Kv7.2/3-mediated current, I_M, as well as inhibition of a voltage-independent K^+ leak current (Halliwell & Adams, 1982; Madison et al., 1987). To determine whether CCh-induced depolarization was dependent on inhibition of I_M, the selective Kv7.2/3 channel blocker XE-991 (10 μM) was bath applied in the presence of synaptic antagonists for 10-15 min prior to application of CCh (25-50μM; Figure 3.6A_1). Bath application of XE-991 depolarized layer II parasubicular neurons by 3.0 ± 0.6 mV (from -61.9 ± 0.8 mV in ACSF to -58.9 ± 1.0 mV in XE-991; $F_{2,8} = 33.95$, $p < 0.01$; N-K, $p < 0.01$; Figure 3.6A_2), and led to decreases in the amplitude of both the fast (from 8.9 ± 0.9 mV in ACSF to 4.1 ± 0.6 mV in XE-991; $F_{2,8} = 33.58$, $p < 0.01$; N-K, $p < 0.01$; Figure 3.6B_2), and medium-duration AHPs (from 6.2 ±

0.7 mV in ACSF to 4.6 ± 0.8 mV in XE-991; $F_{2,8}$ = 4.51, p < 0.05; N-K, p < 0.05) (see also (Storm, 1989; Yoshida & Alonso, 2007). However, additional application of CCh resulted in a further significant depolarization of 3.6 ± 0.8 mV (to -55.3 ± 1.5 mV; N-K, p < 0.01). While XE-991 alone did not alter spike amplitude, the addition of CCh resulted in a reduction of spike amplitude (from 125.5 ± 6.1 mV in XE-991 to 115.4 ± 4.9 mV in CCh; $F_{2,8}$ = 6.16, p < 0.05; N-K, p < 0.05; Figure 3.6B$_3$), indicating that effects of CCh on conductances that mediate action potentials are not due to attenuation of I_M. Therefore, attenuation of I_M cannot fully account for either the depolarization of membrane potential or the effects of CCh on action potential amplitude in parasubicular neurons.

To determine the ionic conductances modulated by cholinergic receptor activation, voltage clamp experiments were conducted in the presence of TTX (0.5 μM) and ZD7288 (50 μM) to block sodium channels and the hyperpolarization-activated mixed cationic current I_h, respectively. Application of ZD7288 and TTX led to an increase in steady-state input resistance (from 79.8 ± 11.4 MΩ in ACSF to 107.9 ± 23.5 MΩ; $F_{3,12}$ = 6.47, p < 0.01; N-K, p < 0.05), and completely abolished inward rectification (from 1.18 ± 0.05 in ACSF to 1.00 ± 0.00 in TTX and ZD7288; $F_{3,12}$ = 7.95, p < 0.01; N-K, p < 0.01). Consistent with the depolarization observed in current clamp experiments, bath application of CCh (50 μM) resulted in an inward current at a holding potential of -60 mV (-40.5 ± 12.1 pA; $F_{3,12}$ = 8.19, p < 0.01; N-K, p < 0.05; Figure 3.7A). The inward current was not associated with a significant increase in the input resistance measured in

response to -60 mV voltage step (from 107.9 ± 23.5 MΩ in ACSF to 119.0 ± 25.9 MΩ in CCh; n.s., $p = 0.12$).

To determine the identity of the currents responsible for cholinergic depolarization in layer II parasubicular neurons, slow voltage ramps were performed from -120 mV to -40 mV (20 mV/s) (Figure 3.7B_1). Digital current subtractions were constructed by subtracting current traces in response to voltage ramps before and after bath application of CCh, and linear regressions were fit to the data between -120 mV and -70 mV to calculate the reversal potential. The CCh-induced current was outward at potentials lower than about -85 mV, and reversed to an inward current at more positive voltages, suggesting that CCh blocked a potassium conductance (Figure 3.7B_2). The CCh-induced current reversed at -83.3 ± 7.0 mV ($n = 5$), and showed a peak inward current at depolarized voltages at -54.4 ± 5.6 mV. Further, the current elicited by CCh showed some rectification at voltages more positive than E_K, suggesting that CCh blocks an inward rectifying potassium current.

It is known that carbachol potently inhibits KCNQ2/3 channels that mediate the M-current, and we also observed that blockade of the M-current with XE-991 depolarized layer II parasubicular neurons (see Figure 3.6), but a further test was conducted to test if CCh acts at least in part in parasubicular neurons through actions on I_M. Slices were treated with CCh (50 μM) for 10-15 min prior to the addition of XE-991 (10 μM) to the bath during perfusion to determine if CCh occludes the effect of XE-991. Although XE-991 did elicit a small inward current in addition to the current induced by CCh, this current was not significant

(-13.3 ± 5.8 pA at -60 mV; N-K, p = 0.32; Figure 3.7A,B). The occlusion of the effects of XE-991 by CCh suggests that the CCh-induced depolarization of layer II parasubicular neurons is mediated at least in part via blockade of I_M.

Carbachol is known to act on K^+ conductances other than I_M, including I_{Kleak} and I_{Kir} currents (Benardo & Prince, 1982b; Carr & Surmeier, 2007; Halliwell & Adams, 1982; Klink & Alonso, 1997a; Shalinsky et al., 2002; Shen et al., 2005; Shen et al., 2007). To determine if CCh acts through the blockade of a K^+ conductance in addition to I_M, voltage ramps were performed during bath perfusion of ACSF containing TTX (0.5 μM), ZD7288 (50 μM), and XE-991 (10 μM) prior to and during application of CCh (50 μM). Application of XE-991 in the presence of TTX and ZD7288 resulted in a significant inward current at a holding potential of -60 mV (-18.1 ± 6.6 pA; $F_{2,16}$ = 18.84, p < 0.01; N-K, p = 0.05; n = 9; Figure 3.8A), and this inward current peaked at -52.0 ± 4.6 mV. Moreover, the current induced by XE-991 reversed at -78.0 ± 3.2 mV, indicating that XE-991 leads to an inward current at holding potentials near rest via a blockade of an outward K^+ conductance (Figure 3.8B). In addition, the subsequent application of CCh resulted in an additional inward current at holding potential of -60 mV (-20.4 ± 5.6 pA; N-K, p < 0.05). The current reversed at -85.3 ± 1.1 mV and showed rectification at voltages positive to E_K, suggesting that the inward current is due to attenuation of an inward rectifying K^+ conductance (Figure 3.8B). Taken together, these data indicate that CCh depolarizes parasubicular neurons through effects on I_M as well as an additional K^+ conductance.

Carbachol induces a Ba^{2+}-sensitive potassium conductance. To help verify if the additional depolarizing current induced by carbachol in the presence of XE-991 is dependent on K^+, voltage ramps were used to determine if CCh would induce a depolarizing current in the presence of Ba^{2+} (200 μM; Figure 3.9A,B). Barium is a wide-acting K^+ channel blocker that attenuates the voltage-independent I_{leak} and the voltage-sensitive I_{Kir}, and has been shown to greatly attenuate cholinergic depolarization in hippocampal neurons (Benson et al., 1988). In the presence of TTX (0.5 μM), ZD7288 (50 μM), and XE-991 (10 μM), to block Na^+, I_h, and I_M currents, respectively, additional bath application of Ba^{2+} (200 μM) increased input resistance in parasubicular neurons, and induced a large inward current at a holding potential of -60 mV (-80.1 ± 37.6 pA; $F_{2,8} = 5.46$, $p < 0.05$; N-K, $p < 0.05$; $n = 5$; Figure 3.9A). Comparison of responses to voltage ramps recorded before and after addition of Ba^{2+} also showed that Ba^{2+} induced a current that reversed at -80.2 ± 1.7 mV, consistent with a block of K^+ conductances. Subsequent application of CCh (50 μM) failed to induce any significant additional current (-11.1 ± 5.3 pA at -60 mV; N-K, $p = 0.72$), and the occlusion of CCh-induced currents by Ba^{2+} is consistent with the idea that CCh depolarizes parasubicular neurons via attenuation of both I_M and an additional K^+ current (Figure 3.9B).

Carbachol blocks an outward K^+ conductance. The reversal potential of the non-I_M CCh-induced inward current was close to the equilibrium potential for the K^+ (-85.3 ± 1.1 mV in 5 mM $[K^+]_O$; Figure 8), and the induction of this current was blocked with the potent K^+ channel blocker Ba^{2+} (Figure 3.9A,B), suggesting

that CCh acts via a blockade of a potassium conductance. To further assess the dependence of the CCh-induced current on K^+, the extracellular concentration of K^+ was altered through addition or subtraction of equimolar substitutions of NaCl in the presence of TTX (0.5 μM), ZD7288 (50 μM), and XE-991 (10 μM). When $[K^+]_O$ was reduced to 3 mM, the reversal potential of the CCh-induced current was shifted to -98.2 ± 6.1 mV (*n* = 4), and raising the $[K^+]_O$ concentration to 7 mM (*n* = 10) and 10 mM (*n* = 6) shifted the reversal potential to -73.8 ± 1.3 mV and -56.8 ± 4.2 mV, respectively. By plotting $[K^+]_O$ concentrations logarithmically, the reversal potential of the CCh-induced current observed in layer II parasubicular neurons shows a linear fit, indicating that the CCh-induced current is likely due to inhibition of a potassium conductance (Ma et al., 2007) (Figure 3.9C).

DISCUSSION

The present study investigated the effects of cholinergic receptor activation on layer II cells of the parasubiculum. Bath application of carbachol resulted in a prominent slow membrane depolarization in ~80% of cells, and was observed in both morphologically-identified stellate and pyramidal neurons. The depolarizing effects of CCh persisted in the presence the of M_2 antagonist methoctramine, as well as in the presence of blockers of synaptic transmission, but were blocked by the nonselective muscarinic receptor antagonist atropine as well as by the M_1 receptor antagonist pirenzepine. The cholinergic depolarization of layer II parasubicular neurons is therefore not due to changes local synaptic activity, but is dependent on activation of M_1 receptors. Cholinergic receptor

activation led to reductions in the amplitude of fast and medium AHPs, as well as increases in action potential duration and decreases in spike amplitude, indicating that acetylcholine modulates conductances associated with action potential generation and repolarization. Voltage-clamp recordings showed that muscarinic depolarization in layer II parasubicular neurons is mediated through inhibition of the muscarinic-dependent current I_M as well as an additional K^+ current. Moreover, the CCh-induced inhibition of the additional K^+ current displayed a voltage-current profile similar to currents carried through Kir2.3 channels observed during muscarinic receptor stimulation in pyramidal neurons in the prefrontal cortex, striatal neurons, sympathetic neurons, as well as mammalian cell lines (Carr & Surmeier, 2007; Rossignol & Jones, 2006; Shen et al., 2007; Uchimura & North, 1990; H. S. Wang & McKinnon, 1996). The mechanisms of the cholinergic depolarization in parasubicular neurons therefore contrasts with cholinergic depolarization in layer II cells of the medial entorhinal cortex which is primarily due to activation of the Ca^{2+}-modulated nonspecific cationic conductance (I_{NCM}) (Klink & Alonso, 1997a; Shalinsky et al., 2002). The cholinergic depolarization of parasubicular neurons may contribute to the genesis of theta-frequency population activity in the parasubiculum by depolarizing neurons to subthreshold voltage ranges at which parasubicular neurons generate intrinsic voltage-dependent, theta-frequency membrane potential oscillations (Glasgow & Chapman, 2007, 2008). Therefore, the cholinergically-mediated depolarization of superficial parasubicular neurons may be an important mechanism that contributes to theta-frequency synchronization, and this may

play an important role in spatial navigation and memory processes associated with the parahippocampal region.

Layer II parasubicular neurons show electrophysiological homogeneity despite morphological differences. Although few studies have examined the electrophysiological correlates of different cell morphologies in the parasubiculum, the results here are in general accordance with previous reports (Funahashi & Stewart, 1997a, 1997b; Menendez de la Prida, Suarez, & Pozo, 2003). Both stellate and pyramidal-like cells were recorded in the superficial layers of the parasubiculum, and in contrast to marked electrophysiological differences between morphological phenotypes found in layer II of the medial entorhinal cortex (Alonso & Llinas, 1989; Klink & Alonso, 1993), few differences in electroresponsiveness were observed between cell types. Here, both cell types showed prominent theta-frequency membrane potential oscillations at subthreshold voltages, indicating that the emergence of oscillatory activity is not specific to one cell type, although the amplitude of oscillations was larger in pyramidal neurons than in stellate neurons. Layer II entorhinal stellate-like neurons are characterized by prominent I_h-dependent inward rectification, non-linear voltage-current relationships, and the presence of subthreshold membrane potential oscillations, whereas nonstellate pyramidal-like neurons display moderate levels of inward rectification and do not show membrane potential oscillations (Alonso & Klink, 1993). In the parasubiculum, however, both cell types show cholinergic depolarization as well as intrinsic theta-frequency

membrane potential oscillations that may contribute to the generation of local field activity (Glasgow & Chapman, 2007).

The parasubicular neurons described here share remarkable similarity with the electrophysiological properties of layer II pyramidal neurons in the entorhinal cortex, including similar levels of inward rectification, input resistances, and spike properties. However, parasubicular neurons can be differentiated from entorhinal pyramidal neurons based on cholinergic responsivity (Klink & Alonso, 1993, 1997b). Both stellate and pyramidal entorhinal neurons respond to cholinergic receptor activation with robust depolarization, however CCh is able to switch pyramidal-like cells from tonic firing to burst firing, and leads to the emergence of Ca^{2+}-mediated low frequency bursting behavior at depolarized voltages (Klink & Alonso, 1997a, 1997b). Although CCh regularly induced strong depolarization in parasubicular neurons, however, burst spiking was never observed in parasubicular neurons during CCh-induced depolarization or when coupled with additional depolarization with constant current injection. This is a clear difference in cholinergic effects on parasubicular and entorhinal neurons.

Stellate and pyramidal neurons in layer II of the parasubiculum share similar electrophysiological properties, and can be delineated from deep layer parasubicular neurons through a number of differences in electroresponsiveness. Deep layer neurons do not show any “sag” response to hyperpolarizing current pulses, and only stellate-like layer V neurons show significant anodal break potentials, whereas superficial layer neurons show both moderate levels of rectification and anodal break potentials (Funahashi & Stewart, 1997a).

Superficial neurons in the presubiculum also differ from those in the parasubiculum in that they are typically characterized by high input resistances, and lack sag response (Fricker, Dinocourt, Eugene, Wood, & Miles, 2009). The similarity of the passive electrical properties of parasubicular layer II stellate and pyramidal neurons, and in their similar responsivity to cholinergic receptor activation suggest that these cell types may play similar roles in the function of the parasubiculum (Funahashi & Stewart, 1997a, 1997b).

Cholinergic modulation of layer II parasubicular neurons is dependent on muscarinic receptors. Five major muscarinic receptor subtypes have been identified in the mammalian central nervous system, and can be categorized into two major families based on intracellular signaling mechanisms and effects on both pre- and postsynaptic domains (Caulfield & Birdsall, 1998). M_1-like receptors, which include M_1, M_3, and M_5, are linked to the α subunits of the G_q/G_{11} signaling cascade, and typically have excitatory effects including membrane potential depolarization. Activation of the G_q/G_{11} pathway leads to activation of phospholipase C (PLC), which in turn hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP_2), and reductions in levels of membrane-bound PIP_2 can lead to inhibition of a number of ion channels through inositol triphosphate (IP_3) and diacylglycerol (DAG) pathways (Brown, Hughes, Marsh, & Tinker, 2007; Sohn et al., 2007; Taylor, Berridge, Cooke, & Potter, 1989; Yuan, Adams, Swank, Sweatt, & Johnston, 2002; Zolles et al., 2006). In contrast, M_2-family receptors, which include M_2 and M_4, typically lead to hyperpolarizing effects on CNS neurons via activation of pertussis toxin-sensitive

G_i or G_O α subunits, which in turn lead to an inhibition of adenylnyl cyclase and subsequent activation of G-protein coupled inward rectifying K^+ channels (GIRKs) (Seeger & Alzheimer, 2001). Autoradiographic studies in both rats and nonhuman primates have demonstrated moderate binding densities of both M_1 and M_2 receptors in the superficial layers of the parasubiculum (Mash & Potter, 1986; Mash, White, & Mesulam, 1988). In the present study, cholinergic depolarization was blocked by bath application of the M_1 receptor antagonist pirenzepine but not by the M_2 antagonist methoctramine. Affinity profiles of muscarinic receptor antagonists have shown that pirenzepine binds to M_1 receptors with up to 35-fold higher affinity than to $M_{2\text{-}5}$, but that it does have moderate affinity for M_4 subtypes (Dorje et al., 1991) so that it is possible that membrane depolarization could be due to stimulation of the M_4 receptor subtype. However, the M_2-preferring antagonist methoctramine is known to have strong additional effects on M_4 receptors (Dorje et al., 1991), but we observed strong membrane potential depolarization in the presence of methoctramine, suggesting that M_4 receptors are not necessary for muscarinic depolarization of membrane potential in layer II parasubicular neurons. The effects of cholinergic stimulation on parasubicular neurons are therefore primarily, if not exclusively, mediated via actions on M_1 receptors.

Cholinergic modulation of ion channels associated with passive cell properties. Although initial reports of cholinergic actions on principal cells in the hippocampus failed to observe consistent effects on the action potential waveform (Benardo & Prince, 1982a; McCormick & Prince, 1986), more recent

studies in the entorhinal cortex and the hippocampus have reported that cholinergic receptor activation is associated with pronounced alterations of action potential shape and duration (Figenschou et al., 1996; Klink & Alonso, 1997b). Muscarinic receptor activation can strongly suppress conductances associated with action potential generation and repolarization, including spike-dependent Ca^{2+} influx through voltage-gated calcium channels (VGCCs), and K^+ channels involved in repolarization such as I_M, and I_K (Mathie, Bernheim, & Hille, 1992; Segal, 1982; Shapiro et al., 2001). In parasubicular neurons, carbachol led to a significant reduction in the amplitude of both fast and medium AHPs, and this was accompanied by a nonsignificant increase in action potential duration (Figure 3.3) (Storm, 1987, 1989). The amplitude of both fast and medium AHPs in both entorhinal and parasubicular neurons are sensitive to blockade of I_M (Chapter 2) (Yoshida & Alonso, 2007), and carbachol acts, in part, through the inhibition of I_M (Madison et al., 1987). Reductions in fAHP amplitude may be due to a cholinergic enhancement of spike afterdepolarizations (ADPs), which are enhanced by Ca^{2+}-mediated inhibition of I_M, and this suggests that cholinergic suppression of I_M may play an important role in modulating spike frequency adaptation (Yoshida & Alonso, 2007; Yue & Yaari, 2004). Functionally, inhibition of I_M and associated reductions in the amplitude of fast and medium AHPs may enhance the amplitude of afterdepolarizations (ADP) and increase repetitive firing. Changes in the number of spikes evoked by steps of positive current were not observed here, but it is possible that changes in AHPs induced by carbachol

may have contributed to the repetitive firing observed here during application of carbachol (Figure 3.2).

Fast AHPs in parasubicular neurons are also reduced during Ca^{2+} channel blockade and in Ca^{2+}-free ACSF indicating that fast AHPs are dependent, in part, on Ca^{2+} entry (Chapter 2), and cholinergic agonists are known to reduce Ca^{2+} currents (Allen & Brown, 1993; Gahwiler & Brown, 1987; Howe & Surmeier, 1995; Klink & Alonso, 1997b). Many of the effects of carbachol are mediated via actions on the M_1 receptor subtype, and M_1 activation can lead to activation of PLCß that is linked to direct and indirect inhibition of high-voltage-activated N- and L-type Ca^{2+} channels (Allen & Brown, 1993; Bernheim, Mathie, & Hille, 1992; Day, Olson, Platzer, Striessnig, & Surmeier, 2002; Howe & Surmeier, 1995; L. Liu et al., 2008; Mathie et al., 1992; Qian & Saggau, 1997). Carbachol may therefore result in an M_1- and PLCß-mediated reduction in spike-evoked Ca^{2+} currents, resulting in spike broadening and attenuation of fast AHPs in layer II parasubicular neurons. Muscarinic receptor activation has also been associated with reductions in fast Ca^{2+}-dependent K^+ currents (Figenschou et al., 1996), and this may contribute to the attenuation of the fAHP observed here.

Muscarinic M_1 receptor activation can modulate hyperpolarization-activated cyclic nucleotide-regulated (HCN) channels that carry the hyperpolarization-activated nonselective cationic current I_h through activation of PLCß (Pian, Bucchi, Decostanzo, Robinson, & Siegelbaum, 2007). In layer II parasubicular neurons, high concentrations of CCh (50 µM) lead to an attenuation of the rectification ratio. However, the rectification ratio of layer II

parasubicular neurons was stable over the course of experiments in which CCh was not applied, indicating that the attenuation was not due to dialyzation of intracellular fluid and a rundown of intracellular signaling products that regulate I_h (Zhou & Lipsius, 1993). Muscarinic activation is also associated with reductions in the rectification ratio, as well as the frequency and strength of membrane potential resonance, in layer II stellate neurons of the entorhinal cortex (Heys et al., 2010). In *Xenopus* oocytes expressing both M_1 receptors and HCN channels, activation of M_1 receptors leads to a positive shift in HCN voltage gating and a slowing of the deactivation kinetics of I_h through a PLCß-mediated mechanism (Pian et al., 2007; Pian, Bucchi, Robinson, & Siegelbaum, 2006), and similar mechanisms could contribute to the reduction in the sag responses in parasubicular neurons.

Interestingly, bath application of carbachol consistently reduced the amplitude of action potentials, however application of XE-991 did not cause a similar reduction, indicating that cholinergic inhibition of I_M does not contribute to decreases in spike amplitude. Muscarinic receptor activation has been linked to reductions in Na^+ currents (Mittmann & Alzheimer, 1998), and M_1 activation of the G_q-coupled intracellular signaling pathway results in mobilization of intracellular Ca^{2+} stores through IP_3. Increases in intracellular Ca^{2+} have been linked to reduction in the number of available voltage-gated Na^+ channels (Bulatko & Greeff, 1995). Additionally, increases in intracellular Ca^{2+} result in elevated levels of protein kinase C (PKC), and PKC is known to phosphorylate voltage-gated Na+ channels (Cantrell, Ma, Scheuer, & Catterall, 1996; Catterall,

2000). Thus, the CCh-induced reduction in spike amplitude in parasubicular neurons may result from an M_1 receptor-dependent increase in Ca^{2+} and PKC resulting in a reduction in the number of voltage-gated Na^+ channels available during the action potential.

Conductances mediating cholinergic depolarization of layer II parasubicular neurons. Acetylcholine and other neurotransmitters can modulate a variety of K^+ conductances (for review, see (Storm, 1990). M_1 receptor stimulation is known to suppress several K^+ conductances including I_M, I_{leak}, I_{AHP}, $I_{K(Ca)}$ and I_{Kir}, and can also lead to activation of a Ca^{2+}-modulated nonspecific cationic current (Benardo & Prince, 1982a; Benson et al., 1988; Carr & Surmeier, 2007; Haj-Dahmane & Andrade, 1996; Halliwell & Adams, 1982; Klink & Alonso, 1997a; Madison et al., 1987; McCormick & Prince, 1986; Shalinsky et al., 2002; Uchimura & North, 1990). Similarly, the present study indicates that muscarinic depolarization in parasubicular neurons is primarily dependent on the inhibition of two K^+ conductances, including suppression of the voltage-dependent K^+ current I_M.

Inhibition of I_M has been implicated in the muscarinic depolarization of numerous cell types. The M-current is a low threshold, non-inactivating, voltage-gated K^+ conductance composed of Kv7 (KCNQ; primarily Kv7.2/3) subunits that was first described in frog sympathetic neurons, and has since been identified in a variety of central neurons (Brown & Adams, 1980; Marrion, 1997; Shapiro et al., 2000; H. S. Wang et al., 1998). The M-current shows prominent time- and voltage-dependence, and is primarily activated at near threshold potentials, and it

therefore provides a powerful mechanism that can modulate neuronal excitability (Delmas & Brown, 2005). Moreover, I_M is inhibited through activation of $G_{q/11}$-coupled neurotransmitters such as acetylcholine, substance P, and some peptides (Brown & Passmore, 2009). Muscarinic agonists stimulate phosphoinositide (PI) turnover (Dutar & Nicoll, 1988), leading to depletion of membrane-bound PIP_2 which directly modulates the open probability of KCNQ channels. The M-current is restored through application of PIP_2 to the inner domain of the plasma membrane, or through resynthesis of PIP_2 by PI4 kinases, as inhibition of PI4 kinases with wortmannin blocks membrane repolarization after muscarinic receptor stimulation (Brown et al., 2007; Li, Gamper, Hilgemann, & Shapiro, 2005; Suh & Hille, 2002; Zhang et al., 2003). The carbachol-induced depolarization of layer II parasubicular neurons may therefore be largely due to reductions in membrane-bound PIP_2, leading to inhibition of the M-current. Application of XE-991 to block the M-current led to depolarization in layer II parasubicular neurons, indicating that parasubicular neurons express KCNQ channels, and that, similar to other neurons, some proportion of KCNQ channels are open at resting membrane potential (Delmas & Brown, 2005). In addition, the inward current induced by XE-991 was occluded by prior application of CCh, indicating that the depolarization induced by CCh is mediated in part by inhibition of I_M (Figure 3.7). When CCh was applied *following* application of XE-991, however, CCh induced an additional depolarization, suggesting that the carbachol-induced depolarization is mediated both by I_M as well as additional conductance. It is unlikely that this result is due to an incomplete block of I_M by

XE-991, because XE-991 evokes a maximal effect at submicromolar concentrations (~0.5 μM) in hippocampal slices, and the concentration used in the current studies (10 μM) should therefore result in a complete block of I_M (Zaczek et al., 1998). Subtractions of currents associated with voltage-ramp protocols were therefore used to identify the additional inward current observed in response to CCh during blockade of I_M. The current was blocked by the K^+ channel blocker Ba^{2+}, and the reversal potential of the carbachol-induced current reversed at -84.7 mV with 5 mM K^+ in the ACSF (near the equilibrium potential for K^+ as predicted by the Nernst equation; -85.3 mV), and the reversal potential of the current shifted consistently as a function of the extracellular K^+ concentration to potentials close to the predicted equilibrium potentials for K^+ (Figure 3.9). The current is therefore attributable to a K^+ conductance, and carbachol therefore affects one or more K^+ conductances in addition to I_M.

The second K^+ conductance is likely to be mediated by inward rectifying K^+ channels (Kir2) which are open at resting potentials and contribute to the regulation of resting membrane potential and integration of synaptic inputs (Stanfield, Nakajima, & Nakajima, 2002). Recent work in invertebrate and vertebrate model systems have demonstrated that Kir2 can be inhibited via M_1 receptor stimulation, and the M_1-mediated depolarization observed here may be due in part through inhibition of Kir2 (Carr & Surmeier, 2007; Chuang, Jan, & Jan, 1997; Du et al., 2004). The current-voltage relationship of the carbachol-induced current in the presence of XE-991 during blockade of I_h and I_{Na} resembled previously characterized I_{Kir} currents (Day et al., 2005), and the

current elicited by carbachol described here typically showed moderate rectification at potentials above E_K. In situ hybridization has also demonstrated that the parasubiculum shows moderate levels of IRK3 mRNA that expresses the Kir2.3 channel protein (Karschin, Dissmann, Stuhmer, & Karschin, 1996). Further, bath application of 200 μM Ba^{2+} completely abolished the carbachol-induced current, and Kir2 channels are effectively blocked by 200 μM Ba^{2+} (Day et al., 2005). Our findings are also consistent with previous reports of muscarinic modulation of Kir2 channels (Carr & Surmeier, 2007; Hsu et al., 1996; Shen et al., 2007; Uchimura & North, 1990). Similar to muscarinic inhibition of the M-current, the open probability of Kir2 channels is gated through binding of PIP_2, and muscarinic depletion of PIP_2 results in the closure of Kir2 channels (Carr & Surmeier, 2007; Stanfield et al., 2002). Therefore, it is likely closure of Kir2 channels contributes to muscarinically-mediated depolarization in layer II parasubicular neurons.

Muscarinic depolarization of other central pyramidal neurons has been attributed to activation of a Ca^{2+}-modulated nonselective cation current (Fisahn et al., 2002; Haj-Dahmane & Andrade, 1996; Shalinsky et al., 2002) and muscarinic receptor activation leads to depolarization in layer II entorhinal neurons that is dependent on activation of a cation channel and the long-lasting inhibition of a K^+ conductance (Klink & Alonso, 1997a; Shalinsky et al., 2002). Interestingly, we observed no evidence of activation of a nonselective cationic current by CCh in layer II parasubicular neurons, indicating that the mechanisms underlying cholinergic depolarization differ between entorhinal and parasubicular neurons.

Functional implications. We have provided evidence that muscarinic receptor stimulation alters both active and passive electrophysiological properties of morphologically-identified layer II parasubicular neurons, and that these effects are mediated via actions on the M_1 receptor subtype. Activation of M_1 receptors leads to a sustained depolarization of resting membrane potential due to inhibition of the muscarinically-sensitive K^+ current I_M as well as inhibition of an inward rectifying K^+ conductance, likely mediated by Kir2 channels. These mechanisms contrast with the depolarizing effects of CCh in layer II of the medial entorhinal cortex which are mediated by M_1 receptor activation of the mixed cationic channel I_{NCM} and long-lasting inhibition of a K+ current (Klink & Alonso, 1997a; Shalinsky et al., 2002).

Parasubicular neurons display intrinsic voltage-dependent theta frequency membrane potential oscillations that are driven through interplay between I_{NaP} and I_h, and these intrinsic oscillations are believed to contribute to local field activity (Glasgow & Chapman, 2007, 2008). However, these rhythmic fluctuations in membrane potential are not typically expressed at resting voltages, and principal cells require positive current injection to depolarize neurons to the subthreshold voltage range where they express the oscillations (Chapter 2). Septal cholinergic projections, which are active during theta-related behavioral states, are important in the generation of network oscillations in the hippocampal formation (Bland, 1986; Buzsaki, 2002), and we have previously reported that layer II of the parasubiculum generates theta-frequency EEG activity that is dependent on cholinergic mechanisms (Glasgow & Chapman,

2007). Therefore, increased cholinergic input from the medial septum during theta states likely serves to depolarize parasubicular neurons to subthreshold voltages, and promote the emergence of membrane potential oscillations *in vivo* (Alonso & Kohler, 1984; Alonso & Llinas, 1989; Bland et al., 2002).

Theta-frequency EEG activity in the hippocampal formation is important for temporal integration of synaptic inputs, and is thought to be generated via rhythmic perisomatic inhibition and synaptic excitation of the distal dendrites (Buzsaki, 2002). Parasubicular neurons are under the control of local inhibitory synaptic inputs from a large number of parvalbumin (PV)-, glutamic acid decarboxylase (GAD)-, and GABA-immunoreactive neurons (Boccara et al., 2010; Funahashi & Stewart, 1998; Kohler et al., 1985). No putative interneurons were reported in the present study, but we have previously recorded voltage-dependent membrane potential oscillations in putative parasubicular interneurons (Chapman & Lacaille, 1999a; Glasgow & Chapman, 2007) and it is likely that muscarinic depolarization may lead to oscillations in local interneurons as well. Hippocampal interneurons show theta-frequency rhythmic spiking resulting in phasic inhibition of principal cells, and this can synchronize the output of principal cells (Klausberger et al., 2003; Klausberger et al., 2004) via the induction of hyperpolarization-activated rebound potentials that can drive cell firing (Chapman & Lacaille, 1999a; Cobb et al., 1995). This mechanism may also synchronize network activity within the parasubiculum, and set the timing of the parasubicular output to the entorhinal cortex.

The superficial layers of the parasubiculum contain a high percentage of cells that fire in relation to local theta activity, and this suggests that network synchronization may be important in regulating the manner in which parasubicular neurons contribute to the computational processing in the entorhinal cortex (Boccara et al., 2010; Cacucci et al., 2004; Taube, 1995b). Further, theta-modulated grid and border cells have recently been reported in both the pre- and parasubiculum (Boccara et al., 2010; Solstad et al., 2008). The hippocampal CA1 region and the anterior thalamus, which contain place and head direction cells that encode contextualized spatial and directional information, respectively, send strong efferents to the parasubiculum (Shibata, 1993; Swanson & Cowan, 1977; van Groen & Wyss, 1990a, 1990b), and many parasubicular neurons fire in relation to both location and head direction ("place-by-direction" cells; Cacucci et al., 2004; Boccara et al., 2010). This suggests that the parasubiculum may serve as an integrator of two complementary spatial signals from the CA1 and anterior thalamus, and further, that parasubicular theta activity may modulate the integration of these synaptic inputs and the timing of the output of the parasubiculum to the entorhinal cortex.

In contrast to the primarily excitatory effects reported here, however, cholinergic receptor activation can also lead to a suppression of excitatory synaptic transmission in the parasubiculum (Glasgow, S. D., Glovaci, I., Karpowicz, L. S., & Chapman, C. A., unpublished observations, see Chapter 4). Increases in cholinergic tone may therefore suppress extrinsic synaptic inputs during theta-related behaviors (Hamam et al., 2007; Hasselmo & Schnell, 1994).

The depolarization of parasubicular neurons coupled with the suppression of synaptic responses may represent a mechanism underlying the generation and maintenance of the spacing of grid cell fields. Recent computational models have suggested that subthreshold membrane potential oscillations encode velocity and grid cell spacing by accentuating discrepancies in frequency between distal dendritic and somatic oscillations to provide a framework that can determine the firing of the cell (Burgess et al., 2007; Hasselmo, Giocomo, & Zilli, 2007). Muscarinic receptor stimulation slows the frequency of membrane potential oscillations in layer II entorhinal cortex cells (Klink & Alonso, 1997b) and the model of interference of theta-frequency membrane potential oscillations suggests that the resulting increase in dendrosomatic differences in oscillatory frequency may increase the spatial resolution of grid cell fields by reducing the spacing between adjacent fields (Burgess, 2008a; Burgess et al., 2007; Hasselmo et al., 2007). Presynaptic inhibition of synaptic transmission may reduce the level of dendritic excitation, and prevent aberrant cell firing evoked by weaker inputs. Therefore, in conjunction with cholinergic suppression of excitatory synaptic transmission, cholinergic depolarization of layer II parasubicular neurons may enhance dendrosomatic interference of theta-frequency membrane potential oscillations, and this mechanism may contribute to the development of more finely-tuned spatial representations in the parahippocampal region during exploration of novel environments.

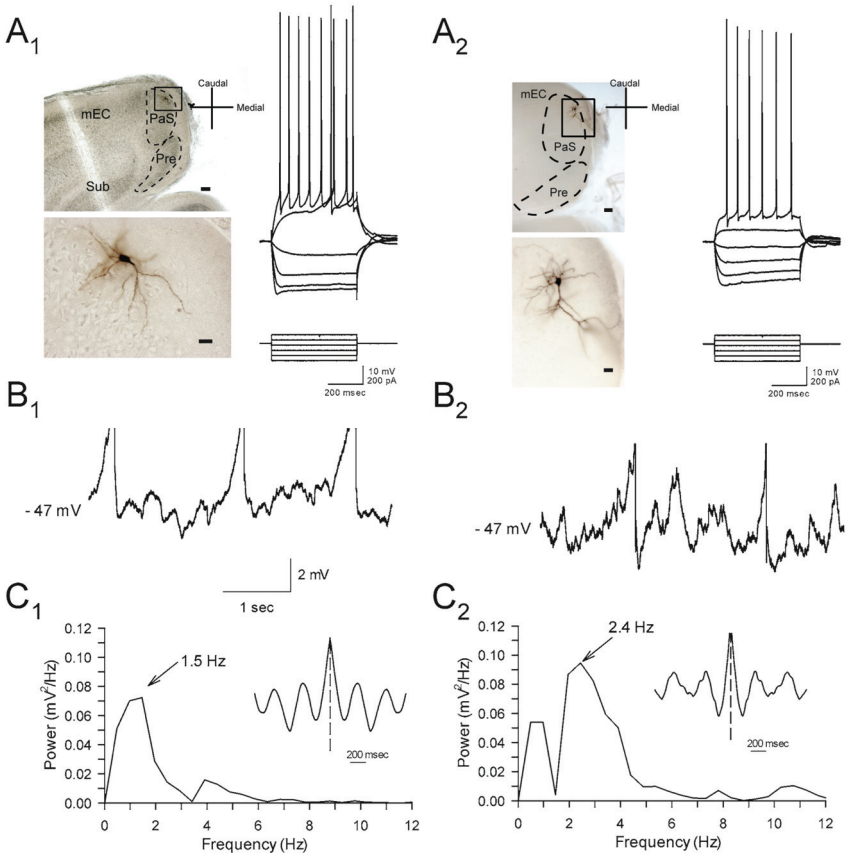
A1
mEC
PaS
Pre
Sub
Caudal
Medial
10 mV
200 pA
200 msec
A2
mEC
PaS
Pre
Caudal
Medial
10 mV
200 pA
200 msec
B1
- 47 mV
2 mV
1 sec
B2
- 47 mV
C1
1.5 Hz
200 msec
Power (mV²/Hz)
Frequency (Hz)
C2
2.4 Hz
200 msec
Power (mV²/Hz)
Frequency (Hz)

Figure 3.1. Layer II parasubicular stellate and pyramidal neurons show similar electrophysiological properties. **A**. Photomicrographs of representative layer II parasubicular stellate cell (A_1) and pyramidal cell (A_2) filled with biocytin show cell bodies located near the border of layer I and II of the parasubiculum (PaS; top panel). Stellate cells show multiple dendrites extending from the cell soma that bifurcate into several major branches. Pyramidal neurons have multiple basal dendrites and typically one primary apical dendrite extending towards layer I. Membrane potential responses to hyperpolarizing and depolarizing current pulses for the cells shown in photomicrographs show similar electrophysiological responses between stellate and pyramidal neurons (A_1 and A_2, right panels). Calibration bars (black) are equal to 100 μm in top panels, and 20 μm in bottom panels. **B**. Both stellate (B_1) and pyramidal (B_2) layer II parasubicular neurons display theta-frequency membrane potential oscillations at near-threshold voltages. Whole-cell current clamp recordings in the same cells as shown in A at near-threshold voltages using steady current injection resulted in 2 to 5 Hz oscillations in both stellate (B_1) and pyramidal (B_2) neurons. Note that action potentials are truncated. **C**. Power spectra for the same cells shown in A show theta-frequency membrane potential oscillations in both stellate (C_1) and pyramidal (C_2) neurons. The rhythmicity of oscillations in both cell types is also reflected in the inset autocorrelograms.

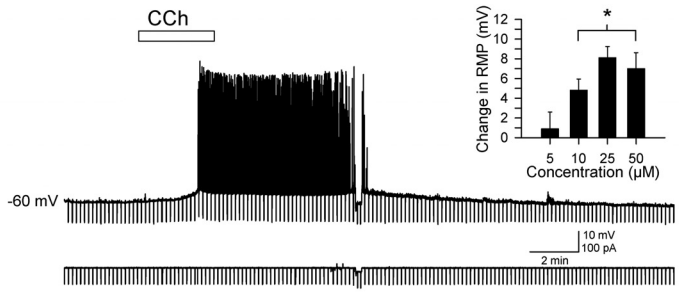
CCh
-60 mV
10 mV
100 pA
2 min
Change in RMP (mV)
0
2
4
6
8
10
12
*
5
10
25
50
Concentration (μM)

Figure 3.2. Carbachol depolarizes parasubicular neurons to threshold voltages. Example of a recording of membrane potential in a parasubicular neuron (same cell as in Figure $1A_1$) in response to a 2 min bath application of 25 μM carbachol (CCh, A_1, white bar). The inset histogram shows the mean level of depolarization induced in response to different concentrations of CCh in separate groups of parasubicular neurons. The lowest concentration of CCh (5 μM) failed to produce a significant depolarization, but higher doses induced significant depolarization of layer II parasubicular neurons (**: $p < 0.01$).

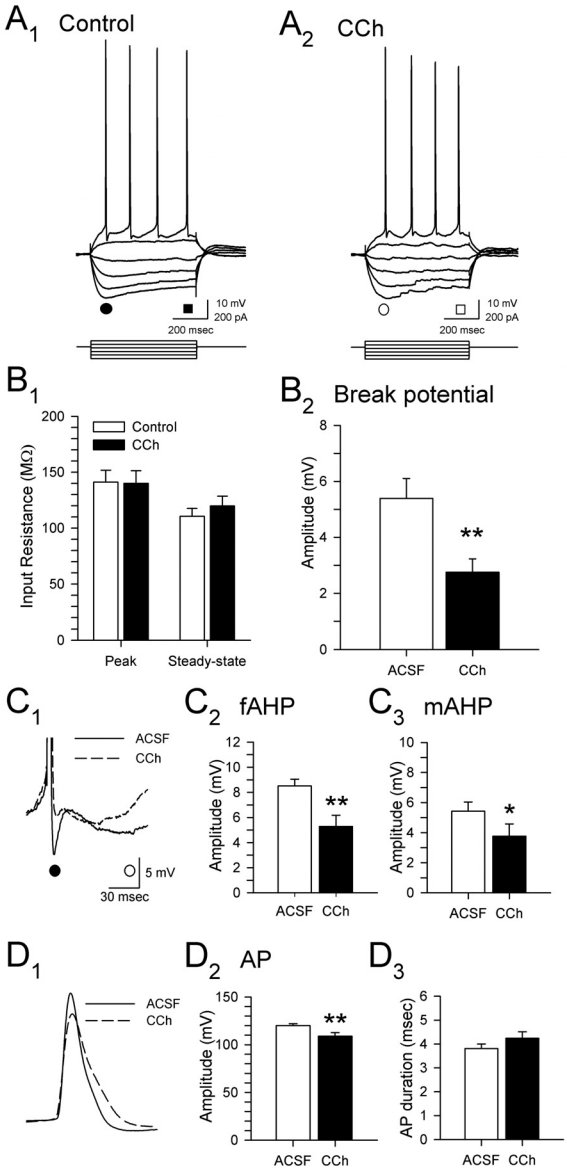
A1 Control
A2 CCh
10 mV
200 pA
200 msec
B1
Input Resistance (MΩ)
Control
CCh
Peak
Steady-state
B2 Break potential
Amplitude (mV)
**
ACSF
CCh
C1
ACSF
CCh
5 mV
30 msec
C2 fAHP
C3 mAHP
Amplitude (mV)
**
*
ACSF
CCh
D1
ACSF
CCh
D2 AP
D3
Amplitude (mV)
AP duration (msec)
**
ACSF
CCh

Figure 3.3. Carbachol has multiple effects on electrophysiological properties of layer II parasubicular neurons. **A**. Membrane voltage responses to hyperpolarizing and depolarizing current pulses in control ACSF (A_1) and 25 μM CCh (A_2). **B**. Group data show that CCh (25 μM) resulted in a slight, non-significant increase in steady-state input resistance (squares in A) during cholinergic receptor activation with CCh compared to control conditions (B_1), but had no effect on peak input resistance (circles in A), and consistently reduced the amplitude of anodal break potentials following -200 pA steps (B_2, **: $p < 0.01$). **C**. Superimposed action potentials (D_1) show a reduction in the amplitude of the fast afterhyperpolarization (D_2; ●, fAHP, **: $p < 0.01$), and a reduction in the amplitude of the medium AHP (C_3; ○, mAHP, *: $p < 0.05$). **D**. Carbachol altered the waveform of the action potential (overlay, C_1), and was associated with a significant reduction in action potential amplitude (C_2; **: $p < 0.01$) and a trend towards increased spike duration (C_3; $p = 0.06$).

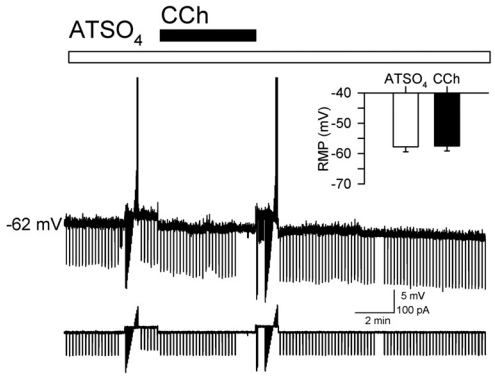
ATSO4
CCh
ATSO4 CCh
-40
-50
-60
-70
RMP (mV)
-62 mV
5 mV
100 pA
2 min

Figure 3.4. Carbachol induces a depolarization of membrane potential in parasubicular neurons that is mediated by activation of muscarinic receptors. **A**. CCh-induced depolarization did not develop when CCh (50 μM; black bar) was coapplied after a 15 min bath application of $ATSO_4$ (1 μM; white bar). Voltage traces of a representative neuron recorded during coapplication of $ATSO_4$ show that CCh failed to induce depolarization. Note current-voltage tests before and after CCh application, and note that action potentials are truncated for clarity in this and subsequent figures. Mean group data of the resting membrane potential in $ATSO_4$ prior to (white bar) and after (black bar) application of CCh demonstrated no significant depolarization, suggesting that the depolarizing effect of CCh is mediated by muscarinic receptor activation (inset).

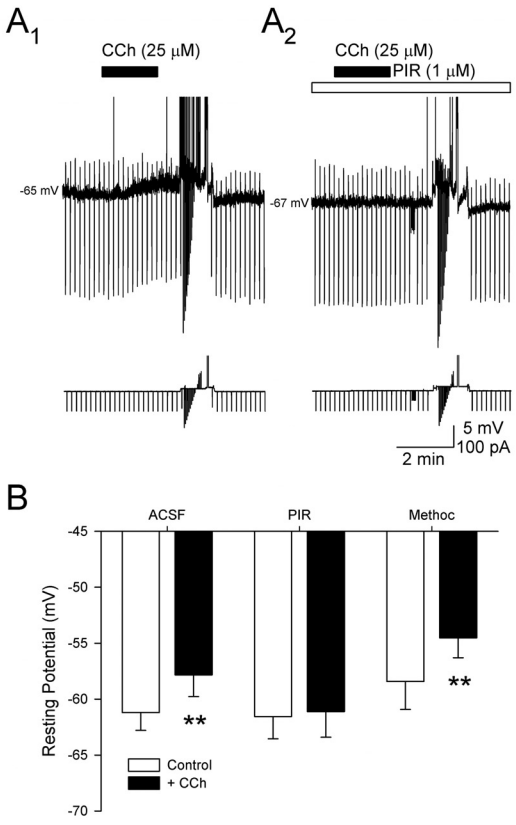
A1
CCh (25 μM)
-65 mV
A2
CCh (25 μM)
PIR (1 μM)
-67 mV
5 mV
100 pA
2 min
B
ACSF
PIR
Methoc
Resting Potential (mV)
-45
-50
-55
-60
-65
-70
**
**
Control
+ CCh

Figure 3.5. Depolarization of layer II parasubicular neurons induced by CCh is mediated by M_1 receptors. **A**. Carbachol-induced depolarization did not develop when CCh (50 μM) was coapplied after a 15 min bath application of the selective M_1 receptor antagonist, pirenzepine (PIR; 1 μM). Voltage traces from a representative parasubicular neuron show that application of CCh alone (black bar) results in a significant depolarization of membrane potential (A_1) that is not observed when CCh is applied in the presence of pirenzepine (A_2; white bar). **B**. Group data showed that the significant depolarization of membrane potential induced by application of CCh (50 μM) was blocked by pirenzepine (PIR). In contrast, the selective M_2 receptor blocker, methoctramine (Methoc; 1 μM) failed to block the CCh-induced depolarization, suggesting that M_2 receptors do not contribute to cholinergic depolarization of layer II parasubicular neurons (**: $p < 0.01$).

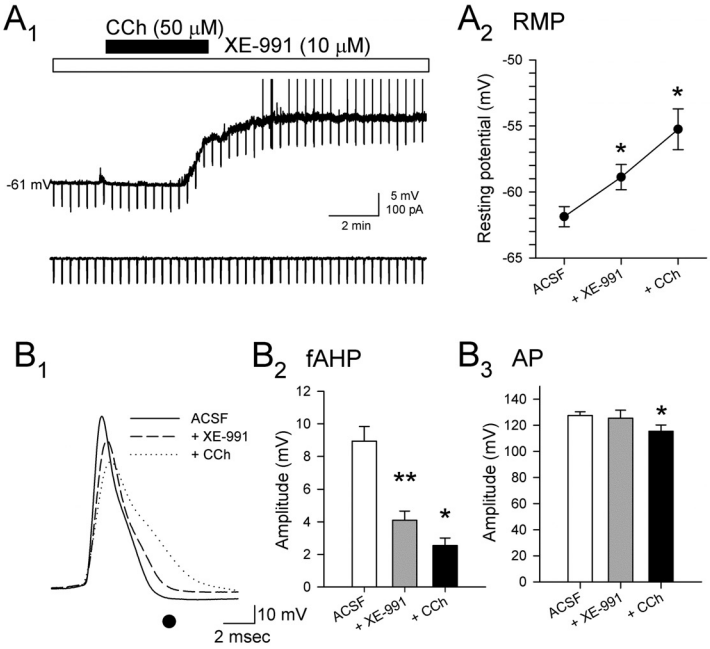
A1
CCh (50 μM)
XE-991 (10 μM)
-61 mV
5 mV
100 pA
2 min
A2 RMP
Resting potential (mV)
-50
-55
-60
-65
ACSF
+ XE-991
+ CCh
B1
ACSF
+ XE-991
+ CCh
10 mV
2 msec
B2 fAHP
Amplitude (mV)
B3 AP
Amplitude (mV)

Figure 3.6. Carbachol-induced depolarization and alterations in spike properties are not mediated solely by inhibition of I_M. **A**. Example of a recording of membrane potential during bath application of the selective Kv7.2/3 antagonist XE-991 (10 μM) and synaptic antagonists (white bar) shows a depolarization. However, further application of CCh (A_1; white bar; 50 μM) resulted in additional depolarization, suggesting that CCh-induced depolarization of parasubicular neurons cannot be fully accounted for by inhibition of I_M. Group data also revealed a significant depolarization induced by CCh (50 μM) during coapplication of XE-991 (10 μM) and synaptic antagonists (A_2; *: $p < 0.05$). **B.** Superimposed voltage traces of action potentials (B_1) demonstrate alterations in the spike waveform during coapplication of XE-991 and CCh (dotted line) compared to XE-991 alone (dashed line), suggesting that CCh-induced conductances other than I_M mediate these changes in parasubicular neurons. Group data show that addition of CCh resulted in additional reductions of amplitude of the fAHP (measured at ● in B_1) compared to XE-991 alone (B_2; *: $p < 0.05$; **: $p < 0.01$). Action potential amplitude (B_3) was not significantly affected by XE-991 (grey bar), but was significantly reduced when CCh was coapplied (black bar; *: $p < 0.05$).

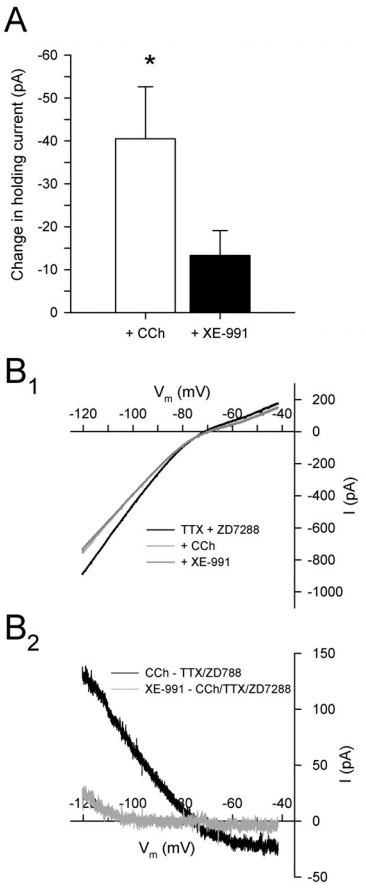
A
Change in holding current (pA)
-60
-50
-40
-30
-20
-10
0
*
+ CCh
+ XE-991
B1
Vm (mV)
-120
-100
-80
-60
-40
200
0
-200
-400
-600
-800
-1000
I (pA)
TTX + ZD7288
+ CCh
+ XE-991
B2
CCh - TTX/ZD788
XE-991 - CCh/TTX/ZD7288
150
100
50
0
-50
I (pA)
-100
-60
-40
Vm (mV)

Figure 3.7. Cholinergic depolarization of parasubicular neurons is dependent in part on blockade of the muscarinically-activated outward K^+ current, I_M. **A**. Group data show that bath application of CCh (50 μM) results in a significant inward current at the holding voltage of -60 mV (-40.5 ± 12.1 pA; *: $p < 0.05$). Addition of the M-current blocker XE-991 (10 μM; black bar) did not result in a significant increase in the membrane current (-13.3 ± 5.8 pA). **B**. Membrane currents during slow voltage ramps from -120 mV to -40 mV in the presence of TTX (0.5 μM) and ZD7288 (50 μM) (black line), and during subsequent bath application of CCh (B_1; light grey line; 50 μM) show that cholinergic receptor activation induces an inward current at voltages near resting membrane potential that reverses around -76 mV, consistent the blockade of an outward current carried by K^+. Further bath application of XE-991 (dark grey line; 10 μM) did not result in any additional membrane current, suggesting that the depolarizing effects of carbachol in parasubicular neurons are mediated at least in part by a suppression of M-current. Current subtractions show that CCh blocks an outward current that reverses at -83.3 ± 7.0 mV ($B_{2;}$ black line), and also occluded membrane currents normally induced by XE-991 (A_2; light grey line).

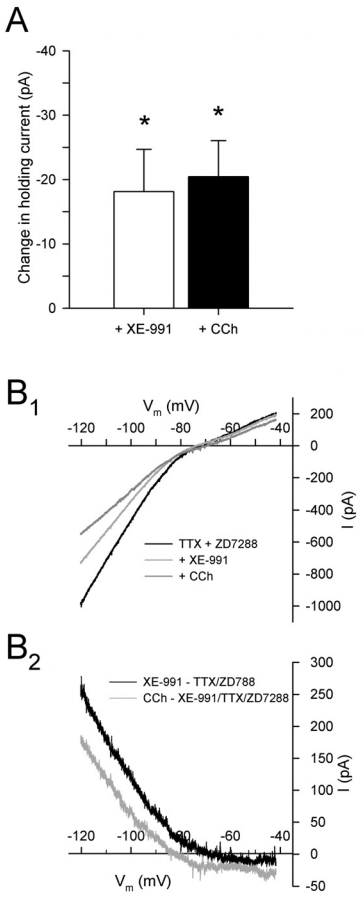
A
Change in holding current (pA)
-40
-30
-20
-10
0
*
*
+ XE-991
+ CCh
B1
Vm (mV)
-120
-100
-80
-60
-40
200
0
-200
-400
-600
-800
-1000
I (pA)
TTX + ZD7288
+ XE-991
+ CCh
B2
XE-991 - TTX/ZD788
CCh - XE-991/TTX/ZD7288
300
250
200
150
100
50
0
-50
I (pA)
-120
-100
-80
-60
-40
Vm (mV)

Figure 3.8. In addition to effects mediated by I_M, cholinergic receptor activation induces an additional inward current that is likely mediated by an attenuation of a K^+ conductance. **A**. Data for the group of cells tested show that bath application of XE-991 (10 μM; -18.1 ± 6.6 pA; white bar) results in an inward shift in the current required at a potential of -60 mV, indicating that layer II cells of the parasubiculum normally express the muscarinically-activated M-current. However, further application of carbachol resulted in an additional inward current (-20.4 ± 5.6 pA; *: $p < 0.05$), suggesting that CCh blocks both I_M and an additional K^+ conductance. **B**. Sample current traces of a parasubicular neuron in response to a slow voltage ramp from -120 mV to -40 mV (B_1) during bath application of TTX (0.5 μM) and ZD7288 (50 μM) (black line), and during subsequent application of XE-991 (10 μM; light grey line), and of carbachol (50 μM; dark grey line; B_1). Current subtractions (B_2) show that XE-991 (black line) induced an inward current at voltages near rest that reversed at -78.0 ± 3.2 mV, and that subsequent co-application of CCh (50 μM; grey line) resulted in an additional inward current that reversed at -85.3 ± 1.1 mV. The estimated equilibrium potential for K^+ was ~ -84 mV, suggesting that the inward currents are mediated by an attenuation of K^+ conductances.

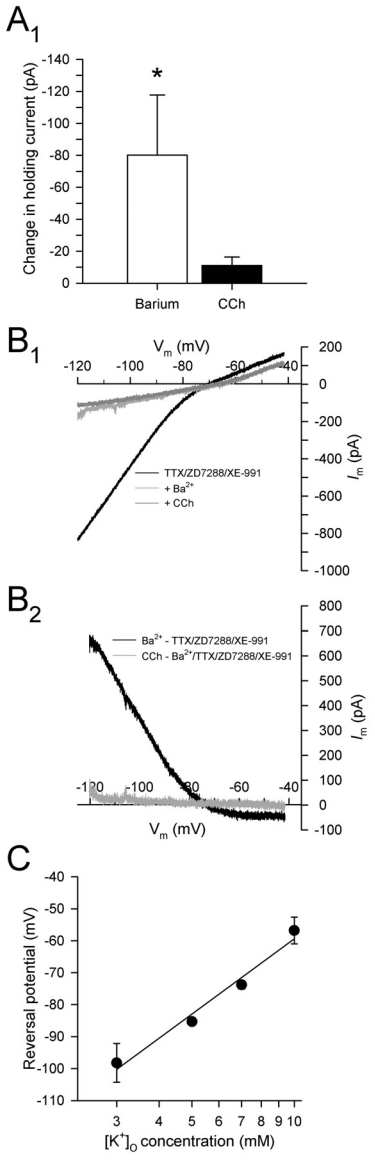
A1
Change in holding current (pA)
-140
-120
-100
-80
-60
-40
-20
0
*
Barium
CCh
B1
Vm (mV)
-120
-100
-80
-60
-40
200
0
-200
-400
-600
-800
-1000
Im (pA)
TTX/ZD7288/XE-991
+ Ba2+
+ CCh
B2
800
700
600
500
400
300
200
100
0
-100
Im (pA)
Ba2+ - TTX/ZD7288/XE-991
CCh - Ba2+/TTX/ZD7288/XE-991
-120
-100
-80
-60
-40
Vm (mV)
C
Reversal potential (mV)
-40
-50
-60
-70
-80
-90
-100
-110
3
4
5
6
7
8
9
10
[K+]o concentration (mM)

Figure 3.9. Cholinergic depolarization in layer II parasubicular neurons is mediated by an inhibition of a Ba^{2+}-sensitive K^+ conductance. **A**. Mean group data show that Ba^{2+} (200 μM; white bar) induces a significant inward current at resting potentials, and that CCh (50 μM; black bar) fails to induce any additional current in the presence of K^+ blockade with Ba^{2+} (*: $p < 0.05$). **B**. Current traces from a representative layer II parasubicular neuron in response to slow voltage ramps from -120 mV to -40 mV (B_1) in the presence of TTX (0.5 μM) and ZD7288 (50 μM) (black line), after the addition of Ba^{2+} (200 μM; light grey line), and after addition of CCh (50 μM; dark grey line). Current subtractions (B_2) show that Ba^{2+} (black line) resulted in a significant inhibition of an outward current at voltages negative to E_K, and subsequent application of CCh (grey line) fails to elicit an additional current, indicating that the inward current at resting potentials induced by CCh is mediated by a K^+ current that is sensitive to Ba^{2+}. **C**. Group data show that the reversal potential of the CCh-induced current in the presence of TTX (0.5 μM), ZD7288 (50 μM), and XE-991 (10 μM) was shifted in relation to the extracellular K^+ concentrations. Substitution of 3 mM $[K^+]_O$ for 5 mM $[K^+]_O$ shifted the mean reversal potential of the CCh-induced current to a more negative voltage, whereas substitution of either 7 mM $[K^+]_O$ or 10 mM $[K^+]_O$ shifted the reversal potential to more positive voltages. The reversal potential of the CCh-induced current showed a linear relationship with the logarithmic

$[K^+]_O$ concentration, indicating that CCh induces an inward current at rest potentials through inhibition of a K^+ current.

CHAPTER 4

CHOLINERGIC SUPPRESSION OF EXCITATORY SYNAPTIC TRANSMISSION IN LAYERS II/III OF THE PARASUBICULUM.

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ABSTRACT

Layer II of the parasubiculum (PaS) receives excitatory synaptic input from the CA1 region of the hippocampus, and sends a major output to layer II of the medial and lateral entorhinal cortex. The PaS also receives heavy cholinergic innervation from the medial septum, and cholinergic receptor activation exerts a wide range of effects in other areas of the hippocampal formation, including presynaptically-mediated suppression of excitatory synaptic responses. Similarly, cholinergic receptor activation is likely to suppress excitatory synaptic input to the layer II/III neurons of the PaS. Field excitatory postsynaptic potentials (fEPSPs) in layer II/III of the PaS were evoked by stimulation of either layer I afferents, or ascending inputs from layer V. Bath-application of the cholinergic agonist carbachol (0.5-10 μM) suppressed the amplitude of fEPSPs evoked by both superficial- and deep layer stimulation, and also enhanced paired-pulse facilitation. Constant bath-application of the $GABA_A$ antagonist bicuculline (25 μM) failed to eliminate the suppression, indicating that the cholinergic suppression of fEPSPs is not due to increased inhibitory tone. The muscarinic receptor antagonist atropine (1 μM) blocked the suppression of fEPSPs, and the selective M_1-preferring receptor antagonist pirenzepine (1 μM), but not the M_2-preferring antagonist methoctramine (1-5 μM), also blocked the suppression, suggesting that the cholinergic suppression of excitatory synaptic responses in the PaS is dependent on activation of M_1–like receptors. Therefore, cholinergic receptor activation suppresses excitatory synaptic input to layer II/III neurons of the PaS, and this suppression is dependent on M_1 receptor activation.

Cells of the parasubiculum (PaS) receive major inputs from the subiculum, CA1 region of the hippocampus, basolateral amygdala and the anterior thalamus, and the single major output of the PaS is to layer II of the entorhinal cortex (Caballero-Bleda & Witter, 1993, 1994; Kohler, 1985, 1986; Shibata, 1993; van Groen & Wyss, 1990a). Layer II entorhinal neurons are thought to play a major role in the sensory and mnemonic functions of the medial temporal lobe because they receive major inputs from sensory and associational cortices, and are also the cells of origin of the perforant path input to the hippocampus (Amaral & Witter, 1989; Swanson & Kohler, 1986). Further, electrical stimulation of the PaS can either inhibit or enhance entorhinal cortex responses to piriform cortex inputs in a time-dependent manner, indicating that activity in the PaS can have strong modulatory effects on how the entorhinal cortex responds to incoming sensory information (Caruana & Chapman, 2004). Therefore, modulation of synaptic transmission within the PaS may affect how layer II entorhinal neurons respond to sensory inputs, and consequently, the PaS may influence the nature of activity within the entorhinal cortex, and within the perforant path output to the hippocampal formation (R. S. Jones, 1994, 1995).

Hippocampal theta-frequency EEG activity is a prominent sinusoidal-like 4-12 Hz rhythm that has been linked to behavioral and neural functions including spatial navigation, sensorimotor integration, and memory consolidation (Bland & Oddie, 2001; Buzsaki, 2002; Hasselmo, 2006). Cholinergic and GABAergic projections that originate in the medial septum and the vertical limb of the diagonal band of Broca (MS/vDBB) play critical roles in the generation of theta

activity in the hippocampal formation (Bland, 1986; Buzsaki, 2002; Stewart & Fox, 1990). Initial studies using anterograde axonal tracing and autoradiography demonstrated that the PaS receives profuse innervation of cholinergic fibers from the medial septum, and also exhibits dense staining of acetylcholinesterase, an enzyme that degrades acetylcholine (Alonso & Kohler, 1984; Geneser-Jensen & Blackstad, 1971; Swanson & Cowan, 1977; van Groen & Wyss, 1990a). In addition, it has recently been demonstrated that the superficial layers of the PaS express cholinergically-dependent theta field oscillations (Glasgow & Chapman, 2007). The firing of many cells in the PaS is modulated by local theta oscillations (Chrobak & Buzsaki, 1994), and recent studies have noted that the superficial layers of the PaS contain theta-modulated place cells (Taube, 1995b), place-by-direction cells (Cacucci et al., 2004), and grid and border cells (Boccara et al., 2010; Hargreaves et al., 2005; Solstad et al., 2008). However, although the firing of parasubicular neurons is known to be dependent in part on cholinergic-dependent theta activity, it is not known how acetylcholine may modulate synaptic transmission within the superficial layers of the PaS.

Acetylcholine is known to exert a wide range of excitatory and inhibitory effects on neurons in other regions of the hippocampal formation (Dickson & Alonso, 1997; Hasselmo & Giocomo, 2006; Klink & Alonso, 1997b; Kunitake, Kunitake, & Stewart, 2004; McCormick & Prince, 1986; Segal & Auerbach, 1997). Carbachol (CCh), a nonspecific cholinergic agonist, has multiple excitatory effects on the intrinsic properties of hippocampal, amygdalar, and entorhinal neurons, mediated through actions on K^+, and Ca^{2+} channels and nonspecific

cationic channels including I_{NCM}, and can lead to membrane depolarization, reductions in afterhyperpolarizations, and changes in repetitive firing properties (Benardo & Prince, 1982b; Chapman & Lacaille, 1999a; Klink & Alonso, 1997b; Washburn & Moises, 1992; Womble & Moises, 1992). Muscarinic receptor activation also results in the depolarization of layer II parasubicular neurons (S.D. Glasgow, Chapter 3), which may contribute to the generation of theta-frequency membrane potential oscillations that are expressed at voltages near the threshold for action potentials (Glasgow & Chapman, 2007, 2008). However, in contrast to these excitatory effects, studies in acute brain slices and freely-moving animals indicate that theta activity is associated with a cholinergic suppression of fEPSPs in the hippocampus (Hargreaves, Cain, & Vanderwolf, 1990; Hasselmo & Schnell, 1994; L. S. Leung, 1980; Wyble, Linster, & Hasselmo, 2000) and entorhinal cortex (Cheong et al., 2001; Hamam et al., 2007; Yun et al., 2000). Recordings in acute brain slices in the CA1 region of the hippocampus and the entorhinal cortex have also shown that the cholinergic suppression of glutamatergic transmission is mediated via reductions of presynaptic transmitter release (Auerbach & Segal, 1996; Cheong et al., 2001; Giocomo & Hasselmo, 2007; Hasselmo & Schnell, 1994; Hounsgaard, 1978; Sheridan & Sutor, 1990; Valentino & Dingledine, 1981; Yun et al., 2000). Increases in cholinergic tone during theta activity may therefore suppress excitatory synaptic transmission in the PaS.

Many of the actions of acetylcholine in the hippocampal formation are mediated via actions on muscarinic receptors (Hasselmo, 2006). Five subtypes

of muscarinic acetylcholine receptors (mAChRs) have been identified, and have been subdivided into two major functional families based on their G-protein coupling (Caulfield & Birdsall, 1998; Hulme, Birdsall, & Buckley, 1990; Wess, Eglen, & Gautam, 2007). The M_1-like receptors, including M_1, M_3 and M_5 subtypes, couple to α subunits of the G_q/G_{11} family and generally have excitatory actions. G_q-coupling activates phospholipase C (PLC), resulting in hydrolysis of phosphatidylinositol biphosphate (PIP_2) and the generation of diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). These pathways lead to activation of protein kinase C and release of intracellular Ca^{2+} stores respectively, (Berridge, 1993), as well as inhibition of K^+ currents including I_M (Shapiro et al., 2000). In contrast, the actions of M_2-like receptors, including the M_2 and M_4 subtypes, are primarily inhibitory. The M_2 and M_4 receptors are coupled to pertussis toxin-sensitive G_i or G_O α subunits, and inhibit adenylnyl cyclase, which leads to activation of G-protein coupled inward rectifying K^+ channels (GIRKs) (Dascal, 1997; Seeger & Alzheimer, 2001). Both M_1 and M_2 receptor subtypes have been implicated in cholinergic suppression of synaptic responses in the hippocampal formation, however the relative involvement of each subtype remains unclear (Auerbach & Segal, 1996; Dutar & Nicoll, 1988; Hounsgaard, 1978; Kremin et al., 2006; Kunitake et al., 2004; Richter, Schilling, & Muller, 1999; Sheridan & Sutor, 1990). Cholinergic suppression of evoked EPSPs in the hippocampus and entorhinal cortex is accompanied by significant increases paired-pulse facilitation, suggesting that acetylcholine suppresses the release of glutamate from presynaptic terminals (Hamam et al., 2007; Hasselmo & Schnell, 1994;

Valentino & Dingledine, 1981; Yun et al., 2000). Both M_1 and M_2 receptors have also both been reported to modulate voltage-gated N- and L-type Ca^{2+} currents (Shapiro et al., 2001), and inhibition of these voltage-dependent Ca^{2+} currents by either receptor subtype could contribute to the presynaptic suppression of transmitter release. Early studies in acute brain slices suggested that presynaptic M_1 receptors mediated decreases in transmitter release at excitatory synapses (Sheridan & Sutor, 1990). More recent work has demonstrated that synaptic responses in the CA1 region of the hippocampus in acute brain slices from M_1 receptor knockout mice show reduced levels of cholinergic suppression as compared to wild-type mice, suggesting that M_1 receptors mediate most of the suppression, but the persistence of a residual suppressive effect suggests that other muscarinic receptor subtypes have a role as well (Kremin et al., 2006). Similarly, Dutar and Nicoll (1988) have reported that gallamine, a muscarinic receptor antagonist with a higher binding affinity for the M_2 receptor subtype, potently blocks cholinergic suppression of EPSPs in the CA1 region. Thus, while it seems clear that the cholinergic suppression of excitatory transmission is mediated by presynaptic mechanisms that regulate transmitter release, the role of the muscarinic receptor subtypes in this suppression is not clear, and it is also not well understood how cholinergic inputs may alter excitatory synaptic transmission within the PaS.

In the present study, I used field potential recordings from acute brain slices maintained in vitro to examine the dose-dependent effects of the muscarinic agonist carbachol on evoked synaptic responses within layer II/III of

the PaS. Carbachol was found to have a powerful suppressive effect on EPSPs evoked by either stimulation of layer I or layer V/VI inputs, and the effect was challenged using antagonists preferring the M_1 and M_2 receptor families to determine the receptor subtypes through which carbachol modulates the synaptic responses.

METHODS

Slice preparation

The methods for slice preparation have been described in detail in previous reports (Glasgow & Chapman, 2008; Kourrich, Glasgow, Caruana, & Chapman, 2008), and were conducted in accordance with the guidelines of the Canadian Council on Animal Care and the Concordia University Animal Research Ethics committee. Acute brain slices were obtained from 4 to 6-week old rats. Briefly, the rat was deeply anesthetized using halothane and decapitated. The brain was quickly extracted and submerged in ice-cold ACSF (4 ºC) containing (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose saturated with 95% O_2 and 5% CO_2 (pH ~7.3; 300-310 mOsm). Horizontal slices (400 µM thick) containing both the hippocampal and retrohippocampal regions were cut using a vibratome (WPI, Vibroslice NVSL), and allowed to recover in room temperature (~22º C) ACSF for ~1.5h. Individual slices were then transferred to nylon net in a temperature-regulated gas-fluid interface chamber (Fine Science Tools). The upper surface of the slice was exposed to a humidified 95%/5% O_2/CO_2 atmosphere, and the chamber was

perfused with warmed oxygenated ACSF at a rate of 1.5 – 2.0 ml/min at 32 ± 0.5º C.

Stimulation and recording

Field potential recording electrodes were pulled from borosilicate glass (1.0 mm OD) using a horizontal puller (Sutter Instruments, P97), and were filled with ACSF (2-6 MΩ). The borders of the superficial layers of the PaS were identified using visible slice landmarks including the angular bundle and layer II of the presubiculum (Funahashi & Stewart, 1997b; Glasgow & Chapman, 2007, 2008). The recording electrode was positioned at the border of layer I/II with the aid of a dissecting microscope (MS5, Leica) at a depth of 50-180 μM below the surface of the slice. Bipolar tungsten stimulating electrodes (FHC Inc.) were placed in layer I of the presubiculum, and layer V of the PaS, approximately 0.4-0.6 mm rostral or medial, respectively, to the recording electrode. Cathodal constant current pulses were delivered using a stimulus generator (WPI, Model A300) and a stimulus isolation unit (Model A360). Evoked field excitatory postsynaptic potentials (fEPSPs) were filtered and amplified (DC-3 kHz, Axon Instr., Axoclamp 2B) in bridge mode, and synaptic responses were digitized (20 kHz, Axon Inst., Digidata 1322A) for storage on computer hard disk using the pClamp 8.2 (Axon Instr.) software package. Stimulation intensities were adjusted to evoke fEPSPs with an amplitude of ~65-75% of the maximal response (typically < 100 μA).

Field potential responses evoked by both deep and superficial layer stimulation were first characterized by examining depth profiles of evoked responses recorded across the entire laminar depth of the PaS. The recording electrode was first positioned in layer I at the edge of the slice, and then moved in ≈50 μm steps toward the deep layers of the PaS along a trajectory perpendicular to the cortical surface (Figure 4.1A). Synaptic responses to five stimulation pulses at 65-75% of maximal stimulation intensity were averaged at each recording site. The amplitude and reversal of evoked responses was examined to infer the origin of the synaptic responses, but current source density analysis (Caruana & Chapman, 2004) was not performed because of the relatively small number of recording locations used (8 sites) and variability in the distance between the recordings sites sampled by the moving electrode.

The effect of cholinergic receptor activation on synaptic responses was assessed using 10 min bath-application of the cholinergic agonist carbamylcholine chloride (CCh, carbachol; 0.5-10 μM). Five evoked synaptic responses were recorded, with an interval of 20 s, for each stimulation site, and drug effects were tested only if baseline fEPSP amplitudes were stable for a 10-20 min period. Application of carbachol for 5 to 10 min was followed by a 20 min washout period. To assess whether increased inhibition contributed to the observed cholinergic suppression of fEPSPs, a group of slices was exposed to CCh during constant bath-application of the $GABA_A$ receptor antagonist bicuculline methiodide (25 μM). The role of muscarinic receptors in the carbachol-induced suppression of fEPSPs was tested using atropine sulfate

($ATSO_4$, 0.5 μM). The receptor subtype that mediates the carbachol-induced suppression of fEPSPs was investigated using the M_1–like receptor blocker pirenzepine (1 or 5 μM) and the M_2–like receptor blocker methoctramine (1 or 5 μM). All drugs were stored as frozen stock solutions and added to ACSF just prior to recordings. Pirenzipine dihydrochloride and bicuculline methiodide were purchased from Ascent Scientific (Princeton, NJ, USA), and all other drugs and chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Changes in paired-pulse facilitation induced by carbachol were examined to determine if the cholinergic suppression of fEPSPs was due to a presynaptic reduction in transmitter release or post-synaptic changes in responsivity. A suppression of transmitter release can lead to increased paired-pulse facilitation due to an enhancement in the pool of readily releasable transmitter during the response to the second stimulation pulse (Zucker & Regehr, 2002). To determine the optimal interval for paired-pulse facilitation, conditioning and test pulses were delivered at interpulse intervals ranging from 10 to 1000 ms. Averages of five responses evoked from stimulation of each pathway were obtained, and the amplitude of the second response in each pair was expressed as a ratio to the amplitude of the response to the first pulse (Chapman & Racine, 1997; Hamam et al., 2007). The maximal facilitation was observed using an interpulse interval of 30 ms, and paired-pulse facilitation ratios for the 30 ms interpulse interval were compared between baseline, drug, and washout conditions.

Data analysis

Evoked postsynaptic responses were averaged across five consecutive sweeps before and after drug treatment, and analyzed using the pClamp 8.2 software package (Axon Instr.). Statistical analyses used *t*-tests for paired samples, and repeated measures ANOVAs with Student Neuman-Keuls tests to assess changes in amplitude of EPSPs. All data were expressed as the mean ± SEM.

RESULTS

Field potentials recorded in layer I/II of the PaS displayed prominent negative-going components in response to stimulation of either layer I of the presubiculum or the deep layers of the PaS. Field potential components at the same latencies reversed in polarity to positive-going responses when the recording electrode was placed deep in layer II/III, consistent with the generation of the field responses by synaptic activation within layers I and II. Stimulation of layer I inputs resulted in a prominent negative deflection in layer II/III (*n* =4; mean peak amplitude: -0.63 ± 0.04 mV; peak latency: 5.2 ± 0.4 ms) (Figure 4.1B*b*-*c*). Evoked potentials in response to superficial stimulation reversed near the border of layers III and IV, and often showed a negative-going putative population spike in deeper portions of layer II/III (Figure 4.1B*d*). Similarly, stimulation of layer V resulted in a major negative field potential component in layers I and II (amplitude: -0.67 ± 0.07 mV; peak latency: 5.5 ± 0.5 ms), which reversed near the border of layers III and IV. There were two major spike-like components: a

very short latency (approximately 1-2 ms) surface-positive, deep-negative spike that likely resulted from direct activation of deep-layer substrates, and a slightly longer latency spike that was negative in superficial layers, with no clear reversal. This latter spike component peaked at latencies near 5.5 ± 0.5 ms on the rising phase of the negative EPSP, and likely reflects a population spike. In addition to the spike components, deep layer stimulation also resulted in an additional short-latency negative component in layer V likely resulting from fast synaptic activation of deep layer neurons via horizontal collaterals (Figure 4.1B*g*). The amplitude and latency of fEPSPs recorded in the superficial layers of the PaS did not differ significantly as a function of stimulation site ($p = 0.73$ and $p = 0.55$, respectively). The present manuscript has focused on changes in the surface-negative synaptic potentials monitored by a stationary recording electrode in layer II/III following stimulation of deep and superficial layer inputs.

Cholinergic effects on excitatory synaptic transmission in layer II/III of the PaS were assessed using constant bath-application of the muscarinic agonist carbachol (CCh; Figure 4.2A). Similar to findings in the superficial layers of the entorhinal cortex (Hamam et al., 2007; Yun et al., 2000), the amplitude of fEPSPs evoked in layer II/III of the PaS by activation of superficial layer inputs was greatly reduced by 10 min bath-application of CCh (10 μM; Fig. 2A, upper traces) to 53.4 ± 6.4 % of baseline levels (-0.58 ± 0.04 mV in baseline vs. -0.30 ± 0.04 mV in CCh; $F_{2,21} = 23.44$, $p < 0.001$; N-K, $p < 0.001$; $n = 14$). Responses were suppressed rapidly during the 10 min bath-application, and returned to baseline levels after 20 min of washout in normal ACSF (104.6 ± 17.9% of

baseline; N-K, n.s., p = 0.83; Figure 4.2B$_1$). Similarly, bath-application of carbachol also suppressed synaptic responses evoked by deep layer stimulation to 69.2 ± 6.2% of baseline levels (-0.57 ± 0.07 mV in ACSF vs. -0.38 ± 0.07 mV in CCh; $F_{2,21}$= 14.29, p < 0.001; N-K, p < 0.001; n = 14), and responses returned to baseline levels after a 20 min washout period (99.7 ± 9.9% of baseline; N-K, n.s., p = 0.94) (Figures 4.2A, lower traces, and 4.2B$_2$). The degree of suppression during bath-application of CCh did not differ between superficial layer and deep layer stimulation (p =0.31). Cholinergic receptor activation therefore results in a reversible suppression of excitatory synaptic transmission in both superficial and deep layer inputs to layer II/III of the PaS.

Lower doses of CCh can also depress synaptic transmission in the hippocampus, subiculum and entorhinal cortex (Auerbach & Segal, 1994, 1996; Cheong et al., 2001; Kunitake et al., 2004; Yun et al., 2000). Ten min bath-application of low doses of CCh (0.5 μM) significantly reduced the amplitude of synaptic responses in layer II/III of the PaS evoked by superficial layer stimulation (-0.74 ± 0.10 mV in ACSF vs. -0.57 ± 0.06 mV in CCh; $F_{2,12}$=4.25, p < 0.05; N-K, p < 0.05; n = 7), but failed to significantly suppress responses evoked by deep layer stimulation (-0.89 ± 0.11 mV in ACSF vs. -0.71 ± 0.09 mV in CCh; $F_{2,12}$=2.77, p = 0.10), suggesting a stronger effect of cholinergic receptor activation within the superficial pathway (Figure 4.2B, grey bars). Sub-micromolar concentrations of carbachol are known to induce a delayed potentiation of field responses in the hippocampus and entorhinal cortex termed muscarinic long-term potentiation (LTP_M) (Auerbach & Segal, 1994, 1996;

Cheong et al., 2001; Yun et al., 2000). However, LTP_M was not observed in parasubicular responses, which returned to baseline levels after a 20 min washout period (92.2 ± 11.1% of baseline in superficial pathway, 99.1 ± 12.4% of baseline in deep layer pathway), and also showed no evidence of potentiation after 30-40 min of washout (n = 4).

Paired-pulse facilitation is a short-term form of synaptic plasticity in which the amplitude of the synaptic response to the second of two stimulation pulses can be enhanced relative to the first response. Paired-pulse facilitation is largely dependent upon mechanisms that control the presynaptic release of transmitter (Creager, Dunwiddie, & Lynch, 1980; Dobrunz & Stevens, 1997) and has been used to assess whether cholinergic modulation of synaptic efficacy is due to pre- or post-synaptic mechanisms (Auerbach & Segal, 1996; Hamam et al., 2007; Hasselmo & Schnell, 1994; Richter et al., 1999). We therefore characterized the properties and optimal interpulse intervals for paired-pulse facilitation in the PaS (Figure 4.2C_1). Paired-pulse stimulation of deep and superficial fibers evoked maximal facilitation effects at an interpulse interval of 30 ms for both superficial (130.9 ± 8.7%; $F_{6,54}$=9.56, p < 0.01; N-K, p < 0.01) and deep layer stimulation (137.5 ± 13.2%; $F_{6,54}$=4.74, p < 0.01; N-K, p < 0.01; n = 10), however interpulse intervals of 20 ms (superficial: 116.7 ± 12.8%, N-K, p < 0.01; deep: 126.4 ± 16.3%, N-K, p < 0.01) and 50 ms (superficial: 122.4 ± 8.4%, N-K, p < 0.01; deep: 129.9 ± 10.3%, N-K, p < 0.01) also showed significant facilitation effects (Figure 4.2C_1). Stimulation of the superficial layer pathway with a shorter interpulse interval of 10 ms resulted in significant paired-pulse inhibition (65.9 ± 17.7 %, N-

K, $p < 0.01$), likely due to fast feed-forward synaptic inhibition, however 10 ms interpulse interval failed to produce similar inhibition in response to deep layer stimulation (88.7 ± 23.0%, N-K, $p = 0.75$). Further, stimulation at longer intervals of 100 to 1000 ms resulted in no significant facilitation. The 30 ms interpulse interval, which induces maximal facilitation effects, was therefore used in subsequent experiments assessing the effects of cholinergic receptor activation on paired-pulse facilitation.

Carbachol has been reported to exert effects on both pre- and postsynaptic mechanisms that affect synaptic function, including postsynaptic membrane depolarization, increases in postsynaptic input resistance, and a decrease in presynaptic terminal transmitter release probability (Cobb & Davies, 2005). To determine whether the cholinergically-mediated synaptic depression is mainly due to reduced transmitter release or due to postsynaptic mechanisms, paired-pulse ratios were monitored before and after exposure to carbachol (Creager et al., 1980; Hamam et al., 2007; Hasselmo & Schnell, 1994; Qian & Saggau, 1997). Bath-application of carbachol consistently reduced the amplitudes of fEPSPs evoked by the first stimulation pulses, and significantly increased paired-pulse facilitation of responses evoked by stimulation of the superficial pathway (116.5 ± 6.9% in ACSF and 156.3 ± 15.7% in CCh, $F_{2,21}$= 4.72, $p < 0.05$; N-K, $p < 0.05$; Figures 4.2C_2 and 4.2C_3). Paired-pulse facilitation in response to deep layer stimulation also increased from 111.3 ± 9.1% to 133.6 ± 7.3% in CCh, but this change was not statistically significant ($F_{2,21}$= 3.28, p = 0.06; Figure 4.2C_2). Following a 20 min washout of carbachol, paired-pulse

ratios returned to baseline values. Further, paired-pulse facilitation ratios were increased in both the superficial (92.3 ± 9.9% in ACSF vs. 106.0 ± 5.6% in CCh; $F_{2,12}$= 3.95, $p < 0.05$; N-K, $p < 0.05$) and deep (116.2 ± 9.5% in ACSF vs. 129.2 ± 9.8% in CCh; $F_{2,12}$= 5.75, $p < 0.05$; N-K, $p < 0.01$) layer pathways during bath-application of 0.5 μM CCh, and returned to baseline levels after 20 min washout (data not shown). Therefore, the depression of responses induced by both low and high doses of CCh is associated with a reversible increase in paired-pulse facilitation. This enhancement of paired-pulse facilitation is consistent with the suppression being mediated by a presynaptic reduction in transmitter release (Egorov, Gloveli, & Muller, 1999; Valentino & Dingledine, 1981; Yun et al., 2000).

Cholinergic receptor agonism is known to depolarize local inhibitory interneurons within the hippocampal formation (Chapman & Lacaille, 1999a; Lawrence, Statland, Grinspan, & McBain, 2006; McQuiston & Madison, 1999; Xiao, Deng, Yang, & Lei, 2009), and it is possible that the suppression of fEPSPs observed here was mediated in part by increased local inhibition of principal neurons. To assess the contribution of local inhibition to the cholinergic suppression observed here, experiments were repeated during constant bath-application of the $GABA_A$-receptor antagonist bicuculline methiodide (25 μM; Figure 4.3). Addition of bicuculline to control ACSF led to an increase in fEPSP amplitudes as well as repetitive population spike discharges in some slices, and stimulation intensity was therefore routinely reduced to minimize repetitive population spikes. Bath-application of CCh in ACSF containing bicuculline reduced the amplitude of fEPSPs evoked by superficial layer stimulation to 19.0

± 2.3% of levels recorded in the presence of bicuculline alone (-1.19 ± 0.28 mV in bicuculline vs. -0.21 ± 0.03 mV in CCh; $F_{2,4}$=13.89, $p < 0.05$; N-K, $p < 0.05$; n = 3), and CCh also reduced in the amplitude of fEPSP evoked by deep layer stimulation to 49.2 ± 12.1% of baseline (-1.33 ± 0.11 mV in bicuculline vs. -0.67 ± 0.20 mV in CCh; $F_{2,4}$=13.47, $p < 0.05$; N-K, $p < 0.05$; Figure 4.3B). These data indicate that increases in local inhibition do not contribute to the cholinergic suppression of synaptic responses in the PaS.

The contribution of muscarinic receptors to the suppression of fEPSPs in the PaS was assessed by adding the muscarinic receptor antagonist atropine sulfate (1 μM) to the bath prior to CCh (10 μM; Figure 4.4). Consistent with a suppressive effect of endogenously released acetylcholine on synaptic responses, the addition of the muscarinic blocker atropine resulted in an overall *increase* in the amplitude of fEPSPs in response to superficial layer stimulation (-0.65 ± 0.07 mV in ACSF vs. -0.91 ± 0.12 mV in atropine; $F_{3,18}$=4.34, $p < 0.05$; N-K, $p < 0.05$; n = 7), and a non-significant increase in response to deep layer stimulation (-0.65 ± 0.10 mV in ACSF vs. -0.82 ± 0.12 mV in atropine; $F_{3,18}$= 2.18, p = 0.12; Figure 4.4B). Further, constant bath-application of atropine for a 10 min period prior to the addition of CCh completely blocked the carbachol-induced suppression of fEPSPs evoked by both superficial (-0.91 ± 0.12 mV in $ATSO_4$ vs. -0.89 ± 0.12 mV in CCh; N-K, p = 0.81) and deep layer stimulation (-0.82 ± 0.12 mV in ATSO4 vs. -0.80 ± 0.14 mV in CCh; *n.s.*). Therefore, the cholinergic suppression of fEPSPs in layer II/III of the PaS evoked by stimulation of both deep and superficial layers is dependent on activation of muscarinic receptors.

To determine which muscarinic receptor subtype contributed to the depression of synaptic responses, either methoctramine, an M_2-like receptor antagonist, or pirenzepine, a M_1-like receptor antagonist, were added to the bathing medium prior to the addition of carbachol. Ten min pre-exposure to the M_2 antagonist, methoctramine (1 μM; *n* = 7), failed to block the suppressive effects of CCh (10 μM) in both superficial (-0.63 ± 0.04 mV in methoctramine vs. -0.21 ± 0.01 mV in CCh; $F_{3,18}$=22.09, p < 0.01; N-K, *p* < 0.01) and deep layer pathways (-0.66 ± 0.04 in methoctramine vs. -0.21 ± 0.02 mV in CCh; $F_{3,18}$=27.16, *p* < 0.01; N-K, p < 0.01; Figures 4.5A and 4.5B). The higher dose of 5 μM methoctramine also failed to block the carbachol-induced suppression of synaptic responses (*n* = 3, Figure 4.5B, black bars), suggesting that M_2 receptors do not mediate the cholinergic suppression of EPSPs.

Pirenzepine has a strong binding affinity for M_1 receptors at a concentration of 1 μM, but higher concentrations of 5 μM also block M_4 receptors (Dorje et al., 1991; Klink & Alonso, 1997a). In our initial experiments, in which pirenzepine was applied for 10 min prior to addition of CCh (10 μM), a dose-dependent effect was observed in which 1 μM pirenzepine (*n* = 6) failed to block the suppression of responses evoked by both superficial (-0.76 ± 0.10 mV in pirenzepine vs. -0.30 ± 0.09 mV in 10 μM CCh; $F_{3,15}$=17.66, *p* < 0.01; N-K, *p* < 0.01; Figure 4.5D_1) and deep layer stimulation (-0.69 ± 0.05 mV in pirenzepine vs. -0.29 ± 0.04 mV in CCh; $F_{3,15}$=20.96, *p* < 0.01; N-K, *p* < 0.01; Figure 4.5D_2), but the higher dose of 5 μM pirenzepine completely blocked the suppression of responses evoked by superficial (*n* = 2; -0.63 ± 0.29 mV in pirenzepine vs. -0.62

± 0.27 mV in CCh) or deep layer inputs (-1.18 ± 0.07 mV in pirenzepine vs. 1.24 ± 0.15 mV in CCh). The large volume of the recording chamber used here can reduce the drug concentration that reaches the slice because the drug solution is diluted as it enters the chamber, and we therefore increased the time that pirenzepine was pre-applied for from 5 min to 20 min, to allow the bath concentration to come closer to the target concentration of 1 μM pirenzepine. Increasing the time of pre-exposure to 20 min, to allow for more selective antagonism of the M_1 receptor subtype, resulted in an overall increase in the amplitude of the fEPSPs in response to superficial layer stimulation (-0.75 ± 0.11 mV in ACSF vs. -1.04 ± 0.26 mV in pirenzepine; $F_{3,12}$=3.48, $p < 0.05$; N-K, $p < 0.05$) but not deep layer stimulation (0.78 ± 0.13 mV in ACSF vs. -1.08 ± 0.30 mV in pirenzepine; $F_{3,12}$= 2.03, $p = 0.16$), and it also resulted in an effective block of the carbachol-induced suppression in both superficial (-0.85 ± 0.20 mV in CCh; N-K, $p > 0.05$) and deep layer stimulation (-1.04 ± 0.32 mV in CCh) (Figure 4.5D). Cholinergic suppression of synaptic transmission in layer II/III of the PaS is therefore dependent on activation of M_1 receptors.

DISCUSSION

The present study investigated the effects of cholinergic receptor activation on evoked excitatory synaptic responses in layer II/III of the PaS using synaptic field potential recordings from acute brain slices. Stimulation of the superficial layers of the presubiculum resulted in a surface-negative field potential component in layer II/III of the PaS Stimulation of deep layers of the

parasubiculum also evoked a negative synaptic component within the superficial layers attributable to activation of ascending inputs, and also evoked a negative synaptic response in the deep layers, likely due to direct activation of horizontal collaterals within the deep layers. Furthermore, we found that bath-application of the nonspecific cholinergic agonist carbachol potently suppressed the amplitude of evoked fEPSPs in layer II/III of the PaS in response to both superficial and deep layer stimulation. The magnitude of the suppression was dose-dependent, and it was not due to increases in local inhibition. Moreover, the suppression was completely blocked by the muscarinic antagonist atropine, and by the M_1 receptor antagonist pirenzepine, but was not affected by bath-application of selective M_2 antagonist, methoctramine. The suppressive effects of carbachol on synaptic transmission are therefore mediated primarily via actions on M_1-like receptors. The cholinergic suppression was associated with an increase in paired-pulse facilitation, suggesting that carbachol likely acts on presynaptic receptors to reduce transmitter release (Creager et al., 1980). These findings are consistent with effects of M_1 receptor activation on glutamate-mediated synaptic transmission in other regions of the hippocampal formation, both in vivo and in vitro, including the subiculum proper, entorhinal cortex and CA1 region of the hippocampus (Cheong et al., 2001; Hamam et al., 2007; Hasselmo & Schnell, 1994; Kremin et al., 2006; Kunitake et al., 2004; L. S. Leung & Vanderwolf, 1980; Yun et al., 2000).

Carbachol-induced suppression is not due to increased inhibitory tone. Carbachol is known to depolarize local inhibitory neurons in the entorhinal cortex

and hippocampus, and increased GABAergic synaptic input can “shunt” excitatory synaptic responses in principal cells (Behrends & ten Bruggencate, 1993; Dickson & Alonso, 1997; McCormick & Prince, 1986; Pitler & Alger, 1992; Reece & Schwartzkroin, 1991). The PaS displays strong GAD, GABA, and parvalbumin immunoreactivity (Boccara et al., 2010; Kohler et al., 1985) and, similar to layer II of the entorhinal cortex, the superficial layers of the PaS show high levels of local inhibition (Funahashi & Stewart, 1997a, 1997b, 1998; R. S. Jones, 1994). Here, cholinergic suppression of synaptic transmission in layer II/III of the PaS was not blocked in the presence of the $GABA_A$ receptor antagonist bicuculline, indicating that changes in levels of fast ionotropic inhibition do not contribute the suppressive effects of carbachol. A pronounced $GABA_B$-mediated late IPSP has been reported in superficial parasubicular neurons in response to presubicular stimulation (Funahashi & Stewart, 1998), and this could act to suppress synaptic transmission during cholinergic receptor agonism. Although the time constant of $GABA_B$–mediated presynaptic inhibition of glutamate release in vivo is consistent with a contribution to the suppression of EPSPs during theta EEG activity (Molyneaux & Hasselmo, 2002; Wyble et al., 2000), $GABA_B$-mediated presynaptic inhibition is most prominent in response to strong repeated synaptic stimulation (J. S. Isaacson, Solis, & Nicoll, 1993), and is therefore unlikely to play a major role in the cholinergic suppression of excitatory synaptic transmission observed here.

Carbachol-induced suppression is mediated by M_1 Receptors. The carbachol-induced reduction in the amplitude of field potentials evoked by

stimulation of both deep and superficial layer inputs to layer II/III of the PaS was completely blocked by application of the muscarinic receptor antagonist atropine, confirming that cholinergic suppression of fEPSPs in the PaS is mediated via actions on muscarinic receptors. Both M_1 and M_2 receptor subtypes have been linked to the suppression of excitatory synaptic transmission in both the hippocampus and the cortex (Auerbach & Segal, 1996; Barral, Galarraga, & Bargas, 1999; Cheong et al., 2001; Colgin, Kramar, Gall, & Lynch, 2003; Dutar & Nicoll, 1988; Fernandez de Sevilla, Cabezas, de Prada, Sanchez-Jimenez, & Buno, 2002; Gulledge & Stuart, 2005; Hamam et al., 2007; Hounsgaard, 1978; Kunitake et al., 2004; Misgeld, Muller, & Polder, 1989; Richter et al., 1999; Sheridan & Sutor, 1990; Yun et al., 2000). The present results indicate that cholinergic suppression of excitatory synaptic transmission in layer II/III of the PaS is mediated primarily via M_1 receptors. Determining the exact receptor subtype responsible for muscarinic suppression of synaptic responses has proven difficult due to the lack of specificity of muscarinic antagonists, but work in other regions of the hippocampal formation, including the CA1 region of the hippocampus, subiculum, and entorhinal cortex also show carbachol-induced suppression of excitatory synaptic transmission that is dependent on activation of M_1–like receptors (Kunitake et al., 2004; Richter et al., 1999; Sheridan & Sutor, 1990). Further, in the CA1 region of the hippocampus, carbachol fails to suppress excitatory synaptic transmission in transgenic mice with a knockout of the M_1 receptor (Kremin et al., 2006). Here, methoctramine, a compound with high affinity binding for M_2 and M_4 muscarinic receptors failed to block the

carbachol-induced suppression of excitatory synaptic transmission in the PaS at both low and high doses. In contrast, the suppressive effect of carbachol was completely blocked after prolonged bath-application of pirenzepine, a muscarinic antagonist that binds preferentially to M_1 receptors. Pirenzepine can also have additional dose-dependent antagonistic effects on the M_4 receptor and, to a lesser extent, the M_3 receptor (Adem & Karlsson, 1997; Dorje et al., 1991), and carbachol has a higher binding affinity for M_2 receptors compared to M_1 receptors, suggesting that carbachol would bind to both receptor subtypes at the concentrations used here (Kunitake et al., 2004). However, either application of 5 μM pirenzepine, or increasing the exposure time for a lower dose of pirenzepine to ensure that bath concentrations reached the more selective dose of 1 μM, effectively blocked the carbachol-induced suppression in both deep and superficial pathways. Taken together, then, the present results indicate that the carbachol-induced suppression of fEPSPs in the PaS is mediated primarily via actions on M_1 receptors that are preferentially blocked by 1 μM pirenzepine.

Cholinergic suppression of presynaptic glutamate release. Although the precise signaling mechanisms that underlie muscarinic suppression of EPSPs in layer II/III of the PaS remain unclear, the cholinergic suppression was associated with a concurrent increase in the paired-pulse ratio in both superficial and deep layer pathways, and this suggests that the suppression is due to presynaptic reduction in transmitter release (Figure 4.2C). Paired-pulse facilitation has been attributed to the summation in the presynaptic terminal of residual calcium from the first pulse with the Ca^{2+} influx evoked by the test pulse, resulting in increased

vesicular mobilization and release of transmitter (Zucker & Regehr, 2002). Here, we observed an increase in paired-pulse facilitation during the cholinergic suppression of EPSPs, and carbachol also induces a similar suppression of EPSPs and increase in paired-pulse facilitation in layers II and V of the entorhinal cortex, the hippocampus, and the piriform cortex (Auerbach & Segal, 1996; Cheong et al., 2001; Hamam et al., 2007; Hasselmo & Bower, 1992; Yun et al., 2000). An increase in paired-pulse facilitation is consistent with a reduction in presynaptic transmitter release in response to the first pulse, resulting in greater facilitation in response to the second pulse due to the larger remaining pool of readily releasable transmitter (Hamam et al., 2007; Manabe, Wyllie, Perkel, & Nicoll, 1993; Yun et al., 2000). The present findings therefore suggest that the cholinergic suppression of excitatory synaptic transmission in layer II/III of the PaS is likely mediated by actions on presynaptic glutamatergic transmission through a reduced glutamate release.

The M_1 receptor antagonist pirenzepine blocked the cholinergic suppression via a presynaptic mechanism, and there are a number of possible pathways through which M_1 receptor activation can modulate EPSPs. In many neurons of the hippocampus and entorhinal cortex, activation of M_1 receptors typically leads to postsynaptic depolarization (Dutar & Nicoll, 1988; Klink & Alonso, 1997a; McCormick & Prince, 1986), but transient local application of carbachol can also result in membrane hyperpolarization in neocortical pyramidal neurons by opening the bicuculline-sensitive K^+ channel, S_K, via IP_3-mediated release of intracellular calcium (Gulledge & Stuart, 2005). Recent work using in situ

hybridization has shown that M_1 receptors are located primarily on postsynaptic dendrites, extrasynaptic membrane, and neuropil on cortical pyramidal neurons (Yamasaki, Matsui, & Watanabe, 2010) but immunocytochemical staining for M_{1-4} has also localized M_1 and M_4 receptors on presynaptic terminals of putative glutamatergic neurons within the striatum (Hersch & Levey, 1995; Levey, Edmunds, Hersch, Wiley, & Heilman, 1995). Therefore, although primarily located postsynaptically, M_1 receptor expression has been detected on the presynaptic density, and this suggests that M_1 receptor activation may reduce presynaptic transmitter release via direct actions on presynaptic terminals.

The ionic mechanisms of the presynaptic suppression in layer II/III of the PaS are not clear. Postsynaptic conductances that affect membrane potential can affect the driving force on EPSPs and associated changes in input resistance could affect EPSP amplitude. In other neurons, acetylcholine is known to modulate several K^+ conductances including inward-rectifying K^+ conductances (Seeger & Alzheimer, 2001) and the muscarinically-activated K^+ current I_M (Benardo & Prince, 1982b; Caulfield, Robbins, Higashida, & Brown, 1993; McCormick & Prince, 1986), and in CA1 hippocampal neurons, postsynaptic M_2 receptors have been linked to the opening of G-protein activated inward rectifying K^+ channels, and this could serve to reduce EPSPs through shunting excitatory synaptic input (Seeger & Alzheimer, 2001). In parasubicular neurons, I have recently shown that carbachol leads to a depolarization of membrane potential through an attenuation of I_M, and the attenuation of an addition inward rectifying K^+ channel (Chapter 3). However, the depolarization of parasubicular neurons

by several millivolts by carbachol is not strong enough to account for the potent suppression of EPSPs observed, and the increased paired-pulse ratio observed also indicates that the muscarinic suppression of excitatory synaptic transmission in layer II/III of the PaS is mediated by reductions in presynaptic glutamate release.

It is known that muscarinic receptor activation exerts actions on N-, P-, and L-type Ca^{2+} currents, and this may lead to significant reductions in transmitter release (Howe & Surmeier, 1995; Toselli & Taglietti, 1995). In hippocampal CA1 neurons, cholinergic receptor activation reduces transmitter release by attenuating presynaptic Ca^{2+} influx via blockade of high-threshold voltage-activated Ca^{2+} currents mediated in part by N-type calcium channels, and are likely to be mediated by a G-protein-coupled signalling pathway similar to adenosine (Qian & Saggau, 1997; Toselli & Taglietti, 1995). Thus, carbachol may suppress synaptic transmission in the superficial layers of the PaS by acting directly on presynaptic M_1 receptors that suppress calcium transients in the presynaptic terminal through inhibition of voltage-activated Ca^{2+} currents, leading to significant reductions in glutamate release.

Cholinergic receptor activation may also modulate presynaptic transmitter release via indirect mechanisms. In hippocampal pyramidal neurons, prolonged depolarization results in Ca^{2+} entry and subsequent synthesis and release of endogenous cannabinoids such as anandamide from the postsynaptic cell, which can serve as powerful retrograde signals to modulate presynaptic activity (Kano, Ohno-Shosaku, Hashimotodani, Uchigashima, & Watanabe, 2009; Kreitzer &

Regehr, 2001; Neu, Foldy, & Soltesz, 2007; Wilson, Kunos, & Nicoll, 2001; Wilson & Nicoll, 2001, 2002). Endocannabinoid receptor-1 (CB1R) activation on presynaptic terminals of both excitatory and inhibitory synapses can reduce transmitter release through adenosine-like actions on N- and P/Q-type Ca^{2+} channels in hippocampal neurons (Foldy, Neu, Jones, & Soltesz, 2006; Guo & Ikeda, 2004; Hoffman, Laaris, Kawamura, Masino, & Lupica, 2010; Kawamura et al., 2006; Misner & Sullivan, 1999; Twitchell, Brown, & Mackie, 1997). Acetylcholine can strongly enhance the endocannabinoid signaling system, and may stimulate synthesis of the endocannabinoid 2-arachidonoylglycerol (2-AG) through $G_{q/11}$-mediated activation of phospholipase C (PLC) (Kano et al., 2009). Moreover, carbachol-induced activation of postsynaptic M_1 and M_3 receptors results in an eight-fold increase in endocannabinoid release in hippocampal neurons (Fukudome et al., 2004; Lau & Vaughan, 2008; Ohno-Shosaku, Maejima, & Kano, 2001; Ohno-Shosaku et al., 2003). The entorhinal cortex, subiculum, and PaS express substantial levels of cannabinoid receptors (Herkenham et al., 1991; Marsicano & Lutz, 1999), and the entorhinal cortex shows CB1R-mediated depolarization-induced inhibition of inhibitory transmission (Morgan, Stanford, & Woodhall, 2008). It is therefore possible that depolarization of layer II parasubicular neurons during cholinergic receptor activation (S. D. Glasgow & C. A. Chapman, unpublished observations) may lead to release of endogenous cannabinoids from the postsynaptic cell, and result in a suppression of presynaptic transmitter release via effects on VDCCs by activation of CB1Rs.

Functional significance. Previous work from our lab has demonstrated that the superficial layers of the PaS generate cholinergic-dependent theta-frequency oscillations, and that both principal cells and interneurons in this region are endowed with intrinsic conductances that lead to theta-frequency oscillations in membrane potential at depolarized voltages (Glasgow & Chapman, 2007, 2008). Cholinergic and GABAergic inputs from the medial septum contribute to the generation of theta-frequency activity throughout the hippocampal formation, and cholinergic inputs to the PaS from the medial septum are likely to be active during theta-related behaviors (Alonso & Kohler, 1984; Baisden, Woodruff, & Hoover, 1984; Bland et al., 1999; Petsche, Stumpf, & Gogolak, 1962a; Swanson & Cowan, 1977). Septal inputs to the hippocampal formation are thought to modulate synaptic transmission during theta activity (Chapman & Racine, 1997; Colgin et al., 2003), and similarly, stimulation of the horizontal limb of the diagonal band of Broca suppresses synaptic potentials in the piriform cortex, and this effect is blocked by cholinergic antagonists (Linster, Wyble, & Hasselmo, 1999). We have shown here that cholinergic septal inputs to the parasubiculum are likely to strongly suppress responses of layer II/III parasubicular neurons to synaptic inputs from deep layers of the parasubiculum and from the hippocampus, subiculum, and other subcortical areas (Giocomo & Hasselmo, 2007; Hasselmo, 2006; Witter et al., 1989). Accordingly, suppression of excitatory synaptic transmission in the superficial layers of the PaS during theta activity may serve to decrease local network activation (Giocomo & Hasselmo, 2007).

The locations of the stimulation electrodes in the present study were chosen to activate fibers originating from the CA1 region of the hippocampus as well as projections from the deep layers of the PaS, both of which terminate in layers I/II of the PaS (Cenquizca & Swanson, 2007; Kohler, 1985; van Groen & Wyss, 1990a, 1990b). Muscarinic receptor activation significantly reduced excitatory synaptic transmission in both pathways, indicating that behavioral states associated with increased cholinergic activation are likely to result in a suppression of synaptic strength in both the extrinsic inputs to the PaS, as well as the intrinsic connections between deep and superficial layers. Layer II cells of the PaS project almost exclusively to layer II of the entorhinal cortex, and layer II entorhinal neurons form the origin of the perforant path (Amaral & Witter, 1989; Caballero-Bleda & Witter, 1993, 1994; Kohler, 1985; van Groen & Wyss, 1990a). Interestingly, while cholinergic activation has consistently been correlated with a reduction of synaptic transmission throughout most of the hippocampal formation, the medial perforant path input to the dentate gyrus is resistant to cholinergic suppression (Schall & Dickson, 2010). This suggests that a generalized suppression of synaptic inputs to the PaS during theta activity, and the resulting suppression of synaptic input from the hippocampus to the entorhinal cortex via the PaS, may reduce the modulation of perforant path activity by hippocampal inputs, and promote the relative strength of extrahippocampal inputs to the hippocampal formation via the entorhinal cortex (Giocomo & Hasselmo, 2007; Hasselmo, 2006; Hasselmo & Giocomo, 2006; Schall & Dickson, 2010).

In vivo recordings from the superficial layers of the PaS have demonstrated a high proportion of theta-modulated cells that are spatially-tuned and fire dependent on an animal's location in space (Boccara et al., 2010; Cacucci et al., 2004; Hargreaves et al., 2005; Solstad et al., 2008; Taube, 1995b). Increases in cholinergic tone in the PaS during theta activity could serve to reduce local excitatory network activity, limiting the contribution of weaker synaptic inputs on parasubicular cell firing, while concurrently enhancing the *relative* impact of stronger synaptic inputs from the CA1 and anterior thalamus. As such, increases in cholinergic tone in the PaS during theta activity may serve to increase the signal to noise ratio for active synaptic inputs due to a reduction in the overall level of network "noise."

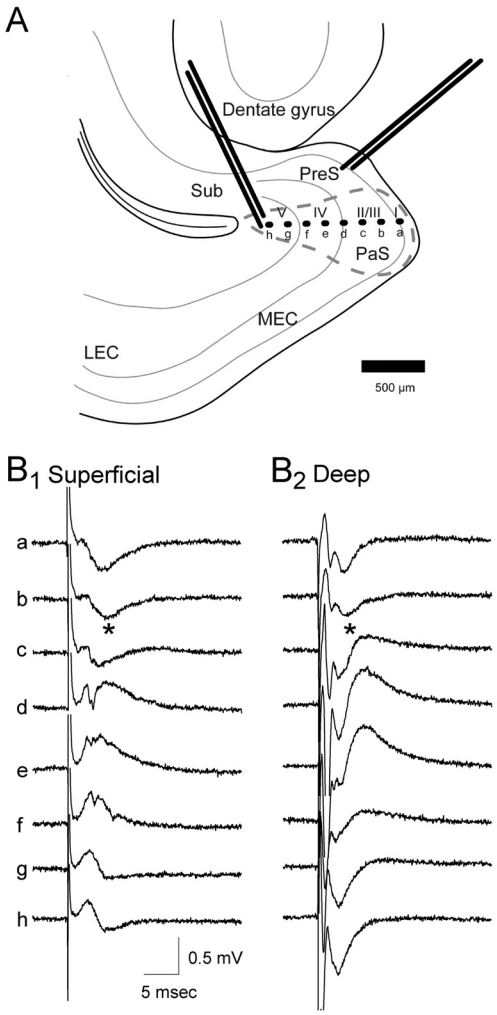
A
Dentate gyrus
PreS
Sub
V
IV
II/III
I
h g f e d c b a
PaS
MEC
LEC
500 µm
B1 Superficial
B2 Deep
a
b
c
d
e
f
g
h
*
*
0.5 mV
5 msec

Figure 4.1. Evoked synaptic field potentials recorded at multiple cortical depths in a representative brain slice of the parasubiculum in response to stimulation of inputs from either the superficial or deep-layers. Both stimulation sites evoked negative potentials in layers I/II. **A**. The location of the two bipolar stimulation electrodes and the locations of the recording sites (●) are shown in a tracing of a representative horizontal section. Lower-case letters (**a** through **h**) indicate the positions of the recording electrode for traces shown in B. **B**. Averaged evoked field potentials (5 sweeps) at multiple cortical depths within the parasubiculum in response to superficial (B_1) and deep (B_2) layer stimulation. Note the surface-negative response in recordings obtained in upper layer II (*, site **b**), and the reversal of the response near the border of layer III and layer IV (site **d**). Stimulation of the superficial layer inputs also evoked an apparent population spike deep in layers II/III (site **d**), and there was also a strong initial spike at this depth in response to deep layer stimulation. Deep layer stimulation also resulted in a very short-latency negative response in layer V (sites **g** and **h**), consistent with activation of horizontal collaterals within layer V.

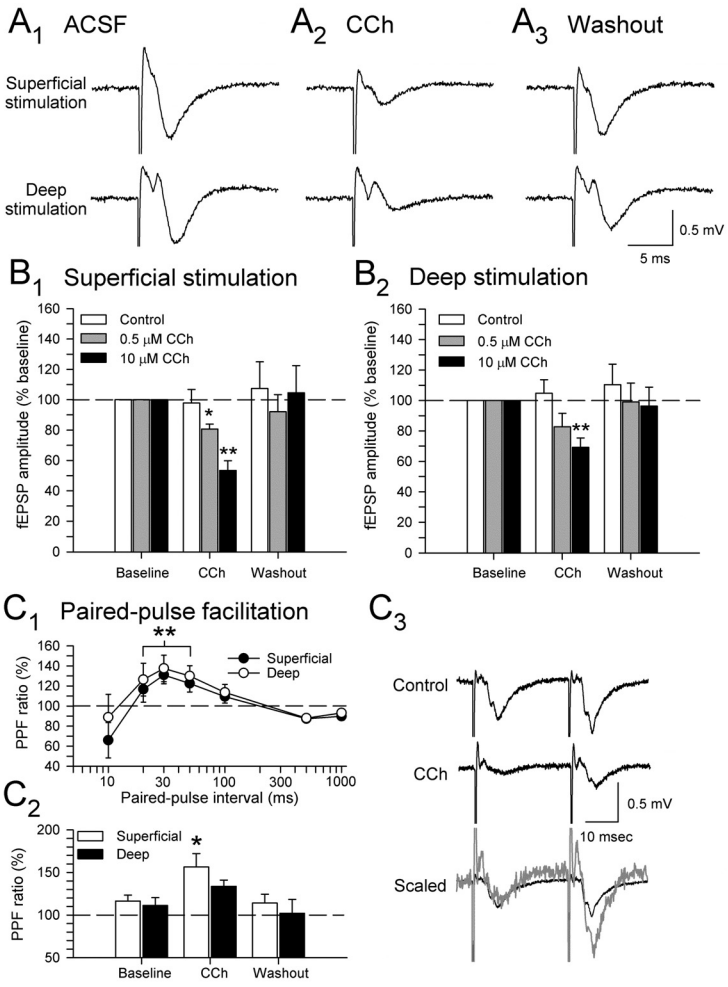
A1 ACSF
A2 CCh
A3 Washout
Superficial stimulation
Deep stimulation
0.5 mV
5 ms
B1 Superficial stimulation
B2 Deep stimulation
Control
0.5 μM CCh
10 μM CCh
fEPSP amplitude (% baseline)
Baseline
CCh
Washout
C1 Paired-pulse facilitation
PPF ratio (%)
Superficial
Deep
Paired-pulse interval (ms)
C2
C3
Control
CCh
0.5 mV
10 msec
Scaled

Figure 4.2. Bath-application of the cholinergic agonist carbachol reduces the amplitude of evoked fEPSPs in layer II/III of the parasubiculum in vitro. **A**. Averaged fEPSP recordings (5 sweeps) evoked by stimulation of either layer I of the presubiculum (A_1, upper traces) or layer V of the parasubiculum (lower traces) are greatly attenuated by 10 min bath-application of 10 μM CCh (A_2). Responses returned to baseline levels after washout (A_3). **B**. Group averages show that both 0.5 μM and 10 μM concentrations of CCh induced a significant and reversible dose-dependent reduction in the amplitude of fEPSPs evoked by both superficial (B_1) and deep layer stimulation (B_2). Asterisks denote significant differences from baseline and washout conditions (*, $p < 0.05$; **, $p < 0.01$). **C**. The cholinergic suppression of fEPSPs was associated with enhanced paired-pulse facilitation, suggesting that the suppression is expressed presynaptically. The maximal amount of paired-pulse facilitation was observed in response to pairs of pulses delivered to the superficial (white circles) and deep (black circles) layer pathways at an interpulse interval of 30 ms, and no facilitation was observed at the 10 ms interval, or intervals above 50 ms (C_1; **, denotes significant differences from single-pulse levels, $p < 0.01$). Group means show a significant and reversible enhancement of paired-pulse facilitation in response to 10 μM CCh for responses evoked by both superficial stimulation (C_2; *, $p < 0.05$), while the enhanced facilitation did not reach statistical significance for deep layer stimulation ($p = 0.06$). Representative averaged responses (5

sweeps) to a pair of superficial stimulation pulses separated by an interpulse interval of 30 ms in control ACSF (C_3, top trace) and CCh (10 μM, C_3, middle trace) have been superimposed and scaled to the amplitude of the first response in control ACSF (C_3, bottom trace) to compare the relative degree of paired-pulse facilitation.

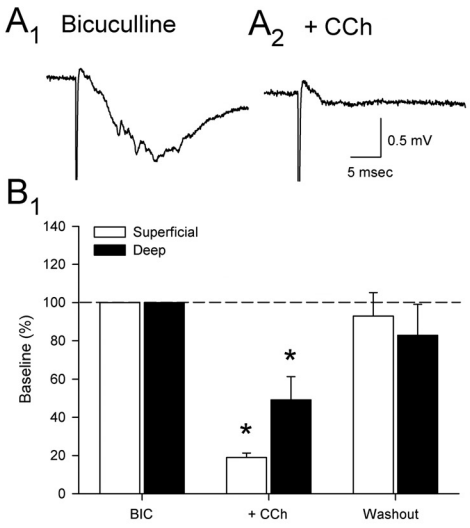
A1 Bicuculline
A2 + CCh
0.5 mV
5 msec
B1
Superficial
Deep
Baseline (%)
140
120
100
80
60
40
20
0
*
*
BIC
+ CCh
Washout

Figure 4.3. Increases in $GABA_A$-mediated synaptic transmission are not required for the cholinergic suppression of fEPSP amplitudes in the parasubiculum. **A**. Averaged sample traces (5 sweeps) of evoked fEPSPs recorded in layer II/III of the parasubiculum in response to superficial stimulation during bath-application of the $GABA_A$ antagonist bicuculline (10 μM; A_1), and with added CCh (10 μM; A_2). Note the increased duration of the EPSP during blockade of $GABA_A$ transmission with bicuculline. **B**. Histograms showing mean EPSP amplitudes indicate that bicuculline failed to block the cholinergic suppression of fEPSP amplitudes in response to both superficial (white bars) and deep (black bars) layer stimulation (*, $p < 0.05$).

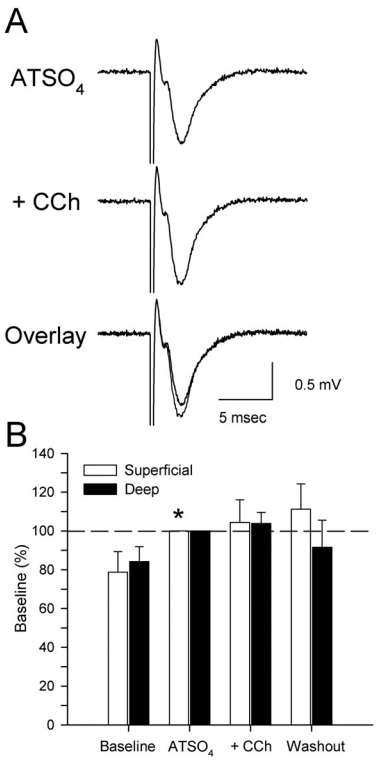
A
ATSO4
+ CCh
Overlay
0.5 mV
5 msec
B
Superficial
Deep
*
Baseline (%)
140
120
100
80
60
40
20
0
Baseline
ATSO4
+ CCh
Washout

Figure 4.4. Cholinergic suppression of fEPSPs is mediated by muscarinic receptors. **A**. Traces show representative fEPSPs evoked by superficial layer stimulation in the presence of the muscarinic receptor antagonist atropine sulfate (A; 0.5 μM; $ATSO_4$, top trace) and after 10 min of added CCh (10 μM; middle trace). Traces are superimposed for comparison (bottom trace). **B.** Mean fEPSP amplitudes shows that application of atropine resulted in an increase in the amplitude of fEPSPs in comparison to baseline responses. The increase was statistically significant for responses evoked by superficial stimulation (*, $p < 0.05$), but not for responses evoked by deep layer stimulation ($p = 0.12$). Atropine also blocked the cholinergic suppression of fEPSPs in response to both superficial and deep layer stimulation.

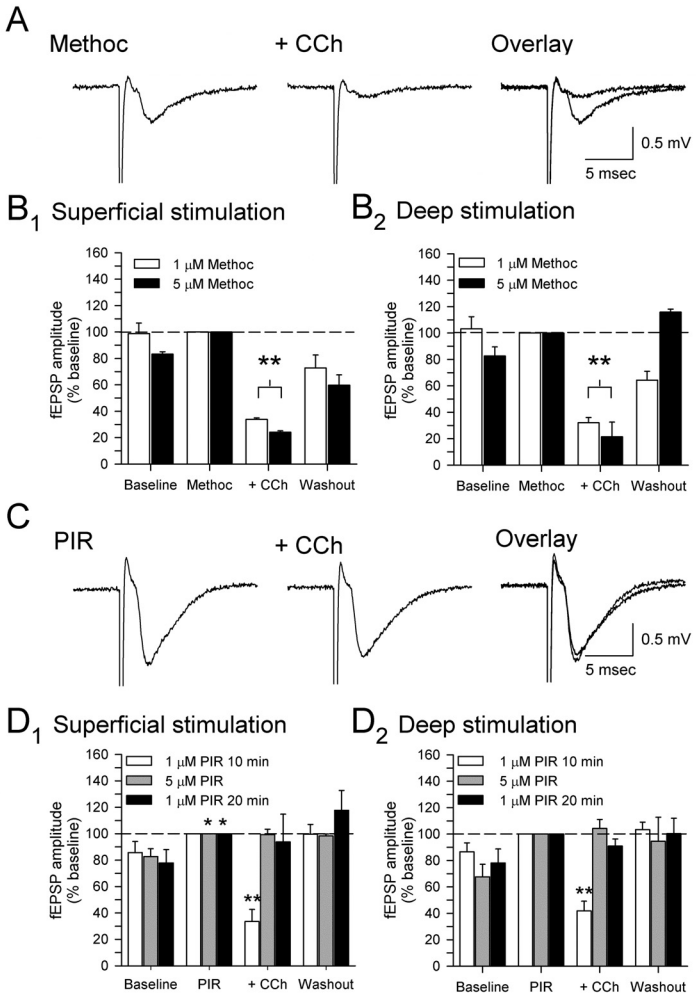
A
Methoc
+ CCh
Overlay
0.5 mV
5 msec
B1 Superficial stimulation
B2 Deep stimulation
1 μM Methoc
5 μM Methoc
fEPSP amplitude (% baseline)
Baseline
Methoc
+ CCh
Washout
C
PIR
+ CCh
Overlay
D1 Superficial stimulation
D2 Deep stimulation
1 μM PIR 10 min
5 μM PIR
1 μM PIR 20 min
Baseline
PIR
+ CCh
Washout

Figure 4.5. The cholinergic suppression of synaptic transmission in layer II/III of the parasubiculum is dependent on M_1-like, but not M_2-like, muscarinic receptors. **A**. Averaged representative traces recorded during constant bath-application of the M_2-like receptor antagonist, methoctramine (Methoc, 1 μM; left trace), and following the addition of CCh (10 μM; middle trace) show that block of M_2 receptors fails to block the cholinergic suppression of fEPSPs evoked by stimulation of superficial layers. Traces are superimposed for comparison (right trace). **B**. Histograms show that both low and high concentrations of methoctramine (1 and 5 μM) failed to block the suppression of fEPSPs evoked by either superficial (B_2) and deep (B_2) layer stimulation. **C**. Traces show representative averaged fEPSPs evoked by superficial layer stimulation after 20 min constant bath-application of M_1-like receptor antagonist, pirenzepine (PIR, 1 μM; A_1; left trace), and 10 min after the addition of CCh (middle trace). Traces are superimposed for comparison (right trace). **D**. Histograms show that a 5 min application of PIR (1 μM) prior to addition of CCh failed to block the suppression of evoked fEPSPs in layer II/III of the parasubiculum in response to both superficial (D_1) and deep (D_2) layer stimulation (white bars; **, $p < 0.01$). However, the cholinergic suppression was completely blocked by 5 min application of a higher dose of pirenzepine (5 μM, grey bars) and was also blocked by a longer, 20-min exposure to a low dose of pirenzepine intended to ensure the bath concentration of the drug approached 1 μM (black bars). Note that

exposure to high concentrations of PIR (5 μM) or extended exposure at 1 μM min also led to a significant increase in the amplitude of baseline fEPSPs recorded in response to stimulation of the superficial layer pathway (*, $p < 0.05$), but the increase was not significant for deep layer inputs.

CHAPTER 5

GENERAL DISCUSSION

The studies included in this thesis have used a number of electrophysiological recording techniques to examine the role of acetylcholine in layer II of the parasubiculum, and assess how cholinergic receptor activation may contribute to the coordination of parasubicular activity during theta-related behaviors. The experiments reported here have shown that parasubicular neurons are endowed with intrinsic conductances to allow for the development of theta frequency membrane potential oscillations that likely contribute to theta EEG activity in the parasubiculum (Chapter 2). Theta activity in the hippocampal formation is mediated, in part, by activation of cholinergic fibers from medial septum (Bland, 2000). The parasubiculum receives a dense cholinergic projection from the medial septum, and it is possible that these fibers are active during theta activity (Stewart & Fox, 1990), but recent data suggests that cholinergic cells in the medial septum may not be active during theta states (Simon, Poindessous-Jazat, Dutar, Epelbaum, & Bassant, 2006). However, theta activity is also associated with increases in levels of acetylcholine in the parasubiculum (Marrosu et al., 1995). The final two experimental chapters described here report that cholinergic receptor activation results in the depolarization of parasubicular neurons (Chapter 3) while concurrently inhibiting presynaptic glutamate-mediated synaptic transmission (Chapter 4). These findings are consistent with the role of acetylcholine within the hippocampal formation, and also show that muscarinic receptor activation modulates parasubicular neurons via novel mechanisms. Taken together, these findings suggest that acetylcholine can play an important role in shaping the network

activity within the parasubiculum. This may contribute to the temporal integration of spatial and directional information relayed from the CA1 region of the hippocampus and the anterior thalamus, respectively, during theta behaviors such as active exploration and REM sleep. Further, given the major output projection from layers II/III of the parasubiculum to projection neurons in the entorhinal cortex, the temporal coordination of activity within the parasubiculum during theta activity is likely to be important in contributing to both entorhinal and hippocampal-dependent spatial processing.

Summary of major findings

The three experimental chapters included in this thesis further clarify the role of the parasubiculum within the hippocampal formation, and underline the important neuromodulatory role of acetylcholine in the parahippocampal area. Cholinergic inputs from the septum to the parasubiculum may increase levels of acetylcholine during theta-related behaviors including active exploration, and it is likely that the mechanisms observed here contribute to mechanisms in the temporal lobe related to spatial navigation.

The second chapter of this thesis examined the ionic conductances that generate intrinsic theta-frequency membrane potential oscillations in layer II parasubicular neurons. Membrane potential oscillations are thought to play major role in the genesis of theta EEG activity in other areas (Bland et al., 2002; Buzsaki, 2002; Buzsaki & Draguhn, 2004; Giocomo & Hasselmo, 2008; Hasselmo et al., 2007). Although a variety of neuron types in the hippocampal

formation show theta-frequency membrane potential oscillations, the conductances that underlie these oscillations differs. In the hippocampus, CA1 principal cells show oscillations that rely on an interaction between Na^+ currents and TEA-sensitive K^+ currents (Garcia-Munoz et al., 1993; L. W. Leung & Yim, 1991), and additional studies have also suggested an important role for the muscarinically-activated K^+ current I_M in these oscillations (Hu et al., 2002, 2007). In contrast, inhibitory interneurons located in str. lacunosum-moleculare show prominent membrane potential oscillations that depend on Na^+ and the voltage-gated A-type K^+ current mediated by $K_{v4.3}$ (Bourdeau et al., 2007; Chapman & Lacaille, 1999b). Layer II stellate cells in the medial entorhinal cortex also show theta-frequency membrane potential oscillations that are mediated by an interaction between I_{NaP} and I_h (Alonso & Llinas, 1989; Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Klink & Alonso, 1993). Here, I built upon the experiments contained in my Masters thesis that first demonstrated that theta EEG activity is generated locally with in the parasubiculum in vivo, and then used intracellular techniques to investigate the mechanisms that generate the oscillations (Glasgow & Chapman, 2007). These experiments showed that parasubicular neurons express voltage-dependent intrinsic oscillations, and also determined the ionic mechanisms that generate these oscillations. To do this, I used advanced intracellular recording techniques, combined with application of pharmacological agents to investigate the ionic conductances that generated theta-frequency oscillations in parasubicular neurons. I found that I_{NaP} and I_h are both required for oscillations in parasubicular neurons, and these ionic

conductances also generate oscillations in entorhinal layer II neurons (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000) through a “push-pull”-like mechanism that controls the overall voltage of the cell. While the persistent nature of I_{NaP} provides tonic excitation, the activation and deactivation kinetics of I_h allow for the generation of rhythmic oscillations when the cell is depolarized to voltages near action potential threshold. As in the entorhinal cortex, intrinsic oscillations in parasubicular neurons are likely to be a major mechanism that promotes theta EEG activity in the parasubiculum (Dickson, Magistretti, Shalinsky, Hamam, et al., 2000; Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Giocomo & Hasselmo, 2008; Hasselmo et al., 2009; Hasselmo, Giocomo, Brandon, & Yoshida, 2010; Zilli, Yoshida, Tahvildari, Giocomo, & Hasselmo, 2009).

Cholinergic inputs from the septum play a major role in promoting theta activity in both the hippocampus (Goutagny et al., 2008; J. D. Green & Arduini, 1954; Petsche, Stumpf, & Gogolak, 1962b; Stewart & Fox, 1990) and entorhinal cortex (Dickson, Trepel, & Bland, 1994; Jeffery et al., 1995). The parasubiculum also receives heavy cholinergic inputs from the medial septum (Alonso & Kohler, 1984; van Groen & Wyss, 1990a; Witter et al., 1989), and my Master’s thesis clearly demonstrated that theta-frequency EEG activity in the parasubiculum is dependent on cholinergic mechanisms (Glasgow & Chapman, 2007). Acetylcholine leads to depolarization of membrane potential in both hippocampal (Benardo & Prince, 1982a; Segal, 1982), subicular (Kawasaki et al., 1999), and entorhinal neurons (Klink & Alonso, 1997b; Shalinsky et al., 2002), and this

suggested that increases in cholinergic tone may lead to the depolarization-induced emergence of theta-frequency membrane potential oscillations in the parasubiculum. In chapter 3 of this thesis, I found that cholinergic receptor activation leads to robust depolarization of membrane potential in the majority of layer II parasubicular neurons. This depolarization was dependent on activation of the M_1 receptor subtype, and was due to inhibition of two K^+ conductances. Muscarinic receptor activation is known to inhibit the muscarinically-activated K^+ current I_M in hippocampal neurons (Brown & Adams, 1980; McCormick & Prince, 1986; Womble & Moises, 1992), and I also found that carbachol also inhibited I_M in parasubicular neurons. Interestingly, I found that muscarinic receptor activation also leads to the blockade of an inward rectifying K^+ current, likely mediated by the Kir 2.3 channel protein. A similar conductance mediates cholinergic depolarization in prefrontal and striatal neurons (Carr & Surmeier, 2007; Shen et al., 2005; Shen et al., 2007; Uchimura & North, 1990; Womble & Moises, 1992), and contrasts with the mechanisms underlying cholinergic depolarization of layer II entorhinal neurons, which depend on the persistent inhibition of a Ba^{2+}-sensitive K^+ conductance and activation of a non-selective cationic current termed I_{NCM} (Klink & Alonso, 1997a; Shalinsky et al., 2002).

In addition to membrane depolarization, muscarinic receptor activation also led to numerous changes in the spike waveform, and other properties of parasubicular neurons, including reductions in afterhyperpolarizations and anodal break potentials. Although initial studies investigating cholinergic modulation of neurons in the hippocampal formation failed to observe any reductions in action

potential amplitude, more recent studies have reported findings similar to those reported here (Figenschou et al., 1996; Klink & Alonso, 1997a). Although the mechanisms are not yet known, this reduction in spike amplitude may be related to inhibition of K^+ current associated with action potential repolarization, although cholinergic modulation of Na^+ currents has been reported (Cantrell et al., 1996; Mittmann & Alzheimer, 1998), and may contribute to the alterations observed here. Further, similar to previous reports in the hippocampus (Azouz, Jensen, & Yaari, 1994; Dutar & Nicoll, 1988), subiculum (Kawasaki & Avoli, 1996) and entorhinal cortex (Klink & Alonso, 1997b; Yoshida & Alonso, 2007), muscarinic receptor activation in layer II parasubicular neurons was also associated with a reduction in both fast and medium duration afterhyperpolarizations. Cholinergic modulation of conductances associated with repolarization and afterhyperpolarization may have increase the excitability of parasubicular neurons by allowing them to rebound and to produce additional spikes more quickly. Increases in the intrinsic excitability of parasubicular neurons through modulation of these conductances may have a profound impact on the pattern of parasubicular synaptic inputs to the entorhinal cortex.

The final experimental chapter investigated the role of cholinergic receptor activation on synaptic transmission within layer II/III of the parasubiculum. I found that carbachol greatly suppressed excitatory synaptic responses to stimulation of either deep or superficial layers. This effect was dependent on M_1 receptors, and was mediated primarily via reductions in presynaptic transmitter release. Although the precise mechanisms of this

suppression remain unclear, it is likely that it serves the function of suppressing excitatory synaptic transmission during theta activity within the hippocampal formation. Therefore, in addition to the depolarizing effect of septal cholinergic input that can lead to theta-frequency membrane potential oscillations, acetylcholine may concurrently mediate a reduction in excitatory synaptic transmission. These mechanisms may govern the manner in which spatial information is integrated within the parasubiculum during theta-related behaviours.

Ionic conductances that mediate membrane potential oscillations and cholinergic depolarization

The ionic conductances that generate theta-frequency membrane potential oscillations in layer II parasubicular neurons are similar to those in stellate neurons of the medial entorhinal cortex and pyramidal neurons in the subiculum (Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Klink & Alonso, 1993; W. T. Wang et al., 2006). Membrane potential oscillations are preferentially expressed by stellate neurons, but not pyramidal neurons, in the medial entorhinal cortex, indicating a functional heterogeneity among these cell types (Alonso & Llinas, 1989; Dickson, Magistretti, Shalinsky, Fransen, et al., 2000; Klink & Alonso, 1993). In contrast, the vast majority of parasubicular neurons, including cells with both stellate and pyramidal morphologies, show membrane potential oscillations (Chapters 2 and 3; Glasgow & Chapman, 2007), indicating that both of these cell types share a powerful mechanism that can

contribute to theta activity, and this may serve to govern the timing of their responsiveness to synaptic inputs, and the timing of their output to the entorhinal cortex during theta-related behaviours (Caballero-Bleda & Witter, 1993; Funahashi & Stewart, 1997a, 1997b; Kohler, 1985; van Groen & Wyss, 1990a; Witter et al., 1989).

Carbachol is known to induce electrophysiologically-distinct responses in morphologically-identified stellate and pyramidal neurons of the medial entorhinal cortex, however many of the parasubicular cells reported here demonstrated slow depolarization of membrane potential similar to that reported in entorhinal stellate neurons (Klink & Alonso, 1997b; Shalinsky et al., 2002). Further, no differences in cell morphology were determined between parasubicular cells that depolarized in response to cholinergic agonism and those that were insensitive to CCh. This suggests that both stellate and pyramidal neurons in layer II/III of the parasubiculum can respond to cholinergic receptor activation with voltage depolarization and alterations in overall excitability, and that both cell types may contribute to the generation of local theta EEG activity. The functional implications of these findings remain unclear because differences in connectivity of stellate and pyramidal neurons are not known, but the homogeneity of intrinsic properties could reflect a functional similarity in the temporal nature of information processing carried out by these cell types.

In addition to depolarizing effects on parasubicular layer II principal cells, it is likely that local interneurons are also depolarized by acetylcholine, leading to an increase in inhibition. A number of different inhibitory cell types are expressed

in the parasubiculum, including cells that are immunopositive for GABA-, glutamic acid decarboxylase (GAD)-, parvalbumin (PV)-, calbindin-, and cholecystokinin (CCK-1) (Boccara et al., 2010; Kohler & Chan-Palay, 1982; Kohler et al., 1985), and activation of GABAergic parasubicular neurons may facilitate rhythmic membrane depolarization through by contributing to IPSP-mediated rebound potentials in principal neurons (Cobb et al., 1995). Parasubicular neurons demonstrate membrane depolarization in response to carbachol in the presence of synaptic blockers, including GABAergic antagonists, indicating that increases in local inhibitory tone are not required for membrane depolarization. Rather, it is likely that cholinergic depolarization of GABAergic neurons facilitates the rhythmicity of activity within parasubicular neurons.

It is also likely that cholinergic modulation of local inhibition play a role in shaping parasubicular-entorhinal synaptic connections. Both the parasubiculum and entorhinal cortex are under considerable inhibitory control (Finch, Tan, & Isokawa-Akesson, 1988; Finch, Wong, Derian, & Babb, 1986), and although previous reports suggest show that parasubicular fibers terminate on the dendrites of entorhinal projection neurons (Caballero-Bleda & Witter, 1994), it is also possible likely that they may also innervate local entorhinal inhibitory interneurons (R. S. Jones, 1990, 1994; R. S. Jones & Buhl, 1993). Cholinergic excitation of principal parasubicular neurons may therefore contribute to increases in local inhibition within the entorhinal cortex, and this may serve as an additional mechanism by which the cholinergic inputs to the parasubiculum during theta can help shape entorhinal output to the hippocampus.

Cholinergic receptor activation may serve as a temporal filter

In addition to the strong membrane depolarization, cholinergic receptor activation also led to a significant suppression of excitatory synaptic transmission in layer II parasubicular neurons, and this may have profound effects on synaptic integration and the role of the parasubiculum in processes mediated by the hippocampal formation during theta-related behaviours. Cholinergic suppression of glutamatergic synaptic transmission has been reported in a number of different brain regions, including the hippocampus (Hasselmo & Schnell, 1994), the entorhinal cortex (Cheong et al., 2001; Hamam et al., 2007; Yun et al., 2000), and the piriform cortex (Hasselmo & Bower, 1991, 1992), and is likely to play a major role in governing synaptic interactions. In the piriform cortex, there is a cholinergic suppression of recurrent collaterals among principal neurons on proximal dendrites, but a concurrent *facilitation* of EPSPs was also observed in response to the layer I inputs from the olfactory bulb (Hasselmo & Bower, 1992). The suppression of activity in the recurrent collaterals is thought to reduce activity-dependent degradation of associations held by those collaterals that might result during processing of novel sensory inputs (Giocomo & Hasselmo, 2007; Hasselmo, 1995, 2005; Hasselmo & Giocomo, 2006). Similarly, the cholinergic suppression of synaptic responses within the hippocampal formation is also thought to serve a similar role during theta activity (Kahle & Cotman, 1989; Kremin & Hasselmo, 2007; Richter et al., 1999). Similar processes may

also occur in the parasubiculum during theta activity, and this may help reduce excitation of local circuitry.

Cholinergic receptor activation led to a profound suppression of excitatory glutamatergic synaptic transmission that was accompanied by an increase in paired-pulse facilitation at an interpulse interval of 30 ms (Chapter 4). The timing of the intervals between 20 and 100 ms at which facilitation effects are observed correspond to the periodicity of gamma and theta frequencies, which suggests that rhythmic activity within the parasubiculum is likely critical in governing how parasubicular neurons respond to synaptic inputs during states when acetylcholine levels are increased. Synaptic inputs that are repeatedly activated at gamma or theta-frequencies may be facilitated, and may also be particularly effective in activating entorhinal neurons if the inputs arrive at the peak of the theta rhythm in the entorhinal cortex. However, there may also be a temporal filtering of synaptic inputs that either arrive the trough of theta oscillations, or which are not rhythmic (Hasselmo, 2005; Singer, 1993). Therefore, although local excitatory synaptic inputs are suppressed during cholinergic activation, short-term synaptic enhancements associated with rhythmic activity may serve to promote the impact of synaptic inputs that arrive at particular phases of the ongoing rhythmic activity during theta. This emphasizes the potential importance of temporal coordination of synaptic inputs during theta-related behaviours.

Similarly, the suppression of excitatory synaptic transmission during cholinergic receptor activation also suggests that temporal summation effects may play a major role in the integration of synaptic inputs during theta activity.

Muscarinic receptor activation has been linked to enhancement of temporal summation of EPSPs in the prefrontal cortex (Carr & Surmeier, 2007), and a similar mechanism may be important in the parasubiculum. Substantial projections from the CA1 and anterior thalamus terminate on parasubicular neurons, and coincident synaptic inputs from these two regions during theta-related increases in cholinergic tone may promote both temporal and spatial summation of EPSPs, and promote cell firing of parasubicular neurons. Conversely, single non-rhythmic inputs, or inputs that arrive at the trough of the local theta cycle, will likely fail to elicit parasubicular neuronal activation, and consequently, will be filtered out. Therefore, inputs that innervate parasubicular neurons simultaneously with other synaptic inputs, or arrive at the peak of the local theta cycle, should show the greatest temporal summation within parasubicular neurons. This type of summation may both promote firing of parasubicular neurons, and may also may promote activity-dependent long-term potentiation (Xu, Ye, Poo, & Zhang, 2006).

Although the muscarinic suppression of glutamatergic synaptic transmission in the parasubiculum was primarily characterized by reductions in the amplitude of evoked EPSPs, muscarinic receptor activation is also associated with slight increases in input resistance in parasubicular neurons. Cholinergic depolarization in layer II parasubicular neurons was mediated via a blockade of K^+ conductances, including I_M, and this was typically associated with an increase in input resistance. Increases in input resistance can lead to an enhancement of dendritic signal propagation, and this could partially offset the

reduction in EPSP amplitudes. Through this mechanism, cholinergic activation during theta could still allow for the propagation of temporally-coordinated dendritic EPSPs to the soma, or conversely, facilitate the backpropagation of somatic EPSPs to the dendrites (Gulledge, Kampa, & Stuart, 2005; Stuart, Spruston, Sakmann, & Hausser, 1997). The combination of backpropagation of action potentials in neocortical neurons and temporally-coordinated EPSPs has been linked to synapse-specific potentiation effects in neocortical pyramidal neurons (Sjostrom & Hausser, 2006), and such a mechanism may be important in activity-dependent synaptic strengthening in the parasubiculum. Moreover, depending on the phase-relation between synaptic inputs and cell firing, this type of mechanism could promote either activity-dependent enhancement or depression of synaptic inputs (Hyman et al., 2003; Orr et al., 2001; Pavlides et al., 1988). In this way, muscarinic modulation of parasubicular neurons during theta activity may allow for input-specific plastic changes to extra-parasubicular inputs.

Cholinergic depolarization in layer II parasubicular neurons was mediated via a blockade of K^+ conductances, including I_M, and increased input resistance in parasubicular neurons during theta-frequency activity could also be expected to enhance the amplitude of membrane potential oscillations associated with theta activity. Increased input resistance associated with inhibition of I_M and the inward rectifying K^+ conductance could increase the amplitude of the voltage response induced by the membrane currents that generate the oscillations. However, similar to the effects in layer II parasubicular neurons, cholinergic

receptor activation in interneurons of str. lacunosum-moleculare is associated with slight increase in input resistance, but does not lead to increases in the power of theta-frequency membrane potential oscillations compared to oscillations observed through constant current-induced depolarization (Chapman & Lacaille, 1999a). Further, in layer II entorhinal stellate neurons, previous findings have failed to report that cholinergic receptor activation results in increases in the amplitude of membrane potential oscillations (Klink & Alonso, 1997b), but rather leads to in a negative shift in the voltage threshold necessary for the occurrence of these oscillations, and a decrease in the frequency of the oscillations, which is consistent with frequency of cholinergically-mediated theta EEG activity *in vivo* (Bland, 1986; Kramis et al., 1975). Taken together, these findings suggest that K^+ conductances associated with increased input resistance do not significantly contribute to the amplitude of theta-frequency membrane potential oscillations in layer II parasubicular neurons.

Functional role of cholinergic activation in the parasubiculum

In comparison to other areas of the hippocampal formation, both behavioral and electrophysiological data regarding the potential function of the parasubiculum in sensory and mnemonic processing is relatively sparse. However, more recent work has suggested that the parasubiculum, through its connections with the entorhinal cortex, hippocampus and anterior thalamus, may play a major role in spatial navigation. Lesions of the parasubiculum result in substantial performance impairments on tasks that assess spatial recognition

and spatial “working memory” (Jarrard et al., 2004; Kesner & Giles, 1998; P. Liu et al., 2001), and these findings suggest that the parasubiculum may be involved in the use and consolidation of spatial information. The parasubiculum receives afferents from both CA1 place cells and head direction cells located in the anterior thalamus, and project to layer II of the entorhinal cortex (Cenquizca & Swanson, 2007; van Groen & Wyss, 1990a, 1990b, 1995; Witter et al., 1989). Given the anatomical connections of the parasubiculum, it is likely that the parasubiculum receives both locational and directional information, and may serve to integrate these spatial inputs. Moreover, the strong connection of the parasubiculum with the projection neurons of the entorhinal cortex suggest that deficits in spatial memory after lesions of the parasubiculum may result in part from a disruption of parasubicular input to entorhinal cortex neurons.

Mnemonic processes of the parahippocampal region may be mediated, in part, via intrinsic cellular mechanisms that allow for maintained activity in local networks, and cells in the entorhinal cortex show persistent firing activity during the delay phase of delayed non-match to sample memory tasks in rats (Young, Otto, Fox, & Eichenbaum, 1997), monkeys (Suzuki, Miller, & Desimone, 1997) and humans (Schon et al., 2005). This suggests that persistent firing may serve as a mechanism underlying short-term working memory function (Fuster, 1997). Cholinergic tone is known to play an important role in mnemonic processes (Hasselmo, 2006), and principal cells in the entorhinal cortex show the development of persistent firing and plateau potentials in response to a suprathreshold stimulus pulse in the presence of cholinergic muscarinic receptor

activation (Egorov, Hamam, Fransen, Hasselmo, & Alonso, 2002). The emergence of this firing pattern relies on cholinergic receptor activation as well as activity-dependent changes in a Ca^{2+}-dependent cationic current, I_{CAN} (Egorov et al., 2002; Tahvildari, Fransen, Alonso, & Hasselmo, 2007; Yoshida & Hasselmo, 2009). Cells in the parasubiculum fire in a location-specific manner during theta-related behaviors (Boccara et al., 2010), and cholinergic receptor activation is likely to contribute to the formation of location-specific spatial firing fields via the induction of persistent cell activity (Yoshida & Hasselmo, 2009). Therefore, increases in cholinergic tone during theta activity may result in the induction of persistent activity in parasubicular neurons, and this may serve as a crucial cellular mechanism underlying the generation of spatial maps within the parasubiculum, and as such, may contribute to the spatial and mnemonic processes associated with the hippocampal formation. However, persistent firing activity has not been investigated in parasubicular neurons in vitro.

Initial studies on the firing activity of location-sensitive parasubicular cells demonstrated that nearly half of all recorded cells showed properties akin to hippocampal place cells (Taube, 1995b). However, further work indicated that the parasubiculum also houses a subset of cells that respond to both locational and directional information, and share similarities with grid and conjunctive cells found in the medial entorhinal cortex (Boccara et al., 2010; Cacucci et al., 2004). Accordingly, it is likely that the cell activity reported by Taube (1995b) reflected the activity of these multiple types of location-sensitive parasubicular neurons, but that the large locational firing fields characteristic of grid cells were not

observed due to the relatively small size of the testing apparatus used in previous studies. This suggests that unit activity of both place and grid cells in the parasubiculum shows discharges in consistent relation to local theta EEG activity.

In both the hippocampus and the entorhinal cortex, theta activity is thought to regulate the firing of location-specific cells, and this firing pattern may mediate aspects of spatial navigation and memory consolidation (Hafting et al., 2008; Hafting et al., 2005; Hasselmo et al., 2009; Maurer, Cowen, Burke, Barnes, & McNaughton, 2006b; O'Keefe & Recce, 1993). Place and grid cells fire in relation to specific phases of the extracellular theta rhythm, and as an animal runs through a place field, the discharge of a specific place or grid cell advances to a progressively earlier phase of the theta cycle as the animal moves through the neuron's receptive field, effectively discharging at a slightly elevated frequency than local theta rhythm (O'Keefe & Recce, 1993; Skaggs et al., 1996). These phase precession effects are observed in hippocampal and entorhinal location-sensitive neurons but, interestingly, are absent in parasubicular neurons (C. Boccara, personal communication). Phase precession effects are thought to arise from spatiotemporal activation patterns between place cells as the animal passes through adjacent receptive fields which, through complex spatiotemporal synaptic interactions between place cells associated with adjacent receptive fields, may serve to encode trajectory-based information. When an animal passes from one receptive field to another, the activity of the first place cell can promote firing of the second neuron such that the phase of firing of the second

neuron is advanced over subsequent cycles of the local theta wave. This may result in a complex spatiotemporal pattern of activity among place cells that is dependent upon synaptic interactions between neurons with adjacent receptive fields, similar to a Hebbian cell assembly or phase sequence (Hafting et al., 2008; Hebb, 1949; Molter & Yamaguchi, 2008; O'Keefe & Recce, 1993; Skaggs et al., 1996). As such, phase precession may reflect temporal information about previous, current, and future locations through the interactions of "assemblies" of place cells. Recordings have demonstrated cell activity that reflects a compressed "replay" of sequences of consecutively activated location-specific cell firing during slow-wave sleep (A. K. Lee & Wilson, 2002; Nadasdy, Hirase, Czurko, Csicsvari, & Buzsaki, 1999) as well as during alert immobility (Foster & Wilson, 2006) and this has also been taken to suggest that sequential activation among assemblies of place cells may represent a mechanism by which memory traces are encoded. Further, the timescale of phase precession across assemblies is consistent with temporal coordination associated with spike-timing-dependent plasticity (Dan & Poo, 2004; Xu et al., 2006), and therefore, it is likely that mechanisms associated with spike-timing dependent plasticity may contribute to the formation of cell assemblies among place cells that code for the representation of trajectories in the environment, (Dragoi & Buzsaki, 2006; Harris et al., 2003; Huxter, Senior, Allen, & Csicsvari, 2008; Maurer et al., 2006a). Since parasubicular neurons fail to show phase precession, it is possible that these cells do not form complex mnemonic representations of trajectories, but rather may serve as a simple integrator of place and directional information, and

in turn, relay this information with high fidelity to upstream areas in the hippocampal formation. Although the extent of the role that the parasubiculum plays in memory function is unclear, it is nevertheless likely that the parasubiculum has an important role in modulating ongoing processing of spatial information in the entorhinal cortex and hippocampus, and in addition contributes the processes associated with memory formation and consolidation in these upstream structures. Clearly though, investigations into the nature of long-term synaptic potentiation and depression effects within the parasubiculum are needed to further investigate synaptic mechanisms of memory within this structure.

The parasubiculum is known to powerfully modulate the responsivity of the entorhinal cortex to synaptic inputs from the piriform cortex, indicating that the parasubiculum can play a strong role in either enhancing or suppressing incoming sensory information (Caruana & Chapman, 2004). Because activity in the parasubiculum can likely modify how the entorhinal cortex reacts to inputs from downstream structures such as the piriform cortex and other sensory regions, parasubicular theta activity is likely to play a major role in coordinating the timing of synaptic activity in entorhinal projection neurons to upstream hippocampal structures. The parasubiculum modulates responses in the entorhinal cortex to piriform cortex stimulation in a time-dependent manner, and stimulation of the parasubiculum between 20 ms and 100 ms prior to stimulation of the piriform cortex can result in an enhancement of EPSPs in the entorhinal cortex (Caruana & Chapman, 2004). The intervals where peak facilitation effects

were observed correspond to gamma- and theta-frequencies, and theta activity in the superficial layers of the parasubiculum is likely phase-locked with theta in the entorhinal cortex (Glasgow & Chapman, 2007). This close synchronization between the parasubiculum and entorhinal cortex may serve to regulate how the entorhinal cortex responds to sensory inputs in relation to inputs received from the parasubiculum. Taken together, network synchronization is likely to play a major role in governing parasubicular cell activity, and may play a crucial role in shaping interactions between the parasubiculum and the entorhinal cortex.

Acetylcholine and plasticity in the parasubiculum

There is a substantial literature that suggests that theta-frequency activity may contribute to induction of activity-dependent long-lasting changes in synaptic transmission, indicating that theta activity in the hippocampal formation could serve as a mechanism contributing to the formation of new memories. Long-term potentiation (LTP) and depression (LTD) are thought to reflect neurophysiological substrates for learning and memory in the brain (Bliss & Collingridge, 1993), and both forms of plasticity have been enhanced in various brain areas by theta activity or theta-like timing of synaptic activation (Larson et al., 1986). Further, the phase of theta is important in the coordination of synaptic inputs, as EPSPs arriving at the peak of theta can lead to LTP, whereas inputs arriving at the trough of theta can result in depression of a pathway (Huerta & Lisman, 1995; Hyman et al., 2003). It is not yet clear, however, how theta-frequency activity in

the parasubiculum may affect the induction of long-lasting changes in synaptic strength.

Theta-frequency EEG activity is associated with increases in levels of acetylcholine in the hippocampus (Monmaur, Collet, Puma, Frankel-Kohn, & Sharif, 1997), and cholinergic neurons are critical for proper memory function. Lesions of septal cholinergic neurons results in significant deficits on navigational tasks (Brandner & Schenk, 1998), and neural grafts of septal cholinergic neurons result in an enhancement of spatial learning (Gage & Bjorklund, 1986), suggesting a crucial role for cholinergic tone in learning and memory processes. In the hippocampus, cholinergic agonists enhance LTP observed in the dentate gyrus and CA1 (Blitzer, Gil, & Landau, 1990; Burgard & Sarvey, 1990; Huerta & Lisman, 1993, 1996), and septal stimulation can also result in the facilitation of synaptic responses in both the dentate gyrus (Fantie & Goddard, 1982) and entorhinal cortex (Chapman & Racine, 1997). Activation of M_1 receptors, as observed here, is known to lead to increases in intracellular Ca^{2+} via an IP_3-dependent pathway (Berridge, 1993), and increases in intracellular Ca^{2+} are necessary for the induction of LTP. This suggests that rises in intracellular Ca^{2+} during cholinergic receptor activation could lead to an enhancement LTP in the parasubiculum. Therefore, increases in cholinergic tone during theta activity may result in an enhancement of synaptic responses, and may contribute to the induction of plastic changes in the hippocampal formation associated with memory function. In a recent study, Issac *et al.* (2009) demonstrated that increases in cholinergic tone during theta-related behaviours may be important

for the maintenance of place cell representations. As mentioned previously, many CA1 pyramidal cells show location-specific firing, and these place fields typically overlap with the other place cells. Cells with overlapping place fields show coincidental cell firing, suggesting that activity-dependent synaptic strengthening between such cells may serve to link them into a network that may represent an area of the environment. Using *in vitro* recording techniques, the time series of action potentials recorded from place cells with overlapping receptive fields was replayed to provide a time-varying pre- and postsynaptic stimulus delivered via postsynaptic current injection and stimulation of presynaptic inputs in CA1 pyramidal neurons. Replay of the firing activity of two place cells with overlapping firing fields was not sufficient to induce any LTP. However, place cells are active when an animal is engaged in active exploration, when hippocampal EEG is characterized by theta-frequency oscillations when cholinergic inputs from the septum are active (Bland, 1986, 2000; E. J. Green, McNaughton, & Barnes, 1990; Jeffery et al., 1995; O'Keefe & Nadel, 1978; Stumpf et al., 1962), and the replay of place cell activity during restoration of cholinergic tone with CCh resulted in the strong induction of NMDAR-dependent LTP, indicating that cholinergic receptor activation can play a permissive role in the strengthening of synaptic connections between place cells. Moreover, these findings suggest that cholinergic tone may contribute to the induction of LTP (Auerbach & Segal, 1996; Blitzer et al., 1990; Carr & Surmeier, 2007; Cheong et al., 2001; Giocomo & Hasselmo, 2007; Huerta & Lisman, 1993, 1995, 1996; Isaac, Buchanan, Muller, & Mellor, 2009; Patil, Linster, Lubenov, & Hasselmo,

1998; Yun et al., 2000). It is also possible that acetylcholine may play an important role in strengthening synaptic connections between layer II parasubicular grid cells with overlapping firing fields, however because parasubicular neurons fail to show phase precession, it is possible that parasubicular networks are resistant to plastic changes, and therefore more experiments are required to test this idea. In addition to shaping synaptic interactions between parasubicular neurons, it is also possible that increases in cholinergic tone may modulate the responsiveness of parasubicular neurons to inputs from the CA1 and anterior thalamus, and may contribute to the possible role of these inputs in the formation and maintenance of spatial representations. Thus, increases in cholinergic tone in the parasubiculum may influence the induction of long-lasting plastic changes in hippocampal and thalamic inputs.

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