Modulation of Synaptic Responses in the Entorhinal Cortex Evoked by Repetitive Stimulation of the Parasubiculum

Daniel W Sparks

A Thesis
In the Department
of
Psychology

Presented in Partial Fulfillment of the Requirements
For the Degree of
Doctor of Philosophy (Psychology) at
Concordia University
Montréal, Québec, Canada

June 22, 2016

CONCORDIA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

This is to certify that the thesis prepared

By: Daniel Sparks

Entitled: Modulation of Synaptic Responses in the Entorhinal Cortex Evoked by Repetitive Stimulation of the Parasubiculum

and submitted in partial fulfillment of the requirements for the degree of

complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the final examining committee:

Dr. Patti Ranahan — Chair

Dr. Jesper Sjostrom — External Examiner

Dr. Richard Courtemanche — External to Program

Dr. Wayne Brake — Examiner

Dr. Mihaela Iordanova — Examiner

Dr. Andrew Chapman — Thesis Supervisor

Approved by

Chair of Department or Graduate Program Director

Dean of Faculty

ABSTRACT

Modulation of Synaptic Responses in the Entorhinal Cortex Evoked by Repetitive Stimulation of the Parasubiculum

Daniel W Sparks, PhD
Concordia University, 2016

The entorhinal cortex provides the hippocampus with the majority of its sensory input. The entorhinal cortex also receives the largest output projection of the parasubiculum, a structure that receives output projections from the hippocampus. The parasubicular projection to the entorhinal cortex could play a critical role in determining how the entorhinal cortex responds to incoming sensory inputs. Therefore, alterations in how the entorhinal cortex responds to and processes sensory input, influenced by its projection from the parasubiculum, could be important for mnemonic processing the hippocampus. Neural activity in brain regions throughout the hippocampal formation, including the parasubiculum and entorhinal cortex, is heavily modulated by the neurotransmitter acetylcholine and the associated theta- and gamma-frequency oscillatory EEG rhythms. However, it is not known how cholinergic activity and synaptic inputs at these frequencies affect communication between the parasubiculum and entorhinal cortex, and how this could influence the processing of sensory inputs within the entorhinal cortex.

The first experiments in this thesis utilized field potential recordings of excitatory postsynaptic potentials (fEPSPs), and found that activation of cholinergic receptors, despite reducing the amplitude of responses to single pulses of stimulation, facilitated entorhinal cortex responses during trains of stimulation of the parasubiculum at both theta- and gamma-frequencies. This effect was found to be reliant upon M_1 muscarinic receptors and was associated with cholinergic reductions in the cationic conductance I_h. In a second series of experiments, intracellular recordings of EPSPs in stellate cells in layer II of the medial entorhinal cortex replicated the findings obtained in field potential recordings, and indicated that the facilitation of train-evoked responses at theta- and gamma-frequencies by cholinergic agonism is due in part to reductions in I_h that lead to increased input resistance and widening of EPSPs. In a third series of experiments, when a second stimulating electrode was placed in layer I of the medial entorhinal cortex to activate sensory input pathways, it was found that trains of stimulation in the

parasubiculum at theta-frequency modulated the strength of subsequent entorhinal cortex responses to incoming layer I sensory inputs. Stimulation of the parasubiculum was found to either suppress or facilitate responses to layer I inputs depending on the interval between parasubicular and layer I stimulation, suggesting that the parasubiculum can influence the ongoing processing of sensory inputs by the entorhinal cortex. When prolonged, repetitive, delivery of theta-frequency parasubicular stimulation trains was paired with single pulses of stimulation of layer I at short intervals after each train, it was found that co-activation of these inputs pathways resulted in a selective and lasting depression of entorhinal cortex responses to layer I stimulation. These results indicate that activation of the parasubiculum can have a lasting impact on how the entorhinal cortex responds to sensory inputs. Overall these results have strong implications for how information is processed in the hippocampal formation, and suggest that parasubicular inputs to the entorhinal cortex can influence the nature of the sensory and associational information that the entorhinal cortex provides to the hippocampus.

ACKNOWLEDGEMENTS

This thesis is the culmination of almost six years of graduate school, following six years of undergraduate work before that and all my high school and elementary school, and it could not have possibly done it without the help of all of my teachers and peers along the way. Specifically, I would like to thank my thesis supervisor Dr. Andrew Chapman, without whose extensive guidance and help in learning electrophysiology, and all of the other skills that have allowed me to grow as a researcher and student, this thesis would not have been possible. I would also like to thank my thesis committee members Drs. Wayne Brake, Mihaela Iordanova, and Richard Courtemanche for their help and guidance in finishing my degree, Drs. Nadia Chaudhri and Peter Shizgal for expanding my breadth as a researcher through collaborations at Concordia, and Dr. Hugo Lehmann, my undergraduate thesis supervisor, without whom I may never have been able to develop my interest in neuroscience and reach graduate school. I would also like to thank current and former fellow Chapman lab members Iulia Glovaci, Dr. Stephen Glasgow, and Dr. Shawnna Barrett for being great colleagues and helping out in the lab whenever I needed it. I would also like to thank my parents Ron and Susan Sparks, and my sister Jenn Sparks, for always supporting my academic endeavours, and my wonderful fiancée Whitney Agnew, who moved with me to Montreal so that I could pursue my dream of obtaining a PhD in neuroscience and has been my greatest supporter. Thank you everyone!

This research was supported by Concordia graduate fellowships from David J. Azrieli and Dominic D'Alessandro, and grants to Dr. Andrew Chapman from the Natural Sciences and Engineering Research Council of Canada.

CONTRIBUTION OF AUTHORS

Chapter 2: Cholinergic receptor activation induces a relative facilitation of synaptic responses in the entorhinal cortex during theta- and gamma-frequency stimulation of parasubicular inputs by Sparks and Chapman.

- **Daniel Sparks:** Designed the experiments, performed electrophysiological recordings, analyzed the data, performed statistics and created the figures, and wrote the manuscript for publication.
- **Andrew Chapman:** Assisted in designing the experiments and writing the manuscript.

Chapter 3: Contribution of I_h to the relative facilitation of synaptic responses induced by carbachol in the entorhinal cortex during repetitive stimulation of the parasubiculum by Sparks and Chapman.

- **Daniel Sparks:** Designed the experiments, performed electrophysiological recordings, analyzed the data, performed statistics and created the figures, and wrote the manuscript for publication.
- **Andrew Chapman:** Assisted in designing the experiments and writing the manuscript.

Chapter 4: Heterosynaptic modulation of evoked synaptic potentials in layer II of the entorhinal cortex by activation of the parasubiculum by Sparks and Chapman.

- **Daniel Sparks:** Designed the experiments, performed electrophysiological recordings, analyzed the data, performed statistics and created the figures, and wrote the manuscript for publication.
- **Andrew Chapman:** Assisted in designing the experiments and writing the manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
APV	2-amino-5-phosphonovaleric acid
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
CCh	carbachol
CREB	cAMP response element-binding protein
Cs^{+}	cesium
EEG	electroencephalographic
EPSC	excitatory postsynaptic current
EPSP	excitatory postsynaptic potential
fEPSP	field excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
I_h	hyperpolarization-activated nonspecific cation current
IK_{ir}	inward-rectifying potassium current
IK_{leak}	leak potassium current
I_M	muscarine-sensitive potassium current
I_{NaP}	persistent sodium current
IP_3	inositol trisphosphate
IPSC	inhibitory postsynaptic current
IPSP	inhibitory postsynaptic potential
K^{+}	potassium
LTD	long-term depression
LTP	long-term potentiation
Mg^{2+}	magnesium
Na^{+}	sodium
NMDA	N-methyl-D-aspartate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PIP_2	phosphatidylinositol 4,5-bisphosphate
R_{in}	input resistance
SEM	standard error of the mean

CHAPTER 1

GENERAL INTRODUCTION

GENERAL OVERVIEW

The ability to acquire and recall information successfully is critically important for normal cognitive processes that influence behaviour in both humans and animals, as being able to learn and remember information enables organisms to successfully navigate and interact with their environment. Researching the neural basis of these learning and memory processes is essential to understanding how these systems normally function, and could be of great importance in treating disorders that affect learning and memory processes such as dementia and Alzheimer's disease.

Some of the earliest research investigating the neural correlates of learning and memory focused on the hippocampus, a brain structure located in the medial temporal lobe of the brain, so named because of its resemblance to a seahorse. The seminal study of patient HM, who received bilateral lesions of his hippocampus as a treatment for medial temporal lobe epilepsy, demonstrated the importance of the hippocampus in learning and memory processes, because after surgery HM was unable to form or recall new memories (Scoville and Milner, 1957). Subsequent studies in both humans and animals have confirmed an essential role for the hippocampus in learning and memory (Kesner, 1973; Squire and Zola-Morgan, 1991; Thompson, 1976; Zola-Morgan and Squire, 1990), and have investigated cellular mechanisms of memory formation, including the permanent strengthening of synaptic connections between neurons using experimental techniques and models such as long-term potentiation (Bliss and Lomo, 1973). Research has also focused on brain regions outside of the hippocampus, including the parahippocampal cortices, a series of brain structures surrounding the hippocampus that serve to provide the hippocampus with sensory input, as well as communicating information processed in the hippocampus to the rest of the cortex (Burwell, 2000; Squire et al., 2004; van Hoesen, 1995; a, 2000). Indeed, closer examination of many studies looking at the neural correlates of learning and memory have revealed that the hippocampus is not solely responsible for these processes, but in fact parahippocampal structures are essential for learning and memory as well (Rempel-Clower et al., 1996; Zola-Morgan et al., 1986). Two brains regions of particular interest and potential importance in the parahippocampal cortex that could play a large role in learning and memory processes are the entorhinal cortex and parasubiculum.

The entorhinal cortex plays a prominent role in the memory network of the hippocampal formation, receiving dense sensory inputs to its superficial layers (layers I-III) from a large

number of cortical and subcortical brain areas (Andersen et al., 1973; Burwell, 2000; Cenquizca and Swanson, 2007; Kerr et al., 2007; Kohler, 1985,1986; Sorensen and Shipley, 1979; Swanson and Cowan, 1977; Witter, 1993; Witter et al., 1989, 2000a), processing this information and sending its outputs to the hippocampus through projections from layers II/III to all of the hippocampal subfields (Amaral, 1993; Burwell et al., 1995; Witter et al., 1989, 2000a). Entorhinal cortex layer II also receives the major output projection of the parasubiculum (Caballero-Bleda and Witter, 1993, 1994; Caruana and Chapman, 2004; van Groen and Wyss, 1990), a structure in the subicular complex which receives projections from a number of cortical and subcortical areas including the CA1 region of the hippocampus, the subiculum, and anterior thalamus (Cenquizca and Swanson, 2007; Swanson and Cohen, 1977; van Groen and Wyss, 1990a and b; Witter et al., 1989). This pattern of connections puts the parasubiculum in the unique position to use information from the hippocampus to modulate how the entorhinal cortex responds to incoming sensory input (Caruana and Chapman, 2004). Together, the connections of these two brain areas provide a possible mechanism for how feedback information from the hippocampus can influence the integration of incoming sensory inputs that arrive in the entorhinal cortex during learning and memory processing.

An important phenomenon observed when measuring neural activity in the hippocampal formation is the presence of oscillatory brain activity during theta (4-12 Hz) and gamma (30-80 Hz) rhythms when an animal is actively exploring its environment (Buzsaki and Draguhn, 2004; Colgin, 2015; Hasselmo et al., 2002; Mitchell and Ranck, 1980). These oscillatory brain rhythms have been associated with successful memory acquisition and expression in both humans and animals (Hasselmo and Stern, 2014). For example, increased theta power during memory acquisition is associated with improved memory recall in human neural imaging experiments (Hsieh and Ranganath, 2013), and the induction of long-term changes in synaptic strength that are associated with neural mechanisms of memory acquisition in animal studies is enhanced by synaptic inputs being stimulated at precise phases of the theta rhythm (Hasselmo et al., 2002). The generation of these rhythms depends on release of the neurotransmitter acetylcholine (Buzsaki, 2002), with increases in cholinergic activity itself also associated with successful performance in learning and memory tasks (Hasselmo, 2006). On a neurophysiological level, rhythmic brain activity associated with increases in acetylcholine causes lasting changes in synaptic strength between structures in the hippocampal formation by making synapses more

susceptible to plasticity induction and coordinating bursts of excitatory cellular activity (Drever et al., 2011; Jerusalinsky et al., 1997).

The projection from the parasubiculum to the entorhinal cortex has been relatively unstudied compared to the rich literature investigating the physiological mechanisms behind synaptic transmission and plastic changes in the hippocampus itself. Therefore, the goal of the current thesis was to investigate the role of acetylcholine and oscillatory brain rhythms in influencing changes in synaptic communication and strength in the parasubicular-entorhinal pathway, as well as investigating how these factors could directly influence how the entorhinal cortex responds to information from its sensory inputs. Investigating these mechanisms could shed light upon how neurotransmitter systems and oscillatory brain rhythms influence the integration of synaptic inputs in the hippocampal formation, and how these factors could have a lasting influence on how the entorhinal cortex processes sensory inputs. These experiments were accomplished using field potential and single-cell recordings of synaptic responses in neurons in layer II of the medial entorhinal cortex evoked in response to stimulation of the parasubiculum and layer I entorhinal cortex, and assessed how synaptic communication in these pathways was modulated by rhythmic neuronal stimulation in the presence of acetylcholine. The following sections of the general introduction review the anatomy and behavioural functions of the entorhinal cortex and parasubiculum, the role that oscillatory brain activity and acetylcholine play in modulating neuronal activity in the hippocampal formation, and how both short and long-term changes in synaptic strength can be influenced by these factors.

Anatomy and Function of the Entorhinal Cortex and Parasubiculum

Located in the medial temporal lobe, posterior to the rest of the hippocampal formation, the entorhinal cortex can be thought of as a transition area between the neocortex and allocortex. Allocortical regions like the hippocampus are three-layered structures that contain a single cell layer with the other two layers reserved for dendritic and axonal processes (Amaral and Witter, 1989). The entorhinal cortex, like much of the rest of the cortex, is a six-layered structure (Insausti et al., 1997), making its laminar organization more reminiscent of neocortical areas. Unlike the neocortex, however, where input projections arrive through the superficial layers, and the deep layers contain the main projection neurons (Douglas, 1967; Forster et al., 2006, Witter et al., 1989), the entorhinal cortex has its main projection neurons located in superficial layers II

and III, with the deep layers integrating inputs from the hippocampus and sending feedback projections to extra-hippocampal cortical areas and the superficial entorhinal cortex (Insausti et al., 1997). The entorhinal cortex also contains the lamina dissecans, or cell-free layer, in layer IV, a laminar feature not usually found in the neocortex, illustrating its architectural uniqueness as a neural structure with characteristics of both the allo and neocortices (Witter et al., 1989). The parasubiculum, also a structure in the hippocampal formation, is located posterior of the hippocampal CA1 region and subiculum, and medial and anterior of the entorhinal cortex (Blackstad, 1956; Kerr, Agster, Furtak, and Burwell, 2007; Witter et al., 1989). Similar to the entorhinal cortex, the parasubiculum resembles the six-layered structure of the neocortex, but with its major projection neurons found in the superficial rather than deep layers (van Groen and Wyss, 1990a; Witter et al., 1989).

While it was initially believed that the entorhinal cortex served largely as a passive relay for information reaching the hippocampus (Douglas, 1967; Raisman et al., 1966), it has since been shown that the entorhinal cortex contains strong afferent and efferent connections to and from the hippocampus as well as to other cortical and subcortical areas (Andersen, Bland, and Dudar, 1973; Burwell, 2000; Cenquizca and Swanson, 2007; Kohler, 1985, 1986; Sorensen and Shipley, 1979; Swanson and Cowan, 1977; Witter et al., 1989; Kerr et al., 2007; Witter, 1993; Witter et al., 2000a). This puts the entorhinal cortex in the important position to integrate and process sensory input to the hippocampus, as well as to receive feedback projections from the hippocampus, essentially making the entorhinal cortex the main locus of information exchange between the hippocampus and the rest of the neocortex (Knierim et al., 2013; Witter et al., 2000a).

The entorhinal cortex has traditionally been divided into two main regions: the medial and lateral entorhinal cortices (Room and Groenwegen, 1986; Lopes da Silva et al., 1990; Burwell et al., 1995; Burwell and Amaral, 1998). The lateral entorhinal cortex is thought to be responsible for processing and providing the hippocampus with information related to individual objects and the content of the environment, such as the location of a specific landmark (i.e. information related to the external environment) (Burwell, 2000; Knierim et al., 2006; Eichenbaum et al., 2012; Knierim et al., 2013). The medial entorhinal cortex, on the other hand, is thought to contribute to more global and contextual egocentric information, such as knowing the position of one's self in an environment, and other information related to spatial memory

processing (i.e. information related to the self) (Burwell, 2000; Knierim et al., 2006; Eichenbaum et al., 2012; Knierim et al., 2013). The medial entorhinal cortex is also thought to be involved in processing the temporal aspects of information, supported by the fact that activity in this area is heavily modulated by theta rhythm EEG activity, which can act to give temporal order to incoming information (Knierim, 2015). Combining information from the medial and lateral divisions of the entorhinal cortex together provides the hippocampus with much of the information required for memory formation.

The information that the entorhinal cortex provides to the hippocampus arrives through inputs from the piriform, perirhinal, and postrhinal cortices, which carry highly processed multimodal sensory information to the entorhinal cortex (Witter et al., 1989, 2000a). The lateral entorhinal cortex receives the majority of the perirhinal cortex inputs, which contains quite mixed sensory information, while the medial entorhinal cortex receives the majority of its inputs from the postrhinal and piriform cortices, which are dominated by visual and olfactory information (Burwell et al., 1995; Burwell and Amaral, 1998; Burwell, 2000; Furtak et al., 2007; Kerr et al., 2007; Witter et al., 2000a). All of these inputs target cells in layers II and III of the entorhinal cortex, usually by synaptic connections on the dendrites of cells in these layers that extend into layer I, where the sensory afferents arrive in the entorhinal cortex (Canto et al., 2008). The entorhinal cortex also receives more direct sensory inputs from unimodal and multimodal association cortices that contain similar information as that provided by the inputs from the perirhinal and postrhinal cortices, although these projections are less strong than the projections from adjacent parahippocampal areas (Burwell, 2000).

Neurons within the superficial layers of the entorhinal cortex project to the hippocampus through a number of pathways, and neurons in the deep layers of the entorhinal cortex receive projections from the hippocampus. The two main pathways from the entorhinal cortex to the hippocampal formation are the perforant path projection from layer II of the entorhinal cortex to the dentate gyrus and the CA3 region of the hippocampus, and the temporoammonic pathway from layer III of the entorhinal cortex to the CA1 region and the subiculum (Craig and McBain, 2015; Kohler, 1986; Witter et al., 1989; Witter et al., 2000b). The major series of connections within the hippocampus itself form the trisynaptic pathway, with strong connections from the dentate gyrus to CA3, from CA3 to CA1, and CA1 to the subiculum. The hippocampus then projects back to the deep layers V and VI of the entorhinal cortex through projections from the

CA1 region and the subiculum (Richter et al., 1999; Witter et al., 2000b). The deep layers of the entorhinal cortex in turn project to cortical and subcortical targets, providing the major output of the hippocampal formation to the rest of the brain (Canto et al., 2008; Rolls, 2013; Swanson and Cohen, 1977; Takehara-Nischiuchi, 2014).

In addition to the dense sensory and associational inputs that it receives, layer II of the entorhinal cortex is also the target of the single major output projection of the parasubiculum, a structure in the subicular formation (Caballero-Bleda and Witter, 1993; Caruana and Chapman, 2004; Kerr et al., 2007; van Groen and Wyss, 1990a). The parasubiculum itself receives projections from area CA1 of the hippocampus, the subiculum, the anterior thalamus, amygdala, mammillary complex, septum, postsubiculum, and retrosplenial cortex (Swanson and Cohen, 1977), and aside from its major projection to the superficial layers of the entorhinal cortex, also sends minor projections to the subiculum, dentate gyrus, CA3 and CA1 region of the hippocampus, and the deep layers of the entorhinal cortex (Andersen et al, 1966a; Kohler, 1986; van Groen and Wyss, 1990a; Witter et al., 1989; van Groen and Wyss, 1990b).

The main parasubicular projection to the entorhinal cortex originates in the superficial layers of the parasubiculum and terminates in layer II of the medial and lateral entorhinal cortex, where it targets interneurons as well as principal cells, innervating both dendrites and cell bodies, and thus providing input onto the neurons that give rise to the perforant path (Caballero-Bleda and Witter, 1994; Ino et al., 2001; Jones and Buhl, 1993; Kohler, 1985). This series of connections between the entorhinal cortex, hippocampus, and parasubiculum, creates a complete circuit of synaptic connections that begins and ends with the superficial layers of the entorhinal cortex (Figure 1.1). These connections provide a means for the hippocampus, through the parasubiculum, to directly influence how the entorhinal cortex responds to and integrates sensory input. This mechanism could influence information processing in the hippocampal formation during active behaviour, perhaps by comparing previously acquired information with current sensory input. Due to the purported role of the medial entorhinal cortex in spatial navigation and memory processes that are governed by rhythmic EEG activity, the rest of this thesis focuses specifically on information related to the medial entorhinal cortex, unless stated otherwise.

Laminar Organization and Cellular Properties of the Entorhinal Cortex and Parasubiculum

The laminar organization of the entorhinal cortex suggests general functions for the cell layers. Layer I of the entorhinal cortex contains only a sparse number of neurons, and primarily contains the axonal projections from sensory and associational inputs arriving in the entorhinal cortex, as well as dendrites of cells from other entorhinal cortex layers, making layer I the locus of many incoming synaptic connections (Caballero-Bleda and Witter, 1993; Insausti et al., 1997). Layer II of the entorhinal cortex consists of two main types of principal neurons: stellate cells, named due to the star-like shape created by their dendritic processes, and pyramidal cells, named for the triangular shape of their somata (Alonso and Klink, 1993; Canto and Witter, 2012; Klink and Alonso, 1993). These layer II principal cells are often grouped into collections of cells referred to as cell islands, with groups of principal cells packed tightly together, with pyramidal cells tending to be located near the border with layer I, while stellate cells are more evenly distributed throughout layer II (Heys et al., 2012; Insausti et al., 1997; Kitamura et al., 2014; Ray et al., 2014; Sun et al., 2015). Stellate cells project to the dentate gyrus via the perforant path, while pyramidal cells target the CA1 region and the contralateral entorhinal cortex (Canto and Witter, 2012; Craig and McBain, 2015; Lingenhohl and Finch, 1991; Ray et al., 2014). Neurons of both principal cell types in layer II have local dendrites as well as dendrites that spread into layers I and III (Lingenhohl and Finch, 1991; Quilichini). Cells in Layer III of the entorhinal cortex are located close to the border with layer II, but can be distinguished by the densely-packed uniform distribution of pyramidal cells as opposed to the clusters found in layer II, with dendrites that spread superficially to layer I and with some processes that reach layer V, and axons that project to CA1 of the hippocampus and the subiculum (Heys et al., 2012; Lingenhohl and Finch, 1991). Following the thin, cell-free lamina dissecans of layer IV (Canto and Witter, 2012), the deep entorhinal cortex layers V and VI contain pyramidal cells with dendrites that spread within their own layers, as well as apical dendrites extending to layer I, and axons projecting within the entorhinal cortex as well as to numerous cortical and subcortical areas (Insausti et al., 1997; Lingenhohl and Finch, 1991; Quilichini et al., 2010).

The neuronal population within the entorhinal cortex that is perhaps the most interesting and extensively studied is the stellate cells of layer II of the medial entorhinal cortex. Stellate cells can be identified as large principal neurons characterized by their spiny dendritic trees and

multiple, equally sized dendrites that stretch over a larger area than pyramidal cells or interneurons in layer II (Alonso and Klink, 1993; Klink and Alonso, 1993; Canto and Witter, 2012). Their proximal dendrites stay within layer II, and distal dendrites extend into layers I and III, while their major axonal projection is sent to the dentate gyrus by perforating the subiculum (Klink and Alonso, 1993; Quilichini et al., 2010; Witter et al., 2000b).

Stellate cells can also be identified by the unique membrane potential properties that they show during electrophysiological recordings. Medial entorhinal layer II stellate cells have a resting membrane potential around -62 mV, and are characterized by having a lower input resistance at rest (< 40 MΩ) than principal cells in other layers of the entorhinal cortex. This is likely due to the influence of Na^+ and K^+ conductances that are partially activated at resting membrane potential, because it has been found that input resistance changes drastically with membrane potential in these neurons, decreasing with hyperpolarization, and increasing with depolarization (Alonso and Klink, 1993; Canto and Witter, 2012; Klink and Alonso, 1993; Quilichini et al., 2010). Stellate cells are also characterized by the presence of intrinsic voltage-dependent membrane potential oscillations at theta frequency (~8.5 Hz) that are initiated when stellate cells are depolarized to around -55 mV (Alonso and Klink, 1993). The idea that stellate cells preferentially processes information at theta-frequency is supported by the fact that layer II stellate cells show membrane potential resonance in response to oscillatory current injection, with an increase in the amplitude of resonant membrane potential oscillations during theta frequency oscillatory current injection (Heys et al., 2010; Tsuno et al., 2013). Subthreshold membrane potential oscillations are believed to occur due to an interaction between the hyperpolarization activated mixed cationic current I_h, and a persistent Na^+ current (I_{NaP}) (Dickson et al., 2000b; Quilichini et al., 2010), which likely are also the conductances responsible for the aforementioned low resting input resistance of these cells (Canto and Witter, 2012; Klink and Alonso, 1993). I_h and other voltage-activated currents sensitive to small changes in membrane potential are likely also responsible for the large inward rectification of membrane potential following hyperpolarizing current injection, and large outward rectification following depolarizing current injection (Canto and Witter, 2012; Dickson et al., 2000b; Klink and Alonso, 1993; Quilichini et al., 2010).

When depolarized by constant current injection to suprathreshold membrane potentials around -50 mV (Alonso and Klink, 1993), stellate cells are characterized by a bursting firing

pattern of action potentials at theta-frequency (Alonso and Klink, 1993; Canto and Witter, 2012; Fernandez et al., 2013). The bursting firing pattern is influenced by theta oscillatory activity that continues at suprathreshold membrane potentials, again suggesting that these cells preferentially respond to theta frequency inputs. Burst firing is influenced by a strong afterdepolarization that occurs after the cell fires an action potential, which helps bring the neuron back to suprathreshold membrane potential levels (Dickson et al., 2000b; Fernandez et al., 2013). However, action potentials in stellate cells also have a shorter duration, and are evoked at a more negative potential than other cell types in the medial entorhinal cortex. These action potential characteristics lead to spike frequency adaptation, where the frequency of action potential firing is reduced over time. Spike frequency adaptation is likely due to the influence of large afterhyperpolarizations following action potential firing, with spike frequency adaptation having the functional consequence of preventing overactivity of entorhinal neurons (Alonso and Klink, 1993; Dickson et al., 2000b; Klink and Alonso, 1993; Tsuno et al., 2013).

Similar to the entorhinal cortex, the parasubiculum contains six cell layers, reminiscent of the neocortex. Layer I of the parasubiculum contains few cells, and mostly contains dendrites and fibers of passage. Unlike the entorhinal cortex, however, the superficial layers of the parasubiculum do not display the same tightly packed clusters of neurons, and there is no obvious border between layers II and III (Funahashi and Stewart, 1997a, 1997b; Kohler, 1985). These superficial layers contain mostly pyramidal-like cells, but also have some stellate cells that are distinguishable by their dendritic arbor. However, membrane potential properties do not differ substantially between the two principal cell types (Funahashi and Stewart, 1997a; Kohler, 1985). Dendrites of both stellate and pyramidal cells are relatively confined to layer II, but also contain dendrites that spread to layer I, and also send their major axonal projection to innervate layer II of the medial and lateral entorhinal cortex (Funahashi and Stewart, 1997a; Kohler, 1985; van Groen and Wyss, 1990). The parasubiculum also contains a cell-free lamina dissecans in layer IV, and the deep layers of the parasubiculum, which contain pyramidal cells, contain weaker projections extending to the deep layers of the presubiculum and entorhinal cortex, as well as to the superficial layers of the parasubiculum (Funahashi and Stewart, 1997a, 1997b).

Principal cells in the superficial layers of the parasubiculum share many properties with layer II medial entorhinal cortex stellate cells, such as a similar resting membrane potential (~64 mV), inward and outward rectification in response to hyperpolarizing and depolarizing current

injection, respectively, but parasubicular neurons have much a higher input resistance than entorhinal stellate neurons (~ 115 MΩ (Funahashi 1997a, Glasgow 2007). Principal cells in the parasubiculum also show subthreshold membrane potential oscillations at theta frequency that rely on I_h and I_{NaP} (Glasgow and Chapman, 2008). However, the burst firing observed in entorhinal stellate cells is not found in principal cells in the parasubiculum (Funahashi 1997a, Glasgow 2007, 2008). Overall, the fact that principal cells in the superficial layers of the parasubiculum have similar properties to layer II entorhinal stellate cells in terms of intrinsic theta-freqeuncy membrane potential oscillations, suggests that these two cell populations may be similarly responsive to theta-frequency activation, which may promote synchronized synaptic communication between these areas.

Behavioural Functions of the Entorhinal Cortex and Parasubiculum

The importance of the entorhinal cortex in memory processes has been demonstrated by a large number of studies. Due to its role as the major input pathway for the hippocampus, a brain area that is itself heavily involved in spatial navigation and memory (e.g. Good, 2002; Morris et al., 1982; O'Keefe and Nadel, 1978; Squire, 2004), many studies have sought to demonstrate a role for the entorhinal cortex in the acquisition and expression of spatial memories. Early lesion studies seemed to confirm a critical role for the entorhinal cortex in memory function, with lesions to the entorhinal cortex leading to significant deficits in spatial memory processing (e.g. Schenk and Morris, 1985; Cho and Kesner, 1996). A common theme of these early studies, however, was that lesions often included surrounding areas of the hippocampal and parahippocampal cortices, such as the subicular complex, or portions of the hippocampus itself, and often employed electrolytic lesions that destroyed fibers of passage to the hippocampus in addition to entorhinal neurons. This lesion method cut the hippocampus off from all sources of sensory input and made it difficult to ascribe behavioural effects observed to only the entorhinal cortex. Even when lesions have been confined to the entorhinal cortex itself, or more specific excitotoxic lesions have been used, inconsistent effects have been found, with studies showing a lack of effect of entorhinal cortex damage on spatial memory performance (Jarrard et al., 2004; Parron et al., 2006). The lack of behavioural effects observed following entorhinal cortex lesions could be due to preservation of sensory projections that target the hippocampus directly (Naber et al., 1999; 2001), and illustrate the difficulty that has been encountered in determining the role of the entorhinal cortex in mnemonic processing in the hippocampal formation

However, more recent studies have suggested that the precise location of lesions in the entorhinal cortex could explain some of the variability in results that has been encountered in determining the role that the entorhinal cortex might play in memory processing. Lesions confined to the dorsal-medial portion of the entorhinal cortex, where grid cells (see below) have the most finely tuned spatial firing patterns, have resulted in consistent effects of entorhinal cortex damage on spatial memory performance (Barry and Bush, 2012; Witter and Moser, 2006). Lesions in this subregion of the entorhinal cortex result in spatial memory deficits, whereas lesions confined to the lateral entorhinal cortex result in consistent object-recognition deficits. This suggests that the entorhinal cortex is indeed involved in a variety of memory processes, and has functionally specialized subregions that determines if a memory deficit will be found, depending on the location of the lesion and the behavioural task employed (Burwell, 2000; Steffenach et al., 2005; Morrissey and Takehara-Nischiuchi, 2014). Overall, this evidence supports a critical role for the entorhinal cortex in memory processes, but with region-specific specialization for different types of memory.

The importance of the medial entorhinal cortex in spatial learning and memory has also been supported by studies investigating the functional firing patterns of entorhinal cortex neurons in awake animals. Perhaps the most important and groundbreaking of these was the study by Hafting et al. (2005) that identified the presence of grid cells in the medial entorhinal cortex, a type of neuron whose firing properties have lead researchers to believe that they are the same stellate cells that have been characterized in vitro (Craig and McBain, 2015; Tsuno et al., 2013). Similar to place cells, which are found most prominently in the hippocampus and fire when an animal is located in a certain spatial location in an environment (O'Keefe and Nadel, 1978), grid cells also fire in a way that is dependent on the animal's location in the environment, indicating that they are modulated by spatial location. Unlike place cells, however, which have a single locus of firing in an environment, grid cells fire at multiple, regularly spaced locations in an environment. Specifically, the firing field of a single grid cell forms a repetitive, hexagonal firing pattern that repeats throughout the environment when the firing rate of a neuron is compared to the physical location of the animal (Fyhn et al., 2004; Hafting et al., 2005). It has been suggested that this grid-like firing pattern could provide egocentric path integration information to the hippocampal formation's spatial navigation and memory system, with the regularly spaced firing of grid cells encoding the distance and direction in which an animal has travelled (Knierim et al.,

2013; Knierim, 2015). This can be contrasted with the allocentric type of information processed by hippocampal place cells that encode information for one specific location in an environment, without taking direction and distance travelled into account. The presence of these cells suggests a significant role for the entorhinal cortex in spatial navigation and memory (Barry and Bush; Moser et al 2008; Buzsaki and Moser, 2013).

Compared to the entorhinal cortex, the behavioural functions of the parasubiculum have been relatively unexplored, with a lack of behavioural studies looking at lesions confined solely to the parasubiculum. A few studies, however, have investigated the effects of combined lesions of either the parasubiculum and presubiculum, or the entire subicular complex. Lesioning the parasubiculum, along with the presubiculum, produces deficits in tasks that measure spatial, but not non-spatial, working memory (Jarrard et al., 2004; Kesner and Giles, 1998), suggesting that, like the entorhinal cortex, the parasubiculum plays an important role in normal spatial learning and memory processes.

It has also been suggested that parasubicular neurons process space and motion-related information, a claim that is supported by cellular recording studies that have found a variety of cells in the parasubiculum with firing patterns that are spatially modulated, including grid, head direction, border, and conjunctive cells (Boccara et al., 2010; Hargreaves et al., 2007). Lesioning the parasubiculum and presubiculum also disrupts place cell firing in the CA1 region, suggesting that the parasubiculum could provide the CA1 region with spatial information through connections with the entorhinal cortex (Knierim et al., 2013; Liu et al., 2004). Overall, this evidence suggests that the parasubiculum plays a major role in spatial navigation and memory systems in the brain through its interaction with other structures in the hippocampal formation.

Acetylcholine and Oscillatory Brain Activity in the Entorhinal Cortex and Parasubiculum

Acetylcholine is a modulatory neurotransmitter that plays an important role throughout the brain in processes related to attention and arousal, and is released in the hippocampal formation during behavioural mobility at times when the animal displays theta frequency EEG activity and is engaged in sensory and mnemonic processing (Bentley et al., 2011; Hasselmo and Sarter, 2011). Acetylcholine is necessary for proper declarative memory acquisition and consolidation (Bentley et al., 2011; Hasselmo, 2006; Picciotto et al., 2012). Behavioural tests in both humans and in animal models have shown that application of a cholinergic antagonist leads

to reduced performance during acquisition and consolidation phases on a variety of memory tasks, as well as decreased levels of neural activity in the hippocampal formation and other brain areas (Bentley et al., 2011; Blokland, 1996; Hasselmo and Sarter, 2011). As well, disruption of cholinergic circuitry by lesioning sources of cholinergic input to areas involved in learning and memory, for example the medial septum, a brain structure that provides the hippocampal formation with dense cholinergic input, has detrimental effects on performance on learning and memory tasks (Hasselmo and Sarter, 2011). The role of acetylcholine in acquiring and consolidating memories is further supported by evidence from studies using microdialysis that have shown increased acetylcholine levels during memory tasks (e.g. Pepeu and Giovannini, 2004). The importance of acetylcholine in normal learning and memory processes is also demonstrated by the fact that disruption or destruction of cholinergic neurons can be seen in the brains of Alzheimer's disease patients, and is thought to be a major cause of the neurological changes and behavioural deficits seen in this disease (Bentley et al., 2011). The role of acetylcholine in learning and memory processes can be explained by the myriad of effects it has on the cellular processes of neurons in the hippocampal formation involved in learning and memory.

Effects of Acetylcholine on Cellular Activity

Acetylcholine is found in high concentrations in both the entorhinal cortex (Mitchell et al., 1982) and parasubiculum (Witter et al., 2000a), and is found primarily in the superficial layers, which receive cholinergic projections from the medial septum (Alonso and Kohler, 1984; van Groen and Wyss, 1990a). Bath application of acetylcholine or cholinergic agonists causes depolarization of neurons throughout the hippocampal formation in the parasubiculum (Glasgow and Chapman, 2013), entorhinal cortex (Heys et al., 2012), subiculum, (Kawasaki et al., 1999) and hippocampus (Dutar and Nicoll, 1988). This indicates that acetylcholine strongly enhances neuronal excitability in the hippocampal area.

In entorhinal cortex stellate cells, cholinergic agonism depolarizes cells by ~4.5 mV through its actions on M_1 muscarinic acetylcholine receptors acting on Ca^{2+}-sensitive mixed cationic conductances (Heys et al., 2012; Klink and Alonso, 1997a; Magistretti et al., 2004). This cholinergic depolarization leads to the induction of voltage-dependent membrane potential oscillations at theta frequency that rely on the currents I_h and I_{NaP} (Dickson et al., 200b). These oscillations begin when the cell reaches a membrane potential higher than -60 mV, and reach

maximal amplitude around -55 mV (Klink and Alonso, 1997b). Cholinergically induced depolarization also leads to increased bursts of action potential firing at intervals that correspond to theta frequency (Klink and Alonso, 1997b). The bursting pattern of firing can be attributed to effects of acetylcholine on action potential properties including reduced action potential amplitude, eliminated action potential afterhyperpolarization, as well as increased afterdepolarizations (Heys et al., 2010; Klink and Alonso 1997b). This modulation of oscillatory activity and induction of bursts of action potentials reflects the relationship between acetylcholine and oscillatory brain rhythms in the entorhinal cortex.

In addition, acetylcholine increases the frequency of action potential firing in the entorhinal cortex, with firing continuing even after the cessation of depolarizing current injection (Klink and Alonso, 1997b). This induction of persistent firing in entorhinal neurons is thought to rely on muscarinic activation of a Ca^{2+} -sensitive cationic conductance. Further, it has been proposed as a possible cellular correlate of working memory, as the persistent and uninterrupted firing could serve to keep information online during the delay phase of a memory task, something necessary for successful behavioural performance related to working memory and for subsequent storage into long-term memory (Hasselmo, 2006; Heys et al., 2012; Magistretti et al., 2004).

In the parasubiculum, acetylcholine also depolarizes principal neurons through M_1 muscarinic receptors, but in this case, acetylcholine does so by acting on the K^+ current I_m and another, unidentified K^+ current (Glasgow and Chapman, 2012; 2013). This results in an increase in steady-state input resistance as well as a shortening of action potential amplitude, and an increase in action potential width (Glasgow and Chapman, 2013). Acetylcholine also reduces inward rectification in response to hyperpolarizing current injection, and initiates theta-frequency membrane potential oscillations in parasubicular cells through actions on currents I_h and I_{NaP} (Glasgow and Chapman, 2012). This suggests that parasubicular cells, like entorhinal stellate cells, are heavily influenced by conductances active at or near resting membrane potential (Glasgow and Chapman, 2013), and also demonstrates the excitatory influence of acetylcholine in the membrane potential of parasubicular neurons.

Cholinergic Effects on Synaptic Responses

Acetylcholine has a strong influence on synaptic communication throughout the hippocampal formation. Numerous studies in the entorhinal cortex (Barrett and Chapman, 2013;

Hamam et al., 2007; Richter et al., 1999; Yun et al., 2000), parasubiculum (Glasgow et al., 2012), striatum (Shen et al., 2007), subiculum (Kunitake et al., 2004), and hippocampus (Auerbach and Segal, 1996) have shown that application of a cholinergic agonist, despite depolarizing principal neurons, suppresses the amplitude of single excitatory postsynaptic potentials induced by electrical stimulation. These effects are likely due to acetylcholine causing a reduction of release of the neurotransmitter glutamate by activation of presynaptic muscarinic receptors on glutamatergic axon terminals (Auerbach and Segal, 1996; Dasari and Gulledge, 2011; Dutar and Nicoll, 1988). This is evidenced by increased paired-pulse facilitation ratios following activation of muscarinic receptors (Cheong et al., 2001; Glasgow et al., 2012; Hamam et al., 2007; Yun et al., 2000), a phenomenon in which the second synaptic response to a pair of pulses is larger than the first due to residual Ca^{2+} in the presynaptic terminal being available following the first pulse, which causes additional transmitter release in response to a second pulse (Yamada and Zucker, 1992). The increased paired pulse ratio following application of acetylcholine could result from acetylcholine reducing transmitter release in response to the first pulse, so that there is increased readily releasable neurotransmitter available for release following the second stimulation pulse.

Despite the reduction in the amplitude of single responses to synaptic stimulation following cholinergic receptor activation, acetylcholine can facilitate synaptic responses in the hippocampal formation in response to repetitive synaptic stimulation. Studies done in the subiculum (Kunitake et al., 2004), prefrontal cortex (Carr and Surmeier, 2007), and striatum (Shen et al., 2007) have shown that, relative to the first response in a train, later responses evoked during short trains of stimulation are enhanced in the presence of acetylcholine, rather than suppressed as individual synaptic responses are. This suggests that a preferential synaptic responsiveness for repetitively active synaptic inputs may occur during periods when acetylcholine is released, and this may function to "filter out" weaker, single synaptic inputs, while enhancing sustained repetitive synaptic input (Hsieh and Ranganath, 2012).

The effects of acetylcholine on repetitive synaptic communication can be explained in part by its actions on the current I_h. I_h is a non-specific cationic current that is unique in that it is activated in response to hyperpolarization, resulting in a large, excitatory inward current, and it also plays a large role in setting the resting membrane potential (Luthi and McCormick, 1998; Nolan et al., 2007). I_h is found in numerous areas throughout the brain, including the prefrontal

cortex, thalamus, and cerebellum (Day et al., 2005; Luthi and McCormick, 1998; Monteggia et al., 2000; Morris et al., 2004; Santoro et al., 2000; Thuault et al. (2013); Yang et al., 1996), and it is also found in principal cells in the hippocampal formation (Bender et al., 2001; Magee, 1999, 2000; Santoro et al., 2000) including parasubicular principal cells (Glasgow and Chapman, 2008, 2013), and medial entorhinal cortex layer II stellate cells (Dickson et al., 2000b; Heys et al., 2010). The partial activation of I_h at resting membrane potential that results in a leak of current plays a large role in setting the low input resistance of these cells, and a resulting reduction in the integration of synaptic inputs (Magee, 2000; van Welie et al., 2006; Ying et al., 2007). This is especially true at distal dendrites, where the concentration of I_h is up to six times greater than in proximal dendrites (Magee, 2001). Reductions in I_h, therefore, can facilitate synaptic communication by increasing input resistance and promoting temporal summation of EPSPs, and this has been shown in studies where block of I_h slows the decay of EPSPs and increases the temporal summation of repetitive synaptic inputs, an effect that is attributable to increased input resistance that also increases neuronal excitability (Day et al., 2005; George et al., 2009; Magee, 1998, 1999, 2000; Rosenkranz et al., 2006; Ying et al., 2007).

Release of acetylcholine can result in an inhibition of I_h that can lead to enhanced integration of synaptic inputs. Cholinergic activation of M_1 muscarinic receptors leads to decreases in cyclic adenosine monophosphate (cAMP) levels, which leads directly to reductions in I_h conductance (Magee, 1998, 2001; Rosenkranz and Johnston, 2006; Yi et al., 2010). There is also evidence that acetylcholine does not influence I_h solely through decreases in cAMP, but also that M_1 receptor activation causes activation of phospholipase C (PLC) leading to decreases in phosphatidylinositol 4,5-bisphosphate (PIP_2) levels , and subsequent inositol trisphosphate (IP_3)-mediated increases in Ca^{2+} (Pian et al., 2006, 2007; Richter et al., 1999), which both result in reductions in I_h. This evidence demonstrates that I_h is a major mechanism through which acetylcholine acts to enhance responses to repetitive synaptic inputs (Magee, 1998, 2001; Rosenkranz and Johnston, 2006; Yi et al., 2010).

The Role of Theta- and Gamma-Frequency EEG Oscillations in the Hippocampal Formation

Electrophysiological recordings of electroencephalographic (EEG) activity have revealed the presence of oscillatory neural rhythms in the hippocampal region that are expressed across a number of behavioural states, such as active exploration of an environment, tasks involving

memory acquisition, and during sleep (Buzsaki and Draguhn, 2004; Buzsaki and Watson, 2012). These oscillations cover a wide range of frequencies from less than 1 Hz to over 100 Hz (Basar et al., 2000; Buzsaki and Watson, 2012). Two of the most prominent brain rhythms that are observed in relation to learning and memory processes are theta (4-12 Hz) and gamma (30-100 Hz) oscillations (Duzel et al., 2010), which are thought to be critical for "chunking" and organizing information in the brain by coordinating synchronous activity among specific populations of cells both within and across brain areas (Colgin, 2013; Hsieh and Ranganath, 2013). These oscillations are also thought to help determine the level of neuronal excitability and how cells integrate synaptic inputs through changes in cellular excitability depending on the phase of the oscillations (Buzsaki and Watson, 2012; Nyhus and Curran, 2010; Penley et al., 2012).

Theta EEG activity has long been associated with learning and memory processes (Hasselmo and Stern, 2014), with theta activity positively correlated with memory acquisition and recall on behavioural memory tasks (Nyhus and Curran, 2010; Colgin, 2013; Hsieh and Ranganath, 2013). One way that theta activity is thought to contribute to learning and memory is by separating the encoding and retrieval phases of memory processes in order to prevent interference between new information being acquired and older, already existing memories (Colgin, 2013; Hasslemo and Stern, 2014; Hiseh and Ranganath, 2013). This segregation of encoding and retrieval processes by theta oscillations can be observed through what occurs at a network level in the hippocampal formation during learning and memory processes. What has been observed is that, at the peak of the network theta rhythm, feedback projections from the CA3 to CA1 regions of the hippocampus, which likely carry information about previously stored information, are suppressed, while sensory inputs from the entorhinal cortex to the CA1 region, which likely carry novel sensory and associational information, are enhanced, with the opposite changes in synaptic strength occurring during the trough of the theta rhythm (Douchamps et al., 2013). This selective enhancement and suppression of synaptic activity depending on the phase of the theta rhythm is accompanied by alterations in acetylcholine levels, with peak acetylcholine found at the peak of the theta oscillations when cells are depolarized, and lowest acetylcholine levels at the trough when cells are least excitable (Douchamps et al., 2013). Overall, this demonstrates how the theta rhythm can organize the encoding and retrieval of memory processes, by reducing interference between encoding and retrieval by timing synaptic activity so

that encoding and retrieval do not take place concurrently, and so that encoding of new information is not contaminated by synaptic activity associated with older memories (Easton, 2013, Hasselmo et al., 2002; Hasselmo, 2006). These ideas are supported behaviourally by the fact that cholinergic antagonism impairs memory encoding, when acetylcholine levels are highest and theta rhythm is at its peak, but not retrieval (Douchamps et al., 2013; Newman et al., 2013).

Another oscillatory EEG rhythm that frequently co-occurs with theta activity, and which is also associated with learning and memory processes, is the gamma EEG rhythm. Gamma oscillations can be divided into slow (30-50 Hz) and fast (80-100 Hz) frequencies, with fast gamma associated with memory acquisition, and slow gamma maximal during memory recall (Colgin et al., 2009; Colgin, 2013, 2015; Easton et al., 2013; Newman et al., 2013; Hasselmo and Stern, 2014). These different frequencies of gamma appear on separate phases of the theta cycle that correspond to encoding and retrieval, with fast gamma maximal during the early descending phase of theta and slow gamma maximal at the theta trough, and seems to be another mechanism that the brain uses to prevent interference between encoding and retrieval processes (Belluscio et al., 2012; Colgin, 2015; Colgin et al., 2009; Lisman and Jensen, 2013). Gamma oscillations are thought to allow sequences of events to be organized temporally, and to link cell assemblies involved in the same neural processes (Colgin et al., 2009; Colgin, 2015). They could also work to bind separate features of a representation into a coherent whole, so that each neuron that fires together during a gamma cycle would represent different features of the same item or experience (Nyhus and Curran, 2010; Buzsaki and Watson, 2012).

Theta and gamma activity are often closely related, with theta oscillations shown to govern gamma activity in multiple cortical areas through a phenomenon known as cross-frequency coupling. This refers to when theta and gamma oscillations co-occur, with lower amplitude and higher frequency gamma oscillations riding on lower-frequency higher amplitude theta oscillations, so that bursts of gamma frequency oscillations always occur at the same phase of the theta oscillations (Canolty and Knight, 2010; Colgin, 2015; Craig and McBain, 2015; Lisman and Jensen, 2013). Cross-frequency coupling between theta and gamma activities is often seen during behavioural tests of learning and memory, with increased cross-frequency coupling associated with improved memory performance (Canolty and Knight, 2010; Lisman and Jensen, 2013; Colgin, 2015; Igarishi et al., 2014). The possible role of cross-frequency

coupling in information processing in the hippocampal formation has led to the idea that the number of gamma oscillations riding on a theta cycle could be related to the number of representations that are maintained in working memory, with a lower frequency of theta activity associated with greater working memory by allowing more gamma oscillations to ride on a single theta cycle (Hsieh and Ranganath, 2013).

Another way in which theta and gamma oscillations could work together to coordinate neural activity relates to the physical space and amount of tissue each rhythm is thought to govern. With higher-frequency oscillations thought to represent local processing, as slower axon conduction velocity limits higher oscillations to smaller volumes of tissue (Buzsaki and Watson, 2012), theta activity could reflect entrainment of neural activity across a greater volume of tissue, or between separate brain areas, while gamma reflects local cortical processing, with cross-frequency coupling reflecting the transfer of information from large-scale brain networks to local cortical processing and vice-versa (Canolty and Knight, 2010). This implies that theta and gamma oscillations can play an essentially similar role in coordinating neural activity, but with theta doing so over a much larger physical area and time course (Chrobak and Buzsaki, 1998). The idea that the gamma rhythm entrains smaller volumes of tissue is supported by evidence from within the entorhinal cortex, where gamma is generated locally in each entorhinal cortex cell layer (Quilichini et al., 2010). Gamma activity in the medial entorhinal cortex, induced by acetylcholine, also shows reduced coherence within layers the further away two sites are recorded from, while theta is coherent throughout the medial entorhinal cortex. This supports the idea that gamma has a more restricted spatial extent as compared to theta (Nyhus and Curran, 2010; Dickson et al., 2000a).

In summary, there is evidence of more specialized roles for different frequencies of neuronal oscillations, with gamma activity representing pieces of information, while theta separates encoding and retrieval processes (Nyhus and Curran, 2010; Lisman and Jensen, 2013). Overall, however, both rhythms play a critical role in coordinating the activity of neuronal populations within and across neural networks.

Generation of Theta and Gamma Frequency EEG Activity

Neural oscillations are thought to be the product an interaction between inhibitory and excitatory neural activity (Buzsaki and Watson, 2012; Colgin, 2013). The traditional view of theta activity in the hippocampal formation in general, and in the medial entorhinal cortex

specifically, is that theta oscillations occur following the activation of cholinergic afferents from the medial septum that terminate in these regions, with depolarization due to cholinergic activation generating an interaction between voltage-dependent conductances that lead to the oscillations (Khakpai et al., 2013; Klink and Alonso, 1997a). The conductance I_h is thought to play a critical role in the generation of the theta rhythm in layer II medial entorhinal cortex stellate cells; I_h becomes activated following hyperpolarization of the resting membrane potential, and the conductance I_h is mediated by an inward Na^+ current that begins to depolarize the cell during each phase of the oscillations. As the cell depolarizes on each phase of the oscillations, another Na^+ current, I_{NaP}, begins to turn on, enhancing the depolarization initiated by I_h. The strong depolarization results in the closure of I_h, and because the net inward current is reduced, the cell begins the hyperpolarizing phase of the oscillation. When the cell becomes hyperpolarized, I_h conductance increases again, initiating the depolarizing phase of the oscillations, and the cycle of membrane potential oscillation continues (Dickson et al., 2000b). The role of I_h in theta oscillations is supported by the fact that knockout of I_h reduces the frequency of these oscillations (Heys et al., 2012).

In addition to the role that cholinergic inputs from the medial septum play in generating the theta rhythm, gamma-aminobutyric acid (GABA)ergic medial septal inputs have been posited to also play a significant role in generating theta oscillations in the hippocampal formation (Hasselmo, 2006). Most oscillations are, in fact, thought to be based primarily on inhibition, with interneurons rhythmically firing and synchronously inhibiting many principal neurons, and causing them to alternate between states of excitation and inhibition (Nyhus and Curran, 2010; Belluscio et al., 2012; Buzsaki and Watson, 2012). It has been suggested that GABA inputs from the medial septum are the pacemaker that drives theta, while acetylcholine modulates the level of neuronal excitability (Colgin, 2013). The role of inhibition in the theta rhythm is supported by the fact that interneurons tend to fire at the trough of theta, so that they are silent at the peak when cells are most excitable (Quilichini et al., 2010). An example of interneurons being involved in theta oscillations are basket cells in the entorhinal cortex, which have been found to play a role in oscillations by discharging synchronously at theta frequency when excited (Heys et al., 2012).

Gamma oscillations are thought to develop from an interaction between inhibitory and excitatory neurons as well (Buzsaki and Watson, 2012; Canolty and Knight, 2010; Nyhus and

Curran, 2010). This idea is supported by the fact that gamma oscillations are abolished by antagonizing either $GABA_A$ or glutamate receptors (Dickson et al., 2000a). The generation of the gamma rhythm through an interaction between principal and inhibitory neurons is thought to occur through a feedback loop where excitatory cells excite interneurons, which then inhibit excitatory neurons, but after time the inhibition decays and excitatory neurons become active, and then the cycle repeats (Lisman and Jensen, 2013). Parvalbumin-containing inhibitory neurons are, in particular, thought to be critical for generating gamma activity (Buzsaki and Watson; Hioki et al., 2013; Lasztoczi and Klausberger, 2014). In addition, the presence of different frequencies of gamma activity could be explained by the involvement of additional types of interneurons for each frequency, of which there are around twenty different types of interneurons in the CA1 region of the hippocampus alone (Colgin, 2015; Craig and McBain, 2015). Theta oscillations could similarly be influenced by an interaction between principal cells and a slower-acting class of interneuron, with interneurons involved in theta activity even setting the firing rate of gamma interneurons, which could explain why gamma interneurons are only active on certain phases of the theta cycle (Nyhus and Curran, 2010). Compared to theta rhythm, less is known about the ionic basis of gamma rhythm generation. Pharmacological studies have suggested, however, that the generation of gamma oscillations also relies on M_1 acetylcholine receptors that are activated by cholinergic input from the medial septum, and in single cells, the generation of gamma rhythm involves an interaction between I_h and a Ca^{2+}-dependent mixed cation current (I_{cat}), with I_{cat} playing a similar role as I_{NaP} does in the generation of theta rhythm oscillations (Dickson et al., 2000a; Fisahn et al., 2002).

Taken together, the evidence presented above shows that acetylcholine induces rhythmic neuronal oscillations that are critically important for proper cognitive function through its depolarizing action on neuronal populations throughout the hippocampal formation. These oscillations involve a complex interplay between excitatory and inhibitory neuronal activity, and their generation prominently features the cation channel I_h, adding importance to this channel in hippocampal formation function on top of the aforementioned role of I_h in integrating synaptic input.

Long-term Synaptic Plasticity in the Entorhinal Cortex and Parasubiculum

An important question asked by early neuroscientists concerned how exactly memories for a specific event could be stored and represented in the brain. Donald Hebb (1949) postulated that collections of cells known as "cell assemblies" that become active during a particular experience form a neural representation of that experience. He predicted that the lasting strengthening of connections among elements of these cell assemblies would be established and enhanced by repeated exposure to the relevant stimulus. This could be caused by repeated activation of the relevant population of neurons in a temporally close manner of correlated pre and postsynaptic activity, which would cause subsequent firing of one cell in the assembly to activate the rest of the assembly, a mechanism that could aid in memory recall (Hebb, 1949). This idea led to the famous phrase "cells that fire together wire together" (Shatz, 1992). It would still be several decades, however, until Hebb's ideas were supported experimentally.

In 1973, Bliss and Lomo delivered high-frequency stimulation to the perforant path input to the dentate gyrus in anaesthetized rabbits, and then compared the size of synaptic responses before and after high-frequency stimulation. Remarkably, they found that their high-frequency stimulation protocol, which consisted of repeated presynaptic activation that resulted in strong post-synaptic firing, experimentally validated Hebb's theory that repeated strong activation of synaptic connections between neurons could cause lasting strengthening of synaptic connections between them. The role of long-term potentiation (LTP), as the discovery became known, was a breakthrough in the study of the neural basis of learning and memory (Shors and Matzel, 1997). Further research led to the discovery of long-term synaptic depression (LTD), which could be induced by repeated low-frequency stimulation of a synaptic pathway (Ito & Kano, 1992). Long-term depression provided an active mechanism for reducing synaptic strength in a cell assembly, a cellular process analogous to forgetting associations and memories that are no longer relevant, (Malenka and Bear, 2004). Links between both LTP and LTD and learning and memory have been supported by studies showing lasting alterations in synaptic strength in the brains of animals that have been trained on learning and memory tasks (Artola et al., 2006; McHugh et al., 1996; Morris et al., 1986; Whitlock et al., 2006).

Mechanisms of Plasticity

In the decades since the initial discovery of LTP, basic mechanisms behind typical stimulation protocols used to induce synaptic plasticity have been elucidated. This includes the

finding that strong, fast depolarization of a postsynaptic cell by presynaptic stimulation tends to lead to LTP, while weaker, prolonged depolarization leads to LTD, with both processes relying on increased Ca^{2+} influx resulting from N-methyl-D-aspartate (NMDA) receptor activation (Sjostrom et al., 2008). Whether LTP or LTD will be produced, however, depends upon the timing and extent of elevations in postsynaptic Ca^{2+} concentration; LTP is induced by strong but brief increases in Ca^{2+} levels, and LTD is induced by smaller but longer-lasting Ca^{2+} increases (Deng and Lei, 2007; Johnston et al., 2003; Feldman, 2012; Sjostrom et al., 2008). These differences in intracellular Ca^{2+} levels result in different postsynaptic processes initiated by Ca^{2+} influx. LTP occurs when sufficient depolarization results in opening of NMDA receptors by removal of the voltage-sensitive magnesium (Mg^{2+}) block within the NMDA receptor ionophore, leading to Ca^{2+} entry into the cell. The resulting increase in Ca^{2+} has a number of effects on the postsynaptic cell, including shorter-term effects like calmodulin-mediated phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and co-activation of protein kinase A (PKA) and protein kinase C (PKC), leading to increased activity and expression of alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, and longer-term changes related to the production of new proteins in the cell nucleus through activation of cAMP response element-binding protein (CREB) protein transcription factors (Adams and Dudek, 2005; Malenka and Bear, 2004). LTD, on the other hand, although also thought to be dependent on activation of NMDA receptors, results from moderate increases in Ca^{2+} levels that lead to dephosphorylation of PKA, without concurrent effects on CaMKII, which leads to reduced AMPA receptor activity and expression at the synapse, and the postsynaptic cell therefore becomes less responsive to subsequent activation of affected synapses (Deng and Lei, 2007; Malenka and Bear, 2004).

While effectively demonstrating a neuronal mechanism that could support Hebb's concept of cell assembly formation, the high-frequency stimulation employed by Bliss and Lomo does not reflect physiological patterns of brain stimulation and activity that are normally seen during memory acquisition (Grover et al., 2009). Many researchers have therefore begun to employ patterns of stimulation that simulate physiologically relevant patterns of activity seen in the brain during memory acquisition such as the theta and gamma rhythms (Zhu et al., 2016). For example, theta-burst stimulation, for which the exact parameters of stimulation vary from study to study, always involves repeated bursts of short trains of pulses, with the intervals between

pulse trains corresponding to the frequency of the theta rhythm. The aim of this pattern of stimulation is to simulate the bursts of action potential firing at theta frequency that are thought to play a major role in mnemonic processing, and theta burst stimulation has been found to be an effective protocol for inducing long-term plasticity in synaptic pathways (Han and Heinemann, 2013; Grover et al., 2009; Yun et al., 2000). This shows the importance of a physiologically relevant frequency of stimulation for the induction of synaptic plasticity (Johnston et al., 2003).

Patterned stimulation protocols can also take advantage of an important concept related to associative plasticity that has emerged more recently: spike-timing-dependent plasticity (Feldman, 2012). Spike-timing-dependent plasticity focuses on the importance of the timing of the firing of two neurons in determining if there is a lasting change in the strength of the connection between them. Spike-timing-dependent LTP can be induced when there is a short temporal delay between presynaptic spikes and subsequent postsynaptic spikes, in which firing of the presynaptic cell predicts firing of the postsynaptic cell (Feldman, 2012), and this fits quite well with Hebb's (1949) idea that synapses should be strengthened when a presynaptic neuron reliably predicts the firing of a postsynaptic neuron. Spike-timing-dependent LTD results when there is a short temporal delay between postsynaptic spikes and subsequent presynaptic firing (Feldman, 2012). Although Hebb did not predict the occurrence of LTD, spike timing dependent LTD is consistent with what one would expect from an elaboration of Hebbian ideas, in that the synaptic connection between a presynaptic and postsynaptic cell is weakened to the extent that the presynaptic neuron does not predict firing of the postsynaptic neuron (Hebb, 1949).

A major mechanism behind the induction of spike-timing-dependent plasticity is back-propagating action potentials, in which action potentials initiated at the axon hillock spread backwards through the dendrites, activating voltage-gated Ca^{2+} channels, and also close K^{+} channels that usually dampen dendritic excitability. The closure of these K^{+} channels makes the dendrites more sensitive to incoming excitatory inputs, and the subsequent enhanced NMDA receptor activation increases Ca^{2+} levels. This serves to enhance the dendritic activation that occurs when presynaptic stimulation is sufficient to activation firing in the postsynaptic neuron, and the resulting promotion of lasting potentiation can allow neurons to communicate more efficiently (Hoffman et al., 1997; Hu et al., 2010; Johnston et al., 2003). Spike-timing-dependent plasticity can be induced both by stimulating a presynaptic pathway with sufficient strength to trigger spikes in the postsynaptic cell, or by injecting current directly into the postsynaptic cell to

cause postsynaptic cell firing coincident with subthreshold presynaptic input (Nevian and Sakmann, 2004; Birtoli and Ulric, 2004, Markram et al., 1997).

Investigating the importance of input timing on synaptic plasticity in the entorhinal cortex in particular, theta-burst stimulation has been shown to be more effective than other stimulation protocols used for the induction of synaptic plasticity, such as high-frequency 100 Hz stimulation (Yun et al., 2002). This may be related to the finding that entorhinal neurons are normally modulated by theta frequency oscillations. It is possible that the initial train of pulses in theta-patterned stimulation may enhance depolarization that could promote NMDA receptor mediated plasticity by reducing fast-acting GABA receptor activation during subsequent bursts in the theta-burst stimulation train (Yun et al., 2002). Thus, while a single burst of stimulation of synaptic inputs to the entorhinal cortex activates both excitatory and inhibitory synapses, the repeated delivery of theta burst stimulation reduces feedforward inhibition, and can result in greater depolarization during theta burst stimulation and therefore greater activation of NMDA receptors that mediate LTP induction (Larson and Munkacsy, 2014). LTD can also be induced in the entorhinal cortex, as low frequency stimulation consistently generates LTD in both superficial and deep layers of the entorhinal cortex (Solger et al., 2004). The fact that LTD occurs when stimulation is delivered around 1-3 Hz shows that LTD in the entorhinal cortex is frequency dependent as well (Johnston et al., 2003), however the relationship of LTD to theta-patterned stimulation has not been determined. The purpose of synaptic potentiation and depression in the entorhinal cortex could be to modulate its responsiveness to particular patterns of sensory input, and this could affect how information is integrated within the entorhinal cortex, and how patterns of activation are projected to the hippocampus.

Heterosynaptic Plasticity

It has been observed that inputs from one neural pathway can transiently modulate the responsiveness of a target region to other inputs that project to the same target area (Caruana and Chapman, 2004; Mouly and Di Scala, 2006; McQuistion, 2010). Interestingly, it has also been shown that lasting changes in synaptic strength can be induced, not only in a pathway that is stimulated strongly, but also at synapses from other projections that terminate onto a common postsynaptic target, a phenomenon referred to as heterosynaptic plasticity (Chistiakova and Volgushev, 2009; Chistiakova et al., 2014; Han and Heinemann, 2013; Larson and Munkacsy, 2014). Heterosynaptic plasticity may act to maintain the overall homeostasis of synaptic weights

to prevent excessive excitation in a neural population (i.e. weaken synaptic connections), and could also serves as a model of associative memory where separate inputs that are active within a short temporal window can become mutually reinforced (Chistiakova and Volgushev, 2009; Chistiakova et al., 2014; Huang et al., 2004; Hulme et al., 2013), leading to lasting associations between the information provided by the separate inputs (i.e. the strengthening synaptic connections).

Mechanisms of heterosynaptic plasticity are thought to share some characteristics with mechanisms that mediate homosynaptic plasticity. These include back-propagating action potentials, where increased Ca^{2+} influx caused by activation of Ca^{2+} spikes in the entire dendritic tree plays a role by priming heterosynaptic inputs for plasticity induction, and insertion of AMPA receptors into dendritic spines to make heterosynaptic dendrites more excitable (Chistiakova and Volgushev, 2009; Chistiakova et al., 2014; Han and Heinemann, 2013). Heterosynaptic plasticity can also involve increased expression of NMDA receptors, whereas homosynaptic plasticity is typically expressed only through increased AMPA receptor expression (Han and Heinemann, 2013). Theta-patterned stimulation can also contribute to heterosynaptic plasticity, indicating the importance of rhythmic neuronal oscillations in coordinating activity and causing lasting changes in synaptic strength between separate synaptic inputs that target a common neuronal population (Bzelot et al., 2015). This evidence indicates the potential importance of heterosynaptic connections in forming associations between different synaptic inputs that target common brain regions, connections that may play an important role in the formation of complete mnemonic representations. However, although layer II of the entorhinal cortex is a target of multiple sensory and associational cortices as well as a target of the parasubiculum, very little is known about heterosynaptic interactions that may govern long-term synaptic plasticity within this cell layer.

Summary of Experimental Chapters

The goal of this thesis was to investigate how the neurotransmitter acetylcholine, in concert with rhythmic brain activity at theta- and gamma-frequencies, may modulate synaptic communication within layer II of the entorhinal cortex, including the parasubicular synaptic inputs to layer II of the medial entorhinal cortex.

The series of experiments described in the second chapter were used to characterize the basic effects of acetylcholine and rhythmic brain stimulation on how the entorhinal cortex responds to stimulation of the parasubiculum, using field potential recordings of synaptic responses in layer II of the entorhinal cortex. It was found that, despite a reduction in the amplitude of responses to single pulses of stimulation following cholinergic agonism, consecutive responses recorded during short trains of stimulation at the frequency of the theta and gamma rhythms were enhanced. This effect was reliant upon M_1 muscarinic receptors and was dependent in part on cholinergic suppression of the cationic current I_h. The results indicate the importance of acetylcholine and rhythmic neuronal activity in synaptic communication in the parasubicular-entorhinal pathway.

The experiments in the third chapter expanded on the results of the second chapter by utilizing intracellular recordings from individual stellate cells in layer II of the medial entorhinal cortex to better understand the mechanisms through which acetylcholine and rhythmic brain stimulation affect synaptic responses in entorhinal cortex neurons. The cholinergic facilitation of consecutive responses recorded during short trains of stimulation that was observed in field potential recordings in Chapter 2 was replicated in the intracellular recordings obtained in Chapter 3. It was also found that the cholinergic facilitation of synaptic responses evoked during theta- and gamma-frequency trains was caused in part by an increase in input resistance and lengthening of the decay time of EPSPs that was attributable to a cholinergic reduction in I_h. These results elucidate the mechanisms behind the cholinergic facilitation of train-evoked responses in the parasubicular-entorhinal pathway, and further demonstrate the importance of cholinergic activity and oscillatory brain rhythms in synaptic communication in this pathway.

The fourth chapter investigated directly how the parasubiculum can modulate how the entorhinal cortex responds to synaptic inputs from sensory projections by measuring the responses of individual neurons in the entorhinal cortex to synaptic stimulation of sensory pathways following rhythmic stimulation of the parasubiculum. Results indicated that trains of parasubicular stimulation at theta rhythm could alter how the entorhinal cortex responds to stimulation in pathways containing synaptic input from sensory areas. Depending on the stimulation parameters, this heterosynaptic modulation had a short-term suppressive or facilitatory effect on responses evoked by stimulation of layer I, demonstrating that the parasubiculum can directly affect how the entorhinal cortex responds to sensory input pathways.

When the same stimulation patterns were given repeatedly at low frequency, a long-term depression of entorhinal cortex responses to layer I inputs was observed, indicating that parasubicular inputs can have lasting effects on how the entorhinal cortex processes incoming sensory inputs. Overall these results indicate that the parasubiculum can directly affect how the entorhinal cortex responsds to sensory inputs.

Overall, this thesis has provided significant insights into the physiological functions of the parasubicular projection to the entorhinal cortex. This is a neural pathway that has been relatively unexplored previously, but which could play a significant role in determining how the hippocampal formation integrates sensory inputs, and therefore have a significant influence on learning and memory processes within the hippocampal formation.

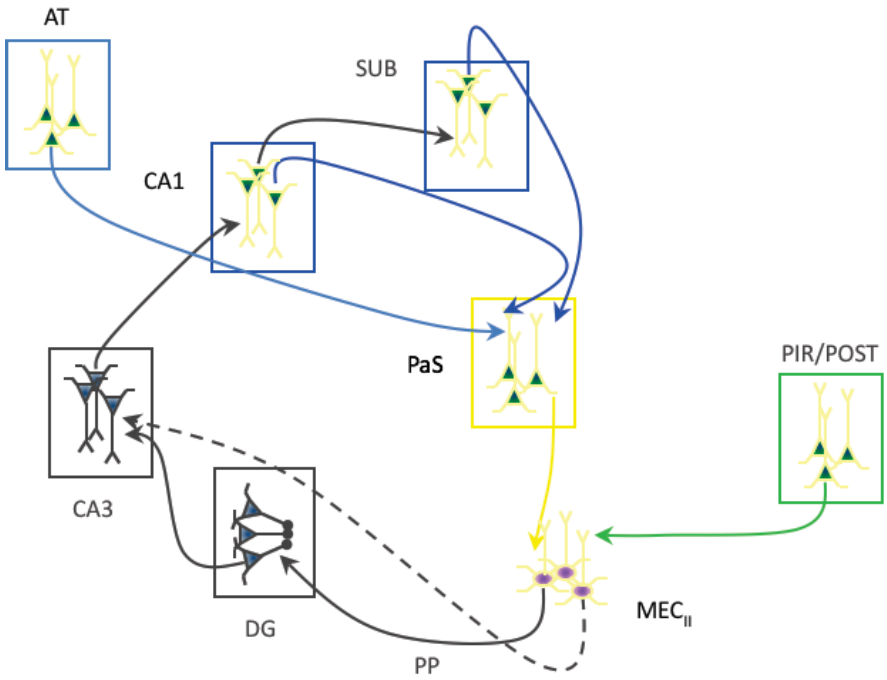

AT
SUB
CA1
PaS
PIR/POST
CA3
DG
PP
MEC$_{II}$

Figure 1.1. Schematic Diagram illustrating the relevant connections of the Parasubiculum (PaS) and other hippocampal formation brain areas. Feedback projections from CA1 and the Subiculum (SUB) to the PaS, and subsequent PaS projections to medial entorhinal cortex layer II (MEC_{II}) complete a closed circuit of synaptic connections within the hippocampal formation. Abbreviations: AT, Anterior Thalamus; CA3, cornu ammonis area 3 of the Hippocampus; DG, Dentate Gyrus; PP, perforant path; PIR/POST, Pifirom and Postrhinal Cortices. Adapted from Caruana & Chapman (2004).

CHAPTER 2

CHOLINERGIC RECEPTOR ACTIVATION INDUCES A RELATIVE FACILITATION OF SYNAPTIC RESPONSES IN THE ENTORHINAL CORTEX DURING THETA- AND GAMMA-FREQUENCY STIMULATION OF PARASUBICULAR INPUTS

Daniel W. Sparks and C. Andrew Chapman

ABSTRACT

The parasubiculum sends its single major output to layer II of the entorhinal cortex, and it may therefore interact with inputs to the entorhinal cortex from other cortical areas, and help to shape the activity of layer II entorhinal cells that project to the hippocampal formation. Cholinergic inputs are thought to contribute to the generation of theta- and gamma-frequency activities in the parasubiculum and entorhinal cortex, and the present study assessed how cholinergic receptor activation affects synaptic responses of the entorhinal cortex to theta-and gamma-frequency stimulation. Depth profiles of field EPSPs in acute brain slices showed a short-latency negative fEPSP in layer II, consistent with activation of excitatory synaptic inputs to layer II. Application of the cholinergic agonist carbachol suppressed synaptic responses and enhanced paired-pulse facilitation. Carbachol also resulted in a marked relative facilitation of synaptic responses evoked during short 5-pulse trains of stimulation at both theta- and gamma-frequencies. Application of the M_1 antagonist pirenzepine, but not the M_2 antagonist methoctramine, blocked the facilitation of responses. Inhibition of the M-current or block of $GABA_B$ receptors had no effect, but the facilitation effect was partially blocked by the NMDA antagonist APV, indicating that NMDA receptors play a role. Application of ZD7288, a selective inhibitor of the hyperpolarization-activated cationic current I_h, almost completely blocked the relative facilitation of responses, and the less potent I_h-blocker Cs^+ also resulted in a partial block. The relative facilitation of synaptic responses induced by carbachol is therefore likely mediated by multiple mechanisms including the cholinergic suppression of transmitter release that enhances transmitter availability during repetitive stimulation, NMDA receptor-mediated effects on pre- or postsynaptic function, and cholinergic modulation of the current I_h. These mechanisms likely contribute to the maintenance of effective synaptic communication within parasubicular inputs to the entorhinal cortex during cholinergically induced rhythmic states.

The superficial entorhinal cortex receives multiple cortical inputs from the piriform, perirhinal, and postrhinal cortices (Burwell et al., 1995; Burwell and Amaral, 1998) and the entorhinal cortex provides the hippocampus with the majority of its cortical sensory input via perforant path inputs from layer II to the dentate gyrus and CA3 region, and layer III inputs to the CA1 region and subiculum (Witter et al., 1989; Amaral, 1993; Burwell et al., 1995). In addition, there is also growing interest in the role of the entorhinal cortex in spatial navigation that has followed the discovery of specialized grid cells in the medial entorhinal cortex that fire in relation to the spatial location of the animal (Hafting et al., 2008; Giocomo et al., 2007; Hargreaves et al., 2007; Hasselmo et al., 2007; Hasselmo, 2008). The entorhinal cortex receives inputs from the subicular complex that also contains place- and head-direction sensitive neurons (Taube, 1995; Cacucci et al., 2004; Jarrard et al., 2004; Boccara et al., 2010). The parasubiculum is a component of the subicular complex that receives major inputs from the subiculum, CA1 region of the hippocampus, basolateral amygdala and the anterior thalamus (Alonso and Kohler, 1984; van Groen and Wyss, 1990), and sends its only major output projection to layer II of the entorhinal cortex (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994; Caruana and Chapman, 2004). The parasubicular input to the entorhinal cortex may therefore contribute to the modulation of sensory inputs carried by the entorhinal cortex to the hippocampus (Caruana and Chapman, 2004), and could also contribute to the activity of grid cells in the entorhinal cortex. However, the basic synaptic physiology of the projection from the parasubiculum to the entorhinal cortex, and how it responds to repetitive synaptic activation that is likely to occur during spatial navigation, is not well known.

Electroencephalographic (EEG) activity in the entorhinal cortex and parasubiculum is dominated by theta (4-12 Hz) and gamma (30-80 Hz) rhythms during active exploration of the environment (Mitchell et al., 1980; Chrobak and Buzsaki, 1998). Theta activity is also thought to help govern the activities of grid and place cells in the hippocampal region by helping to time the interactions between cells (Taube et al., 1995; Hasselmo et al., 2007; Boccara et al., 2010), and theta has also been linked to mechanisms thought to contribute to learning and memory including induction of long-term synaptic potentiation (Buzsaki, 2002; Hasselmo, 2006). Both the entorhinal cortex and parasubiculum receive strong cholinergic inputs from the medial septum (Gaykema et al., 1990), and acetylcholine is thought to play a major role in the generation of theta and gamma activities in both regions (Mitchel et al. 1980; Dickson et al. 2000a; Glasgow

and Chapman, 2007). Acetylcholine can help generate theta activity by depolarizing neurons to near-threshold voltages at which principle neurons generate intrinsic theta-frequency oscillations in membrane potential (Klink and Alonso 1997; Dickson et al. 2000b; Hasselmo 2006; Glasgow and Chapman, 2008), and in the hippocampus, similar mechanisms contribute to theta-frequency firing of inhibitory interneurons that can synchronize the activity of principal cells (Chapman and Lacaille, 1999; Buzsaki 2002).

In addition to the marked increases in network synchrony and neuronal excitability induced by cholinergic activation, the cholinergic agonist carbachol (CCh) is also well-known to result in a strong suppression of excitatory synaptic transmission in the entorhinal cortex (Richter et al., 1999; Hamam et al., 2007; Yun et al., 2000), parasubiculum (Glasgow et al., 2012), subiculum (Kunitake et al., 2004), and hippocampus (Auerbach and Segal, 1996). The cholinergic suppression of excitatory synaptic transmission among intrinsic excitatory connections has been proposed to help reduce interference between representations carried by these connections at times when memories for new information are being formed (Hasselmo, 2006). The cholinergic suppression of synaptic responses may also serve to offset increased firing associated with cholinergic depolarization to help prevent overactivation of cortex (Klink and Alonso, 1997; Funahashi and Stewart, 1998; Gloveli et al., 1999)

Theta and gamma frequency activities co-occur in the hippocampal region (Chrobak and Buzsaki, 1998) and there has been substantial interest in how repetitive stimulation at these frequencies may modulate synaptic transmission (Castro-Alamancos and Connors, 1996; Kunitake et al., 2004; Carr and Surmeier, 2007). Although CCh causes a reduction in the amplitude of EPSPs evoked by a single pulse, the amplitude of EPSPs evoked during short trains of stimulation is enhanced by CCh in both the subiculum and prefrontal cortex (Kunitake et al., 2004; Carr and Surmeier, 2007). This effect is thought to be due to the closure of K^+ channels which can enhance temporal summation due to increased input resistance, and the concurrent suppression of transmitter release that increases the readily releasable pool of neurotransmitter available later in the train (Yamada and Zucker, 1992; Hamam et al., 2007). Thus, although CCh induces an overall suppression of synaptic responses during the trains, the responses to consecutive pulses in the train are maintained or facilitated, suggesting that acetylcholine may serve to help maintain the strength of active synaptic responses during cholinergic induced rhythmic states.

In the present study we have characterized field excitatory synaptic responses in layer II of the entorhinal cortex in vitro evoked by short trains of theta- and gamma-frequency stimulation of the parasubiculum, and we have also examined how these responses are modulated by CCh. Carbachol resulted in an overall suppression of synaptic responses during trains, but the amplitude of responses to each pulse during the trains was facilitated relative to the amplitude of the first response. Mechanisms that mediate the relative facilitation effect were investigated by targeting receptors and conductances linked to theta- and gamma-frequency activity. We examined the role of the muscarinic sensitive K^+ current I_M which may contribute to depolarization of layer II neurons (Yoshida and Alonso, 2007), depolarization-dependent enhancement of N-methyl-D-aspartate (NMDA) components of synaptic responses (Jones, 1990; Markram and Segal, 1990; Auerbach and Segal, 1996; Khateb et al., 1997; Aramakis et al., 1999), inhibition of gamma aminobutyric acid (GABA) release via $GABA_B$ autoreceptors (Jones and Buhl, 1993; Davies and Collingridge, 1996; Funahashi and Stewart, 1998), and modulation of the hyperpolarization-activated cationic current I_h that contributes to theta rhythmicity in the entorhinal cortex (Castro-Alamancos and Connors 1996; Magee, 1998; Dickson et al., 2000b; Carr et al., 2007).

METHODS

Slice Preparation

Experiments were conducted in accordance with the guidelines of the Canadian Council on Animal care. Acute horizontal brain slices were obtained from 4 to 7-week-old Long-Evans rats after anesthetization with halothane and decapitation. Brains were removed and submerged in artificial cerebrospinal fluid (ACSF, 4 ºC) consisting of (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% O2/5% CO_2. Slices were cut 400 μm thick using a vibratome (WPI, Vibroslice NVSL), and recovered at room temperature for at least an hour. Individual slices were then placed on a nylon net in a gas-fluid interface chamber at 32 ºC (Fine Science tools, North Vancouver, BC, Canada) and were visualized with a Leica MS5 microscope. Slices continued to be exposed to 95% O_2/5% CO_2, and were perfused with ACSF at a rate of 1-1.5 ml/min for the duration of the experiment.

Stimulation and Recording

Field excitatory postsynaptic potentials (fEPSPs) were recorded in layer II of the medial entorhinal cortex using glass pipettes obtained using a horizontal puller (Flaming/Brown, Model P97) and filled with ACSF. Cathodal constant current pulses (0.1 ms duration, 75-150 μA) were delivered to the parasubiculum via a bipolar tungsten electrode (FHC., Bowdoin, ME) using a stimulus generator (WPI, Model A300) and a stimulus isolation unit (Model A360). Evoked fEPSPs were filtered and amplified (DC-3 kHz, Axon Instr., Axoclamp 2B) and digitized (20 kHz, Axon Instr., Digidata 1322A) for storage on computer hard disk using the pClamp 8.2 (Axon Instr.) software package.

Depth Profile Recordings. Fibers leaving portions of the parasubiculum proximal to the presubiculum terminate in the medial entorhinal cortex closest to the parasubiculum, while fibers leaving the parasubiculum at more rostral locations travel a longer distance to more distal regions of the entorhinal cortex (Caballero-Bleda and Witter, 1993). The stimulation electrode was therefore routinely placed in the middle depths of the parasubiculum, at sites proximal to the presubiculum. The distribution of synaptic responses to parasubicular stimulation throughout the depth of the medial entorhinal cortex has not been described previously in vitro. To characterize the synaptic response, depth profiles of fEPSPs were obtained by recording at ~100 μm intervals across the depth of the medial entorhinal cortex, perpendicular to the cortical surface and at sites proximal to the parasubiculum. Five samples of evoked responses were obtained at each depth. The amplitudes and reversals of field potentials were analyzed to infer the origin of the synaptic responses but current source density analysis (e.g., Caruana and Chapman, 2004) was not conducted due to the moderate number of recording sites and slight variations in spatial intervals between sites. The largest negative-going field potential component was consistently observed in layer II and with smaller responses in upper layer III, and responses for subsequent recordings were therefore recorded in layer II.

Effects of Carbachol on Evoked Responses. The effect of the cholinergic receptor agonist carbachol (CCh; Sigma-Aldrich, St. Louis, MO) on synaptic responses evoked by single pulses, pairs of pulses and short trains of stimulation was assessed by recording responses in normal ACSF, following addition of CCh, and following washout in normal ASCF. Single fEPSPs were recorded every 10 seconds during a 15-20 minute baseline period to ensure that responses remained stable, and then baseline paired-pulse and train-evoked responses were

recorded. Following baseline recordings, CCh was applied for 10-15 minutes, and paired-pulse and train-evoked responses recorded again. Initial tests used either 0.5 or 10 μM CCh, but 10 μM was used routinely in subsequent experiments. Responses were also obtained following a 10-15 minute washout period in normal ACSF. Paired-pulse tests employed pairs of stimulation pulses delivered at a range of ascending inter-pulse intervals between 5 and 1200 ms. There was an interval of 10 sec between each pair, and 5 samples were obtained at each interval. Synaptic responses to theta- and gamma-frequency stimulation were assessed using short, 5-pulse trains delivered at 10 and 33 Hz. Ten responses were obtained for both 10 and 33 Hz using a 10 sec interval between trains.

Pharmacology. Mechanisms mediating the relative facilitation of synaptic responses during theta- and gamma-frequency stimulation were assessed by applying CCh in the presence of synaptic receptor antagonists or blockers of membrane conductances. Tests in normal ACSF were repeated after 15-20 min application of blockers or antagonists, after 20 min addition of CCh, and after washout of CCh in the presence of the blocker or antagonist. All drugs were stored as frozen stock solutions and mixed with ACSF immediately before perfusion. The muscarinic receptors involved in the CCh-induced modulation of responses were investigated using the M_1 receptor antagonist pirenzepine (1 μM, Ascent Scientific, Princeton, NJ), or the M_2 antagonist methoctramine (10 μM, Sigma-Aldrich). The role of NMDA glutamate receptors was assessed using the antagonist APV (50 μM, Ascent Scientific), and the $GABA_B$ receptor blocker CGP55845 (1 μM, Tocris Bioscience, Bristol, UK) was used to assess the possible role of $GABA_B$ autoreceptors. The role of the hyperpolarization-activated cationic current I_h was investigated by application of the I_h blockers ZD7288 (10 μM, Ascent Scientific) and Cs^+ (1 mM, Sigma-Aldrich), and the role of I_M was evaluated using application of XE 991 (10 μM, Ascent Scientific).

Data Analysis

Peak amplitudes of averaged evoked synaptic potentials were measured relative to the baseline prior to each stimulus pulse (Axon Instr., pClamp 8.2), and group averages were expressed as the mean ± SEM. Paired-pulse ratios were calculated by expressing the amplitude of the second response in each pair relative to the response evoked by the first pulse. For very short interpulse intervals, in which the second response overlapped the first response, the amplitude of the second response was measured relative to the baseline prior to the second

stimulation pulse, and paired-pulse ratio was calculated relative the amplitude of responses evoked by conditioning pulses at other intervals. Effects of CCh on paired-pulse facilitation were assessed using matched-samples t-tests.

Effects of CCh and of pharmacological antagonists or conductance blockers on train-evoked responses were assessed by normalizing responses to the amplitude of the first response evoked in the presence of the antagonist or blocker. Repeated measures ANOVAs assessed changes in train-evoked responses induced by addition of the antagonist or blocker to normal ACSF, and by the addition of CCh in the presence of the antagonist or blocker.

The effects of CCh on relative changes in EPSP amplitudes during the 5-pulse trains were assessed by normalizing responses evoked during the trains to the amplitude of the first responses in each train. Repeated measures ANOVAs compared relative response amplitudes during the trains before and after application of CCh, and mixed ANOVAs compared responses evoked following CCh application in normal ACSF versus in the presence of blockers. Planned comparisons relative to the first responses in trains were also used in both models to test for changes in amplitudes of responses evoked by each pulse in the trains.

RESULTS

Cholinergic Suppression of Single EPSPs

Synaptic field potential responses evoked in layer II of the entorhinal cortex by parasubicular stimulation have been observed previously (Jones, 1990; Caruana and Chapman, 2004; Tolner et al., 2007) but the localization of responses within layer II and the variation in responses with cortical depth has not been well characterized in vitro. Field potential responses were therefore recorded at multiple cortical depths in the medial entorhinal cortex in response to single-pulse stimulation of the parasubiculum at mid-cortical depths at sites proximal to the presubiculum (Caballero-Bleda and Witter, 1993). There was some variability between slices, but a strong negative-going response located in the layer II/III area was always found, and was flanked by smaller positive-going responses in layer I and in the deep layers (n = 10; Figure 2.1A). This pattern is consistent with the induction of an active current sink in layer II and passive current sources in the adjacent cortical layers (Caruana and Chapman, 2004), and is also consistent with the known anatomy of the major output of the parasubiculum to layer II of the medial entorhinal cortex (Caballero-Bleda and Witter, 1993).

The effects of cholinergic receptor activation on entorhinal synaptic responses were assessed by comparing fEPSPs evoked by single stimulation pulses before, during and after application of CCh (0.5 or 10 μM). Application of 0.5 μM CCh resulted in no significant change in EPSP amplitude (1.21 ± 0.24 versus 1.21 ± 0.21 mV; $n = 9$; $t_8 = 0.02$, $p = 0.99$), but 10 μM CCh ($n = 11$) significantly reduced the fEPSP amplitude from 0.76 ± 0.16 to 0.37 ± 0.05 mV ($n = 11$; $t_{10} = 2.90$, $p < 0.05$; Figure 2.1B). Responses returned to baseline values following 5 to 10 min washout in normal ACSF. This strong suppression of synaptic responses evoked in layer II of the entorhinal cortex by single stimulation pulses delivered to the parasubiculum is similar the cholinergic suppression effects observed within the parasubiculum (Glasgow et. al., 2012) and in for other synaptic pathways within the entorhinal cortex (Auerbach and Segal, 1996; Richter et al., 1999; Yun et al., 2000; Hamam et al., 2007).

Cholinergic Effects on Train-Evoked Responses

The effect of 10 μM CCh on trains of stimulation pulses delivered at theta- and gamma-frequencies was tested by delivering short five-pulse trains at either 10 and 33 Hz respectively ($n = 7$) (0.5 μM CCh had no significant effect on train-evoked responses in 9 slices, not shown). During 33 Hz stimulation in normal ACSF, there was a slight facilitation of the amplitude of the response to the second pulse in the train, but subsequent pulses in the train evoked progressively smaller EPSPs such that the last EPSP was 56.8 ± 23.9 % of the amplitude of the first EPSP. Following application of CCh, the amplitude of the response to the first pulse in the train was strongly suppressed, just as in single pulse tests ($p < 0.01$), but all subsequent responses to stimulation pulses in the train showed a maintained facilitation relative to the response to the first pulse (Figure 2.2A). Raw amplitudes of responses to the last pulses in the train were similar in normal ACSF and in the presence of CCh (Figure 2.2A_3), but the relative amplitude of these responses was much greater in CCh as compared to normal ACSF (124.3 ± 34.9% versus 56.8 ± 23.9 %; $F_{1,18} = 31.90$, $p < 0.01$; Figure 2.2A_4).

Results were very similar for tests with 10 Hz trains; responses in normal ACSF showed a marked depression in amplitude during the train and, although CCh induced a strong overall suppression of synaptic responses ($p < 0.01$), there was a maintenance of response amplitudes during the train such that the relative amplitude of responses was significantly facilitated in CCh as compared to normal ACSF (112.2 ± 17.7 % in CCh vs. 70.1 ± 19.2 % in normal ACSF; $F_{1,18} = 16.65$, $p < 0.05$; Figure 2.2B). The amount of facilitation tended to be larger during gamma- than

during theta-frequency stimulation, with peak facilitation effects in response to the second pulse reaching 152.4 ± 22.0 % for 33 Hz and 118.1 ± 11.3 % for 10-Hz stimulation. In control experiments in which responses to 10- and 33-Hz trains were monitored over three time periods in normal ACSF, there was no significant change in the amplitudes of response evoked during the trains for either 10-Hz ($F_{2,12} = 0.05$, $p = 0.95$; $n = 5$) or 33-Hz stimulation trains ($F_{2,12} = 0.04$, $p = 0.96$).

The cholinergic suppression of single fEPSPs is mediated by reduced transmitter release, and this leads to enhanced paired-pulse facilitation ratios due to a larger pool of readily releasable transmitter during the response to the second pulse (Yamada and Zucker, 1992; Hamam et al., 2007). Similar to previous results for piriform cortex inputs (Chapman and Racine, 1997; Bouras and Chapman, 2003) paired-pulse facilitation of parasubicular inputs to the entorhinal cortex was greatest at intervals of 20-30 ms that correspond to the period of gamma activity, and the amount of facilitation declined as interpulse interval was increased to 100 ms (the period of 10 Hz theta activity). Results obtained here replicated the finding that 10 μM CCh increases paired-pulse facilitation at intervals near 30 ms ($n = 11$; $t_{10} = -2.50$, $p < 0.05$; Richter et al. 1999; Hamam et al. 2000), and also showed that facilitation is enhanced by CCh at intervals up to 100 ms ($t_{10} = -2.40$, $p < 0.05$; Figure 2.2C). The lower concentration of 0.5 μM CCh had no significant effect. This facilitation of paired-pulse ratios by 10 μM CCh across a wide range of interpulse intervals is consistent with a role for presynaptic reductions in transmitter release in the facilitation of responses during theta and gamma frequency trains. In addition, CCh also modified paired-pulse effects at the 5 ms interval, such that paired-pulse inhibition in normal ACSF was converted to paired-pulse facilitation in CCh ($t_{10} = -6.57$, $p < 0.01$), and this could be due to either enhanced neurotransmitter availability, or to altered fast synaptic inhibition. Changes in paired-pulse responses during subsequent tests using receptor blockers were consistent with changes in train-evoked responses, and are therefore not reported.

Muscarinic Receptors

The receptors involved in the relative facilitation of train-evoked responses by CCh were investigated using constant bath application of muscarinic antagonists prior to addition of CCh. The M_2 receptor antagonist methoctramine (10 μM) did not significantly affect the amplitude of the first responses in trains ($n = 7$, $p = 0.22$ and 0.08 for 33 Hz and 10 Hz, respectively). Similar to effects observed in normal ACSF, subsequent addition of CCh suppressed the amplitude of the

first responses during 33 Hz and 10 Hz trains ($p < 0.05$ for both conditions), and increased the relative amplitude of subsequent responses during the trains (Figure 2.3A,B; 33 Hz, $F_{1,18} = 9.00$, $p < 0.05$; 10 Hz, $F_{1,18} = 10.62$, $p < 0.05$). The amount of facilitation induced by CCh in methoctramine was similar to that observed in normal ACSF, including a somewhat larger facilitation effect during 33 Hz trains (compare Figures $2.2A_4$,B_4 and $2.3A_4$,B_4). The cholinergic modulation of train-evoked responses is therefore not dependent on methoctramine-sensitive M_2 receptors.

Although there was a trend for the M_1 receptor blocker pirenzepine (1 μM) to result in an increase in basal synaptic transmission during both 33 Hz and 10 Hz trains, the effect was not statistically significant ($n = 6$, $p = 0.12$ and 0.19, respectively; see also Glasgow et al., 2012). However, pirenzepine prevented the CCh-induced reduction in fEPSP amplitude during both 33 Hz and 10 Hz trains ($p = 0.73$ and 0.44, respectively), and it also blocked changes in the relative size of responses induced by CCh during 33 Hz and 10 Hz trains ($F_{1,15} = 3.54$, $p = 0.12$, and $F_{1,15} = 4.03$, $p = 0.10$ respectively; Figure 2.3C,D). The significant block of the CCh-induced facilitation effect by pirenzepine, as compared to that observed in normal ACSF (33 Hz, $F_{1,11} = 20.12$, $p < 0.01$; 10 Hz, $F_{1,11} = 8.95$, $p < 0.05$), indicates that M_1 receptors likely mediate the CCh-induced relative facilitation effect.

M-Current

The cholinergic depolarization of entorhinal layer II neurons is mediated in part by a non-specific cation current I_{NCM} (Shalinsky et al., 2002) but reductions in the muscarinic sensitive K^+ current I_M are also thought to contribute (Yoshida and Alonso, 2007). The possible contribution of I_M-mediated depolarization to the relative facilitation effect was therefore tested by determining if the CCh-induced facilitation effect could be occluded by bath application of the M-current blocker XE991 (10 μM, $n = 5$). Application of XE991 led to a non-significant increase in the amplitude of the first responses in 33 Hz and 10 Hz trains ($p = 0.07$ and 0.13, respectively), consistent with an increase in input resistance. Application of CCh in the presence of XE991 resulted in both an overall decrease in the amplitude of fEPSPs during the trains ($p < 0.01$ for both conditions), as well as a strong relative facilitation of responses (33 Hz, $F_{1,12} = 12.51$, $p < 0.05$; 10 Hz, $F_{1,12} = 12.66$, $p < 0.05$; Figure 2.4). There was no significant difference in the size of the relative facilitation effects in XE991 versus in normal ACSF (33 Hz, $F_{1,10} =$

0.02, $p = 0.90$; 10 Hz, $F_{1,10} = 0.00$, $p = 1.00$; compare with Figure 2.2), indicating that the relative facilitation induced by CCh is not dependent on reductions in I_M.

$GABA_B$ Receptors

Reductions in GABA release at inhibitory synapses caused by activation of $GABA_B$ autoreceptors is known to contribute to the facilitation of postsynaptic responses during theta-burst stimulation (Arai and Lynch, 1992; Staubli and Otaky, 1994; Staubli et al., 1999), and we therefore assessed the role of $GABA_B$ receptors in the relative facilitation induced by CCh using the $GABA_B$ antagonist CGP55845 (1μM, $n = 7$). Addition of CGP55845 to normal ACSF had no significant effect on train-evoked responses ($p = 0.48$ and 0.44 for 33 and 10 Hz trains, respectively). Application of CCh in the presence of CPG55845 resulted in an overall suppression of train-evoked responses ($p < 0.01$ for both conditions), and also induced a relative facilitation of responses during the trains (33 Hz, $F_{1,18} = 21.39$, $p < 0.01$; 10 Hz, $F_{1,18} = 14.78$, $p < 0.05$; Figure 2.5). The size of the relative facilitation was similar to that observed in normal ACSF for both 33 and 10 Hz trains (compare Figures $2.2A_4$,B_4 with Figure $2.5A_4$, B_4; 33 Hz, $F_{1,12} = 1.31$, $p = 0.27$; 10 Hz, $F_{1,12} = 0.08$, $p = 0.79$), indicating that the cholinergic modulation of train-evoked responses is not dependent on $GABA_B$ autoreceptors.

NMDA Glutamate Receptors

The depolarization of entorhinal layer II neurons by CCh could enhance NMDA receptor-mediated components of synaptic responses (Jones, 1990; Jones and Buhl, 1993), and the NMDA receptor antagonist APV (50 μM) was therefore used to assess the role of NMDA receptors in the relative facilitation of responses induced by CCh. Application of APV alone resulted in no significant change in train-evoked responses ($n = 9$; $p = 0.19$ and 0.94 for 33 Hz and 10 Hz conditions, respectively), and also did not affect the size of the overall suppression in fEPSP amplitudes during the trains ($p < 0.01$ for both 33 and 10 Hz). Application of CCh in the presence of APV resulted in a significant relative facilitation of responses during both 33 and 10 Hz trains ($F_{1,24} = 25.74$, $p < 0.01$ and $F_{1,24} = 47.38$, $p < 0.01$, respectively; Figure 2.6); however, the size of relative facilitation was reduced compared to results in normal ACSF for 33 Hz trains ($F_{1,14} = 5.45$, $p < 0.05$). The reduction was not significant for 10 Hz trains ($F_{1,14} = 3.10$, $p = 0.10$), although there was a significant decrease in the relative facilitation of the second pulse when APV was present compared to just CCh ($p < 0.05$). The relative facilitation of train-evoked

responses is therefore partially dependent on NMDA receptor activation, and NMDA receptors appear to contribute most consistently to the relative facilitation observed during 33 Hz trains.

The I_h Current

The hyperpolarization-activated inward cationic current I_h plays a major role in the generation of subthreshold theta-frequency oscillations in layer II neurons (Dickson et al., 2000b). The current I_h is modulated by muscarinic receptor activation (Buzsaki, 2002; Dickson et al., 2000b), and I_h has been shown to amplify synaptic responses evoked during 10 Hz stimulation in the sensorimotor cortex (Castro-Alamancos and Connors 1996). We therefore used the selective I_h blocker ZD7228 (10 μM), to assess the role of I_h in the relative facilitation induced by CCh. The application of ZD7288 itself, while not causing a change in the degree of relative facilitation of evoked trains, led to an increase in the amplitude of responses during both 33 and 10 Hz trains ($n = 8$; $p < 0.05$), an effect that has been described previously (Chevaleyre and Castillo, 2002). Subsequent application of CCh resulted in suppression of synaptic responses during 33 Hz and 10 Hz trains ($p < 0.01$), but the CCh-induced relative facilitation of responses evoked during the trains was strongly blocked (33 Hz, $F_{1,21} = 0.73$, $p = 0.42$; 10 Hz $F_{1,21} = 1.36$, $p = 0.28$; Figure 2.7A,B), and was significantly reduced as compared to the facilitation observed in normal ACSF (33 Hz, $F_{1,13} = 6.72$, $p < 0.05$; 10 Hz, $F_{1,13} = 8.41$, $p < 0.05$). The current I_h therefore plays an important role in the facilitation effects induced during application of CCh.

Similar results were obtained with the less potent I_h blocker Cs^+ (1 mM). Application of Cs^+ alone increased synaptic responses during 33 Hz and 10 Hz trains ($n = 7$, $p < 0.05$), and addition of CCh suppressed basal synaptic transmission during 33 and 10 Hz trains ($p < 0.05$ and $p < 0.01$, respectively). Addition of CCh resulted in a significant relative facilitation of responses during the trains (33 Hz, $F_{1,18} = 10.61$, $p < 0.05$; 10 Hz, $F_{1,18} = 14.93$, $p < 0.05$), however, there was a significantly smaller degree of facilitation when comparing the degree of facilitation for the second ($p = 0.09$), and third ($p = 0.06$) pulses for the 33 Hz condition, and the second ($p = 0.06$) and fourth ($p = 0.06$) pulses for the 10 Hz condition before and after application of CCh to a medium already containing Cs^+, as illustrated in Figure 2.7 C and D. When compared to normal ACSF the amount of facilitation during 33 Hz was smaller than that observed without Cs^+ present ($F_{1,12} = 5.60$, $p < 0.05$; compare Figure 2.2), although the amount of facilitation induced during 10 Hz trains did not differ overall in Cs^+ and in normal ACSF ($F_{1,12} = 1.03$, $p =$

0.33). Results obtained with Cs^+ are therefore consistent with a role for I_h in the CCh-induced facilitation of train-evoked responses.

DISCUSSION

The single major output projection of the parasubiculum targets layer II of the entorhinal cortex, which plays a pivotal role in providing the hippocampal formation with cortical sensory input (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994; Caruana and Chapman, 2004) and cholinergic receptor activation is thought to contribute the theta and gamma activities in both the entorhinal cortex (Mitchell and Ranck, 1980; Dickson et al., 2000a) and parasubiculum (Glasgow et al., 2007). The present study has used field potential recordings in vitro to characterize the effect of the cholinergic agonist carbachol on single entorhinal fEPSPs evoked by parasubicular stimulation, and has examined mechanisms contributing to the cholinergic modulation of synaptic responses during short trains of theta- and gamma-frequency stimulation. Field potentials recorded at multiple depths in the entorhinal cortex following single-pulse stimulation of the parasubiculum showed a negative-going fEPSP located in layer II and upper layer III, with smaller positive potentials in layer I and in deeper layers, consistent with previous reports of evoked responses in vivo and at single depths in vitro (Jones, 1990; Scharfman et al., 1998; Tolner et al., 2007) and with the known anatomy of the parasubicular projection to layer II of the entorhinal cortex (Cabalerro-Bleda and Witter, 1993; Caruana and Chapman, 2004). Constant bath application of CCh resulted in a strong suppression of synaptic responses in layer II of the entorhinal cortex evoked by single stimulation pulses delivered to the parasubiculum, and a concurrent increase in paired-pulse facilitation ratio. This is consistent with the cholinergic suppression of transmitter release that has been shown previously in other synaptic inputs to the entorhinal cortex as well as in the CA1 region, subiculum, and parasubiculum (Auerbach and Segal, 1996; Richter et al., 1999; Yun et al., 2000; Cheong et al., 2001; Kunitake et al., 2004; Carr and Surmeier, 2007; Hamam et al., 2007; Glasgow et al., 2012). The suppression of synaptic transmission may prevent overexcitability during cholinergic depolarization (Klink and Alonso, 1997; Funahashi and Stewart, 1998; Gloveli et al. 1999) and may also serve to reduce interference between representations by suppressing activity within recurrent connections of the hippocampal formation (Haas and White, 2002; de Curtis and Pare, 2004).

Cholinergic inputs to the parasubiculum and entorhinal cortex are thought to contribute to the induction of theta and gamma EEG activities that often co-occur (Gaykema et al., 1990; Buzsaki, 2002; Hasselmo, 2006), and the present study assessed how cholinergic receptor activation may affect repetitive synaptic responses at these frequencies. Application of CCh resulted in an overall suppression of responses evoked during short trains of stimulation at 10 and 33 Hz but, instead of the strong decrements in responses to consecutive pulses that are observed in normal ACSF, the smaller responses evoked in the presence of CCh were much more robust, and were either maintained or facilitated in response to consecutive pulses in the trains. Theta and gamma-frequency EEG activity that is sensitive to cholinergic antagonists is generated in the entorhinal cortex and parasubiculum as animals navigate through the environment (Dickson et al., 2000a; Dickson and de Curtis, 2002; Brazhnik et al., 2003, 2004), and the firing of parasubicular neurons is paced in part by these rhythms (Taube, 1995; Boccara et al., 2010). The relative facilitation of synaptic responses during theta activity could help maintain synaptic communication between the parasubiculum and entorhinal cortex during the cholinergic suppression of transmitter release (Brenowitz and Trussel, 2001; Kunitake et al., 2004; Carr and Surmeier, 2007). This may help maintain parasubicular contributions to the responsiveness of the entorhinal cortex to other sensory inputs (Caruana and Chapman, 2004) and may also promote synaptic communication among cells in the parasubiculum and entorhinal cortex involved in spatial navigation (Taube, 1995; Hargreaves et al., 2007; Hafting et al., 2008; Boccara et al., 2010).

Receptor-Mediated Effects

Both the cholinergic suppression of EPSPs and the relative facilitation of responses during trains were blocked by pirenzepine, but not methoctramine, suggesting that the effects require activation of M_1 receptors. This is a tentative conclusion because of possible effects of antagonists on multiple receptor subtypes (Caulfield and Birdsall, 1998), and there is good evidence for a role of M_4 receptors in cholinergic suppression in the hippocampus (Dasari and Gulledge, 2011), but the present results are consistent with similar evidence for the M_1 receptor-dependent suppression of synaptic responses in the entorhinal cortex (Richter et al., 1999), parasubiculum (Glasgow et al., 2012), subiculum (Kunitake et al., 2004) and hippocampus (Kremin et al., 2006). The cholinergic suppression of EPSPs is mediated by reduced transmitter release that also leads to an increase in paired-pulse facilitation due to the larger readily

releasable pool of transmitter available during the second pulse (Yamada and Zucker, 1992; Hamam et al., 2007). The relative facilitation of evoked responses observed here in CCh is almost certainly dependent to a large extent on a larger pool of readily releasable transmitter maintained during the trains (Brenowitz and Trussel, 2001).

Synaptic responses can be enhanced during theta-burst stimulation due to reductions in GABA release at inhibitory synapses caused by activation of $GABA_B$ autoreceptors (Davies and Collingridge, 1996; Staubli et al., 1999). However, the $GABA_B$ receptor blocker CGP55845, although associated with a slight non-significant facilitation of responses when applied alone, caused no change in the relative facilitation of responses induced by CCh. The relatively mild stimulation trains of five single pulses may have been insufficient to evoke substantial activation of presynaptic $GABA_B$ receptors.

The NMDA receptor blocker APV partially blocked the relative facilitation of train-evoked responses induced by CCh for both theta- and gamma-frequency trains. Woodhall et al. (2001) have shown that activation of presynaptic NMDA autoreceptors on terminals onto layer II and V entorhinal cells can strongly facilitate transmitter release and, further, that APV eliminates the facilitation of EPSCs that is observed during 3 Hz stimulation of layer V neurons. We did not observe an effect of APV alone on train-evoked responses, but it is possible that repetitive stimulation in the presence of CCh could result in the activation of NMDA autoreceptors and an enhancement of transmitter release during the responses observed here.

In addition, activation of cholinergic receptors can enhance NMDA currents in hippocampus and cortex (Markram and Segal, 1990; Aramakis et al., 1999), and the cholinergic depolarization of entorhinal layer II neurons (Klink and Alonso, 1997; Hamam et al., 2007) could result in a depolarization-dependent enhancement of NMDA receptor-mediated components of the EPSP. Stronger theta-patterned stimulation trains are thought to be effective for the induction of long-term synaptic potentiation due to an enhancement of NMDA receptor-mediated currents (Arai and Lynch, 1992), and NMDA receptors also contribute to the facilitation of responses to 5-pulse 100 Hz trains in the subiculum (Kunitake et al., 2004).

The M-Current and I_h

Application of the M-current blocker XE991 depolarizes stellate and non-stellate neurons in layer II of the entorhinal cortex (Yoshida and Alonso, 2007) and CCh-induced suppression of I_M could enhance EPSPs by increasing input resistance and by a depolarization-dependent

enhancement of NMDA currents. Application of the I_m blocker XE991 alone caused a clear increase in EPSPs that may have resulted in part from increased input resistance (Day et al., 2005; Carr and Surmier, 2007; Carr et al., 2007). However, application of XE991 did not occlude the relative facilitation of train-evoked responses induced by CCh, indicating that the facilitation effect is does not rely on I_M-dependent mechanisms. Carbachol-induced depolarization can also be mediated by muscarinic activation of the nonspecific cation current I_{NCM} mediated by transient receptor potential channels (Shalinsky et al., 2002); intracellular recordings will be needed to more directly assess the role of cholinergic depolarization on the facilitation effects observed here.

The hyperpolarization-activated mixed cationic current I_h is known to contribute to the generation of theta-frequency membrane potential oscillations in layer II entorhinal neurons, and muscarinic depolarization can induce I_h-dependent membrane potential oscillations that may interact with repetitive synaptic inputs (Castro-Alamancos and Connors, 1996; Klink and Alonso 1997; Dickson et al., 2000b). However, the application of CCh is also known to suppress theta-frequency membrane potential resonance in entorhinal layer II cells through an *inhibition* of I_h at depolarized potentials (Heys and Hasselmo, 2010), and the inhibition of I_h can also enhance temporal summation of EPSPs by increasing input resistance (Chevaleyre and Castillo, 2002; Rosenkranz and Johnson, 2006). The I_h blockers ZD7288 and Cs^+ were used here to determine if blocking I_h would occlude further CCh-induced changes in train-evoked responses. Application of either ZD7288 or Cs^+ resulted in an increase the amplitude of synaptic responses (see also Chevaleyre and Castillo, 2002), and the I_h blockers also inhibited the relative facilitation of responses induced by CCh. Application of ZD7288, the more potent and selective I_h blocker, also caused a more potent block of facilitation effects. Modulation of I_h currents following CCh application may therefore contribute substantially to the relative enhancement of synaptic responses during trains.

The facilitation of EPSPs induced by block of I_h with ZD7288 is not likely to be due to increased transmitter release because paired-pulse facilitation ratio was not affected, and postsynaptic factors such as increased input resistance are therefore likely involved. *Increases* in I_h reduce summation of EPSPs due to reduced input resistance in various cell types including neurons in layer V of the entorhinal cortex (Magee, 2000; Rosenkranz and Johnston, 2006; van Welie et al., 2006; Liu and Shipley, 2008), and reductions in I_h induced by application of either

CCh or ZD7288 may contribute to the facilitation effects observed here (Magee, 1998; van Welie et al., 2006; Day et al., 2005). Although a relative reduction in the amount of I_h expressed at depolarized voltage may contribute, application of ZD7288 alone had no substantial effect on the relative facilitation of response (Figure 2.7A_4,B_4), and CCh may have a much more powerful effect of the summation of repetitive synaptic inputs through the depolarization of neurons into the voltage range at which I_h is able to contribute to oscillations and resonance in membrane potential (Heys et al., 2010). Carbachol induces theta-frequency membrane potential oscillations in layer II entorhinal neurons that are dependent on activation of I_h (Gloveli et al., 1999; Dickson and Alonso 2000b), and CCh also induces gamma-frequency population activity in layer II neurons (Dickson, et al., 2000a). Activation of synaptic inputs to the entorhinal cortex synchronizes the phase of gamma oscillations among neurons (Dickson, et al., 2000a; Dickson and de Curtis, 2002), and stimulation of parasubicular inputs may also synchronize activity in principal neurons through the activation of inhibitory inputs that may induce synchronous activation of I_h (Jones, 1990; Jones and Buhl, 1993; Chapman and Lacaille, 1997). Trains of stimulation used here may therefore have helped to synchronize oscillatory mechanisms within the entorhinal cortex, and synaptic responses may have been enhanced by the associated intrinsic conductances including I_h. In the sensorimotor cortex, activation of I_h currents is thought to be a major contributor to the augmenting of synaptic responses during 10 Hz stimulation (Castro-Alamancos and Connors 1996).

Conclusion

Field potential recording techniques have been used in the present manuscript to describe a novel M_1 receptor-dependent suppression of synaptic responses in parasubicular inputs to layer II of the entorhinal cortex, and to investigate associated mechanisms that mediate a relative facilitation of synaptic responses evoked during short theta- and gamma-frequency trains. The maintenance and facilitation of synaptic responses during the trains is likely to be largely dependent on a maintained pool of readily releasable transmitter, but we also show here that additional factors including modulation of I_h, and activation of NMDA glutamate receptors, also contribute to the train-evoked responses. Intracellular recordings will be required to determine the relative contributions of these mechanisms to the facilitation of synaptic responses.

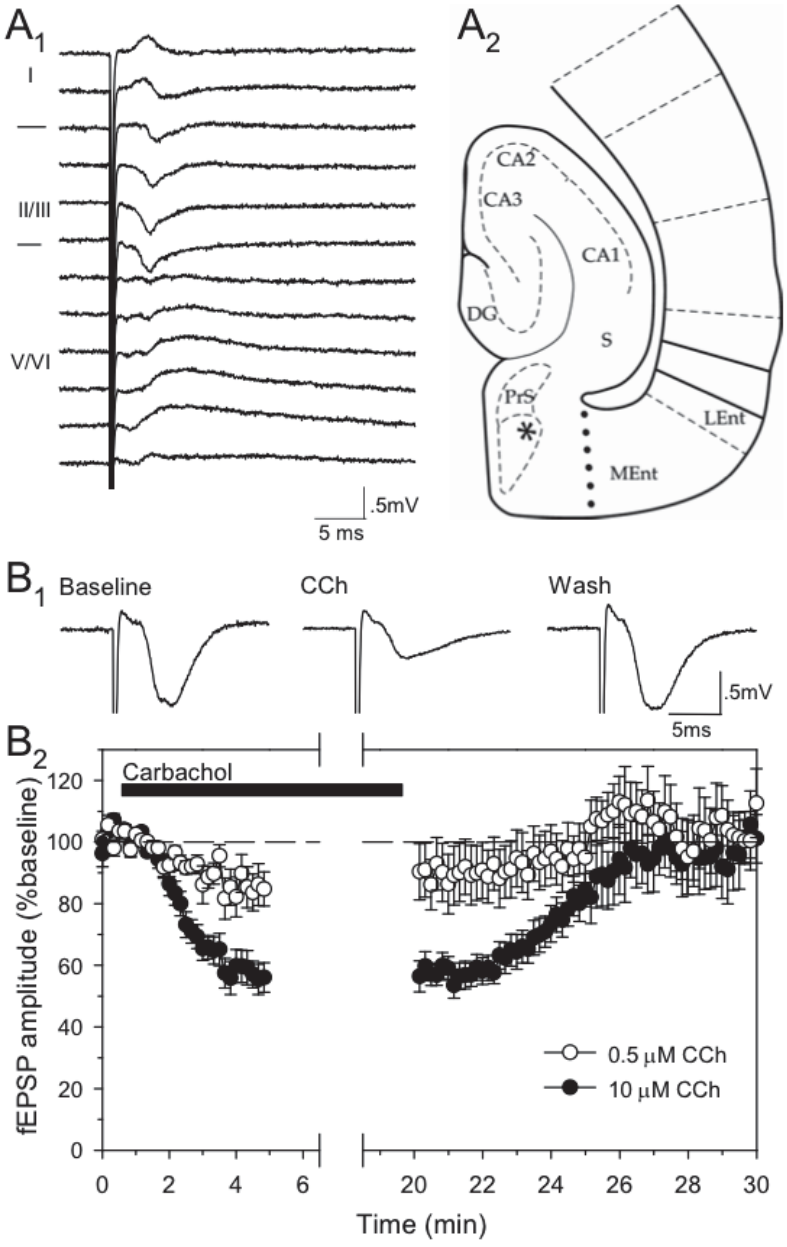

A1
I
II/III
V/VI
.5mV
5 ms
A2
CA2
CA3
CA1
DG
S
PrS
LEnt
MEnt
B1
Baseline
CCh
Wash
.5mV
5ms
B2
Carbachol
fEPSP amplitude (%baseline)
120
100
80
60
40
20
0
0
2
4
6
20
22
24
26
28
30
Time (min)
0.5 μM CCh
10 μM CCh

Figure 2.1. Field excitatory postsynaptic potentials (fEPSPs) at multiple depths in the medial entorhinal cortex evoked by stimulation of the parasubiculum (PaS). **(A)** Recordings of fEPSPs, which were obtained starting at the surface of the entorhinal cortex and moving toward the deep layers at intervals of 100 μm, are shown for a representative slice (A_1). Locations of electrodes are shown schematically on a horizontal section taken from the atlas of Paxinos and Watson (1998, A_2). The asterisk indicates the location of the stimulation electrode, and the dotted line indicates the trajectory of recording electrode locations. Responses typically showed a small positive-deflection in layer I, and a larger negative-going response in layers II/III that reversed into a positive-going response in the deeper layers. This is consistent with the activation of synaptic targets of the parasubiculum in layer II of the entorhinal cortex. **(B)** Application of the cholinergic agonist carbachol (CCh) suppresses the amplitude of fEPSP in the entorhinal cortex evoked by single pulses of stimulation delivered to the parasubiculum. Averaged field EPSPs evoked by parasubicular stimulation were obtained before, during, and after application of 10 μM CCh (B_1). Application of CCh (bar) resulted in a significant reduction in the amplitude of fEPSPs in slices exposed to 10 μM CCh (B_2). The gap in recordings reflects the time at which responses to trains of stimulation were obtained.

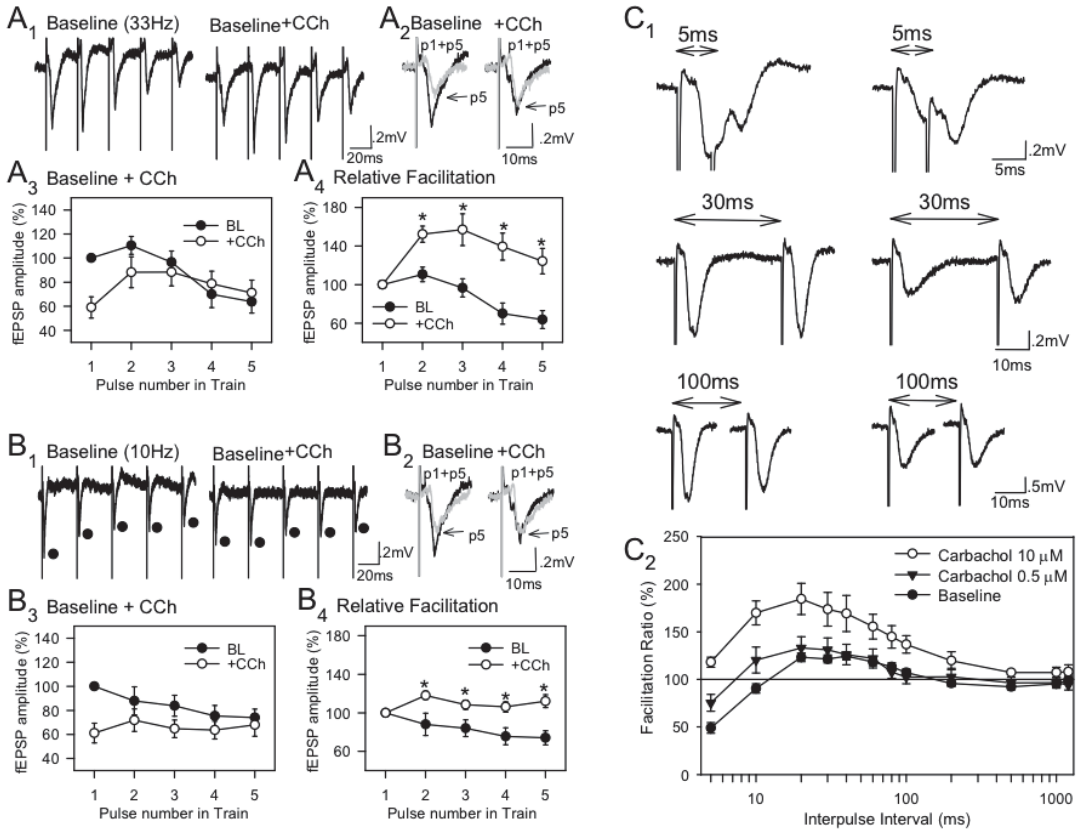

A1 Baseline (33Hz)
Baseline+CCh
A2 Baseline +CCh
p1+p5
p5
.2mV
20ms
10ms
A3 Baseline + CCh
A4 Relative Facilitation
fEPSP amplitude (%)
BL
+CCh
Pulse number in Train
B1 Baseline (10Hz)
B2 Baseline +CCh
B3 Baseline + CCh
B4 Relative Facilitation
C1
5ms
30ms
100ms
.5mV
C2
Carbachol 10 μM
Carbachol 0.5 μM
Baseline
Facilitation Ratio (%)
Interpulse Interval (ms)

Figure 2.2. The cholinergic agonist carbachol (CCh) attenuates the decline in fEPSPs observed during short trains of repetitive stimulation at frequencies within the gamma (33 Hz) and theta (10 Hz) bands. **(A)** Sample recordings of responses to 5-pulse trains of stimulation at 33 Hz are shown for a representative slice before and after application of 10 μM CCh (A_1). The response to the first pulse in the train has been superimposed with the response to the fifth pulse (p1 + p5) at an expanded time scale for both baseline responses and responses in CCh (A_2). Arrows indicate the peak of the response to the fifth pulse. Normalization of responses to the amplitude of the first response in the train in normal ACSF shows that CCh suppressed the amplitude of the first response in the train (A_3). However, application of CCh results in a marked facilitation of the amplitudes of responses expressed as a percentage of the amplitude of the first response in the train (A_4). **(B)** A similar facilitation of train-evoked responses is observed in response to trains of 10-Hz stimulation pulses. Conventions are as in A, and circles indicate the amplitudes of responses evoked by each pulse. **(C)** Application of CCh resulted in an increase in paired-pulse facilitation ratios at interpulse intervals of 5–100 ms. Representative responses recorded before and after CCh application are shown for intervals of 5, 30, and 100 ms (C_1). Mean paired-pulse ratios were enhanced at all intervals from 5 to 100 ms by 10 μM CCh, but 0.5 μM CCh had a non-significant effect (C_2). Note the peak facilitation observed at interpulse intervals of 20–30 ms.

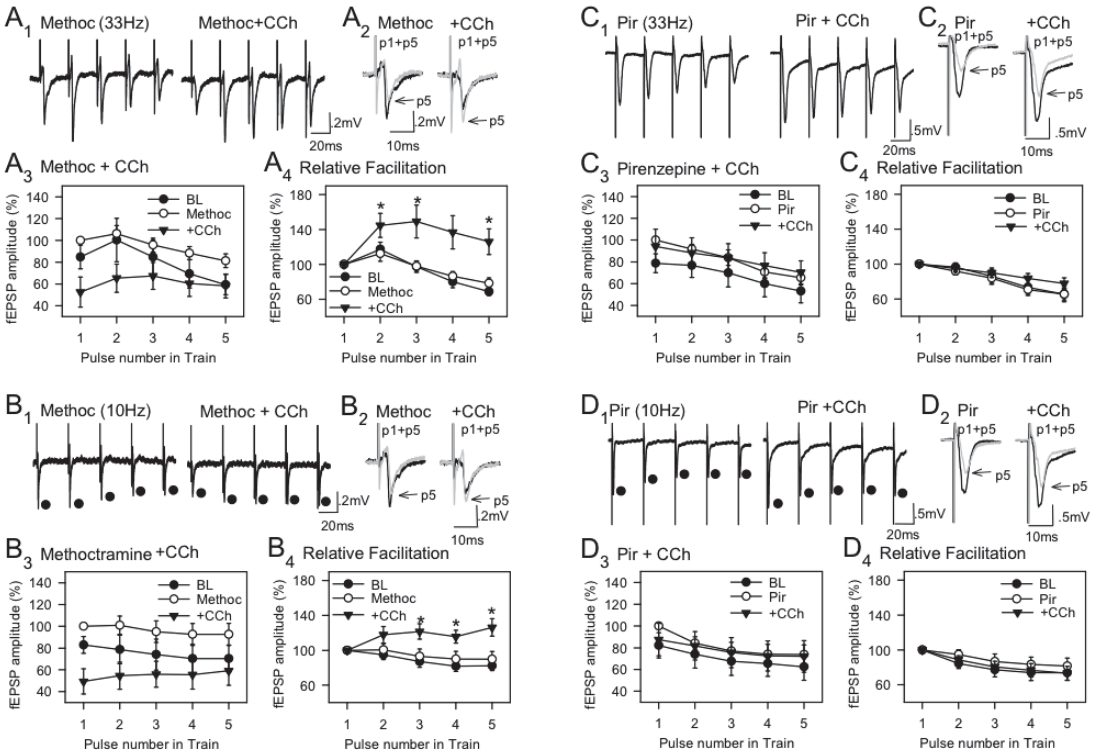

A1 Methoc (33Hz)
Methoc+CCh
A2 Methoc
+CCh
p1+p5
p5
2mV
20ms
10ms
A3 Methoc + CCh
A4 Relative Facilitation
fEPSP amplitude (%)
BL
Methoc
+CCh
Pulse number in Train
B1 Methoc (10Hz)
Methoc + CCh
B2 Methoc
+CCh
B3 Methoctramine +CCh
B4 Relative Facilitation
C1 Pir (33Hz)
Pir + CCh
C2 Pir
+CCh
5mV
.5mV
C3 Pirenzepine + CCh
C4 Relative Facilitation
Pir
D1 Pir (10Hz)
Pir +CCh
D2 Pir
+CCh
D3 Pir + CCh
D4 Relative Facilitation

Figure 2.3. The muscarinic M_1 receptor antagonist pirenzepine, but not the M_2 receptor antagonist methoctramine, blocks both the CCh-induced suppression of responses to single stimuli, and the relative facilitation of responses induced by CCh during both 33- and 10-Hz stimulus trains. Conventions in this and subsequent figures are as in Fig. 2.2A, B. **(A, B)** Application of the M_2 receptor blocker methoctramine (10 μM) alone had no significant effect on basal synaptic transmission, and the addition of CCh resulted in strong effects on train-evoked responses similar to those observed following CCh-application in normal ACSF (see Fig. 2). **(C, D)** Application of the M_1 receptor blocker pirenzepine (1 μM) completely blocked both the suppression of basal synaptic transmission, and the relative facilitation of synaptic responses observed during both 33- and 10-Hz trains, suggesting that these effects are mediated by M_1 receptor activation.

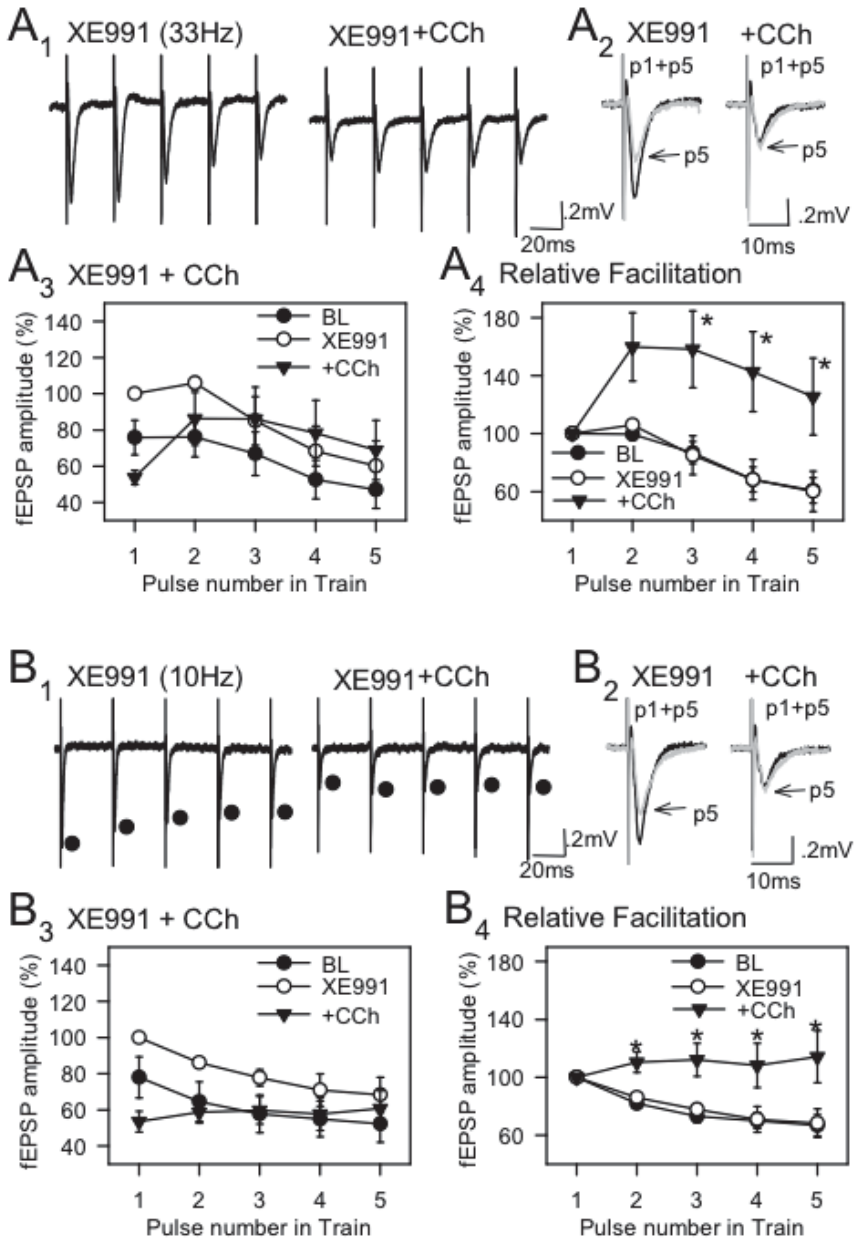

A1 XE991 (33Hz)
XE991+CCh
.2mV
20ms
A2 XE991
+CCh
p1+p5
p5
.2mV
10ms
A3 XE991 + CCh
BL
XE991
+CCh
fEPSP amplitude (%)
Pulse number in Train
A4 Relative Facilitation
B1 XE991 (10Hz)
XE991+CCh
B2 XE991
+CCh
B3 XE991 + CCh
B4 Relative Facilitation

Figure 2.4. The M-current receptor antagonist XE991 has no effect on the CCh-induced relative facilitation of synaptic transmission. **(A, B)** Addition of XE991 alone was associated with non-significant increases in the amplitude of responses evoked by both 33- and 10-Hz trains ($p = 0.07$ and 0.13, respectively; A_3, B_3). However, in comparison to results obtained in normal ACSF (see Fig. 2), XE991 had no significant effect on either the overall suppression of responses induced by CCh (A_3, B_3), or the relative facilitation of responses during the trains (A_4, B_4). The M-current therefore plays no significant role in the relative facilitation of responses induced by CCh.

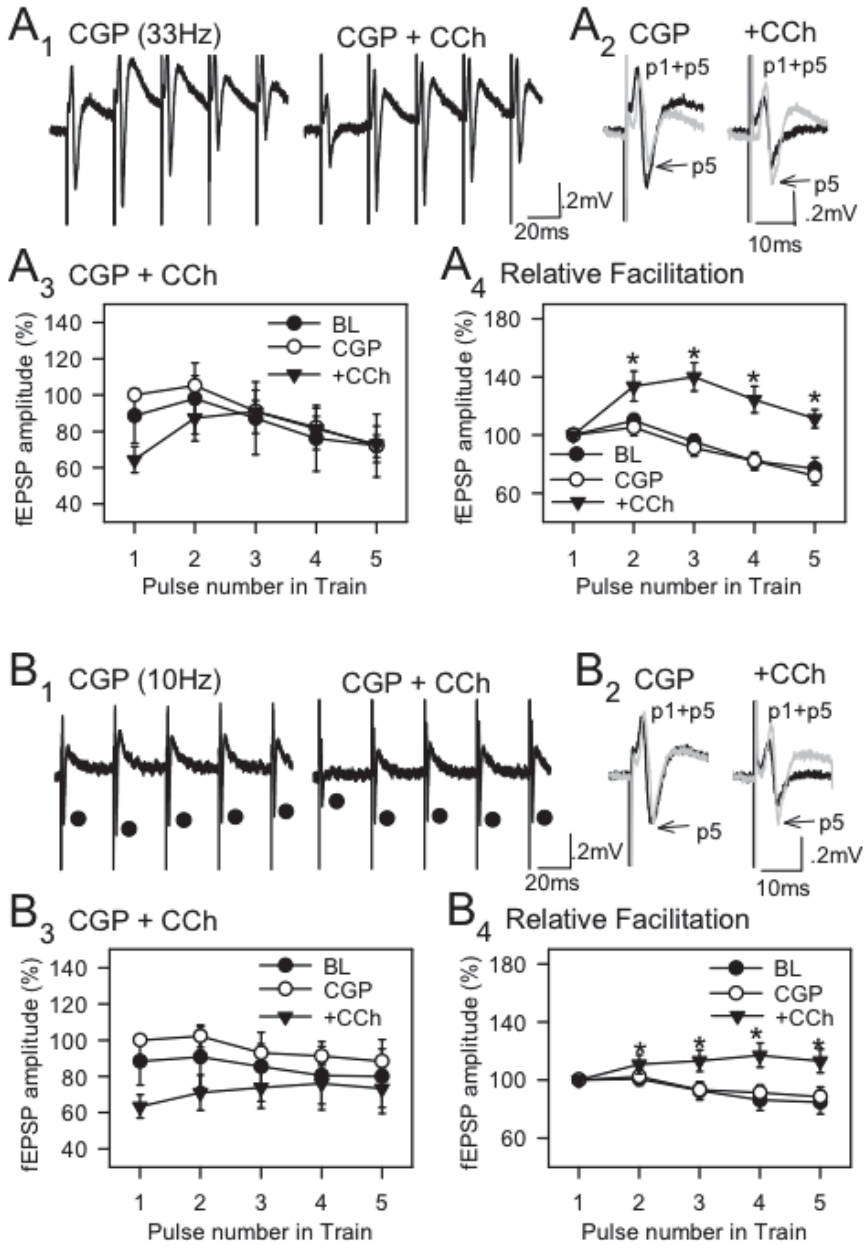

A1 CGP (33Hz)
CGP + CCh
A2 CGP
+CCh
p1+p5
p5
.2mV
20ms
10ms
A3 CGP + CCh
A4 Relative Facilitation
fEPSP amplitude (%)
BL
CGP
+CCh
Pulse number in Train
B1 CGP (10Hz)
CGP + CCh
B2 CGP
+CCh
B3 CGP + CCh
B4 Relative Facilitation

Figure 2.5. The $GABA_B$ receptor antagonist CGP55845 does not significantly affect the relative facilitation of train-evoked responses induced by CCh. **(A, B)** Application of CGP55845 alone had no effect on train-evoked responses (A_3, B_3) and, in comparison to results obtained in normal ACSF (see Fig. 2), CGP55845 also had no significant effect on the overall suppression of responses induced by CCh (A_3, B_3), and on the relative facilitation of responses during the trains (A_4, B_4). Modulation of synaptic transmission dependent on $GABA_B$ receptors therefore does not play a significant role in the facilitation of train-evoked responses induced by CCh.

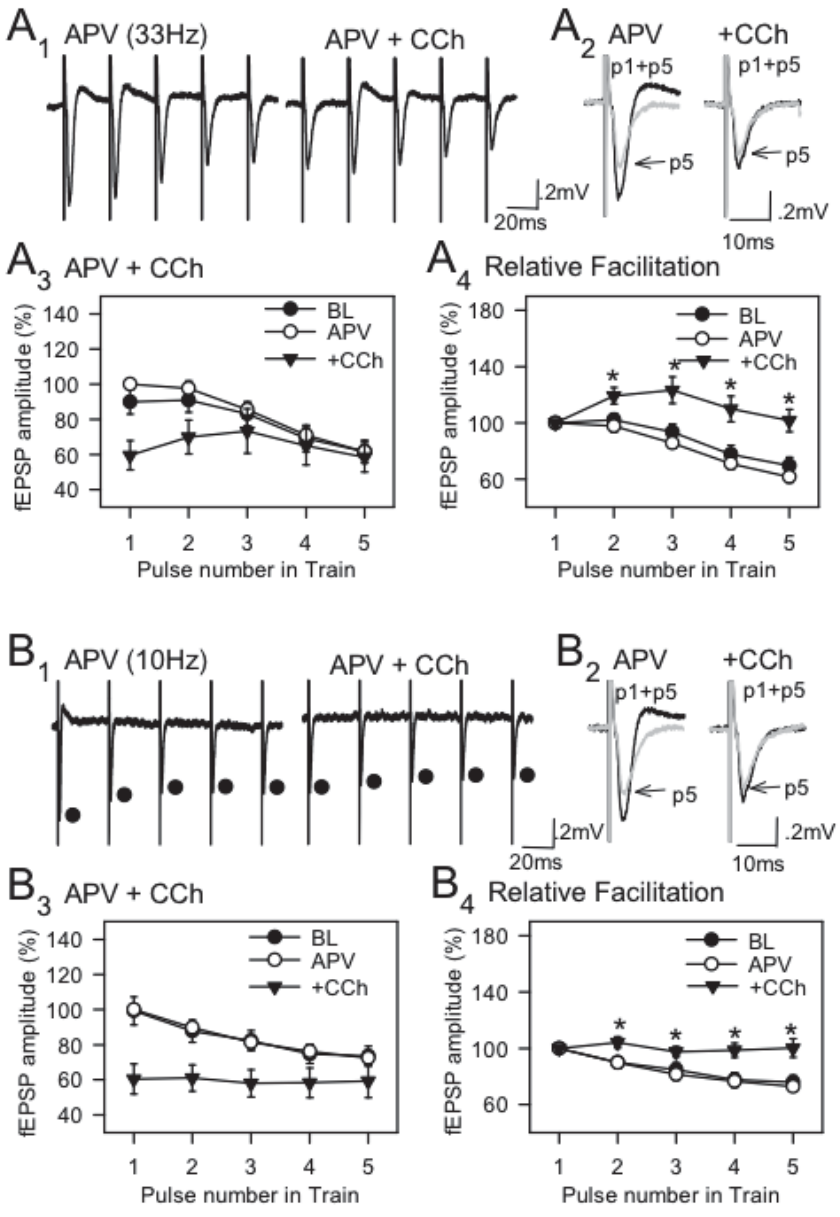

A1 APV (33Hz)
APV + CCh
.2mV
20ms
A2 APV
+CCh
p1+p5
p5
.2mV
10ms
A3 APV + CCh
fEPSP amplitude (%)
BL
APV
+CCh
Pulse number in Train
A4 Relative Facilitation
B1 APV (10Hz)
APV + CCh
B2 APV
+CCh
B3 APV + CCh
B4 Relative Facilitation

Figure 2.6. The NMDA glutamate receptor antagonist APV partially blocked the relative facilitation of train-evoked responses induced by CCh. **(A, B)** Application of APV alone had no significant effect on responses evoked by 33- and 10-Hz trains, and also did not significantly affect the overall suppression of train-evoked responses induced by CCh (A_3, B_3), but APV significantly suppressed the relative facilitation of responses induced by CCh during a 33-Hz stimulation in comparison to the facilitation effect observed in normal ACSF (A_4). A trend toward reduced facilitation during 10-Hz trains was not significant (B_4). The relative facilitation of synaptic responses during 33-Hz trains is therefore dependent in part on the activation of NMDA receptors, and NMDA receptors may also contribute to facilitation effects during 10-Hz trains.

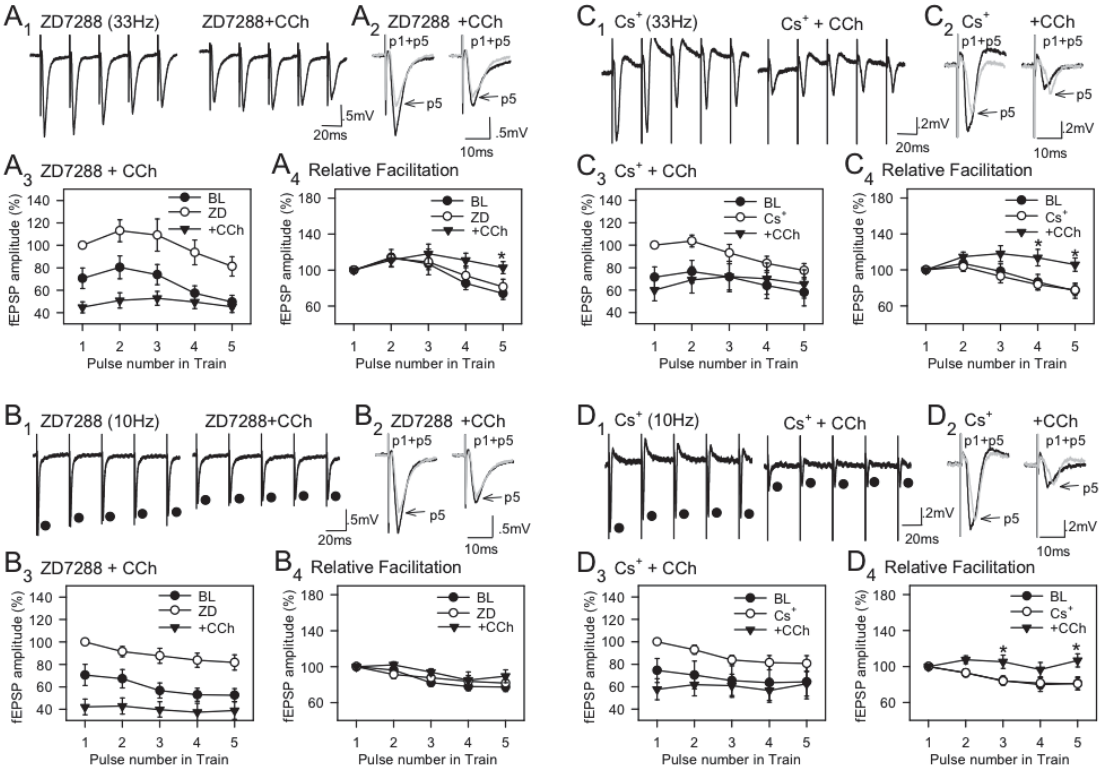

A1 ZD7288 (33Hz)
ZD7288+CCh
A2 ZD7288 +CCh
p1+p5
p5
5mV
20ms
.5mV
10ms
A3 ZD7288 + CCh
A4 Relative Facilitation
BL
ZD
+CCh
fEPSP amplitude (%)
Pulse number in Train
C1 Cs+ (33Hz)
Cs+ + CCh
C2 Cs+
+CCh
2mV
C3 Cs+ + CCh
C4 Relative Facilitation
Cs+
B1 ZD7288 (10Hz)
ZD7288+CCh
B2 ZD7288 +CCh
B3 ZD7288 + CCh
B4 Relative Facilitation
D1 Cs+ (10Hz)
Cs+ + CCh
D2 Cs+
+CCh
D3 Cs+ + CCh
D4 Relative Facilitation

Figure 2.7. The CCh-induced facilitation of synaptic responses during short trains of stimulation is strongly blocked by the selective I_h blocker ZD7288, and is also partially blocked by the less potent I_h inhibitor Cs^+. **(A, B)** Constant bath application of ZD7288 alone resulted in a significant overall increase in the amplitude of train-evoked responses (A_3, B_3). Subsequent addition of CCh resulted in an overall suppression of evoked responses similar to that observed in normal ACSF (A_3, B_3), but the relative facilitation of responses induced by CCh in normal ACSF was blocked in the presence of ZD7288 (A_4, B_4). **(C, D)** Application of Cs^+, like ZD7288, resulted in an increase in the amplitude of basal synaptic transmission, and did not affect the overall suppression of synaptic responses induced by CCh (C_3, C_4). The presence of Cs^+ significantly reduced the amount of facilitation induced by CCh during 33-Hz trains as compared to that induced by CCh in normal ACSF (C_4), and Cs^+ also partially reduced the facilitation effect during 10-Hz trains, such that the amount of facilitation was significantly suppressed for responses evoked by the second and fourth pulses in the trains. The CCh-induced facilitation of synaptic responses evoked during theta and gamma-frequency stimulation trains is therefore mediated in part by the hyperpolarization-activated current I_h.

CHAPTER 3

CONTRIBUTION OF I_h TO THE RELATIVE FACILITATION OF SYNAPTIC RESPONSES INDUCED BY CARBACHOL IN THE ENTORHINAL CORTEX DURING REPETITIVE STIMULATION OF THE PARASUBICULUM

Daniel W. Sparks and C. Andrew Chapman

ABSTRACT

Neurons in the superficial layers of the entorhinal cortex provide the hippocampus with the majority of its cortical sensory input, and also receive the major output projection from the parasubiculum. This puts the parasubiculum in a position to modulate the activity of entorhinal neurons that project to the hippocampus. These brain areas receive cholinergic projections that are active during periods of theta and gamma-frequency EEG activity. The purpose of this study was to investigate how cholinergic receptor activation affects the strength of repetitive synaptic responses at these frequencies in the parasubicular-entorhinal pathway and the cellular mechanisms involved. Whole-cell patch clamp recordings of layer II medial entorhinal neurons were conducted using an acute slice preparation, and responses to 5-pulse trains of stimulation at theta and gamma-frequency delivered to the parasubiculum were recorded. The cholinergic agonist carbachol suppressed the amplitude of single synaptic responses, but also produced a relative facilitation of synaptic responses evoked during stimulation trains. The NMDA glutamate receptor blocker APV did not significantly reduce the relative facilitation effect. However, the I_h channel blocker ZD7288 mimicked the relative facilitation induced by carbachol, suggesting that carbachol-induced inhibition of I_h could produce the effect by increasing dendritic input resistance. Inward-rectifying and leak K^+ currents are known to interact with I_h to affect synaptic excitability. Application of the K^+ channel antagonist Ba^{2+} depolarized neurons and enhanced temporal summation, but did not block further facilitation of train-evoked responses by ZD7288. The I_h-dependent facilitation of synaptic responses can therefore occur during reductions in IK_{ir} associated with dendritic depolarization. Thus, in addition to cholinergic reductions in transmitter release that are known to facilitate train-evoked responses, these findings emphasize the role of inhibition of I_h in the integration of synaptic inputs within the entorhinal cortex during cholinergically-induced oscillatory states, likely due to enhanced summation of EPSPs induced by increases in dendritic input resistance.

The superficial layers of the entorhinal cortex provide the hippocampus with the majority of its sensory input via layer II projections to the dentate gyrus, and layer III projections to CA1 and the subiculum, (Witter et al., 1989; Amaral, 1993; Burwell et al., 1995). The entorhinal cortex obtains its inputs from cortical sensory areas including the perirhinal, postrhinal, and piriform cortices (Burwell et al., 1995; Burwell and Amaral, 1998), and layer II entorhinal neurons also receive a direct projection from the parasubiculum (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994; Caruana and Chapman, 2004). The parasubiculum is a structure that receives inputs from the CA1, subiculum, basolateral amygdala, and anterior thalamus (van Groen and Wyss, 1990), and parasubicular projections to the entorhinal cortex provide a pathway that may modulate the nature of entorhinal sensory inputs to the hippocampus (Caruana and Chapman, 2004; Sparks and Chapman, 2013).

Both the entorhinal cortex and parasubiculum receive dense cholinergic projections from the medial septum (Gaykema et al., 1990). Acetylcholine plays a role in the generation of theta and gamma rhythm EEG activities in both the entorhinal cortex and parasubiculum (Mitchell and Ranck, 1980; Dickson et al. 2000a; Glasgow and Chapman, 2007; Tsuno et al., 2013) in part by depolarizing neurons to near-threshold voltage levels (Klink and Alonso 1997; Dickson et al. 2000b; Hasselmo 2006; Glasgow and Chapman, 2008). Theta and gamma activities co-occur and are thought to contribute to mechanisms of spatial navigation and learning and memory in the hippocampal formation, entorhinal cortex and parasubiculum (Buzsaki, 2002; Hasselmo, 2006; Igarashi et al., 2014; Yamamoto et al., 2014) and the coordination of firing among place and grid cells (O'Keefe and Recce, 1993; Taube, 1995; Hasselmo et al., 2007; Boccara et al., 2010).

Our previous findings have shown that the cholinergic agonist carbachol can enhance synaptic responses in the entorhinal cortex recorded in response to short trains of stimulation pulses delivered at theta and gamma frequencies (Sparks and Chapman, 2013). Although muscarinic receptor activation suppresses synaptic responses to single stimulation pulses in the entorhinal cortex (Richter et al., 1999; Hamam et al., 2007; Glasgow et al., 2012; Barrett and Chapman, 2013), similar to what has been found following cholinergic agonism in the hippocampal formation (Auerbach and Segal, 1996; Yun et al., 2000; Kunitake et al., 2004), responses to later pulses in the stimulation trains show a relative facilitation (Sparks and Chapman, 2013). Similar facilitation effects have been shown in other areas of the hippocampal formation (Kunitake et al., 2004; Carr and Surmeier, 2007). This relative facilitation of synaptic

transmission implies that signals received at these frequencies are maintained during cholinergic suppression of transmitter release (Brenowitz and Trussel, 2001; Kunitake et al., 2004; Carr and Surmeier, 2007), promoting synaptic communication during spatial learning and navigation (Taube, 1995; Hargreaves et al., 2007; Hafting et al., 2008; Boccara et al., 2010). In addition to the cholinergic suppression of transmitter release that can facilitate train-evoked responses by increasing the availability of readily releasable neurotransmitter, we found that the relative facilitation effect in the entorhinal cortex was dependent upon the non-specific cationic current I_h, and was reduced by NMDA receptor blockade (Sparks and Chapman, 2013), but the field potential recordings used did not allow changes in intracellular EPSPs or cellular input resistance to be assessed.

I_h is a hyperpolarization-activated non-specific cationic current that contributes to generation of theta EEG activity and synaptic integration in the hippocampal formation (Magee, 1998; Dickson et al., 2000b; Carr et al., 2007) and the timing of firing of place cells and grid cells (Giocomo et al., 2007; Barry et al., 2012; Hasselmo and Brandon, 2012). Activation of I_h leads to depolarization of membrane potential, but the associated reductions in dendritic input resistance can decrease the width of EPSPs and reduce temporal summation of synaptic inputs (Magee, 1998). Conversely, closure of I_h can enhance synaptic communication through increases in input resistance (Magee, 1998; Day et al., 2005; Rosenkranz and Johnston, 2006; Garden et al., 2008). Because muscarinic receptor activation can reduce I_h through phospholipase C-mediated depletion of PIP_2 (Pian et al., 2006, 2007), muscarinic induced reduction in I_h can enhance temporal summation of synaptic responses through changes in dendritic input resistance (Magee, 1998; Kunitake et al., 2004).

Membrane potential is influenced by I_h, inwardly rectifying and leak K^+ channels (Meuth et al., 2006), and changes in currents carried by IK_{ir} and IK_{leak} can lead to changes in membrane potential that can modulate I_h. The hyperpolarizing influence of outward currents mediated by K^+ channels helps to maintain membrane potential within a range at which I_h can remain active (George et al., 2009), and closure of potassium channels can lead to depolarization-induced reductions in I_h that can enhance temporal summation via increased dendritic input resistance (Day et al., 2005; Carr and Surmeier, 2007). Muscarinic receptor activation results in membrane potential depolarization in entorhinal cells that can lead to reductions in inward rectifying K channels (Yun et al., 2000; Glasgow and Chapman, 2013), and effects of cholinergically-induced

changes in I_h on train-evoked responses may therefore be dependent in part upon changes in K^+ channels (Day et al., 2005; Carr and Surmeier, 2007; Shen et al., 2007).

The present study used recordings of intracellular EPSPs in layer II entorhinal neurons to investigate mechanisms mediating the cholinergic facilitation of entorhinal synaptic responses during brief, 5-pulse trains of theta- and gamma-frequency stimulation of the parasubiculum. Our previous results showed that NMDA receptors contribute to the relative facilitation of field potential recordings induced by the cholinergic agonist carbachol (CCh; Sparks and Chapman, 2013; see also Aramakis et al., 1999; Woodhall et al., 2001), and we therefore assessed the effects of NMDA receptor blockade on the CCh-induced facilitation of intracellular EPSPs. The contribution of I_h to the relative facilitation effect was assessed using the I_h blocker ZD7288, and by using Ba^{2+} to determine if effects of I_h might be restricted by blocking inward rectifying or leak K^+ channels. Results show a strong role for I_h that is related to changes in input resistance that is not dependent on Ba^{2+}-sensitive K^+ channels.

METHODS

Slice preparation

Guidelines of the Canadian Council on Animal Care were followed in all experiments. Acute horizontal brain slices were obtained from 4 to 8-week-old male Long-Evans rats after halothane anaesthetization and decapitation. Brains were removed and submerged in artificial cerebrospinal fluid (ACSF, 4 ºC) consisting of (in mM): 250 sucrose, 2 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 26 $NaHCO_3$, 0.5 $CaCl_2$ and 10 dextrose saturated with 95% O_2/5% CO_2. Slices were cut 300 μm-thick using a vibratome (WPI, Vibroslice NVSL, Sarasota, FL, USA), and recovered at room temperature for 1 to 1.5 hours in ACSF containing 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose. Individual slices were placed on a nylon net in a gas-fluid interface chamber at 32 ºC with a 95% O_2/5% CO_2 atmosphere and were perfused with ACSF at a rate of 1 to 1.5 ml/min. Slices were visualized using a Leica DM-LFS microscope (Concord, ON, CA) with a long-range water immersion objective (40X) and differential interference contrast optics.

Stimulation and recording

Whole-cell patch clamp recordings were obtained using borosilicate glass pipettes (1.0 mm OD, 0.5 mm ID, 4-8 MΩ) made with a horizontal puller (Sutter Instr., Navato, CA, USA,

Model P97). These were filled with a patch solution 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 adjusted to 7.20–7.26 using KOH; 270–280 mOsm). Pipettes were lowered to make contact with layer II medial entorhinal cortex neurons located close to the border with layer I. Gentle suction was applied in voltage-clamp mode to obtain a seal of at least 1GΩ and stronger suction was applied to rupture the membrane. Cells rested for at least 10 minutes before recordings were taken. Series resistance was compensated by adjusting the discontinuity in voltage responses to -100 pA pulses, and cells were considered acceptable if series resistance was less than 30 MΩ (19.8 ± 1.5 MΩ), and if resting membrane potential was less than -50 mV. Input resistance was measured using 500 ms hyperpolarizing current injections of -100 pA (0.1 Hz) at both resting potential and at -70 mV. Peak input resistance was measured via the largest voltage change following negative current injection relative to resting membrane potential, and steady-state input resistance was measured just prior to the end of the current pulse. Rectification ratio was calculated as the ratio between peak and steady-state input resistances.

Excitatory postsynaptic potentials (EPSPs) were recorded in layer II of the medial entorhinal cortex following stimulation of mid to deep layers of the parasubiculum using 0.1 ms constant current pulses delivered via a tungsten bipolar electrode (FHC., Bowdoin, ME, USA) using a stimulus generator (WPI, Model A300) and a stimulus isolation unit (Model A360). Evoked EPSPs were filtered and amplified (DC-10 kHz, Axoclamp 200B, Molecular Devices, Sunnyvale, CA, USA) and digitized (20kHz, Digidata 1322A, Molecular Devices) for storage on computer hard disk using the pClamp 8.2 (Molecular Devices) software package.

Effects of carbachol on evoked responses

The effect of the cholinergic receptor agonist carbachol (Sigma-Aldrich, St. Louis, MO) on synaptic responses was assessed by recording responses in normal ACSF and following 10 min bath application of CCh (10 μM). Constant current injection was used to hold cells at subthreshold voltages near resting membrane potential to avoid contamination of synaptic responses by action potentials, and recordings were also obtained at a more hyperpolarized potential of -70 mV at which I_h is enhanced. Synaptic responses were evoked by short 5-pulse trains of theta- and gamma-frequency stimulation at 10 and 33 Hz. Ten responses were obtained at each frequency with a 10 sec interval between trains.

The role of NMDA glutamate receptors in the relative facilitation effect was assessed by recording train-evoked responses in normal ACSF, after 10 min application of the NMDA glutamate receptor antagonist APV (50 μM, Ascent Scientific, Princeton, NJ, USA), and 10 min following addition of CCh in the presence of APV. The role of I_h was assessed following the same protocol using the I_h blocker ZD7288 (10 μM, Ascent Scientific). The dependence of the effects of I_h blockade with ZD7288 on K^+ channels was assessed by applying ZD7288 in the presence of the K^+ channel blocker Ba^{2+} (200μM, Sigma-Aldrich). In experiments where barium was used, both PO_4 and SO_4 were removed from the ACSF. All drugs were stored as frozen stock solutions and mixed with ACSF immediately before testing.

Data analysis

Peak amplitudes of averaged evoked synaptic potentials were measured relative to the pre-train membrane potential (Axon Instr., pClamp 8.2), and group averages were expressed as the mean ± SEM. The single exponential decay time constant (τ) of EPSPs was quantified during the 100 ms period just after the peak of single evoked EPSPs. Drug effects on resting membrane potential, action potential width, and afterhyperpolarizations were assessed and significant effects reported. The effects of CCh on train-evoked responses were assessed by normalizing amplitudes of all responses to the amplitude of the first response in the train obtained prior to CCh administration (in either normal ACSF, APV or ZD7288). Repeated measures ANOVAs assessed changes in train-evoked responses induced by addition of the antagonist or blocker to normal ACSF, and by the addition of CCh in the presence of the antagonist or blocker. Planned comparisons of the amplitudes of responses to each pulse in the train before and after drug administration were also conducted. The effects of CCh on *relative* changes in the amplitudes of EPSPs evoked during the 5-puse trains were also assessed by re-normalizing responses to the amplitudes of the first responses within each respective recording condition. Analogous repeated measures ANOVAs and planned comparisons were also conducted on the renormalized data.

RESULTS

Effects of acetylcholine on train-evoked responses

The effects of 10 μM carbachol (CCh) on train-evoked responses were assessed at resting potential (-59.9 ± 0.8 mV) and at a hyperpolarized potential of -70 mV using negative current injection (n = 8). Application of CCh depolarized neurons to -55.2 ± 1.1 mV and synaptic

responses were recorded before and after CCh application at the same membrane potential to avoid contamination by action potentials. Carbachol also led to an increase in the decay time constant of EPSPs from 12.3 ± 3.5 to 18.9 ± 4.5 ms ($p = 0.04$). Carbachol also decreased the amplitude of action potentials (83.6 ± 5.5 mV vs. 93.0 ± 5.7 mV, $p = 0.02$) as well as the amplitude of the fast afterhyperpolarization (19.5 ± 7.6 vs. 27.2 ± 8.3 mV, $p = 0.04$; see Klink and Alonso, 1997).

During 33 Hz stimulation in normal ACSF, the amplitude of the second response in the train was facilitated to 131.6 ± 8.8 % of first response, but the final response declined to 114.0 ± 10.9 % (Figure 3.1A$_3$, closed symbols). Application of CCh resulted in a marked suppression of the amplitude of the first pulse in the train (54.4 ± 14.9 % of baseline), but responses were well maintained during the train so that by the last pulse there was no significant suppression of the response relative to normal ACSF ($p = 0.11$) (Figure 3.1A$_3$, open symbols). Thus, although CCh resulted in a suppression of responses to single stimulation pulses, there was greater relative growth in responses during the trains, and peak relative response amplitudes reached 211.6 ± 22.7 % of baseline when CCh was present versus 141.2 ± 12.5 % in normal ACSF ($F_{1,7} = 12.53$, $p = 0.01$; Figure 3.1A$_4$).

Responses during theta-frequency stimulation showed less facilitation than responses to gamma-frequency stimulation: the response to the second pulse increased to 121.1 ± 7.9 % of the first pulse, and then declined to 109.8 ± 11.5 % for the fifth pulse. This may result from lesser temporal summation during the lower-frequency theta frequency stimulation (Figure 3.1B). The effects of CCh on responses were similar to those observed for gamma-frequency stimulation; carbachol suppressed train-evoked responses but the amplitudes of responses grew during the train and there was a relative facilitation in CCh as compared to normal ACSF (180.2 ± 29.0 vs. 119.3 ± 9.4 %; $F_{1,7} = 7.93$, $p = 0.03$; Figure 3.1B).

Control experiments (not shown) showed similar baseline responses, and a trend towards overall *increases* in train-evoked responses with repeated tests (gamma, $F_{1,4} = 5.52$, $p = 0.08$, $n = 5$; theta, $F_{1,4} = 4.05$, $p = 0.11$) with no relative changes in responses during trains (gamma, $F_{1,4} = 0.11$, $p = 0.77$; theta $F_{1,4} = 0.77$, $p = 0.43$). In addition, there were no significant differences in the amplitudes of responses to initial pulses in gamma- and theta-frequency trains during baseline or CCh recordings (initial responses during theta stimulation were 100.7 ± 5.7 % [$p =$

0.80] and 92.4 ± 11.5 % [$p = 0.27$] of responses during gamma stimulation, respectively), suggesting no prolonged effects of stimulation.

Voltage-dependent effects on the modulation of train-evoked responses by CCh were assessed by hyperpolarizing cells to -70 mV, and two effects were observed in normal ACSF. First, amplitudes of single evoked EPSPs were increased during hyperpolarization (3.96 ± 0.61 vs. 2.32 ± 0.24 mV, $t_{15} = 4.19$, $p < 0.001$; Figure 3.1A_1, B_1), most likely due to an increase in driving force (Palmer and Stuart, 2009). Hyperpolarization also led to a greater decline in amplitudes by the end of the trains (Figure 3.1A_3,B_3; gamma, $F_{1,14} = 6.39$, $p = 0.02$; theta, $F_{1,14} = 4.71$, $p = 0.049$), and less relative growth in responses during trains (Figure 3.1A_4,B_4; gamma, $F_{1,14} = 4.62$, $p = 0.049$; theta $F_{1,14} = 6.04$, $p = 0.03$). In addition, although hyperpolarization did not affect percent reductions in responses to the first pulses in trains induced by CCh, and significant relative facilitation effects were also observed (gamma, $F_{1,7} = 11.02$, $p = 0.01$; theta, $F_{1,7} = 8.06$, $p = 0.03$; Figure 3.1A_4,B_4), hyperpolarization did reduce the *size* of the relative facilitation effects for theta-frequency stimulation ($F_{1,14} = 5.26$, $p = 0.04$). A similar trend for gamma-frequency trains was not statistically significant ($F_{1,14} = 2.67$, $p = 0.125$).

Though reduced transmitter release is likely to drive much of the CCh-induced reduction in the amplitudes of train-evoked responses (Richter et al., 1999; Yun et al., 2000; Hamam et al., 2007), the relative increases in the amplitudes of train-evoked responses and the greater relative facilitation at depolarized versus hyperpolarized potentials observed here could involve postsynaptic effects including changes in input resistance (Shen et al., 2007). Inspection of traces suggests that the increased growth of train-evoked responses induced by carbachol is likely due to enhanced temporal summation due to slower decay of EPSPs (Figure 3.1A_1,B_1), and this is consistent with a trend towards larger effects of CCh on facilitation induced by gamma versus theta frequency stimulation (Figure 3.1A_4 vs. B_4).

CCh can enhance temporal summation of EPSPs by reducing I_h to enhance dendritic input resistance (Magee, 1998; Magee, 2000; Day et al., 2005). Here, we found that although CCh did not significantly increase peak somatic input resistance (from 81.0 ± 11.3 to 87.4 ± 14.4 MΩ, $p = 0.36$; see also Hamam et al., 2007) there was a significant increase in rectification ratio (0.82 ± 0.03 vs. 0.90 ± 0.02, $p < 0.001$) suggesting that a CCh-induced reduction in I_h resulted in enhanced temporal summation (Figure 3.2C_2). Conversely, the reduction in train-evoked responses during hyperpolarisation (that was associated with reduced in input resistance; 81.0 ±

11.3 vs. 61.5 ± 11.8 MΩ; $p = 0.01$) may also be driven by greater activation of I_h at hyperpolarized potentials (Magee, 1998, Chevaleyre and Castillo, 2002). Results are therefore consistent with a role of I_h in promoting the relative facilitation of EPSPs induced by CCh in entorhinal neurons.

Effects of NMDA receptor blockade on train-evoked responses

Because cholinergic receptor activation and depolarization can enhance NMDA currents (Aramakis et al., 1999) and because we previously found that NMDA receptor activation contributed in part to the relative facilitation of entorhinal field EPSPs (Sparks and Chapman, 2013), we also assessed the effect of the NMDA receptor antagonist APV (50 μM) on CCh-induced changes in intracellular train-evoked responses. APV itself had no significant effect on resting membrane potential or action potential properties, and subsequent application of CCh had similar effects as did CCh alone (data not shown).

Application of APV alone also had no significant effect on the amplitudes of single evoked EPSPs or train-evoked responses (gamma, $F_{1,5} = 1.05$, $p = 0.35$; theta, $F_{1,5} = 0.26$, $p = 0.63$; $n = 6$). The effects of CCh in the presence of APV were similar to those observed in normal ASCF; CCh significantly decreased the amplitude of initial EPSPs and also led to a relative facilitation of train-evoked responses (gamma, $F_{3,15} = 3.92$, $p = 0.03$; theta, $F_{3,15} = 3.47$, $p = 0.04$; Figure 3.3A_3,B_3). APV led to a significant decrease in EPSP decay time constant from 18.5 ± 3.9 to 13.0 ± 2.2 ms ($p = 0.03$), and subsequent application of CCh increased the time constant to 18.0 ± 4.9 ms ($p = 0.02$). The relative facilitation effects induced by CCh in the presence of APV tended to be smaller than in normal ACSF (peak facilitation of 163.4 ± 25.5 vs. 206.2 ± 22.1% for gamma, and 133.1 ±13.6 vs. 177.1 ± 23.8% for theta), but in contrast to previous results obtained using field potential recordings (Sparks and Chapman, 2013), the reduction in relative facilitation was not statistically significant ($p = 0.22$ and 0.10, respectively).

Effects of block of I_h on train-evoked responses

The hyperpolarization-activated cationic current I_h is reduced by muscarinic receptor activation (Pian et al., 2007; Glasgow and Chapman, 2013), and we previously found that I_h plays a role in the effects of CCh on train-evoked responses in the entorhinal cortex (Sparks and Chapman, 2013). We therefore applied the specific I_h channel blocker ZD7288 (50 μM, $n = 9$) and obtained recordings at rest and at -70 mV where I_h is more active. Resting membrane potential was reduced by application of ZD7288 from -60.8 ± 1.0 to -67.0 ± 2.3 mV and

subsequent application of CCh depolarized cells to -58.6 ± 2.0 mV. ZD7288 decreased fast afterhyperpolarizations (1.5 ± 0.8 vs. 7.4 ± 0.8 mV; $p < 0.01$; see: Dickson et al., 2000; Atherton et al., 2010), and subsequent CCh application reduced action potential amplitude (59.9 ±1.9 vs. 75.5 ± 3.7 mV, $p = 0.048$).

Application of ZD7288 resulted in an increase in responses to initial stimulation pulses (see also Chevaleyre and Castillo, 2002; Sparks and Chapman, 2013), and also resulted in a strong overall enhancement of train-evoked responses (gamma, $F_{1,8} = 8.15$, $p = 0.04$; theta, $F_{1,8} = 7.07$, $p = 0.03$) (Figure 3.4$A_{2,}B_2$). ZD7288 also mimicked the relative facilitation of responses induced by CCh during both gamma and theta frequency stimulation (gamma, $F_{1,8} = 21.88$, $p < 0.001$; theta, $F_{1,8} = 10.68$, $p = 0.01$; Figure 3.4$A_{3,}B_3$) suggesting that, in addition to modulation of transmitter release by CCh, that postsynaptic suppression of I_h could contribute to the relative facilitation induced by CCh (Sparks and Chapman, 2013). EPSP decay time constant was increased by ZD7288 (from 16.7 ± 2.7 to 27.7 ± 5.0 ms, $p = 0.01$) consistent with increased dendritic input resistance. Subsequent application of CCh led to suppression of responses to the first pulses in the trains ($p = 0.03$), and a strong further relative facilitation of responses to gamma and theta-frequency stimulation (gamma, $F_{3,24} = 11.44$, $p < 0.001$; theta, $F_{3,24} = 9.09$, $p < 0.001$) with no added effect on decay time constant ($p = 0.77$), consistent with additional facilitation of responses due to the CCh-induced suppression of transmitter release (Yun et al., 2000; Hamam et al., 2007; Barrett and Chapman, 2013).

The sizes of increases in the amplitudes of train-evoked responses induced by ZD7288 were similar at both resting and hyperpolarized membrane potentials (gamma, $F_{1,18} = 1.35$, $p = 0.27$; theta, $F_{1,18} = 0.70$, $p = 0.80$), and there was also an increase in relative facilitation at hyperpolarized potentials following application of ZD7288 (gamma, $F_{1,8} = 9.58$, $p = 0.02$; theta: $F_{1,8} = 7.17$, $p = 0.03$; Figure 3.4A_3B_3), with a trend towards smaller increases at hyperpolarized potentials, consistent with enhancement of train-evoked responses by block of I_h at both rest and at hyperpolarized potentials. In addition, CCh induced a similar pattern of changes at both potentials, with somewhat smaller relative facilitation effects induced by theta-frequency stimulation ($F_{1,18} = 5.41$, $p = 0.03$) than by gamma frequency stimulation ($F_{1,18} = 1.35$, $p = 0.26$) at hyperpolarized potentials when compared to rest. This could be due to reduced input resistance at hyperpolarized potentials ($p = 0.01$), and is similar to the changes in relative facilitation seen during CCh application alone (Figure 3.1).

The facilitation of train-evoked responses induced by ZD7288 was associated with a significant increase in peak input resistance (from 71.7 ± 6.8 to 107.2 ± 10.3 MΩ, $p = 0.012$) and an elimination of the hyperpolarization-induced sag in voltage responses to current steps (Figure 3.5A; rectification ratio 0.90 ± 0.02 vs. 1.04 ± 0.01, $p < 0.001$). Block of I_h channels by ZD7288 are therefore likely to have enhanced train-evoked responses through an increase in input resistance. Application of CCh following ZD7288 application did not result in an additional increase in peak input resistance ($p = 0.97$) or rectification ratio ($p = 0.33$) (Figure 3.5C).

Role of K^+ currents in I_h-mediated relative facilitation

Because closure of K_{ir} channels can depolarize neurons to reduce I_h activation and enhance I_h-dependent facilitation of train-evoked responses (Day et al., 2005; George et al., 2009), we tested their role in the CCh-induced relative facilitation effect by applying the K^+ channel antagonist Ba^{2+} (200 μM, $n = 7$). Application of Ba^{2+} increased action potential width (2.17 ± 0.37 vs. 1.24 ± 0.47 ms, $p = 0.01$) and firing frequency (7.6 ± 1.1 vs. 5.8 ±1.0, $p < 0.01$), reduced fast afterhyperpolarization amplitude (8.0 ±3.3 vs. 14.0 ± 2.6 mV, $p = 0.01$), and depolarized resting potential from -58.6 ± 1.0 to -52.9 ± 1.4 mV. Subsequent co-application of ZD7288 was associated with increased action potential width (2.17 ± 0.37 vs. 3.06 ±0.39 ms, $p = 0.01$) and repolarization to -59.9 ± 1.7 mV.

Although Ba^{2+} had no significant effect on the peak amplitudes of initial responses in trains, it caused a strong increase in the amplitudes of train-evoked responses both during gamma- and theta-frequency trains (gamma, $F_{4,24} = 7.37$, $p < 0.001$; theta, $F_{4,24} = 4.79$, $p = 0.01$; Figure 3.6A_2,B_2; see also Day et al., 2005), and also caused strong relative facilitation effects (Figure 3.6A_3,B_3; gamma, $F_{1,6} = 13.10$, $p = 0.01$; theta, $F_{1,6} = 11.06$, $p = 0.02$). However, Ba^{2+} did not prevent further increases in train-evoked responses induced by ZD7288 (gamma, $F_{4,24} = 10.35$, $p = < 0.001$; theta, $F_{4,24} = 4.297$, $p = 0.01$). The increased train-evoked responses induced by Ba^{2+} alone were associated with an increase in input resistance (123.6 ± 16.5 vs. 95.5 ± 10.0 MΩ, $p = 0.026$), an increase in EPSP decay time constant (48.4 ±11.5 vs. 20.9 ± 5.7 ms, $p = 0.02$), and a maintained hyperpolarization-induced sag in voltage responses to negative current steps (0.85 ± 0.03 vs. 0.84 ± 0.04, $p = 0.73$). The addition of ZD7288 caused no further change to peak input resistance or decay time constant, but did eliminate the hyperpolarization induced sag (0.84 ± 0.04 vs. 1.04 ± 0.04, $p = 0.016$). This suggests that I_h in entorhinal neurons remains active at

depolarized potentials, and that closure of I_h channels can contribute to relative facilitation effects even during block of K^+ channels.

DISCUSSION

The present study used intracellular recordings to investigate mechanisms mediating our previous finding that cholinergic receptor activation causes a relative facilitation of synaptic responses evoked during gamma- and theta-frequency trains of stimulation delivered to parasubicular inputs to layer II of the medial entorhinal cortex (Sparks and Chapman, 2013). Activation of cholinergic receptors results in a reduction in transmitter release that is likely to account for much of the facilitation effect by increasing transmitter availability later in the trains (Yun et al., 2000; Hamam et al., 2007; Barrett and Chapman, 2013). In addition, our data are consistent with the relative facilitation of synaptic responses by carbachol being due in part to closure of I_h channels. The relative facilitation induced by CCh was associated with a reduction in hyperpolarization-induced inward rectification characteristic of I_h (Dickson et al., 2000b; Heys et al., 2012), and the facilitation effect was mimicked by block of I_h using the antagonist ZD7288. Application of ZD7288 blocked the sag in voltage responses to negative current steps and slowed decay of EPSPs, suggesting that a CCh-induced reduction in I_h that increases input resistance and enhances temporal summation is a possible mechanism contributing to the relative facilitation effect. Blocking K^+ channels with Ba^{2+} facilitated train-evoked responses, but did not occlude the effects of ZD7288, indicating that the effects of modulation of I_h on synaptic responses is not dependent on changes in IK_{ir} or K_{leak} (Day et al., 2005; George et al., 2009).

The parasubiculum sends its major projection to layer II of the entorhinal cortex (van Groen and Wyss, 1990; Caballero-Bleda and Witter, 1993, 1994; Caruana and Chapman, 2004) which provides the hippocampus with the majority of its cortical sensory input (Witter et al., 1989; Amaral, 1993; Burwell et al., 1995), and the parasubicular-entorhinal projection is therefore well positioned to modulate activity of entorhinal afferents to the hippocampus (Caruana and Chapman, 2004; Sparks and Chapman, 2013). Cholinergic inputs from the medial septum to these areas contribute to gamma and theta EEG activities (Mitchell and Ranck, 1980; Gaykema et al., 1990; Dickson et al. 2000a; Glasgow and Chapman, 2007; Tsuno et al., 2013) which are associated with functions of learning and memory (Buzsaki, 2002; Hasselmo, 2006) and spatial navigation (O'Keefe and Recce, 1993, Taube, 1995; Hasselmo et al., 2007; Boccara

et al., 2010; Igarashi et al., 2014; Yamamoto et al., 2014). The relative facilitation effect, in which CCh enhances temporal summation despite a reduction in the amplitude of single synaptic responses, may therefore contribute to cognitive function by helping maintain the strength of repetitive synaptic inputs to the entorhinal cortex during cholinergically induced rhythmic states (Hamam et al., 2007; Glasgow et al., 2012; Sparks and Chapman, 2013). Facilitation effects may similarly help maintain the ability of the parasubiculum to provide the entorhinal cortex with rhythmic synaptic inputs that reflect the activity of both hippocampal and subcortical areas (van Groen and Wyss, 1990). This may help to modulate the sensitivity of the entorhinal cortex to sensory inputs in a way that is dependent upon ongoing hippocampal processing (Caruana and Chapman, 2004), and which may also help to modulate the activity of spatial grid cells in the entorhinal cortex based on inputs form the CA1 region and anterior thalamus (van Groen and Wyss, 1990; Hargreaves et al., 2007).

Cholinergic effects on train-evoked responses

Despite a reduction in responses to single pulses of stimulation, carbachol resulted in a marked increase in relative growth of responses evoked during trains of gamma- and theta-frequency stimulation that appears to involve changes in the conductance of I_h. I_h is a non-specific cationic current that is known to contribute to the generation of EEG oscillations in the entorhinal cortex and other areas of the hippocampal formation (Magee, 1998; Dickson et al., 2000b; Carr et al., 2007) and can be modulated by muscarinic receptor activation (Dickson et al., 2000b; Pian et al., 2006, 2007). The relative facilitation induced by CCh in field potential recordings was dependent upon I_h (Sparks and Chapman, 2013), and previous research has shown that CCh can suppress I_h (Heys et al. 2012) and that suppression of I_h can enhance temporal summation of ESPSs through increased input resistance (Magee, 1998; Day et al., 2005). We found here that application of CCh significantly reduced the I_h-mediated sag in voltage responses to negative current injection. Further, hyperpolarization caused reductions in temporal summation which are likely due in part to reduced input resistance associated with a hyperpolarization-induced increase in I_h. We found that CCh induced only a small non-significant increases in somatic input resistance (Hamam et al. 2007; but see Heys et al. 2010), but I_h is about six times larger in dendrites (Magee, 1998), and so CCh-induced changes in I_h may enhance temporal summation of dendritic EPSPs while having a lesser effect on somatic input resistance.

We tested if CCh acts through I_h to enhance summation of train-evoked responses by applying the specific I_h-channel antagonist ZD7288 to determine if it could mimic the effects of CCh. Application of ZD7288 abolished inward rectification and increased input resistance (Dickson et al., 2000b) and also mimicked the effects of CCh application on train-evoked responses by leading to a marked relative facilitation. ZD7288 induced significant facilitation effects at both hyperpolarized and depolarized potentials, consistent with a contribution of I_h to synaptic integration at a range of membrane potentials. These findings are consistent with other work showing that reductions in I_h increase summation of train-evoked responses via increases in input resistance (Magee, 1998; Day et al., 2005; Rosenkranz and Johnston, 2006; Garden et al., 2008). In addition to the depolarizing effect of I_h, which can lead to its own deactivation (Day et al., 2005), cholinergic inputs to the entorhinal cortex are also likely to reduce I_h through membrane depolarization (Klink and Alonso, 1997; Hamam et al., 2007) and through reductions in I_h via hydrolysis of PIP_2 (Pian et al., 2006 and 2007). The resulting reductions of I_h in dendrites is therefore likely to shape the integration of synaptic inputs by increasing input resistance and slowing the decay of EPSPs, which would lead to an enhancement of temporal summation (Magee, 1998). Synchronous oscillatory activity associated with gamma and theta EEG rhythms might also contribute to dendritic depolarization to enhance inactivation of I_h to enhance summation of EPSPs in a phase-dependent manner (Dickson et al., 2000b; Heys et al., 2010).

We also previously found using field potential recordings that ZD7288 resulted in an overall increase in train-evoked responses. However, a significant increase in the initial field response in the trains resulted in no significant relative facilitation effect (Sparks and Chapman, 2013). Others have observed transient increases in single synaptic field potentials in response to ZD7288 (Chevaleyre and Castillo, 2002), but the stable initial response and enhanced temporal summation during block of I_h observed in the present study is more consistent with the literature (e.g., Magee, 2000). In addition, the present results show that ZD7288 hyperpolarizes entorhinal neurons, and that relative facilitation effects are reduced at hyperpolarized potentials, and this may have contributed to smaller relative facilitation effects observed in field potential recordings as compared to intracellular recordings in cells held near threshold.

Although ZD7288 has a non-specific inhibitory effect on T-type Ca^{2+} channels (Sanchez-Alonso et al., 2008), and I_h can also regulate presynaptic transmitter release through effects on T-

type Ca^{2+} channels (Huang et al., 2011) these effects are unlikely to contribute substantially to the results obtained here. Presynaptic I_h inhibits synaptic transmission onto layer III entorhinal neurons by depolarizing terminals and helping maintain inactivation of T-type Ca^{2+} channels that contribute to transmitter release (Huang et al., 2011). Here, ZD7288 did not significantly increase or decrease responses to initial stimulation pulses in trains, and the increased EPSP decay time constants observed following carbachol and ZD7288 are also better explained by postsynaptic factors than by presynaptic alterations in T-type Ca^{2+} channels. In addition, however, it is possible that the inhibition of I_h with ZD7288 or carbachol might contribute to the relative facilitation by enhancing postsynaptic Ca^{2+} spikes (Tsay et al., 2007).

Application of CCh during blockade of I_h with ZD7288 led to a reduction in the amplitude of train-evoked responses, but also enhanced the relative amount of temporal summation during the trains. This increase in relative facilitation is likely due to cholinergic suppression of transmitter release, which increases the transmitter availability during responses later in the train (Richter et al., 1999; Yun et al., 2000; Hamam et al., 2007; Barrett and Chapman, 2013). This could be tested in future using local pressure-application of glutamate to hold transmitter levels constant.

Inward rectifying and leak potassium channels

Inward rectifying and leak potassium channels can interact with I_h to influence membrane potential and synaptic integration (Day et al., 2005; Meuth et al., 2006; George et al., 2009). The hyperpolarizing influence of IK_{ir} that helps maintain resting potential also maintains activation of I_h to limit temporal summation of EPSPs (George et al., 2009). Cholinergic receptor activation can reduce IK_{ir} through depleting PIP_2 (Carr and Surmeier, 2007), and depolarization induced by block of K_{ir} and K_{leak} channels can lead to deactivation of I_h, increased input resistance, and enhanced temporal summation (Day et al., 2005). Thus, reductions in IK_{ir} induced during cholinergically induced rhythmic states may lead to reductions in I_h that could contribute to the I_h-dependent enhancement of temporal summation in entorhinal neurons.

To assess the contribution of changes in K^+ channels to relative facilitation effects induced by I_h blockade with ZD7288, we tested the effects of ZD7288 in the presence of the K^+ channel antagonist Ba^{2+} at a dose that is known to target K_{ir} and K_{leak} channels (Day et al., 2005). Consistent with previous findings (Day et al., 2005; Carr and Surmeier, 2007), blockage of K^+ channels induced a marked relative facilitation of train-evoked responses at both gamma and theta frequencies, likely due increased dendritic input resistance and partial inhibition of I_h

(George et al., 2009). Subsequent application of ZD7288 caused a further increase in the amplitude of train-evoked responses, indicating that, although cholinergic depolarization mediated by closure of K^+ channels may contribute to synaptic facilitation effects, I_h remains active even during block of IK_{ir} and IK_{leak}, and that cholinergic receptor activation may act on I_h independent of effects on K^+ channels.

Conclusion

The intracellular recordings shown here build upon our previous observations that the cholinergic agonist CCh induces a frequency-dependent enhancement of synaptic communication in parasubicular inputs to layer II of the entorhinal cortex (Sparks and Chapman, 2013). We found that the relative facilitation effect was not significantly dependent on NMDA glutamate receptors. CCh application resulted in a reduction in I_h, and the effects of CCh were mimicked by the I_h blocker ZD7288, suggesting that cholinergic reductions in I_h and resulting increases in dendritic input resistance and temporal summation contribute to the facilitation effect. The effects of I_h blockade were not prevented by blocking inward rectifying or leak K^+ channels, suggesting that effects of I_h on integration of synaptic inputs is not dependent on these channels in entorhinal neurons, and are expressed at depolarized membrane potentials (Day et al., 2005; George et al., 2009). These results are consistent with a growing literature indicating the importance of I_h in modulating integration of repetitive synaptic inputs in cortical neurons.

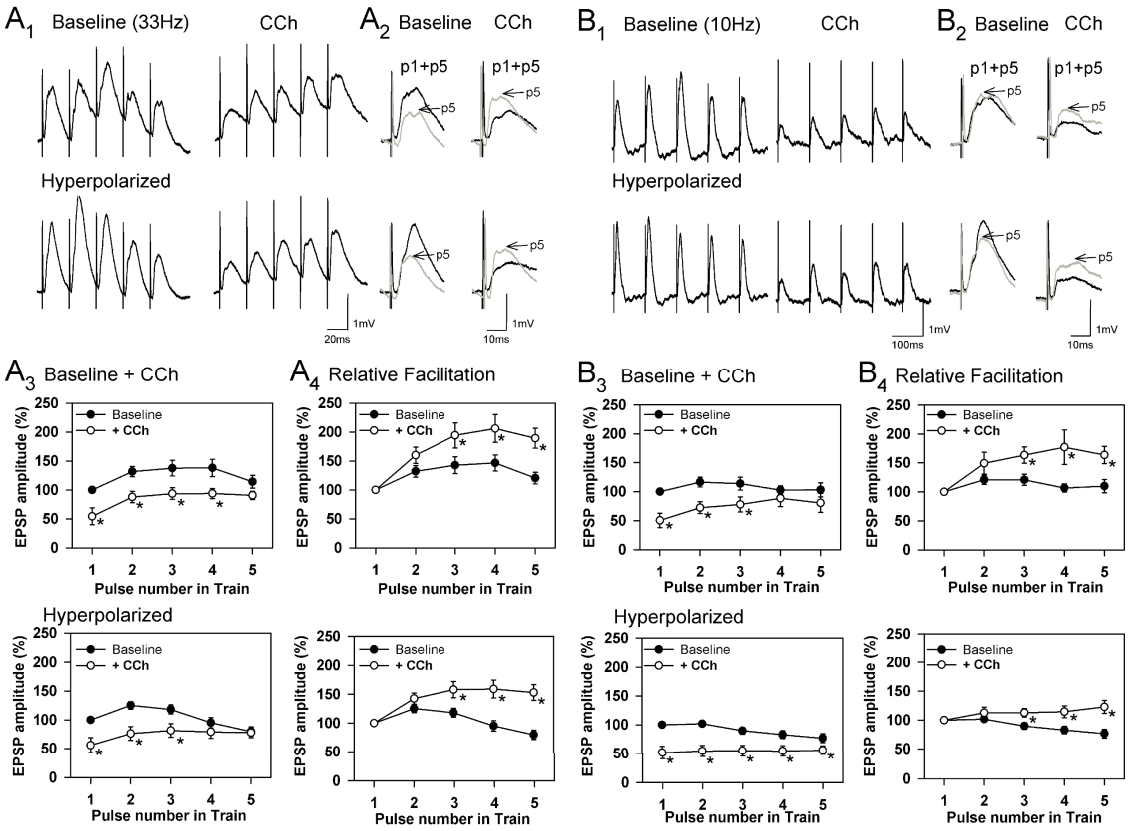

A1 Baseline (33Hz)
CCh
A2 Baseline
CCh
p1+p5
p5
Hyperpolarized
1mV
20ms
10ms
B1 Baseline (10Hz)
CCh
B2 Baseline
CCh
100ms
A3 Baseline + CCh
A4 Relative Facilitation
B3 Baseline + CCh
B4 Relative Facilitation
Baseline
+ CCh
EPSP amplitude (%)
Pulse number in Train

Figure 3.1. The cholinergic agonist carbachol (CCh) results in a relative facilitation of excitatory postsynaptic potentials (EPSPs) during five-pulse trains of stimulation at gamma (33 Hz) and theta (10 Hz) frequencies. **A.** Sample traces of responses evoked by 33-Hz stimulation are shown before and after application of 10 μM CCh at both resting potential and during hyperpolarization to 70 mV (A_1). Responses to the first and fifth pulses in the trains (p1 + p5) are superimposed at an expanded timescale for both baseline and CCh conditions (A_2). Peak responses to the fifth pulses are indicated by arrows. Mean response amplitudes normalized to the initial response during baseline trains (A_3) show that CCh suppressed train- evoked responses at both rest and hyperpolarized potentials, and renormalization of responses to the amplitude of initial responses within each testing condition (A_4) shows that carbachol was associated with a much greater relative growth in responses during the trains. **B.** Carbachol induced a similar relative facilitation of synaptic responses during theta-frequency stimulation. The size of the relative facilitation effect was reduced by hyperpolarization.

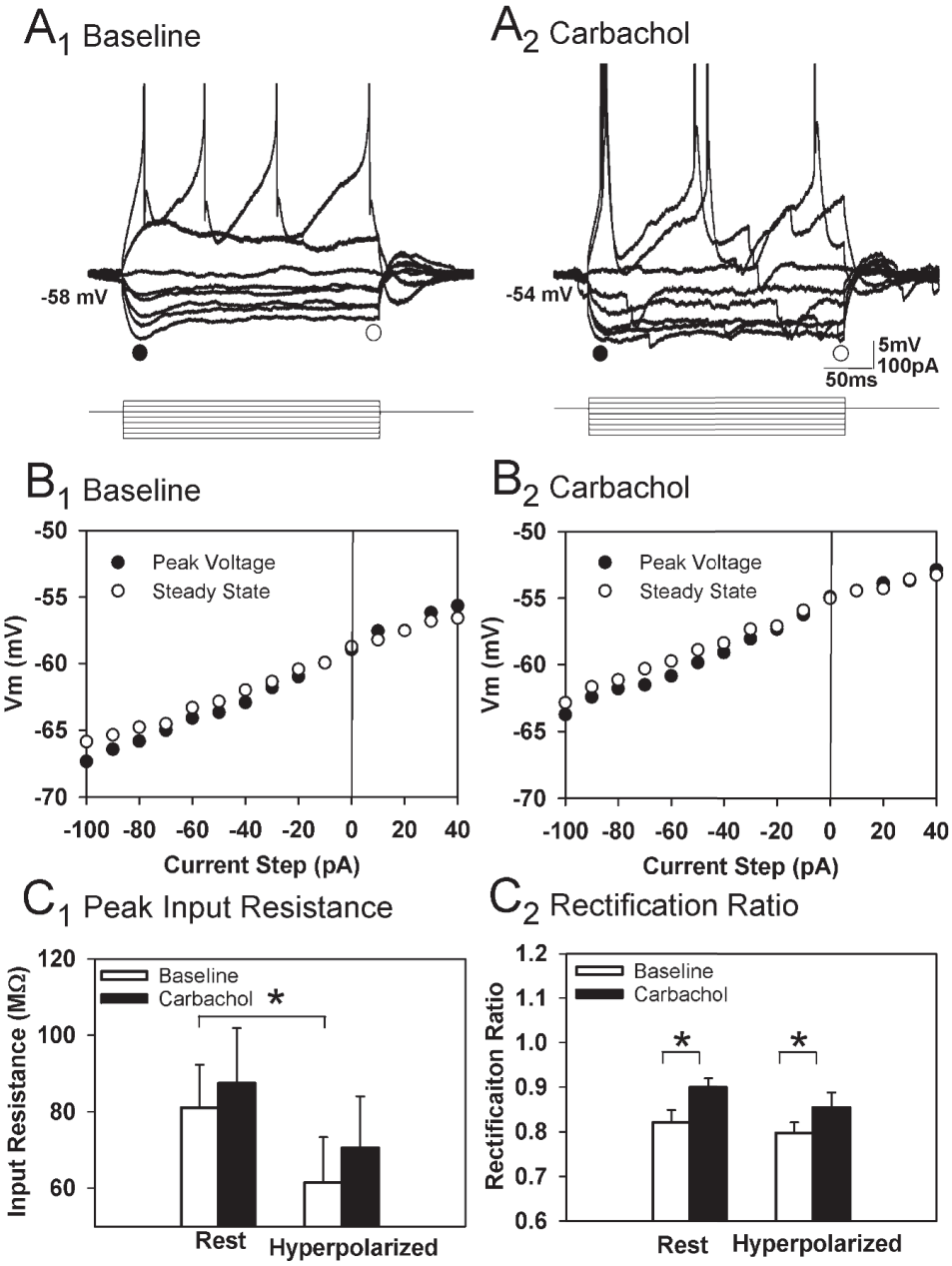

A1 Baseline
A2 Carbachol
-58 mV
-54 mV
5mV
100pA
50ms
B1 Baseline
B2 Carbachol
Peak Voltage
Steady State
Vm (mV)
Current Step (pA)
C1 Peak Input Resistance
C2 Rectification Ratio
Baseline
Carbachol
Input Resistance (MΩ)
Rectificaiton Ratio
Rest
Hyperpolarized

Figure 3.2. Application of carbachol (CCh) reduces hyperpolarization- induced inward rectification. **A.** Sample traces show inward rectification during negative current steps (A_1) that is reduced by CCh (A_2). Circles indicate latencies at which peak and steady-state inputs resistance were quantified. **B.** Representative IV plots showing larger peak voltage response compared to steady-state at the most hyperpolarized potentials (B_1), an effect that is reduced following CCh application (B_1). C. Peak input resistance was not significantly increased by CCh at rest or at hyperpolarized membrane potentials (B_1) but the reduction in rectification was reflected by an increase in rectification ratio (peak vs. steady state input resistance; B_2).

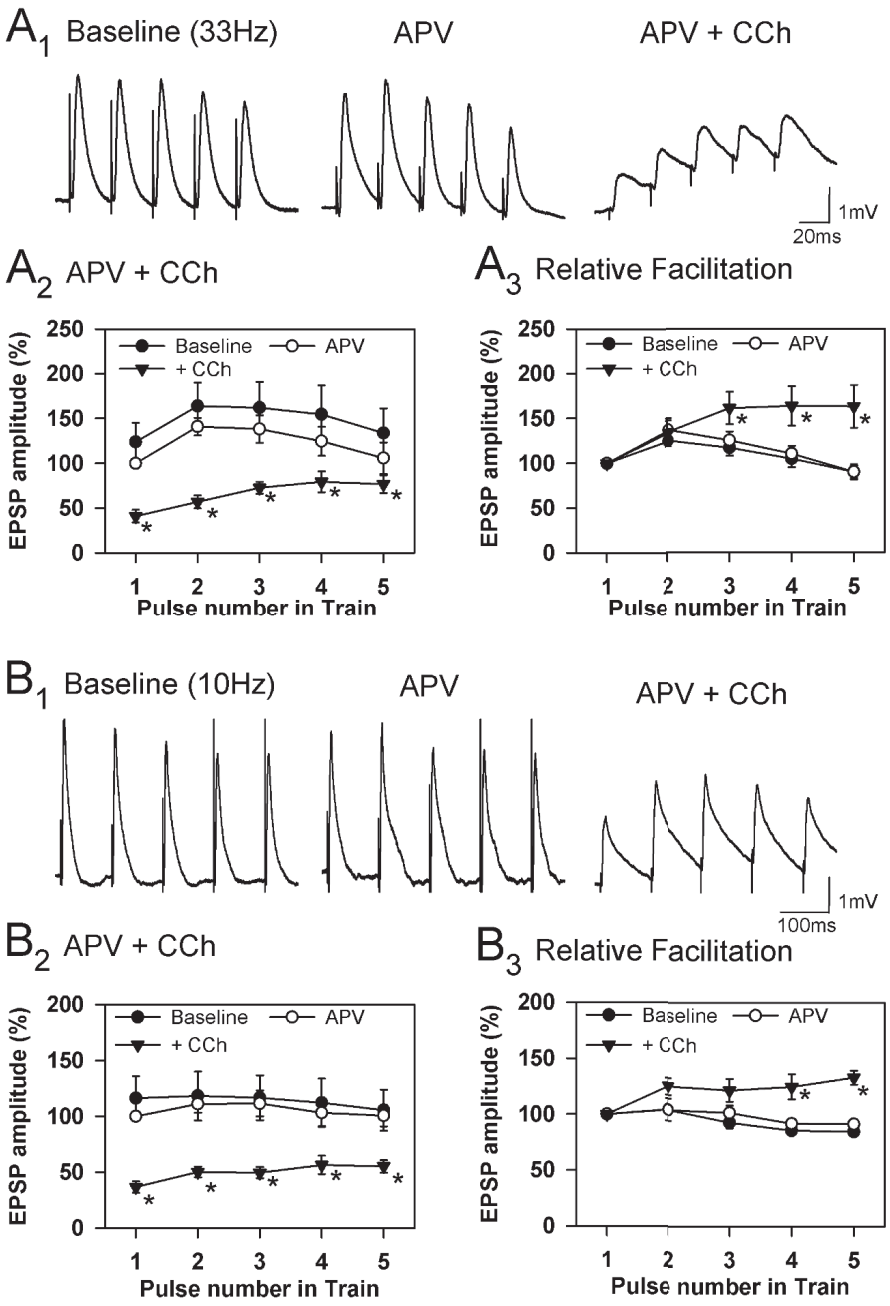

A1 Baseline (33Hz)
APV
APV + CCh
1mV
20ms
A2 APV + CCh
A3 Relative Facilitation
Baseline
APV
+ CCh
EPSP amplitude (%)
Pulse number in Train
B1 Baseline (10Hz)
APV
APV + CCh
1mV
100ms
B2 APV + CCh
B3 Relative Facilitation
Baseline
APV
+ CCh
EPSP amplitude (%)
Pulse number in Train

Figure 3.3. The NMDA glutamate receptor antagonist APV does not attenuate the relative facilitation of train-evoked responses induced by CCh. **A and B.** Application of APV by itself had no significant effect on the amplitude of train-evoked responses. Addition of CCh resulted in a reduction in the overall amplitude of train-evoked responses (A_2 and B_2) that was associated with a relative facilitation of train-evoked responses (A_3 and B_3), indicating that NMDA receptors are not required for the relative facilitation effect induced by CCh.

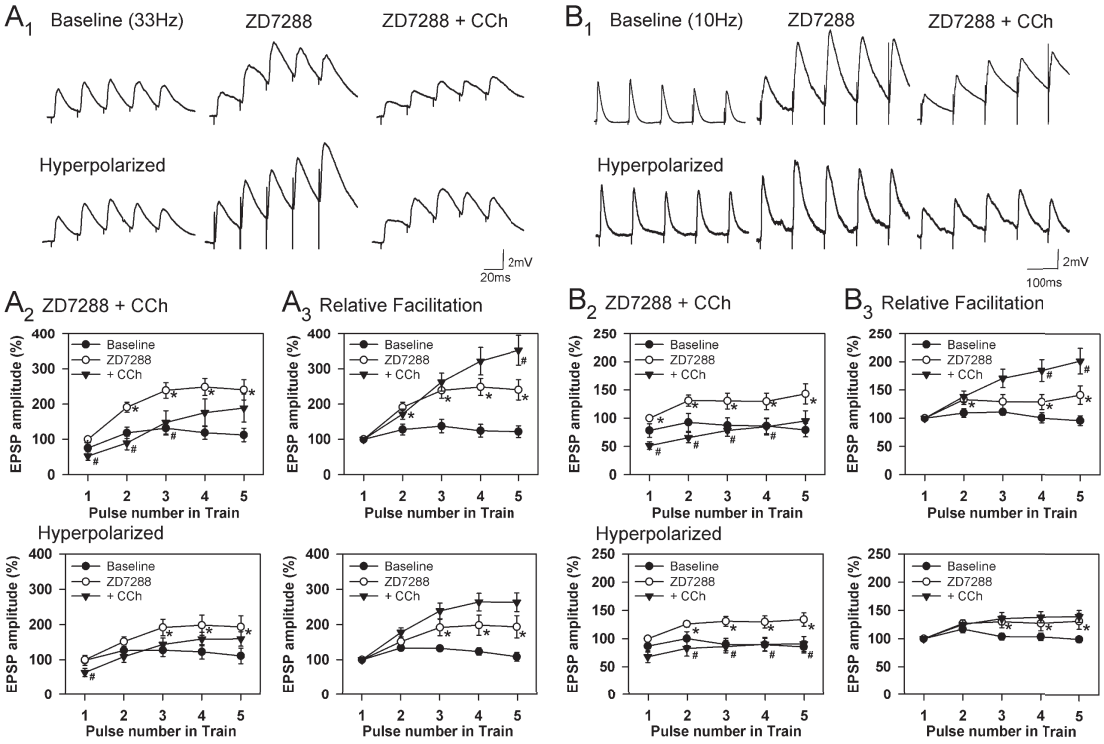

A1 Baseline (33Hz)
ZD7288
ZD7288 + CCh
Hyperpolarized
2mV
20ms
B1 Baseline (10Hz)
ZD7288
ZD7288 + CCh
Hyperpolarized
2mV
100ms
A2 ZD7288 + CCh
A3 Relative Facilitation
B2 ZD7288 + CCh
B3 Relative Facilitation
Baseline
ZD7288
+ CCh
EPSP amplitude (%)
Pulse number in Train
Hyperpolarized

Figure 3.4. Application of the selective I_h antagonist ZD7288 mimics the effects of CCh on the relative facilitation of train-evoked responses. **A and B.** Application of ZD7288 alone increased the amplitudes of train-evoked responses (A_2 and B_2), and also induced a relative facilitation of synaptic responses similar to that induced by CCh alone (A_3 and B_3). Application of CCh in the presence of ZD7288 caused a reduction in the amplitudes of initial responses in the trains, and a further increment in relative facilitation likely due to enhanced transmitter availability later in the trains (A_3 and B_3). Significant changes from baseline to ZD7288 are indicated by*, and significant changes induced by addition of CCh are indicated by # ($p < 0.05$).

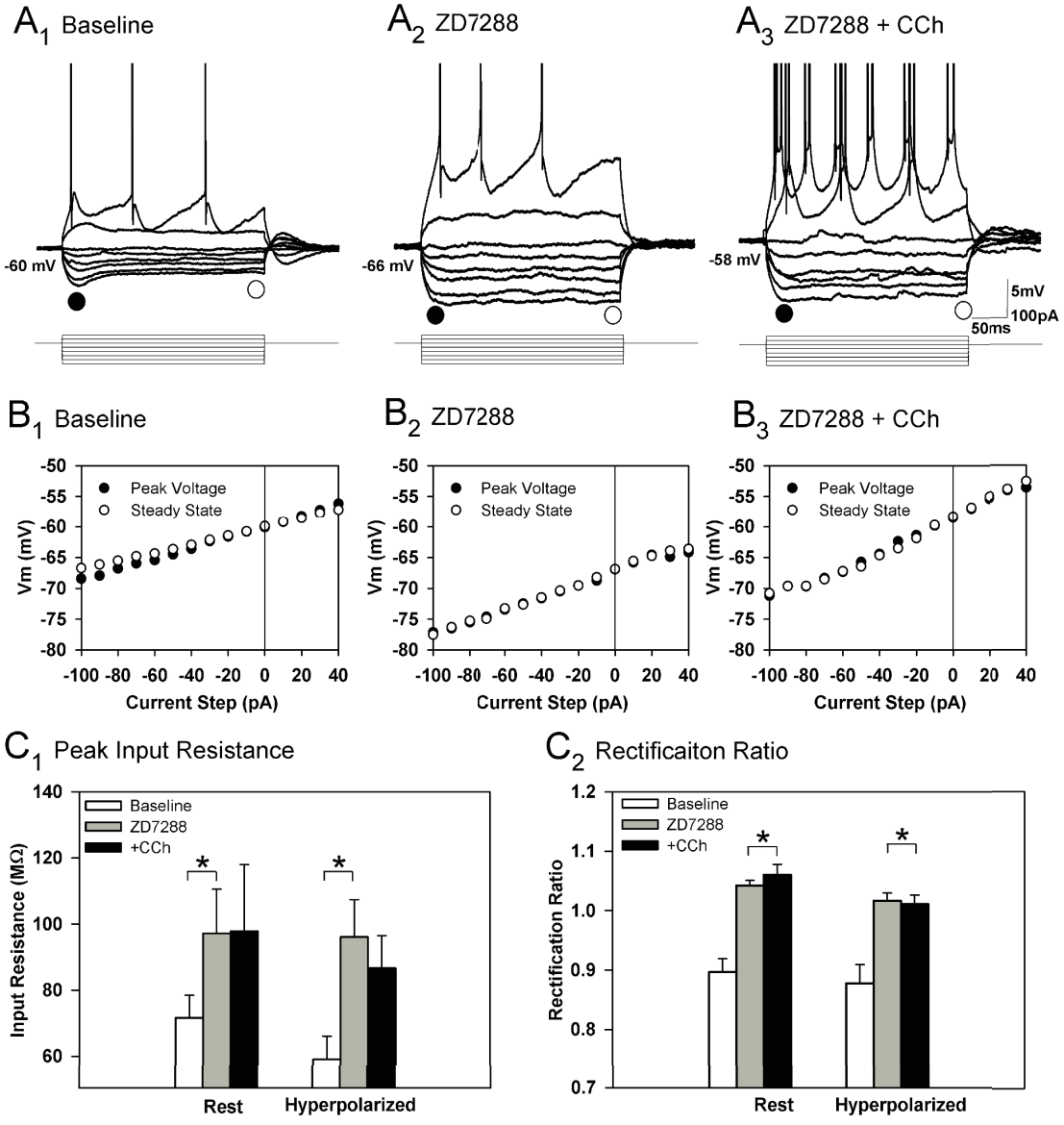

A1 Baseline
A2 ZD7288
A3 ZD7288 + CCh
-60 mV
-66 mV
-58 mV
5mV
100pA
50ms
B1 Baseline
B2 ZD7288
B3 ZD7288 + CCh
Peak Voltage
Steady State
Vm (mV)
Current Step (pA)
C1 Peak Input Resistance
C2 Rectificaiton Ratio
Baseline
ZD7288
+CCh
Input Resistance (MΩ)
Rectification Ratio
Rest
Hyperpolarized

Figure 3.5. Blocking I_h with ZD7288 increases input resistance and blocks hyperpolarization-induced inward rectification. **A and B.** The sag in voltage responses to negative current injection observed in normal ACSF (A_1) was blocked by the I_h inhibitor ZD7288 (A_2), with no further change induced by CCh (A_3). C. Mean data show that application of ZD7288 resulted in significant increases in peak input resistance and rectification ratio.

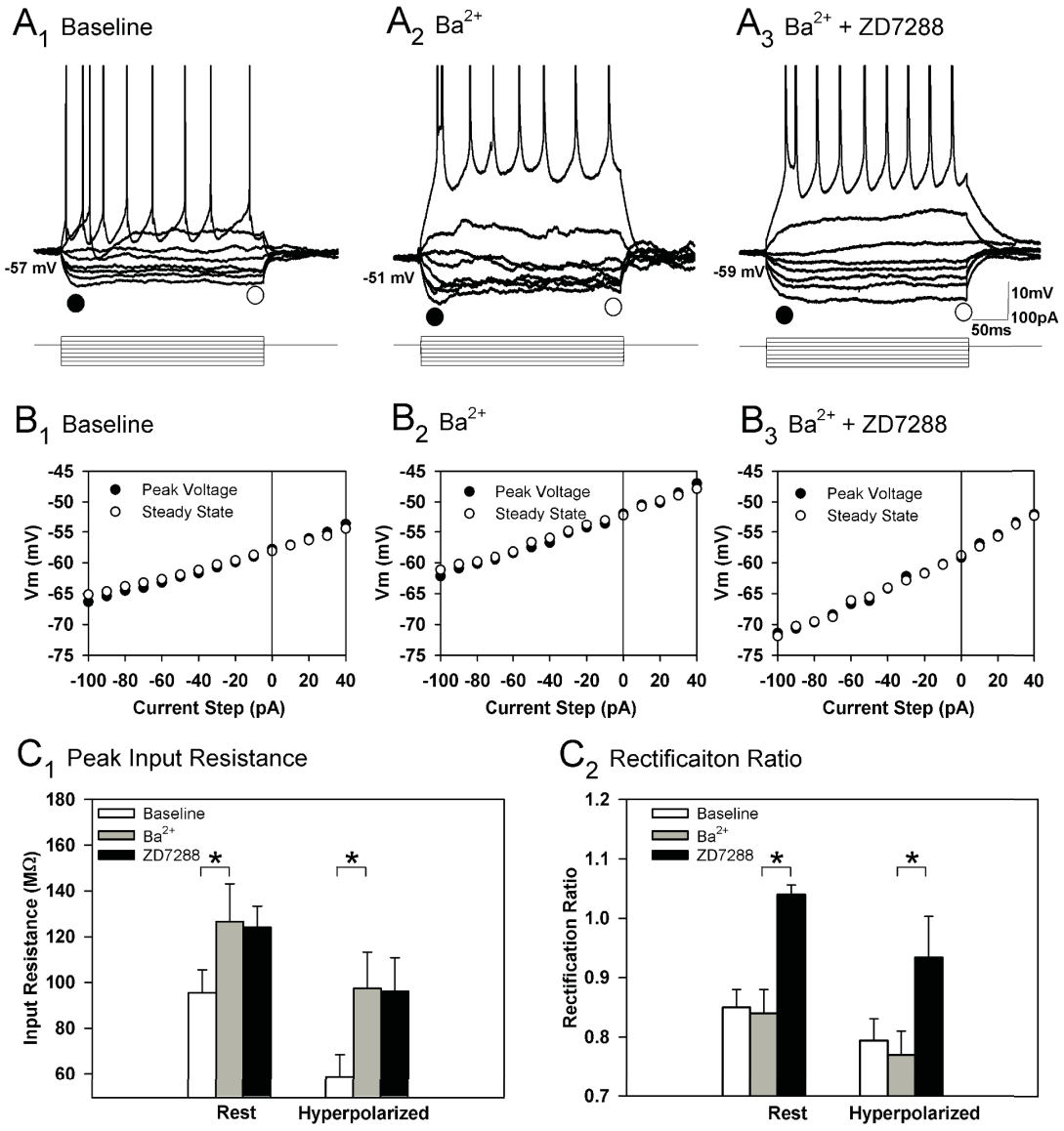

A1 Baseline
A2 Ba2+
A3 Ba2+ + ZD7288
-57 mV
-51 mV
-59 mV
10mV
100pA
50ms
B1 Baseline
B2 Ba2+
B3 Ba2+ + ZD7288
Peak Voltage
Steady State
Vm (mV)
Current Step (pA)
C1 Peak Input Resistance
C2 Rectificaiton Ratio
Baseline
Ba2+
ZD7288
Input Resistance (MΩ)
Rectification Ratio
Rest
Hyperpolarized

Figure 3.6. Blocking K_{ir} and K_{leak} channels with barium (200 μM) increases input resistance with no effect on hyperpolarization-induced inward rectification. **A and B.** Application Ba^{2+} resulted in larger voltage responses to negative current injection, and subsequent application of the I_h antagonist ZD7288 abolished inward rectification. **C.** Application of Ba^{2+} significantly increased peak input resistance with no effect on rectification ratio. Subsequent application of ZD7288 resulted in no further increase in input resistance, and reduced inward rectification.

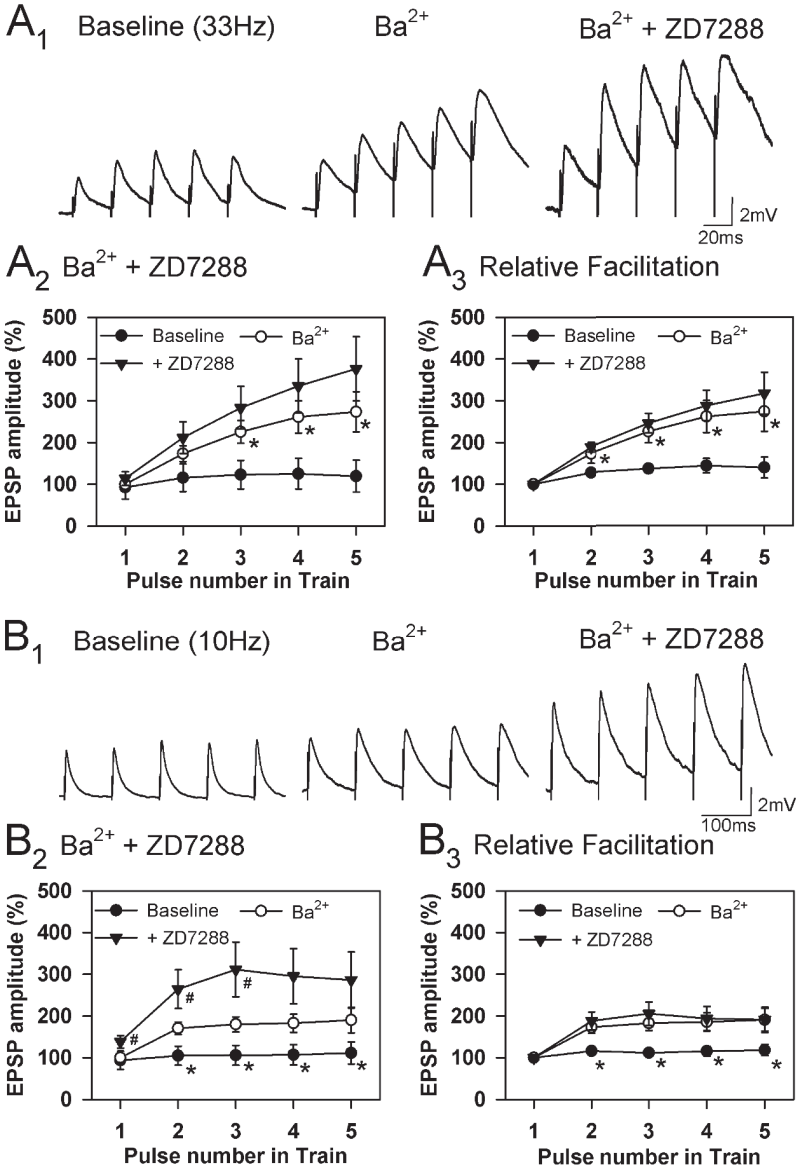

A1 Baseline (33Hz)
Ba2+
Ba2+ + ZD7288
2mV
20ms
A2 Ba2+ + ZD7288
A3 Relative Facilitation
Baseline
Ba2+
+ ZD7288
EPSP amplitude (%)
Pulse number in Train
B1 Baseline (10Hz)
Ba2+
Ba2+ + ZD7288
2mV
100ms
B2 Ba2+ + ZD7288
B3 Relative Facilitation
Baseline
Ba2+
+ ZD7288
EPSP amplitude (%)
Pulse number in Train

Figure 3.7. Blockade of inward rectifying potassium channels (K_{ir}) with Ba^{2+} enhances train-evoked responses, but does not prevent increases in train-evoked responses induced by block of I_h with ZD7288. **A and B.** Application of Ba^{2+} increased train-evoked responses, and addition of ZD7288 caused further significant increases in train evoked responses (A_2 and B_2). The relative degree of facilitation of responses during the trains was similar in the presence of Ba^{2+} and following addition of ZD7288 (A_3 and B_3). Significant changes from baseline to Ba^{2+} are indicated by *, and significant changes induced by addition of ZD7288 are indicated by # ($p < 0.05$).

CHAPTER 4

HETEROSYNAPTIC MODULATION OF EVOKED SYNAPTIC RESPONSES IN LAYER II OF THE ENTORHINAL CORTEX BY ACTIVATION OF THE PARASUBICULUM

Daniel W. Sparks and C. Andrew Chapman

ABSTRACT

The superficial layers of the entorhinal cortex receive sensory and associational cortical inputs, and provide the hippocampus with the majority of its cortical sensory input. The parasubiculum, which receives input from multiple hippocampal subfields, sends it single major output projection to layer II of the entorhinal cortex, suggesting that it may modulate processing of synaptic inputs to the entorhinal cortex. Indeed, stimulation of the parasubiculum can enhance entorhinal responses to synaptic input from the piriform cortex in vivo. Theta EEG activity contributes to spatial and mnemonic processes in this region, and the current study assessed how stimulation of the parasubiculum with either single pulses, or short 5-pulse theta-frequency trains, may modulate synaptic responses in layer II entorhinal stellate neurons evoked by stimulation of layer I afferents in vitro. Parasubicular stimulation pulses or trains supressed responses to layer I stimulation at intervals of 5 ms, and parasubicular stimulation trains facilitated layer I responses at a train-pulse interval of 25 ms. This suggests that firing of parasubicular neurons during theta activity may heterosynaptically enhance incoming sensory inputs to the entorhinal cortex. Bath application of the I_h blocker ZD7288 enhanced the facilitation effect, suggesting that cholinergic inhibition of I_h may contribute. In addition, repetitive pairing of parasubicular trains and layer I stimulation induced a lasting depression of entorhinal responses to layer I stimulation. These findings provide evidence that theta activity in the parasubiculum may promote heterosynaptic modulation effects that may alter sensory processing in the entorhinal cortex.

The entorhinal cortex and hippocampus are known to play important roles in spatial navigation and mnemonic processing (Brun et al., 2002; Fyhn et al., 2007; Hargreaves et al., 2007; Jarrard et al., 2004; Knierim et al., 2013; Moser et al., 2015; O'Reilly et al., 2014; Rolls, 2013), and the entorhinal cortex plays a major role within the hippocampal formation by providing the hippocampus with the majority of its cortical sensory input (Amaral, 1993; Burwell et al., 1995; Witter et al., 1989, 2000). The superficial layers of the entorhinal cortex receive converging inputs from multiple sensory and associational cortical areas via the piriform, perirhinal and postrhinal cortices (Burwell et al., 1995, Burwell and Amaral, 1998, Burwell, 2000, Witter et al., 2000). Layer II of the entorhinal cortex also receives the single major output projection of the parasubiculum, a brain area that receives inputs from area CA1 of the hippocampus and the subiculum, as well as inputs from the anterior thalamus, mammillary complex, amygdala, and postsubiculum (Cenquizca and Swanson, 2007; Swanson and Cohen, 1977; van Groen and Wyss, 1990a and b; Witter et al., 1989). Because the parasubiculum projects to both stellate and pyramidal neurons in layer II of the entorhinal cortex (Caballero-Bleda and Witter, 1994), and also receives inputs from hippocampal output regions, the parasubiculum is in a unique position to be able to affect the nature of entorhinal cortex responses to sensory inputs that arrive in layer II in a way that may be dependent upon ongoing processing within the hippocampal formation.

It has been shown previously that activation of parasubicular inputs to the entorhinal cortex can modulate the responsiveness of the entorhinal cortex to inputs from the piriform (olfactory) cortex (Caruana and Chapman, 2004). Using field potential recordings in anaesthetised rats it was found that stimulation of the parasubiculum with a single conditioning pulse can result in either a *suppression* of subsequent synaptic responses evoked by piriform cortex stimulation at a short interval 5 ms, or a *facilitation* of synaptic responses at longer intervals of 20 to 100 ms. The heterosynaptic suppression of entorhinal responses at the short, 5 ms interval was attributed to activation of fast feedforward inhibitory circuitry common to both input pathways. The heterosynaptic facilitation of synaptic responses at longer intervals near 25 ms corresponds to the period of slow-gamma frequency EEG activity (Colgin, 2015), and was attributed to common postsynaptic mechanisms that could include depolarization-dependent enhancement of NMDA receptor activation (Berretta and Jones 1996; Woodhall et al., 2001), summation with inward I_h currents induced by the initial inhibition (Dickson et al., 2000a), or

suppression of inhibitory $GABA_A$ transmission by activation of presynaptic $GABA_B$ autoreceptors (Deisz and Prince 1989; Jones and Buhl 1993; Metherate and Ashe 1994).

In addition to short-term heterosynaptic effects, the parasubiculum is likely to have an important role in modulating the induction of long-lasting changes in synaptic strength within the entorhinal cortex that may contribute to lasting changes in sensory or mnemonic processing (Yun et al., 2000, 2002). The induction of both long-term synaptic potentiation and long-term depression have been observed in piriform cortex inputs to the entorhinal cortex (Bouras and Chapman, 2003; Chapman and Racine, 1997; Kourrich et al., 2008), and the induction of these lasting synaptic changes is enhanced by patterns of stimulation related to theta and gamma rhythms (Alonso et al., 1990; Chapman and Racine, 1997; Yun et al., 2000; see also Johnston et al., 2003; Judge and Hasselmo, 2004). Heterosynaptic interactions in the induction of lasting synaptic plasticity are also common in the hippocampal formation (Chistiakova and Volgushev, 2009; Habib et al., 2013; Han and Heinemann, 2013; Judge and Hasselmo, 2004; Levy and Steward, 1983; Saudargiene et al., 2014) and strong activation patterns modelled on theta-patterned stimulation are known to contribute to lasting plasticity (Han and Heinemann, 2013; Hoffman et al., 2002). It is not known, however how rhythmic activity in parasubicular inputs may affect long-term plasticity in the entorhinal cortex.

Neural activity within the hippocampal formation, including the parasubicular-entorhinal pathway, is modulated by theta (4 - 12 Hz) and gamma (30 - 80 Hz) frequency EEG activities (Chrobak and Buzsaki, 1998; Colgin et al., 2009; Glasgow and Chapman, 2013; Klink and Alonso, 1993; Mitchell and Ranck, 1980). These oscillatory rhythms have been associated with aiding encoding and recall of memories through the coordination of population neuronal activity (Buzsaki, 2002; Chrobak and Buzsaki, 1998; Hasselmo, 2006), including the activity of spatially-tuned place cells and grid cells (Boccara et al., 2010; Hasselmo et al., 2007; Igarashi et al., 2014; Lisman and Jensen, 2013; Moser et al., 2015; O'Keefe and Recce, 1993). We have previously found that single EPSPs in the entorhinal cortex evoked by stimulation of the parasubiculum are suppressed following application of a cholinergic agonist, but that cholinergic activation is also associated with much greater growth in the amplitude of consecutive synaptic responses during short trains of theta and gamma frequency stimulation relative to the growth in responses that is observed in normal ACSF (Sparks and Chapman, 2013, 2014). This is consistent with a potential role of cholinergic inputs to the entorhinal cortex in promoting

processing of synaptic afferents that are active during theta and gamma EEG activity (Dickson et al., 2000b; Glasgow and Chapman, 2007; Mitchell and Ranck, 1980). This cholinergic facilitation of rhythmic parasubicular synaptic input to the entorhinal cortex was found to be due in part to a reduction in the cationic conductance I_h, which also increases input resistance and enhances dendritic integration during repetitive synaptic activation in the hippocampal formation (Carr and Surmeier, 2007; Day et al., 2005; Dickson et al., 2000a; Garden et al., 2008; Magee, 1998; Rosenkranz and Johnston, 2006; Sparks and Chapman, 2014).

Here we have investigated mechanisms through which activation of the parasubiculum can heterosynaptically modulate the strength of layer I inputs to the entorhinal cortex using intracellular recordings from layer II medial entorhinal stellate neurons in vitro. Short-term heterosynaptic effects were assessed by stimulating the parasubiculum with either single pulses or with short 5-pulse, 10 Hz trains of pulses, and by then evoking synaptic responses with layer I stimulation at intervals of 5 to 1000 ms. Short-term heterosynaptic suppression at the 5 ms interval was investigated by assessing the contribution of $GABA_A$ transmission, and by assessing depolarization-induced changes in driving force during the EPSP. The role of reductions in I_h in the heterosynaptic facilitation effect was also assessed (Dickson et al., 2000a; Magee, 1998). We then investigated how theta-frequency activity in parasubicular efferents may contribute to lasting synaptic changes in the entorhinal cortex by determining if repetitive theta-frequency stimulation of the parasubiculum combined with single-pulse stimulation of layer I could result in lasting modifications in the strength of synaptic inputs from layer I.

METHODS

Slice preparation

Guidelines of the Canadian Council on Animal Care were followed. Acute horizontal brain slices were obtained from 4 to 8-week-old male Long-Evans rats after halothane anaesthetization and decapitation. Brains were removed and submerged in artificial cerebrospinal fluid (ACSF, 4 °C) consisting of (in mM): 250 sucrose, 2 KCl, 1.25 NaH_2PO_4, 7 $MgCl_2$, 26 $NaHCO_3$, 0.5 $CaCl_2$ and 10 dextrose saturated with 95% O_2/5% CO_2. Slices were cut 300 μm-thick using a vibratome (Leica VT1200, Leica Biosystems, Concord, ON, Canada) and recovered at room temperature for 1 - 1.5 h in ACSF containing 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 10 dextrose. Individual slices were placed in a recording

chamber at 32 °C and were perfused with ACSF at a rate of 1 - 1.5 ml/min. Slices were visualized using a Leica DM-LFS microscope with a long-range water immersion objective (40X) and differential interference contrast optics.

Stimulation and recording

Whole-cell patch-clamp recordings were obtained using borosilicate glass pipettes (1.0 mm OD, 0.5 mm ID, 2 - 5MΩ) made with a horizontal puller (Sutter Instr., Novato, CA, USA, Model P97). These were filled with a solution containing (in mM): 140 K-gluconate, 5 NaCl, 2 MgCl, 10 HEPES, 0.5 EGTA, 2 ATP-Tris, and 0.4 GTP-Tris (pH adjusted to 7.20 - 7.3 using KOH; 270 - 280 mOsm). Pipettes were lowered to make contact with layer II medial entorhinal cortex neurons located close to the border with layer I. Gentle suction was applied in voltage-clamp mode to obtain a seal of at least 1 GΩ and stronger suction was applied to rupture the membrane. Cells rested for 10 to 15 minutes before recordings were taken. Series resistance was compensated by adjusting the discontinuity in voltage responses to 100 pA pulse steps, and cells were considered acceptable if series resistance was less than 30 MΩ (10.3 ± 0.5 MΩ), and if resting membrane potential was less than -50 mV (-62.7 ± 0.4 mV). Input resistance was measured using 500 ms hyperpolarizing current injections of -100 pA at resting potential. Peak input resistance was measured via the largest voltage change following negative current injection relative to resting membrane potential (46.1 ± 2.6 MΩ). Putative stellate cells, identified by their large inward voltage response to hyperpolarizing current injection (Alonso and Klink, 1993; Dickson et al., 2000a), were targeted in this study due to their strong expression of I_h which is known to play a role in both intrinsic theta frequency oscillations (Dickson et al., 2000a) and the integration of synaptic inputs (Magee, 1998).

EPSPs were recorded following stimulation of mid to deep layers of the parasubiculum and layer I of the entorhinal cortex using 0.1 ms constant current pulses delivered via a tungsten bipolar electrode (FHC, Bowdoin, ME, USA) using a stimulus generator (WPI, Model A300) and isolation unit (Model A360). Projections from the superficial layers of the parasubiculum pass through the deep layers of the parasubiculum and entorhinal cortex before ascending to reach layer II (Caballero-Bleda and Witter, 1993). Stimulation intensity (75 - 150 μA) was set to evoke EPSPs of approximately 50 to 75% of maximal responses in order to avoid ceiling effects on synaptic responses and to limit activation of neighbouring regions. Evoked EPSPs were filtered and amplified (DC- 10 kHz, Axoclamp 200B, Molecular Devices, Sunnyvale, CA, USA)

and digitized (20 kHz, Digidata 1322A, Molecular Devices) for storage on computer hard disk using the pClamp 8.2 (Molecular Devices) software package.

Short-term plasticity

Constant current injection was used to hold cells at subthreshold voltages near resting membrane potential to avoid contamination of synaptic responses by action potentials. For homosynaptic paired pulse tests, pairs of pulses were delivered to the parasubiculum at intervals of 5, 25, 100, or 1000 ms. For heterosynaptic tests, synaptic responses were evoked by either a single pulse or by a short 5-pulse 10 Hz train of stimulation in the parasubiculum, followed by a single pulse in layer I at an interval of 5, 25, 100, or 1000 ms. Ten responses were obtained for each condition with a 10 s interval between trials.

The contribution of I_h to suppression and facilitation effects was assessed by monitoring responses before and after a 10-min bath application of the I_h blocker ZD7288 (10 μM, Ascent Scientific, Princeton, NJ, USA). The role of inhibitory synaptic transmission was assessed by blocking $GABA_A$ transmission with bicuculline methiodide (25 μm, Sigma-Aldrich, St. Louis, MO, USA) and by blocking $GABA_B$ receptors with CGP55845 (1μM, Tocris Bioscience, Bristol, UK). NMDA receptors were blocked with APV (50 μM, Ascent Scientific). All drugs were stored as frozen stock solutions.

Long-term plasticity

To assess heterosynaptic modulation of induction of lasting synaptic plasticity in layer I inputs, baseline responses to single pulses delivered to the parasubiculum and layer I were recorded alternately every 20 seconds for 10 minutes prior to delivery of patterns of repetitive stimulation. Patterned stimulation was followed by a 30-minute follow-up period. In the first two groups tested, five-pulse 10 Hz parasubicular stimulation trains were delivered once every two seconds for 15 min, and each train was followed by a single layer I pulse at an interval of either 5 or 25 ms. Control groups received either, repetitive 0.5 Hz delivery of 10 Hz parasubicular trains without subsequent pulses in layer I, repetitive 0.5 Hz delivery of single pulses to layer I without the parasubicular trains, or continued delivery of test-pulses every 20 sec alternately to the parasubiculum and layer I without patterned stimulation. In separate groups, to determine if postsynaptic cell firing combined with layer I stimulation might enhance plasticity effects, a depolarizing current step (20 ms, 500 pA) was delivered concurrently with layer I pulses either 5 or 25 ms following theta-frequency parasubicular trains. Control groups

received depolarizing current injections alone, without concurrent layer I stimulation, either 5 or 25 ms following theta-frequency parasubicular trains.

Data analysis

Peak amplitudes of averaged evoked synaptic potentials were measured relative to the pre-stimulation membrane potential, and group averages were expressed as the mean ± standard error of the mean. Paired-pulse ratios were calculated by expressing the amplitude of the second response in each pair relative to the response evoked by the first pulse. For the 5 ms inter-pulse interval, in which responses overlapped, the amplitude of the second response was measured after first subtracting the trace obtained in response to a single parasubicular stimulation pulse. Changes in layer I responses induced by prior parasubicular stimulation were assessed relative to the amplitude of layer I responses with no preceding parasubicular stimulation. Repeated-measures ANOVAs, as well as planned comparisons for each interpulse interval ($\alpha < 0.05$), were used to assess changes in response amplitudes and changes in paired-pulse ratios between baseline and drug conditions.

Induction of long-term synaptic plasticity was assessed by calculating mean responses recorded during the ten-minute baseline period, one minute immediately following patterned stimulation, and during the last five minutes of recordings. One-way ANOVAs were used to assess the effects of patterned stimulation on the amplitudes of synaptic responses, and planned comparisons between the baseline and one minute following patterned stimulation, and between the baseline and the last five minutes of the recording period were also conducted.

RESULTS

Homosynaptic paired-pulse tests

Homosynaptic paired pulse tests, in which pairs of pulses were delivered to the parasubiculum at intervals of 5 to 1000 ms, were used to assess inhibitory and facilitatory mechanisms initiated by stimulation of the parasubiculum. Similar to previous findings (Caruana and Chapman, 2004), a significant paired-pulse suppression was found at the 5 ms inter-pulse interval (76.1 ± 7.3 % of baseline, $p = 0.03$; Figure 4.1A, $n = 5$). Paired-pulse facilitation was also observed at the 25 ms interval (138.8 ± 8.0 %, $p < 0.01$; Figure 4.1A$_1$), likely due largely to summation of presynaptic Ca^{2+} and increased transmitter release (Yamada and Zucker, 1992; Caruana and Chapman, 2004). No significant changes in paired-pulse ratios

were found at longer inter-pulse intervals of 100 and 1000 ms in this group of cells ($p > 0.05$; Figure 4.1A), although the facilitation was significant for the 100 ms interval when combining all cells in Figure 4.1 ($p = 0.01$). The strong synaptic facilitation observed at the 25 ms interval indicates the potential importance of neuronal firing at the period of the gamma rhythm for enhancing synaptic responses in this pathway (Hamam et al., 2007).

We then assessed the role of fast synaptic inhibition in the paired-pulse suppression observed at the 5 ms interval. Block of $GABA_A$ receptors with bicuculline methiodide strongly increased the amplitude of single EPSPs (154.0 ± 10.8 % of baseline, $n = 6$; $F_{1,10} = 5.5$, $p = 0.01$; Figure 4.1B_1), but had no effect on paired-pulse ratio at any interval ($p > 0.05$; Figure 4.1B_2). This indicates that EPSP amplitude is strongly affected by synaptic inhibition, but that fast $GABA_A$ transmission does not mediate the paired-pulse suppression effect. The $GABA_B$ antagonist CGP55845 was also applied to assess the role of $GABA_B$ autoreceptors in paired-pulse facilitation at longer intervals through presynaptic inhibition of GABA release (Davies and Collingridge, 1998). However, no significant change in paired-pulse ratio was found at any inter-pulse interval tested ($n = 7$, $p > 0.05$; data not shown), indicating that $GABA_B$ receptors are not required for the facilitation.

We next applied the I_h antagonist ZD7288, because reductions in I_h contribute to the facilitation of responses during repetitive theta and gamma-frequency stimulation (Sparks and Chapman, 2014) and I_h is also known to play an important role in synaptic integration in the hippocampal formation (Carr and Surmeier, 2007; Magee, 1998). Application of ZD7288 caused an overall increase in the amplitude of EPSPs (118.9 ± 7.4 % of baseline for single pulses, $n = 6$; $F_{1,10} = 5.7$, $p = 0.04$; Figure 4.1C_1), likely due to the associated increase in input resistance (from 31.6 ± 2.7 to 46.0 ± 5.1 MΩ). However, ZD7288 caused no significant change in paired-pulse ratios at any interval ($p > 0.05$; Figure 4.1C_2). The stability of paired-pulse facilitation ratios during pharmacological block of I_h suggests that I_h plays a lesser role as compared to presynaptic mechanisms that enhance transmitter release (Yamada and Zucker, 1992).

Because paired-pulse suppression at the 5 ms interval was not dependent on $GABA_A$ receptors, we investigated whether reductions in driving force on EPSPs might contribute (Spruston, 2008); the second EPSP in each pair occurs during a period of depolarization caused by the first EPSP, and this could reduce the electrotonic driving force on the second EPSP. We found that there was greater paired-pulse suppression when the amplitude of the initial response

was larger (r = -0.53, $p < 0.01$), suggesting that depolarization during the initial EPSP could play a role. To assess this, single stimulation pulses were delivered during constant current injections used to set membrane potential at levels from -10 mV to +10 mV from rest (n = 7). The relationship between membrane potential and EPSP amplitude was generally linear, and depolarization of cells by 5 mV reduced EPSP amplitudes to 81.6 ± 6.7 % of values at rest (p = 0.02; Figure 4.2A). This result was replicated in voltage clamp to provide increased control of membrane potential; EPSCs were increased by 24.9 ± 6.0 % when hyperpolarized by -10 mV ($p < 0.01$), and were supressed by 12.2 ± 5.3 % when depolarized by 5 mV (p = 0.047, Figure $4.2B_{1,4}$).

Because a depolarization-induced increase in driving force on synaptic inhibition may have contributed to the reduction of EPSCs, we repeated tests in the presence of bicuculline to block inhibitory synaptic potentials. Bicuculline increased the amplitudes of EPSCs (129.7 ± 8.1 % of baseline, p = 0.01, Figure $4.2B_2$), but the reduction in EPSC amplitude during depolarization of membrane potential to +5 mV was maintained, suggesting that reductions in EPSC amplitudes during depolarization result more from a reduction in the driving force on EPSPs rather than increased driving force on synaptic inhibition.

Further reductions in EPSC amplitude were not observed when cells were depolarized from 5 mV to 10 mV from rest in bicuculline, perhaps due to depolarization-dependent enhancement of NMDA receptor-mediated currents (Grover and Yan, 1999). The AMPA-mediated EPSC was therefore isolated by addition of the NMDA receptor antagonist APV. Amplitudes of mixed EPSCs were reduced only slightly at hyperpolarized potentials by addition of APV ($p > 0.05$), and showed larger reductions at depolarized potentials (75.1 ± 8.1 % at resting potential, p = 0.04; Figure $4.2B_3$), indicating the presence of a depolarization-dependent NMDA component of the mixed EPSC. Depolarization-induced *reductions* in the mixed EPSC are therefore likely to be mainly due to reductions in the driving force on the AMPA receptor-mediated components of the response.

Heterosynaptic stimulation

We also examined the effects of parasubicular stimulation on responses evoked in layer II neurons by layer I stimulation to investigate heterosynaptic effects observed previously in vivo (Caruana and Chapman, 2004). Either single conditioning pulses, or trains of five pulses at 10 Hz, were delivered to the parasubiculum, and a single pulse was then delivered to layer I at an

interval of 5, 25, 100, or 1000 ms. There was a significant decrease in the size of responses to layer I pulses delivered 5 ms after single parasubicular pulses (71.7 ± 5.4 % of baseline, $p < 0.01$, $n = 5$; Figure 4.3A$_1$), an effect that is likely due in part to the reduced driving force on EPSPs that contributes to homosynaptic paired-pulse suppression. No significant changes in EPSPs were observed at other inter-pulse intervals ($p > 0.05$). However, following parasubicular trains, there was both a suppression in layer I responses at the 5 ms interval (77.7 ± 4.2 %, $p < 0.01$), as well as a facilitation of layer I responses evoked 25 ms following the trains (117.8 ± 8.5 %, $p = 0.02$; Figure 4.3A$_2$) indicating that theta-frequency stimulation of the parasubiculum can facilitate entorhinal responses to layer I inputs.

We assessed the contribution of both $GABA_A$ and $GABA_B$-mediated transmission to heterosynaptic suppression and facilitation effects. Bicuculline increased the amplitudes of responses to layer I stimulation (153.8 ± 11.0 % of baseline, $n = 6$; $p < 0.05$; Figure 4.3B). Bicuculline did not block the suppression of layer I responses observed 5 ms after single parasubicular pulses ($p > 0.05$; Figure 4.3B$_1$), but it did abolish the suppression of responses observed 5 ms after *trains* of parasubicular stimulation (109.7 ± 6.9 % vs. 89.4 ± 6.3 %, $p = 0.03$; Figure 4.3B$_2$), perhaps due to increased recruitment of synaptic inhibition by entrainment of interneurons during theta-frequency stimulation (Bitzenhofer et al., 2015; Tamas et al., 2004). Block of $GABA_B$ receptors with CGP55845 had no significant effect on responses to layer I stimulation, or on the modulation of layer I responses by parasubicular stimulation ($p > 0.05$, $n = 7$; not shown), suggesting that $GABA_B$ receptors do not play a substantial role in these heterosynaptic effects.

Repetitive synaptic stimulation can enhance dendritic integration by depolarization-induced reductions in the current I_h, which increases dendritic input resistance (Carr and Surmeier, 2007; Magee, 1998), and we therefore assessed if heterosynaptic facilitation of responses at the 25 ms interval would be affected by block of I_h. Blocking I_h with ZD7288 increased responses to layer I stimulation with single pulses (119.6 ± 10.0, $n = 6$; $p < 0.05$) and also increased layer I responses evoked after either single pulses or trains of parasubicular pulses (Figure 4.3C). Effects of I_h blockade on facilitation ratios were strongest at the 25 ms interval: ZD7288 significantly increased the facilitation ratio for layer I responses evoked 25 ms following parasubicular trains (138.3 ± 5.5 % of baseline, $p = 0.01$; Figure 4.3C$_2$), and the facilitation ratio for layer I responses evoked 25 ms after single parasubicular pulses was also

increased non-significantly by ZD7288 (114.1 ± 4.7 %, $p = 0.051$; Figure 4.3C_1). The greater increases in heterosynaptic facilitation induced by ZD7288 for trains of stimulation as compared to single pulses is consistent with the I_h-dependent facilitation of entorhinal responses to 10 Hz parasubicular stimulation we have observed previously (Sparks and Chapman, 2013, 2014), and is also consistent with enhanced dendritic integration during repetitive stimulation mediated by reductions in I_h that has been observed by others (Carr and Surmeier, 2007; Magee, 1998).

Long-term synaptic plasticity

We then assessed whether heterosynaptic suppression and facilitation effects induced by theta-frequency stimulation of the parasubiculum could modulate induction of long-term synaptic plasticity in layer I inputs to the entorhinal cortex. Low frequency repetitive paired-pulse stimulation of layer I using a 30 ms inter-pulse interval can lead to long-term synaptic depression (Bouras and Chapman, 2003; Kourrich and Chapman, 2003; Kourrich et al, 2008), and theta frequency stimulation is known to be effective in the induction of long-term potentiation in the entorhinal cortex (Yun et al., 2002). We therefore assessed the effects of repetitive delivery of trains of 10 Hz parasubicular stimulation followed by layer I stimulation pulses at intervals of either 5 or 25 ms, in the induction of lasting changes in the strength of synaptic inputs to layer I. Repetitive train-pulse stimulation for 15 min using either the 5 ms or 25 ms interval resulted in a lasting depression of responses evoked by layer I stimulation, but only a transient depression of responses evoked by the parasubiculum. Responses to layer I test pulses were reduced to 64.7 ± 13.2 % of baseline levels 30 min after stimulation using the 5 ms interval ($p = 0.03$, $n = 8$; Figure 4.4$A_{1,2}$) and were reduced to 66.6 ± 8.5 % 30 min after stimulation using the 25 ms interval ($p < 0.01$, $n = 9$; Figure 4.4$B_{1,2}$). Responses to test pulses delivered to the parasubiculum did not show significant changes other than a transient reduction in responses immediately after train-pulse delivery at the 5 ms interval (71.2 ± 11.6 % of baseline, $p = 0.04$; Figure 4.4A_3). Responses in a control group that received single pulses every 20 sec instead of train-pulse stimulation remained stable ($p > 0.05$, Figure 4.4$A_{2,3}$ and 4$B_{2,3}$ open symbols, $n = 8$).

To determine if the depression of layer I responses was due to a heterosynaptic interaction, rather than to repetitive stimulation of either pathway alone, control tests were conducted in which parasubicular trains were delivered without layer I stimulation, or in which layer I pulses were delivered without parasubicular trains. Results showed no significant lasting

changes in either input pathway in response to either 0.5 Hz delivery of parasubicular trains alone ($n = 8$, $p > 0.05$; Figure 4.4C_1), or 0.5 Hz delivery of layer I pulses alone ($n = 8$, $p > 0.05$; Figure 4.4C_2). Thus, although repetitive stimulation of either input pathway has no lasting effects when delivered alone, our results indicate that repetitive theta frequency stimulation of the parasubiculum can heterosynaptically promote the induction of long-term synaptic depression in layer I inputs when the layer I inputs are activated at short intervals following parasubicular trains.

Heterosynaptic plasticity can be enhanced by firing in the postsynaptic cell (Chistiakova and Volgushev, 2009; Chistiakova et al., 2014), and we therefore assessed whether addition of 20 ms, depolarizing current pulses at the onset of layer I stimulation could enhance LTD induction by train-pulse pairs. LTD of responses evoked by layer I stimulation was observed following patterned stimulation using either the 5 ms train-pulse interval (66.1 ± 7.6 %, $p < 0.01$, $n = 9$) or the 25 ms interval (57.4 ± 7.1 %, $p < 0.01$, $n = 8$) (Figure 4.5$A_{1,2}$ and $B_{1,2,}$ closed symbols). Responses to parasubicular test pulses showed no lasting effects ($p > 0.05$; Figure 4.5A_3 and B_3, closed symbols) indicating that the LTD was specific to layer I inputs. Control tests also showed that combined delivery of parasubicular trains and current pulses without the layer I stimulation did not result in LTD ($p > 0.05$; Figure 4.5$A_{2,3}$ $B_{2,3}$, open symbols). Overall, however, these experiments showed that the LTD induced by repetitive train-pulse stimulation combined with current pulses was similar to that observed without the current pulses ($p > 0.05$; compare Figures 4.4 and 4.5), indicating that added depolarization to reliably induce action potentials during heterosynaptic stimulation does not add to the LTD induced.

DISCUSSION

The purpose of the present study was to assess short-term heterosynaptic effects of the parasubiculum on layer I inputs to layer II stellate cells of the entorhinal cortex, and to determine how these effects may modulate induction of long-term synaptic plasticity in layer I inputs. Stimulation of the parasubiculum suppressed synaptic responses in the entorhinal cortex evoked after an interval of 5 ms. The synaptic suppression in paired-pulse tests appeared to be due mainly to a depolarization-dependent reduction in the driving force on AMPA receptor mediated currents (Figure 4.2), but synaptic inhibition via $GABA_A$ receptors contributed to the suppression induced following short trains of parasubicular stimulation (Figure 4.3). Heterosynaptic

facilitation effects were observed 25 ms after trains of parasubicular stimulation, but not after single pulses, indicating that theta frequency stimulation can promote synaptic facilitation. Repeated pairings of short 10 Hz parasubicular trains with layer I stimulation 5 or 25 ms after each train induced long-term synaptic depression in entorhinal responses to layer I stimulation, likely due to sustained, moderate increases in postsynaptic calcium (Feldman, 2012; Malenka and Nicoll, 1993). These findings illustrate the importance of rhythmic neuronal activity at theta- and gamma-frequencies in the integration of synaptic input in the entorhinal cortex (Sparks and Chapman, 2013, 2014), and also emphasize the potential role of the parasubiculum in modulating entorhinal cortex responsiveness to sensory inputs (Caruana and Chapman, 2004).

Short-term synaptic suppression evoked by parasubicular stimulation

Paired-pulse stimulation can be used to investigate inhibitory and facilitatory mechanisms that are induced by the first pulse in each pair that depend upon presynaptic factors that affect transmitter release, activation of inhibitory inputs, and postsynaptic voltage-dependent conductances (Yamada and Zucker, 1992; Yejun et al., 2002; Yun et al., 2000). Similar to findings in this pathway in vivo (Caruana and Chapman, 2004) paired-pulse stimulation of the parasubiculum induced a suppression of entorhinal responses at the 5 ms inter-pulse interval, and facilitation at the 25 ms interval. We previously proposed that the suppression at the 5 ms interval was likely due to activation of GABAergic projections to the entorhinal cortex by parasubicular stimulation (Caruana and Chapman, 2004). However, block of $GABA_A$ transmission, despite increasing the overall amplitude of EPSPs, likely by blocking both spontaneous synaptic inhibition, and fast shunting inhibition evoked by each stimulation pulse, did not block paired-pulse suppression of parasubicular inputs to the entorhinal cortex. Similarly, $GABA_A$ blockade did not block *heterosynaptic* paired-pulse suppression of layer I responses caused by conditioning stimulation of the parasubiculum. We then tested if the suppression might be due to a reduction in the driving force on the AMPA-mediated EPSPs due to depolarization of the neuron in response to the first pulse in the pair (Abrahamsson et al., 2012; Bush and Sejnowksi, 1994; Spruston, 2008; see also Economo et al., 2014). We found that membrane hyperpolarization enhanced EPSP amplitudes and that depolarization suppressed EPSP amplitudes, supporting the idea that depolarization during the initial EPSP resulted in a suppression of the driving force on the second EPSP. EPSCs were also reduced at depolarized potentials in voltage clamp tests. In these tests, blocking $GABA_A$ receptors and NMDA receptors

did not affect the membrane-potential dependent modulation of EPSC amplitude, suggesting that paired-pulse suppression of EPSPs is likely due mainly to changes in driving force on AMPA receptor-mediated currents and is not likely dependent on an increase in driving force on the $GABA_A$-mediated Cl^- conductance, or affected by depolarization-dependent increase in NMDA-mediated currents.

Theta EEG activity is associated with strong effects on synaptic transmission in the entorhinal cortex (Fernandez et al., 2013; Sparks and Chapman, 2014), and we therefore assessed the heterosynaptic effects of theta-frequency stimulation of the parasubiculum on layer I responses. Ten Hz trains of stimulation delivered to the parasubiculum led to a strong suppression of layer I inputs 5 ms after the train, but in contrast to paired-pulse results, the inhibitory effect induced by trains was blocked by bicuculline. This suggests that theta-frequency activation of the parasubiculum recruits activation of interneurons that are entrained by theta activity and are activated shortly after each stimulation pulse (Bitzenhofer et al., 2015; Tamas et al., 2004).

Short-term facilitation evoked by parasubicular stimulation

Short-term synaptic facilitation effects may enhance transmission from the parasubiculum to entorhinal cortex during rhythmic EEG activities, and we found here that stimulation of the parasubiculum induced a facilitation of synaptic responses evoked 25 ms later. Depolarization-induced reductions in I_h could contribute to paired-pulse facilitation by increasing dendritic input resistance (Carr and Surmeier, 2007; Dickson et al., 2000a; Magee, 1998). In paired-pulse tests, blocking I_h with ZD7288 resulted in a significant overall increase in EPSP amplitude, but had no effect on paired-pulse ratio at the 25 ms interval. Homosynaptic paired-pulse facilitation at this interval, then, is more likely to be due to enhanced transmitter release due to summation of presynaptic Ca^{2+} transients (Yamada and Zucker, 1992), rather than to changes in dendritic input resistance induced by reductions in I_h, which may require longer repetitive activation of inputs (Magee, 1998; Medinilla et al., 2013; Sparks and Chapman, 2014)

Heterosynaptic paired-pulse tests, in which a pulse was delivered to layer I at various intervals after parasubicular stimulation, were used to assess how parasubicular inputs might modulate entorhinal responsiveness to incoming cortical sensory inputs. Summation of presynaptic Ca^{2+} transients cannot contribute to heterosynaptic paired-pulse facilitation, and no significant facilitation of layer I inputs was found with a 25 ms inter-pulse interval. However,

when 10 Hz trains of stimulation were delivered to the parasubiculum, there was a facilitation of responses to layer I inputs at the 25 ms inter-pulse interval. In addition, blocking I_h with ZD7288 led to a significant increase in the heterosynaptic facilitation effect. These results point to an important role of theta-frequency oscillatory activity in enhancing synaptic input to the entorhinal cortex, and they also suggest that reductions in I_h resulting from repetitive stimulation, as compared to stimulation with single pulses, plays an important role in the facilitation effect (Day et al., 2005; Magee, 2000; Sparks and Chapman, 2014). Synaptic inputs to layer I may be especially sensitive to this effect due to the greater density of I_h on distal dendrites that are targeted by layer I inputs (Canto and Witter, 2012, Canto et al., 2012; Kerr et al., 2007; Magee, 2001).

Long-term synaptic plasticity

We were also interested in how the parasubiculum might promote long-term changes in the responsiveness of the entorhinal cortex to sensory inputs. Other pathways within the hippocampal formation show strong heterosynaptic interactions in the induction of lasting plasticity, including modulation of CA3-CA1 synapses by inputs to CA1 from the entorhinal cortex (Han and Heinemann, 2013; Judge and Hasselmo, 2004; Saudargiene et al., 2014), interactions between ipsilateral and contralateral projections from the entorhinal cortex to the dentate gyrus (Levy and Steward, 1983), and CA1 responses to inputs from the septum and CA3 (Habib et al., 2013). Frequencies of stimulation associated with theta and gamma EEG activities are also effective in the induction of lasting changes in synaptic strength (Han and Heinemann, 2013; Grover et al., 2009; Yun et al., 2000). Here, to test for the induction of lasting synaptic plasticity, we combined repetitive delivery of 5-pulse parasubicular trains every 2 sec for 15 min with delivery of single stimulation pulses to layer I at short intervals after each train. We found that this pattern of stimulation, using either a 5 or 25 ms train-pulse interval, resulted in long-term synaptic depression of layer I inputs, and only a transient suppression of responses to parasubicular stimulation. Control conditions in which parasubicular trains were delivered alone, or in which single pulses were delivered to layer I, resulted in no changes, indicating that the LTD of layer I inputs was due to the interaction of parasubicular trains with subsequent activation of layer I inputs.

Similar heterosynaptic LTD was observed when layer I pulses were delivered either 5 or 25 ms after the parasubicular trains, suggesting that the occurrence of synaptic suppression and

facilitation effects during the layer I responses does not alter the magnitude of LTD induced. The LTD induced at the 5 ms interval is consistent with findings in the hippocampus in which repetitive stimulation at intervals that result in paired-pulse suppression results in LTD (Doyere et al., 1996, Thiels et al., 1994) and this is consistent with an anti-Hebbian suppression of active synapses that do not drive cell firing (Feldman, 2012). In contrast, we have also previously found that LTD in layer I inputs to the entorhinal cortex is effectively induced using repetitive paired-pulse stimulation at a 30 ms interval that results in *facilitation* of layer I responses (Bouras and Chapman, 2003; Kourrich and Chapman, 2003; Kourrich et al, 2008), and this effect was attributed to prolonged moderate increases in postsynaptic Ca^{2+} that can induce LTD (Malenka and Nicoll, 1993). Here, the similar heterosynaptic LTD induced by layer I activation during both synaptic suppression and facilitation suggests that both patterns may have promoted moderate increases in Ca^{2+} levels. The absence of any long-term changes in the strength of response to parasubicular stimulation indicates that these inputs are specific to layer I synapses, and that parasubicular inputs to the entorhinal cortex are more resistant to induction of LTD as compared to layer I inputs.

Depolarizing current injection to evoke action potentials can enhance induction of both homosynaptic and heterosynatic plasticity by initiating backpropagating action potentials that enhance dendritic Ca^{2+} (Chistiakova and Volgushev, 2009; Chistiakova et al., 2014; Feldman, 2012). In our first tests of LTD induction, layer I inputs were set to evoke EPSPs below action potential threshold, and we therefore repeated these tests with the addition of depolarizing current steps overlapping with the layer I stimulation pulses delivered after each parasubicular train. Control tests in which depolarizing pulses were applied without layer I stimulation did not result in significant LTD, indicating that synaptic activation is required. The addition of depolarizing pulses to initiate action potentials did not enhance LTD effects, suggesting that the heterosynaptic LTD effect is not enhanced by consistent association with cell firing.

Conclusion

The parasubiculum selectively targets layer II of the entorhinal cortex, and the present study has demonstrated strong inhibitory and facilitatory effects of this pathway that may modulate the responsiveness of the entorhinal cortex to incoming sensory information arriving via layer I inputs. Short-lasting inhibitory effects are related to reductions in driving force on the EPSP following single pulses, and are mediated by $GABA_A$ synaptic transmission following

trains of parasubicular stimulation. Short-term facilitatory effects were observed at an interval of 25 ms after parasubicular stimulation, and are likely dependent on postsynaptic effects including depolarization-induced reductions in dendritic I_h conductance. In addition, theta-frequency activation of the parasubiculum was found to contribute to the induction of heterosynaptic long-term depression of layer I inputs to the entorhinal cortex. This suggests that activity in parasubicular inputs to the entorhinal cortex during theta-frequency activity may lead to reductions in the strength of layer I inputs that are consistently co-active with inputs from the parasubiculum, and may result in lasting reductions in the responsiveness of the entorhinal cortex to patterns of sensory input.

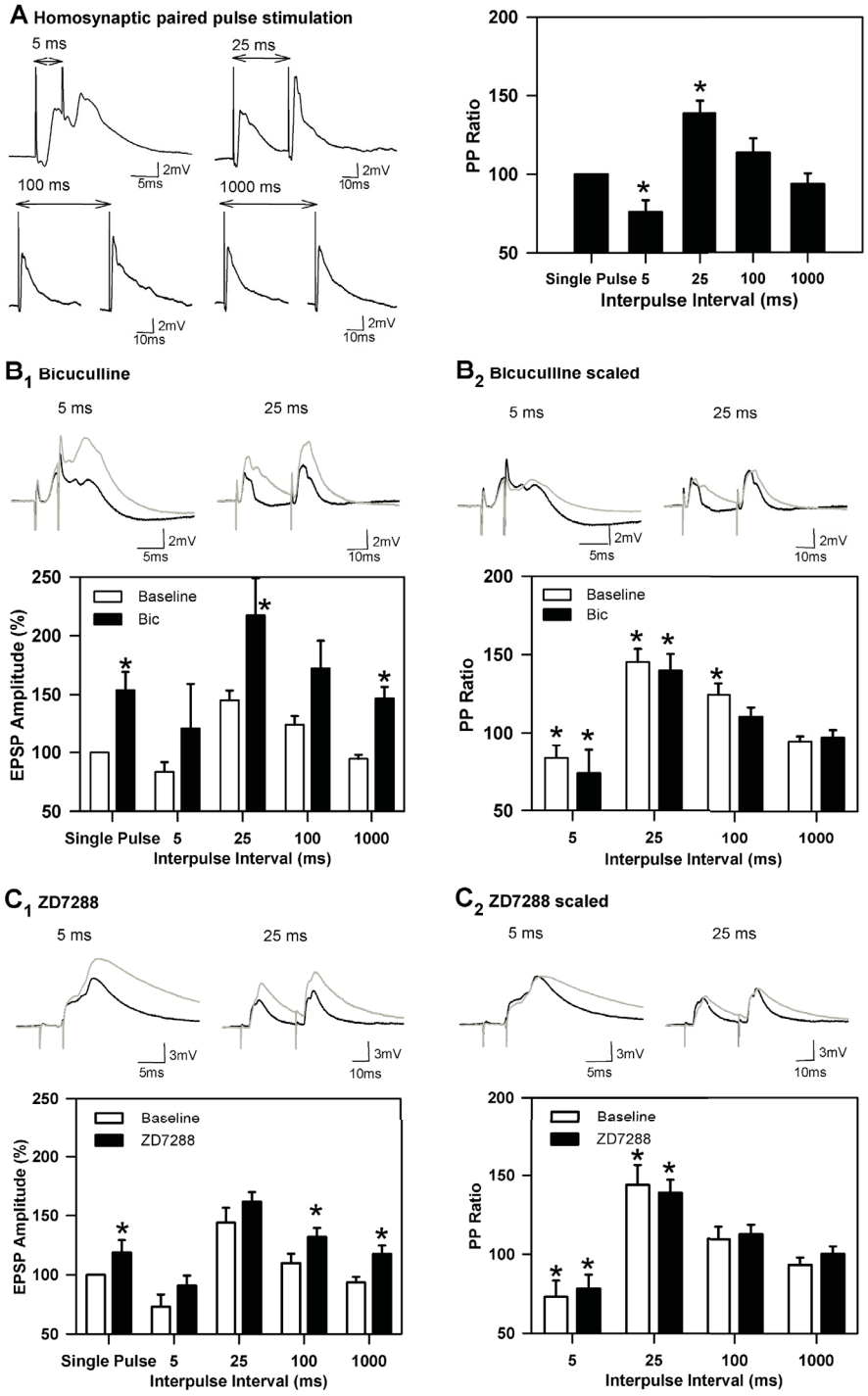

A Homosynaptic paired pulse stimulation
5 ms
25 ms
100 ms
1000 ms
2mV
5ms
2mV
10ms
PP Ratio
Single Pulse 5 25 100 1000
Interpulse Interval (ms)
B1 Bicuculline
B2 Bicuculline scaled
Baseline
Bic
EPSP Amplitude (%)
C1 ZD7288
C2 ZD7288 scaled
ZD7288
3mV

Figure 4.1. Stimulation of the parasubiculum results in time-dependent suppression and facilitation of synaptic responses in the entorhinal cortex. **A.** Paired-pulse stimulation of the parasubiculum results in paired-pulse suppression of entorhinal synaptic responses at the 5 ms interpulse interval, and paired-pulse facilitation at an interval of 25 ms, with no significant paired-pulse facilitation at longer intervals (*, $p<0.05$, $n = 5$). **B.** Block of $GABA_A$-mediated inhibition with bicuculline ($n = 6$) significantly increases the amplitude of EPSPs (B_1), but does not alter paired-pulse ratios at any interval (B_2), indicating that the paired-pulse suppression is not dependent upon $GABA_A$ synaptic transmission. Traces recorded in bicuculline (grey traces) are superimposed on traces recorded in normal ACSF (solid traces). Traces recorded in bicuculline in B_2 have been scaled to the amplitude of the response to the first pulse in each pair. **C.** Blocking I_h currents with ZD7288 ($n = 6$) led to an overall increase in EPSP amplitudes (C_1), but did not alter paired-pulse ratios at any interval tested (C_2), suggesting that the paired-pulse facilitation is not dependent upon depolarization-induced reductions in I_h that can increase dendritic input resistance.

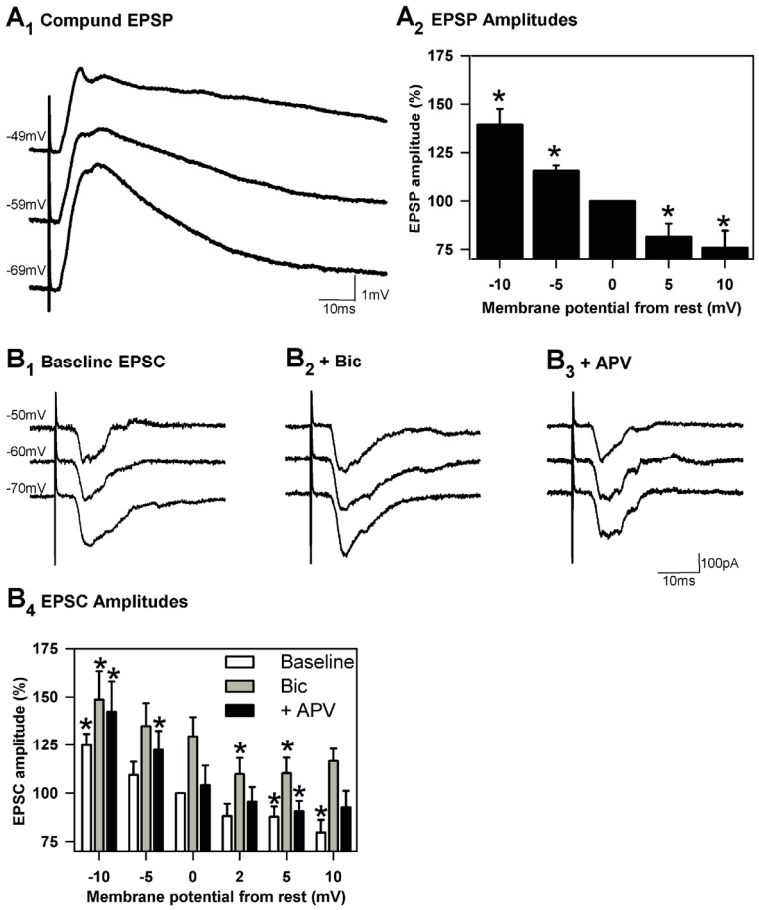
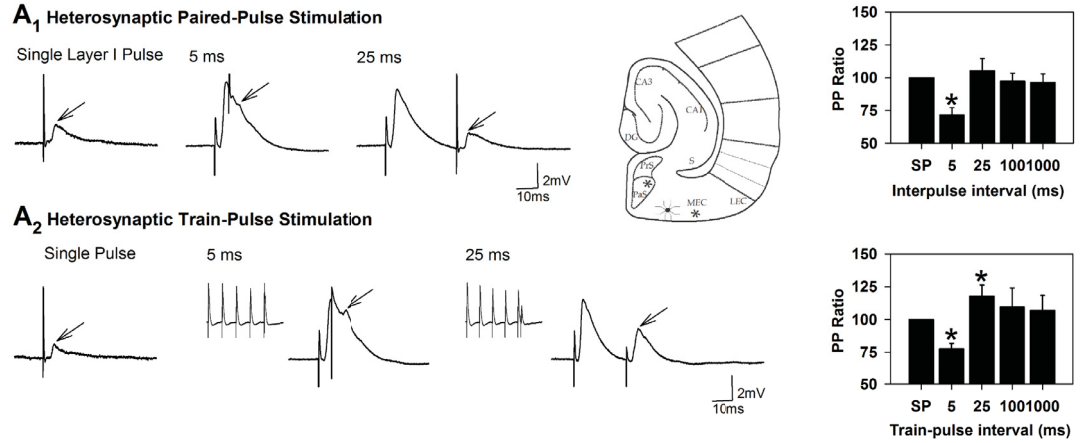
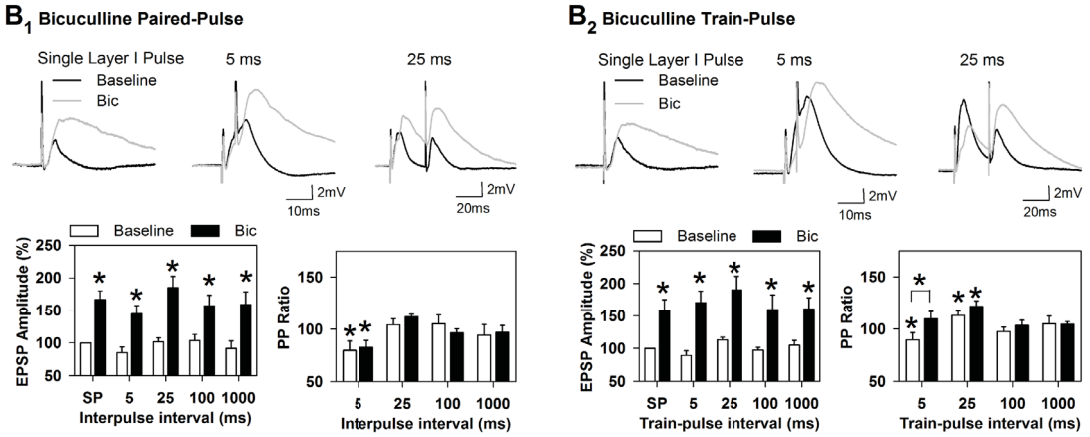

A1 Compund EPSP
-49mV
-59mV
-69mV
1mV
10ms
A2 EPSP Amplitudes
EPSP amplitude (%)
175
150
125
100
75
-10
-5
0
5
10
Membrane potential from rest (mV)
B1 Baseline EPSC
-50mV
-60mV
-70mV
B2 + Bic
B3 + APV
100pA
10ms
B4 EPSC Amplitudes
EPSC amplitude (%)
Baseline
Bic
+ APV
-10
-5
0
2
5
10
Membrane potential from rest (mV)

Figure 4.2. Paired-pulse suppression at the 5 ms interpulse interval is likely due in part to depolarization-induced reductions in the driving force on AMPA-mediated EPSPs during the first synaptic response. **A.** Representative traces recorded as cells were held at varying membrane potentials using constant current injection illustrate the effect of membrane potential on EPSP amplitude ($n = 7$) (A_1). Hyperpolarization of membrane potential increased EPSP amplitude, and membrane depolarization reduced EPSP amplitude (*$p<0.05$; A_2). **B.** In voltage clamp, hyperpolarization of membrane potential increased EPSC amplitudes, and depolarization reduced EPSC amplitudes in normal ACSF (B_1). Addition of bicuculline (Bic; B_2) to block $GABA_A$ receptors increased EPSC amplitudes, but depolarization-dependent reductions in EPSC amplitude were maintained (B_2) suggesting that increased driving force on $GABA_A$ inhibition does not contribute. Subsequent application of APV to block NMDA receptors reduced EPSCs primarily at depolarized holding potentials (B_3), and the depolarization-dependent reduction in EPSC amplitude was maintained (B_4), indicating that depolarization causes reductions in the isolated AMPA-mediated EPSC.


A1 Heterosynaptic Paired-Pulse Stimulation
Single Layer I Pulse
5 ms
25 ms
2mV
10ms
CA3
CA1
DG
S
PaS
MEC
LEC
PP Ratio
SP 5 25 1001000
Interpulse interval (ms)
A2 Heterosynaptic Train-Pulse Stimulation
Single Pulse
5 ms
25 ms
2mV
10ms
PP Ratio
SP 5 25 1001000
Train-pulse interval (ms)


B1 Bicuculline Paired-Pulse
Single Layer I Pulse
Baseline
Bic
5 ms
25 ms
2mV
10ms
2mV
20ms
Baseline
Bic
EPSP Amplitude (%)
SP 5 25 100 1000
Interpulse interval (ms)
PP Ratio
5 25 100 1000
Interpulse interval (ms)
B2 Bicuculline Train-Pulse
Single Layer I Pulse
Baseline
Bic
5 ms
25 ms
2mV
10ms
2mV
20ms
Baseline
Bic
EPSP Amplitude (%)
SP 5 25 100 1000
Train-pulse interval (ms)
PP Ratio
5 25 100 1000
Train-pulse interval (ms)

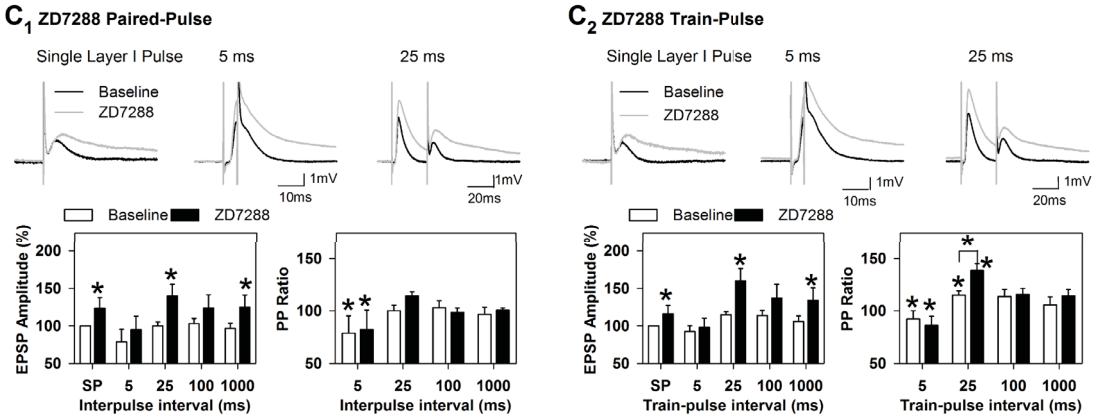

C1 ZD7288 Paired-Pulse
Single Layer I Pulse
Baseline
ZD7288
5 ms
25 ms
1mV
10ms
1mV
20ms
Baseline
ZD7288
EPSP Amplitude (%)
SP 5 25 100 1000
Interpulse interval (ms)
PP Ratio
5 25 100 1000
Interpulse interval (ms)
C2 ZD7288 Train-Pulse
Single Layer I Pulse
Baseline
ZD7288
5 ms
25 ms
1mV
10ms
1mV
20ms
Baseline
ZD7288
EPSP Amplitude (%)
SP 5 25 100 1000
Train-pulse interval (ms)
PP Ratio
5 25 100 1000
Train-pulse interval (ms)

Figure 4.3. Synaptic responses to layer I stimulation in the entorhinal cortex are modulated heterosynaptically by stimulation of the parasubiculum. **A.** Representative traces show responses of the entorhinal cortex to layer I stimulation at various intervals following either single pulses of stimulation in the parasubiculum (n = 5) (A_1) or 5-pulse trains of 10 Hz stimulation (A_2). A schematic diagram from the atlas of Paxinos and Watson (1998) shows the positions of stimulating electrodes (*) in the parasubiculum (PaS) and medial entorhinal cortex (MEC). When single conditioning pulses were delivered to the parasubiculum there was a significant paired-pulse suppression of responses to layer I stimulation at the 5 ms interval, but no significant facilitation effects at longer intervals (A_1). Theta-frequency trains of stimulation of the parasubiculum resulted in both a suppression of responses to layer I stimulation at the 5 ms interval, and a facilitation of responses at the 25 ms interval (A_2). Arrows indicate responses evoked by layer I stimulation. Inset traces in A_2 show the train-pulse responses. **B.** $GABA_A$ receptor antagonism with bicuculline (n = 6) increased the amplitude of EPSPs. $GABA_A$ receptor antagonism did not block the paired-pulse suppression of layer I responses induced by single pulses of parasubicular stimulation (B_1), but did block the heterosynaptic suppression induced by trains of parasubicular stimulation (B_2), suggesting that heterosynaptic activation of inhibitory interneurons plays a stronger role following trains of parasubicular stimulation. **C.** Application of ZD7288 to block I_h channels (n = 6) increased the amplitude of EPSPs. Paired-pulse suppression induced by single parasubicular stimulation pulses was not affected by ZD7288 (C_1), but the facilitation of layer I responses induced by trains of parasubicular stimulation at the 25 ms interval was significantly increased during block of I_h (C_2), indicating that reduction in I_h can contribute to the heterosynaptic facilitation of responses evoked by activation of layer I inputs to the entorhinal cortex.

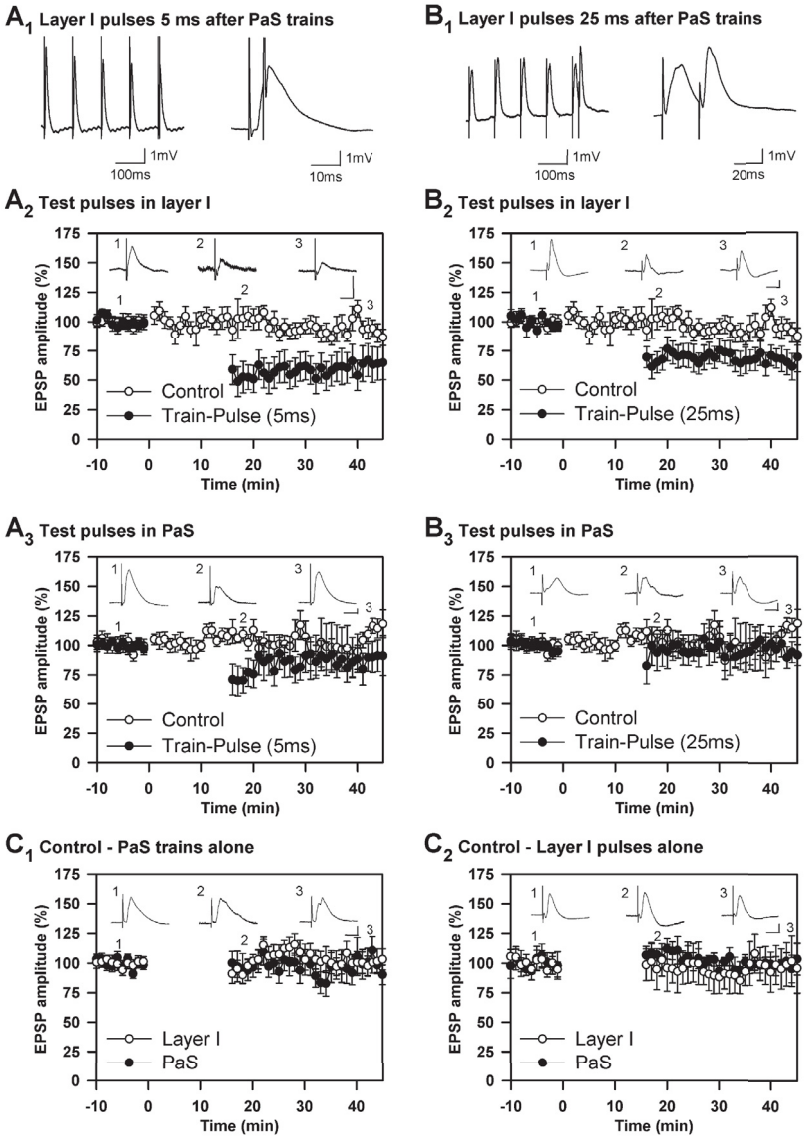

A1 Layer I pulses 5 ms after PaS trains
1mV
100ms
1mV
10ms
B1 Layer I pulses 25 ms after PaS trains
1mV
100ms
1mV
20ms
A2 Test pulses in layer I
B2 Test pulses in layer I
A3 Test pulses in PaS
B3 Test pulses in PaS
EPSP amplitude (%)
Control
Train-Pulse (5ms)
Train-Pulse (25ms)
Time (min)
C1 Control - PaS trains alone
C2 Control - Layer I pulses alone
Layer I
PaS

Figure 4.4. Repetitive pairing of theta-frequency parasubicular stimulation with single pulses of layer I stimulation 5 or 25 ms intervals after each train results in long-term synaptic depression of layer I inputs to the entorhinal cortex. **A.** An example of train-pulse conditioning stimulation with a 5 ms train-pulse (n = 8) interval is shown in A_1, and the trace has been expanded at right to illustrate the layer I response evoked 5 ms after the fifth pulse in the train. Fifteen minutes of train-pulse stimulation at 0.5 Hz resulted in long-term depression of responses to layer I stimulation (A_2, closed symbols), but no lasting change in responses to parasubicular stimulation (A_3, closed symbols). Responses in both pathways were stable in a control group that did not receive conditioning stimulation ($A_{2,3}$, open symbols). Inset traces show representative responses recorded at the indicated times (calibration bars indicate 1 mV and 10 ms in all panels). **B.** A similar pattern of results was observed in a group of cells that received repetitive train-pulse stimulation with a 25 ms interval between the parasubicular train and layer I pulse (n = 9), indicating that heterosynaptic long-term depression is induced at both train-pulse intervals. **C.** Control conditions in which either repetitive parasubicular trains (n = 8, C_1) or repetitive 0.5 Hz layer I pulses (n = 8, C_2) were delivered alone, resulted in no significant change responses to either parasubicular (closed symbols) or layer I test pulses (open symbols, and inset traces), indicating that combined repetitive stimulation is require for the LTD effect.

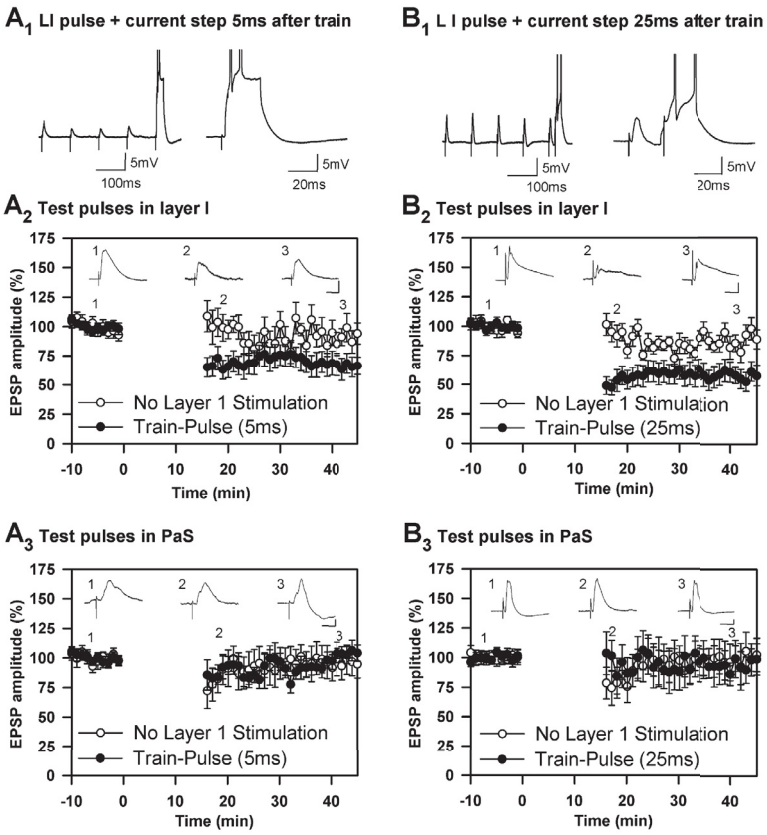

A1 LI pulse + current step 5ms after train
5mV
100ms
5mV
20ms
B1 L I pulse + current step 25ms after train
5mV
100ms
5mV
20ms
A2 Test pulses in layer I
EPSP amplitude (%)
No Layer 1 Stimulation
Train-Pulse (5ms)
Time (min)
B2 Test pulses in layer I
EPSP amplitude (%)
No Layer 1 Stimulation
Train-Pulse (25ms)
Time (min)
A3 Test pulses in PaS
EPSP amplitude (%)
No Layer 1 Stimulation
Train-Pulse (5ms)
Time (min)
B3 Test pulses in PaS
EPSP amplitude (%)
No Layer 1 Stimulation
Train-Pulse (25ms)
Time (min)

Figure 4.5. The addition of depolarizing current pulses to promote cell firing does not enhance the amount of long-term synaptic depression induced by repetitive train-pulse stimulation of the parasubiculum and layer I inputs. **A.** Cells (n = 9) received repetitive conditioning stimulation at 0.5 Hz consisting of 5-pulse 10 Hz parasubicular trains followed 5 ms later by both a single layer I stimulation pulse and a 20 ms depolarizing current step (A_1). Conditioning stimulation resulted in a long-term depression of Layer I responses (A_2, closed symbols), but there was no lasting depression when parasubicular trains and current steps were delivered without layer I pulses (A_2, open symbols). Parasubicular responses showed no lasting changes in amplitude (A_3). Conventions are as in Figure 4.4. **B.** Tests using a 25 ms interval (n = 8) between parasubicular trains and the layer I stimulation applied during the depolarizing current step also showed a lasting depression of responses to layer I stimulation with no lasting changes in responses to parasubicular stimulation.

CHAPTER 5

GENERAL DISCUSSION

Summary of Major Findings

This thesis utilized a number of in vitro electrophysiological techniques to investigate how the parasubiculum modulates cellular activity in layer II of the medial entorhinal cortex, and how the neurotransmitter acetylcholine and oscillatory brain rhythms at theta- and gamma-frequencies influence this cellular activity. The experiments performed have produced a number of interesting insights concerning the mechanisms governing synaptic communication in the parasubicular-entorhinal pathway.

First, it was shown that, despite a reduction in the amplitude of single EPSPs evoked in the entorhinal cortex by parasubicular stimulation in the presence of a cholinergic agonist, responses during *repetitive* stimulation at theta and gamma frequencies were enhanced (Chapter 2). This relative facilitation was due to activation of M_1, rather than M_2, muscarinic receptors, and effects were found to be due in part to reductions in the cationic current I_h, with no substantial contribution of NMDA or $GABA_B$ receptors, or the muscarinic receptor dependent current I_m. These findings suggest a role for acetylcholine and repetitive synaptic activation during theta and gamma frequency brain rhythms in facilitating synaptic communication in the parasubicular-entorhinal pathway.

The second major set of experiments (Chapter 3) indicated that the enhancements of synaptic responses to repetitive stimulation at theta and gamma frequency observed in the first set of experiments were due partly to a cholinergic-mediated decrease in I_h, which leads to increased temporal summation of synaptic responses due to an increase in input resistance and increase in the duration of EPSPs. Additional effects of cholinergic agonism suggested that facilitation of presynaptic transmitter release also played a role in the relative facilitation, but that the inward-rectifying K^+ current I_{Kir} did not interact with I_h to produce these effects. These findings demonstrated the mechanisms through which increases in acetylcholine and theta and gamma rhythms may facilitate synaptic responses during of repetitive activation patterns in the parasubicular-entorhinal pathway.

The final experimental chapter of this thesis demonstrated how oscillatory inputs at theta-rhythm from the parasubiculum to the entorhinal cortex could cause heterosynaptic changes in how the entorhinal cortex responds to sensory inputs (Chapter 4). It was found that a train of parasubicular stimulation can heterosynaptically suppress the amplitude of a subsequent entorhinal cortex response to stimulation of layer I inputs when there is a short interval of 5 ms

between the parasubicular train and the layer I stimulation pulse, an effect that was reliant on $GABA_A$ receptors. When the stimulation interval was increased to 25 ms, an I_h-dependent facilitation of entorhinal cortex responses to stimulation of layer I inputs was found, which was enhanced by I_h block. In order to test if the parasubiculum can have lasting effects on how the entorhinal cortex responds to sensory input pathways, the same stimulation patterns were repeated for a prolonged period of time at low frequency, and a lasting depression of layer I inputs to the entorhinal cortex was found. Overall, these results demonstrate that parasubicular inputs to the entorhinal cortex may have effects on short term processing of cortical inputs to the entorhinal during theta and gamma activity, as well as a lasting influence on how the entorhinal cortex responds to sensory inputs that may represent online processing of inputs to the hippocampus.

The results obtained in this thesis are consistent with a number of findings from other areas of the hippocampal formation obtained from earlier research. It has been shown previously that excitatory responses to single pulses of stimulation are suppressed following cholinergic agonism in the entorhinal cortex (Barrett and Chapman, 2013; Hamam et al., 2007; Richter et al., 1999; Yun et al., 2000), parasubiculum (Glasgow et al., 2012), striatum (Shen et al., 2007), subiculum (Kunitake et al., 2004), and hippocampus (Auerbach and Segal, 1996), but that responses to repetitive stimulation are enhanced by cholinergic agonists in the striatum (Shen et al., 2007), subiculum (Kunitake et al., 2004), and prefrontal cortex (Carr and Surmeier, 2007). This enhancement of responses to repetitive stimulation is due to increases in input resistance and widening of EPSPs that are caused by cholinergic reductions in I_h (Day et al., 2005; George et al., 2009; Magee, 1998, 1999, 2000; Rosenkranz et al., 2006; Ying et al., 2007). The similar findings in the present thesis confirm the role of I_h closure and oscillatory synaptic input at physiological-relevant frequencies in enhancing synaptic communication between hippocampal formation structures.

The present findings also support the importance of the parasubicular input to the entorhinal cortex in modulating entorhinal function (Caruana and Chapman, 2004), which could play an important role in how the entorhinal cortex processes sensory information within the hippocampal formation during mnemonic processes (Amaral, 1993; Burwell et al., 1995; Witter et al., 1989, 2000a). Overall, the results obtained from the experiments reported in this thesis suggest that cholinergic activity and associated oscillatory brain rhythms play an important role

in determining how the entorhinal cortex responds to inputs from the parasubiculum, and how parasubicular inputs can shape entorhinal cortex responses to incoming sensory inputs. This modulation of entorhinal cortex responses to sensory input by the parasubiculum could play an important role in ongoing sensory processing during spatial learning and memory processes in the hippocampal formation. Parasubicular inputs, likely influenced by ongoing hippocampal activity that reaches the parasubiculum from the CA1 region and the subiculum, therefore likely influencing how the entorhinal cortex integrates sensory inputs that are then sent to the hippocampus.

The Influence of Acetylcholine and Oscillatory Brain Activity on Synaptic Communication in the Entorhinal Cortex

The experiments in this thesis investigated the role of cholinergic activity and oscillatory brain rhythms in determining how the entorhinal cortex integrates synaptic inputs from the parasubiculum. The goal of the first two experimental chapters was to determine how cholinergic agonism and patterned synaptic stimulation at theta- and gamma-frequencies could modulate medial entorhinal cortex layer II responses to stimulation of the parasubiculum. This was accomplished first using field potential recordings from populations of entorhinal cortex neurons, and then using single cell recordings of layer II medial entorhinal cortex stellate cells.

A hallmark of neural activity during active exploration of an environment and engagement in learning and memory tasks is increased release of acetylcholine in the hippocampal formation due to activation of cholinergic basal forebrain projections (Alonso and Kohler, 1984; van Groen and Wyss, 1990a). This increase in acetylcholine levels, and the induction of rhythmic theta- and gamma-frequency oscillations that subsequently occur (Buzsaki, 2002), can help to facilitate synaptic communication between brain areas within the hippocampal formation (Colgin, 2013; Hsieh and Ranganath, 2013). The findings of the present study support this idea because, despite a reduction in the amplitude of responses to single pulses of stimulation in the entorhinal cortex following parasubicular stimulation in the presence of a cholinergic agonist, responses to repetitive trains at theta- and gamma-frequency were facilitated, with the amplitudes of responses maintained or slightly enhanced over the course of the train, rather than decreasing in amplitude with each successive response. These results were found for both field potential recordings and intracellular single cell recordings. This suggests that

oscillatory brain activity during periods of elevated acetylcholine levels, a phenomenon that is seen when an animal is actively exploring its environment and engaged in learning and memory processes (Buzsaki, 2002; Buzsaki and Draguhn, 2004; Colgin, 2015; Hasselmo et al., 2002; Mitchell and Ranck, 1980), helps to maintain the strength of synaptic communication in the parasubicular-entorhinal pathway. This could represent a type of filter mechanism that preferentially processes relevant, strong inputs that repeat at these frequencies, while filtering out arrhythmic background noise (Hsieh and Ranganath, 2013).

To investigate the mechanisms through which cholinergic receptor activation could produce the relative facilitation effect, antagonists for multiple neurotransmitter receptors and ion channels were applied prior to cholinergic agonism to see if they could block or mimic the effects of cholinergic agonism on evoked synaptic responses. The relative facilitation effect was found to rely on activation of M_1, rather than M_2, muscarinic receptors following cholinergic agonism. We also tested the effects of blocking potential downstream targets of M_1 receptor activation, including NMDA and $GABA_B$ receptors, as well as I_m and I_h currents. There was no effect on the relative facilitation effect induced by cholinergic agonism following antagonism of NMDA receptors, $GABA_B$ receptors, or I_m. However, results showed that the current I_h plays an important role in mediating the relative facilitation effect.

The hyperpolarization-activated cation channel I_h is a major factor in the generation of oscillatory activity in numerous hippocampal formation pathways, including the ones investigated in the present thesis. I_h is known to be intimately involved in the generation of theta-rhythm membrane potential oscillations in layer II medial entorhinal cortex stellate cells through a voltage-dependent interaction with the persistent Na^+ current I_{NaP} (Dickson et al., 2000b; Heys et al., 2012). I_h is also a channel involved in facilitating synaptic input in the hippocampal formation through its close association with acetylcholine (Magee, 1998, 2001; Rosenkranz and Johnston, 2006). This is likely due to the fact that closure of I_h through activation of cholinergic inputs, or through depolarization caused by repetitive synaptic input, leads to increased temporal summation of repetitive inputs through increased input resistance in dendrites and widening of EPSPs, indicating that this channel plays a critical role in integrating synaptic inputs (Day et al., 2005; George et al., 2009; Magee, 1999, 2000; Ying et al., 2007). Although it may seem counterintuitive that reductions in I_h aid in the integration of synaptic inputs, and that I_h is also involved in the generation of intrinsic theta frequency membrane potential, during the peak of

the theta oscillation, a time when the cell is most excitable, I_h is actually turned off (Dickson et al., 200b). Therefore, at the point where the I_h current is lowest during theta oscillations, the cell is actually the most excitable (Colgin, 2013). I_h is thought to be reduced following M_1 muscarinic receptor activation through either cAMP or PLC mediated pathways (Pian et al., 2006, 2007; Richter et al., 1999; Yi et al., 2010), although the exact intracellular mechanism has not been studied in layer II medial entorhinal stellate cells and would be an important question to address in future studies.

The results obtained in the current thesis when I_h was blocked were somewhat inconsistent, where, in field potential recordings, block of I_h both lead to increases in the basal amplitude of EPSPs, but also occluded the majority of the effect of cholinergic agonism on train-evoked responses when a cholinergic agonist was subsequently applied. However, when I_h was blocked in single cell recordings, there was a mimicking of the effects of cholinergic agonism, where I_h block lead to a relative facilitation of train-evoked responses. The reason for the contradiction between the two studies is likely due to the fact that membrane potential cannot be controlled in field potential recordings, and block of I_h is known to hyperpolarize the membrane (Day et al., 2005; Dickson et al., 2000b; Heys et al., 2012). When the resting membrane potential was hyperpolarized in the intracellular recordings, the relative facilitation effect was largely abolished, suggesting that I_h block in the field potential study may have hyperpolarized the membrane potential of the cell population being recorded, leading to the lack of a facilitation observed following I_h block in that study. The findings from the single cell recordings are also more in line with what is typically found in the literature where temporal summation is enhanced following I_h block (e.g. Day et al., 2005; Magee, 2000). These results confirm that frequency-dependent modulation of train-evoked responses in the parasubicular-entorhinal pathway occurs through mechanisms that involve M_1 muscarinic receptor induced closure of the cation channel I_h, while demonstrating the important role that cellular membrane properties play in these effects.

Factors other than I_h are likely to play a role in facilitating responses to repetitive synaptic inputs in the parasubicular-entorhinal pathway, however. When a cholinergic agonist was applied following I_h block in the single cell recordings, it led to further increases in relative facilitation, suggesting that cholinergic effects on presynaptic transmitter release also play a role in the observed relative facilitation. Blocking inward-rectifying K^+ channels (I_{Kir}) also had an effect of increasing temporal summation of theta- and gamma-rhythm trains independent of I_h,

although it had been shown previously that an interaction between I_h and K^+ channels could occur (Day et al., 2005). As well, the I_{NaP} channel that is also involved in theta oscillations in the medial entorhinal cortex (Dickson et sl., 2000b) has not been investigated in a paradigm similar to the one used in the present thesis. Therefore, investigation of presynaptic mechanisms of the relative facilitation effect, which could be accomplished using a picospritzer to control glutamate release onto postsynaptic cells, as well investigating other ion channels and their interaction with I_h should be studied in the future. This could help to determine if factors other than muscarinic receptor mediated closure of I_h plays a role in integrating oscillatory input following increases in cholinergic activity.

In order to determine how cholinergic agonism and I_h block led to the relative facilitation of theta- and gamma-rhythm trains, membrane potential properties were analyzed in single cell recordings following application of a cholinergic agonist or an I_h channel blocker. Following application of the cholinergic agonist, membrane voltage responses to hyperpolarizing current steps revealed that the characteristic inward sag that reflects the presence of I_h channels (Alonso and Klink, 1993) was abolished following cholinergic agonism. However, cholinergic agonism did not affect the peak somatic input resistance of the cells, an effect that we believed a likely candidate to explain the relative facilitation of train-evoked responses following cholinergic agonism, as blocking I_h is known to greatly increase input resistance and the size of EPSPs (Day et al., 2005; George et al., 2009; Magee, 1998, 1999, 2000; Rosenkranz et al., 2006; Ying et al., 2007). However, when I_h was blocked, there was both an abolition of the inward sag as well as an increase in peak input resistance. The fact that a significant change in peak input resistance was observed following block of I_h with ZD7288, but not following cholinergic agonism, is likely due to the more total block of I_h found when using an I_h antagonist. The reductions in the I_h-mediated sag in voltage responses induced by cholinergic agonism suggests that cholinergic agonism acts partly through I_h to induce a relative facilitation of train-evoked responses at theta- and gamma-frequency in the parasubicular-entorhinal pathway by increasing the input resistance of neurons and increasing the width of EPSPs.

The functional significance of these findings is that, during active exploration of an environment or during a learning and memory task, acetylcholine and oscillatory brain rhythms may help to determine which synaptic inputs are integrated by the postsynaptic cells and passed on by the parasubiculum to the medial entorhinal cortex. This could determine how the

entorhinal cortex processes incoming sensory input. For example, one can consider the parasubicular-entorhinal projection as a feedback pathway because the entorhinal cortex projects to the hippocampal region, and the activity patterns within the hippocampal region may feedback onto the entorhinal cortex via projections from the parasubiculum to the entorhinal cortex (Swanson and Cohen, 1977). During exploration of an environment, when memory encoding and retrieval processes are likely to be engaged, the theta- and-gamma rhythms could play a role in coordinating the activity of neurons involved in mnemonic processes, so the timing of inputs could determine which information in the hippocampus is functionally effective in activating the parasubiculum and subsequently the entorhinal cortex. This could influence how the entorhinal cortex then processes incoming sensory stimuli that may affect which information is passed on the hippocampus and to integrated with ongoing memory processing there.

Heterosynaptic Modulation and the Role of the Parasubiculum in the Hippocampal Formation

The final experimental chapter of this thesis looked at how parasubicular inputs to the entorhinal cortex modulate entorhinal cortex responses to stimulation of incoming sensory inputs in layer I. In order to do this, the parasubiculum was stimulated at varying intervals before stimulation of layer I inputs to the entorhinal cortex, and the effect that parasubicular stimulation had on entorhinal cortex responses to layer I stimulation was analyzed. In support of previous research from our lab using in vivo experiments, showing that stimulation of the parasubiculum can modulate responses of the entorhinal cortex to stimulation of the piriform cortex (Caruana and Chapman, 2004), it was found that parasubicular stimulation suppressed entorhinal cortex responses to layer I stimulation at a short 5 ms interval, and facilitated entorhinal cortex responses to layer I stimulation at a longer 25 ms interval. A significant facilitation effect was only observed when layer I stimulation was preceded by a *train* of stimulation at theta frequency in the parasubiculum, rather than a single parasubicular pulse, and this demonstrates the importance of repetitive oscillatory activity in modulating synaptic communication in the entorhinal cortex. The heterosynaptic suppression observed at the short 5 ms interval after parasubicular trains was found to rely upon $GABA_A$ receptors, while I_h was found to contribute to the heterosynaptic facilitation observed at the longer interval, indicating that the timing of synaptic input relative to the peak of rhythmic synaptic activation may have different effects on

the integration of sensory inputs by the postsynaptic neuron. These results indicate the importance of the parasubicular-entorhinal projection in determining how the entorhinal cortex responds to incoming sensory input, and the importance of rhythmic neuronal activity in mediating these effects.

The long-term effect of parasubicular stimulation on entorhinal cortex responses to sensory and associational cortical inputs was also investigated by repeating the pairing of parasubicular trains at theta-frequency with layer I pulses for a sustained period of time at low frequency. This procedure led to a lasting depression of entorhinal cortex responses to layer I inputs. This occurred regardless of whether or not the interval between parasubicular and layer I stimulation was the 5 ms interval, which had previously resulted in a suppression of the entorhinal response evoked after each train, or the 25 ms interval, which resulted in a facilitation of the entorhinal response evoked after each train. This effect is likely due to sustained, moderate increases in intracellular Ca^{2+} that appears to result in a lasting synaptic depression regardless of whether the short-term effect of a particular inter-stimulation interval is to suppress or facilitate layer I inputs (Deng and Lei, 2007; Johnston et al., 2003; Feldman, 2012; Sjostrom et al., 2008). This theory could be tested in the future using Ca^{2+} imaging techniques or Ca^{2+} chelators to measure directly how changes in Ca^{2+} levels relate to the observed plasticity effects. These results indicate that, in additional to short-term modulatory effects, rhythmic inputs from the parasubiculum may cause lasting alterations in how the entorhinal cortex processes sensory inputs by reducing entorhinal cortex responsiveness to sensory input pathways.

Taken together, the results from these experiments examining the interactions between the parasubiculum and layer I inputs to the entorhinal cortex indicate that the parasubiculum can have both short-term and lasting effects on how the entorhinal cortex responds to sensory input. The anatomical connections of the parasubiculum put it in a position to both influence cellular activity in the entorhinal cortex, as well as to receive input from the hippocampus and other brain areas (Caballero-Bleda and Witter, 1993; Caruana and Chapman, 2004; Ino et al., 2001; Jones and Buhl, 1993; Kerr et al., 2007; Kohler, 1985; Swanson and Cohen, 1977; van Groen and Wyss, 1990a). It has been theorized that the different projections from the entorhinal cortex to different hippocampal subfields, as well as direct sensory projections to the hippocampus that bypass the entorhinal cortex, could represent different sources of sensory input that allow the comparison of expected and actual sensory input that could influence mnemonic processing by

determining which information is relevant and which should be ignored, or to update current mnemonic representations (Colgin, 2013; Lisman and Grace, 2005; Rolls, 2013). The parasubicular-entorhinal pathway is unique in that its anatomical organization puts it in a position to act as a feedback loop beginning and ending with entorhinal cortex neurons that project to the hippocampus, with the parasubiculum likely modulating the sensitivity of the entorhinal cortex to sensory inputs in a way that is dependent on ongoing hippocampal processing. The activity of these connections therefore could reflect the ongoing processing of information in the hippocampal formation, influencing how the entorhinal cortex processes sensory inputs through synaptic inputs from the parasubiculum that contain information from the hippocampus itself.

Concluding Statement

The parasubiculum is in a unique position to influence how the entorhinal cortex, the main source of sensory input to the hippocampus, processes input from the many sensory and associational cortical areas that project to it (Caruana and Chapman, 2004). Major factors that could influence synaptic communication in the parasubicular-entorhinal pathway are increases in levels of the neurotransmitter acetylcholine and the co-occurring induction of oscillatory brain rhythms, both of which are known to have heavy influence on neuronal activity throughout the hippocampal formation (Buzsaki, 2002; Buzsaki and Watson, 2012; Hasselmo, 2002). The present thesis indicates that it is indeed the case that neural activity in the parasubicular-entorhinal pathway is modulated by acetylcholine and by electrical stimulation at the frequencies of theta and gamma rhythms, as both factors played a major role in enhancing synaptic communication in this pathway. These results indicate that the high levels of acetylcholine measured in the hippocampal formation in dialysis studies (Pepeu and Giovannini, 2004), as well as oscillatory brain rhythms observed in EEG recordings (Buzsaki, 2002; Chrobak and Buzsaki, 1998; Colgin, 2011, 2013, 2015), play a major functional role in modulating synaptic communication in the parasubicular-entorhinal pathway. Results also show that acetylcholine can enhance repetitive synaptic transmission through modulation of the cation channel I_h. It was also shown that these factors could have long-term heterosynaptic effects in which parasubicular inputs to the entorhinal cortex influence how the entorhinal cortex responds to inputs containing sensory information that arrive via layer I.

In summary, the pathway from the parasubiculum to the entorhinal cortex is a potentially critically important pathway for proper neural function in the hippocampal formation, due to the influence that the parasubiculum can have in modulating how the entorhinal cortex integrates incoming sensory projections and provides input to the hippocampus. Despite this, the factors that influence synaptic communication in this pathway have been relatively unexplored. The present thesis, therefore, sought to elucidate some of the mechanisms that affect synaptic communication in the parasubicular-entorhinal pathway. The present findings that the parasubicular-entorhinal pathway is heavily modulated by cholinergic activity and rhythmic synaptic inputs provides a strong foundation upon which future studies can investigate the cellular mechanisms and behavioural correlates of neural activity in this pathway.

REFERENCES

Abrahamsson T, Cathala L, Matsui K, Shigemoto R, DiGregorio DA (2012) Thin dendrites of cerebellar interneurons confer sublinear synaptic integration and a gradient of short-term plasticity. Neuron 73:1159-1172.

Adams JP, Dudek SM (2005) Late-phase long-term potentiation: Getting to the nucleus. Nat Rev Neurosci 6:737-743.

Alonso A, deCurtis M, Llinas R (1990) Postsynanptic hebbian and non-hebbian long-term potentiation of synaptic efficacy in the entorhinal cortex in slices and in the isolated adult guinea pig brain. P Natl Acad Sci USA 87:9280-9284.

Alonso A, Klink R (1993) Differential electroresponsiveness of stellate and pyramidal-like cells of medial entorhinal cortex layer II. J Neurophysiol 70:128-143.

Alonso A, Kohler C (1984) A study of the reciprocal connections between the septum and the entorhinal area using anterograde and retrograde axonal transport methods in the rat brain. J Comp Neurol 225:327-343.

Amaral DG (1993) Emerging principles of intrinsic hippocampal organization. Curr Opin Neurobiol 3:225-229.

Amaral DG, Witter MP (1989) The three-dimensional organization of the hippocampal formation: A review of anatomical data. Neuroscience 31:571-591.

Andersen P, Bland BH, Dudar JD (1973) Organization of the hippocampal output. Exp Brain Res 17:152-168.

Andersen P, Holmqvist B, Voorhoeve PE (1966b) Excitatory synapses on hippocampal apical dendrites activated by entorhinal stimulation. Acta Physiol Scand 66:461-472.

Arai A, Lynch G (1992) Factors regulating the magnitude of long-term potentiation induced by theta pattern stimulation. Brain Res 598:173–184.

Aramakis VB, Bandrowski AE, Ashe JH (1999) Role of muscarinic receptors, G-proteins, and intracellular messengers in muscarinic modulation of NMDA receptor-mediated synaptic transmission. Synapse 32:262–275.

Artola A, von Frijtag JC, Fermont PCJ, Gispen WH, Scharma LH, Kamal A, Spruijt BM (2006) Long-lasting modulation of the induction of LTD and LTP in rat hippocampal CA1 by behavioural stress and environmental enrichment. Eur J Neurosci 23:261-272.

Atherton JF, Kitano K, Baufreton J, Fan K, Wokosin D, Tkatch T, Shigemoto R, Surmeier DJ, Bevan MD (2010) Selective participation of somatodendritic HCN channels in inhibitory but not excitatory synaptic integration in neurons of the subthalamic nucleus. J Neurosci 30:16025–16040.

Auerbach JM, Segal M (1996) Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus. J Physiol 492:479-493.

Barrett SG, Chapman CA (2013) Contribution of muscarinic M1 receptors to the cholinergic suppression of synaptic responses in layer II of the entorhinal cortex. Neuroscience 554:11-15.

Barry C, Bush D (2012) From A to Z: A potential role for grid cells in spatial navigation. Neural Systems & Circuits 2:1-8.

Barry C, Heys JG, Hasselmo ME (2012) Possible roles of Acetylcholine in regulating spatial novelty effects on theta rhythm and grid cells. Front Neural Circuits 6:1–13.

Basar E, Basar-Eroglu C, Karakas S, Schurmann M (2000) Brain oscillations in perception and memory. Int J Psychophysiol 35:95-124.

Belluscio MA, Mizuseki K, Schmidt R, Kempter R, Buzsaki G (2012) Cross-frequency phase-phase coupling between theta and gamma oscillations in the hippocampus. J Neurosci 32:423-435.

Bender RA, Brewster A, Santoro B, Ludwig A, Hofmann F, Biel M, Baram TZ (2001) Differential and age-dependent expression of hyperpolarization-activated cyclic nucleotide-gated cationchannel isoforms 1-4 suggests evolving roles in the developing rat hippocampus. Neuroscience 106:689-698.

Bently P, Driver J, Dolan RJ (2011) Cholinergic modulation of cognition: Insights from human pharmacological functional neuroimaging. Prog Neurobio 94:360-388.

Berretta N, Jones RSG (1996) Tonic facilitation of glutamate release by presynaptic *N*-methyl-$_D$-aspartate autoreceptors in the entorhinal cortex. Neuroscience 75:339-344.

Birtoli B, Ulrich D (2004) Firing mode-dependent synaptic plasticity in rat neocortical pyramidal neurons. J Neurosci 24:4935-4940.

Bizenhofer SH, Sieben, K, Siebert KD, Spehr M, Hanganu-Opatz IL (2015) Oscillatory activity in developing prefrontal networks results from theta-gamma-modulated synaptic inputs. Cell Rep 11:486-497.

Blackstad TW (1956) Commissural connections of the hippocampal region in the rat, with special reference to their mode of termination. J Comp Neurol 105:417-537.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331-356.

Blokland A (1996) Acetylcholine: A neurotransmitter for learning and memory? Brain Res Rev 21:285-300.

Boccara CN, Sargolini F, Thoresen VH, Solstad T, Witter MP, Moser EI, Moser MB (2010) Grid cells in pre- and parasubiculum. Nat Neurosci 13:987-994.

Bouras R, Chapman CA (2003) Long-term synaptic depression in the adult entorhinal cortex in vivo. Hippocampus 13:780–790.

Brazhnik ES, Muller RU, Fox SE (2003) Muscarinic blockade slows and degrades the location-specific firing of hippocampal pyramidal cells. J Neurosci 23:611–621.

Brazhnik ES, Ramie B, Muller RU, Fox SE (2004) The effects on place cells of local scopolamine dialysis are mimicked by a mixture of two specific muscarinic antagonists. J Neurosci 24:9313–9323.

Brenowitz S, Trussel LO (2001) Minimizing synaptic depression by control of release probability. J Neurosci 21:1857–1867.

Brun VH, Otnaess MK, Molden S, Steffenach HA, Witter MP, Moser MB, Moser EI (2002) Place cells and place recognition maintained by direct entorhinal-hippocampal circuitry. Science 296:2243-2246.

Burwell RD (2000) The parahippocampal region: corticocortical connectivity. Ann N Y Acad Sci 911:25-42.

Burwell RD, Amaral DG (1998) Perirhinal and postrhinal cortices of the rat: Interconnectivity and connections with the entorhinal cortex. Journal Comp Neurol 391:293-321.

Burwell RD, Witter MP, Amaral DG (1995) Perirhinal and postrhinal cortices of the rat: a review of neuroanatomical literature and comparison with findings from the monkey brain. Hippocampus 5:390–408.

Bush PC, Sejnowski TJ (1994) Effects of inhibition and dendritic saturation in simulated neocortical pyramidal cells. J Neurophysiol 71:2183-2193.

Buzsaki G (2002) Theta oscillations in the hippocampus. Neuron 33:325-340.

Buzsaki G, Draguhn A (2004) Neuronal oscillations in cortical networks. Science, 304:1926-1929.

Buzsaki G, Moser EI (2013) Memory, navigation and theta rhythm in the hippocampal-entorhinal system. Nat Neurosci 16:130-138.

Buzsaki G, Watson BO (2012) Brain rhythms and neural syntax: Implications for efficient coding of cognitive content and neuropsychiatric disease. Dialogues Clin Neurosci 14:345-367.

Bzelot M, Bocchio M, Kasugai Y, Fischer D, Dodson PD, Ferraguti F, Capogna M (2015) Hippocampal theta input to the amygdala shapes feedforward inhibition to gate heterosynaptic plasticity. Neuron 87:1290-1303.

Caballero-Bleda M, Witter MP (1993) Regional and laminar organization of projections from the presubiculum and parasubiculum to the entorhinal cortex: an anterograde tracing study in the rat. J Comp Neurol 328:115-129.

Caballero-Bleda M, Witter M.P (1994) Projections from the presubiculum and the parasubiculum to morphologically characterized entorhinal-hippocampal projection neurons in the rat. Exp Brain Res 101:93-108.

Cacucci F, Lever C, Wills TJ, Burgess N, O'Keefe J (2004) Theta- modulated place-by-direction cells in the hippocampal formation in the rat. J Neurosci 24:8265–8277.

Canolty RT, Knight RT (2010) The functional role of cross-frequency coupling. Trends Cogn Sci 14:506-515.

Canto CB, Koganezawa N, Breed P, Moser EI, Witter MP (2012) All layers of medial entorhinal cortex receive presubicular and parasubicular inputs. J Neurosci 32:17620-17631.

Canto CB, Witter MP (2012) Cellular properties of principal neurons in the rat entorhinal cortex. II. The medial entorhinal cortex. Hippocampus 22:1277-1299.

Canto CB, Wouterlood FG, Witter MP (2008). What does the anatomical organization of the entorhinal cortex tell us? Neural Plast 2008:1-18.

Carr DB, Andrews GD, William BG, Lavin A (2007) a2-Noradrenergic receptors activation enhances excitability and synaptic integration in rat prefrontal cortex pyramidal neurons via inhibition of HCN currents. J physiol 584:437–450.

Carr DB, Surmeier DJ (2007) M1 muscarinic receptor modulation of Kir2 channels enhances temporal summation of excitatory synaptic potentials in prefrontal cortex pyramidal neurons. J Neurophysiol 97:3432-3438.

Caruana DA, Chapman CA (2004). Stimulation of the parasubiculum modulates entorhinal cortex responses to piriform cortex inputs in vivo. J Neurophysiol 92:1226-1235.

Castro-Alamancos MA, Connors BW (1996) Cellular mechanisms of the augmenting response: short-term plasticity in a thalamacortical pathway. J Neurosci 16:7742–7756.

Caulfield MP, Birdsall NJM (1998) International union of pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Am Soc Pharmacol Exp Ther 50:279–290.

Cenquizca LA, Swanson LW (2007) Spatial organization of direct hippocampal field CA1 axonal projections to the rest of the cerebral cortex. Brain Res Rev 56:1-26.

Chapman CA, Lacaille JC (1999) Cholinergic induction of theta- frequency oscillations in hippocampal inhibitory interneurons and pacing of pyramidal cell firing. J Neurosci 19:8637–8645.

Chapman CA, Racine RJ (1997) Converging inputs to the entorhinal cortex from the piriform cortex and medial septum: Facilitation and current source density analysis. J Neurophysiol 78:2602–2615.

Cheong MI, Yun SH, Mook-Jung I, Joo I, Huh K. Jung MW (2001) Cholinergic modulation of synaptic physiology in deep layer entorhinal cortex of the rat. J Neurosci Res 66:117-121.

Chevaleyre V, Castillo PE (2002) Assessing the role of Ih channels in synaptic transmission and mossy fiber LTP. PNAS 99:9538–9543.

Chistiakova M, Bannon NM, Bazhenov M, Volgushev M (2014) Heterosynaptic plasticity: Multiple mechanisms and multiple roles. Neuroscientist 20:483-498.

Chistiakova M, Volgushev M (2009) Heterosynaptic plasticity in the neocortex. Exp Brain Res 199:377-390.

Cho YH, Kesner RP (1996) Involvement of entorhinal cortex or parietal cortex in long-term discrimination memory in rats: Retrograde amnesia. Behav Neurosci 110:436-442.

Chrobak JJ, Buzsaki G (1998) gamma oscillations in the entorhinal cortex of the freely behaving rat. J Neurosci 18: 388-398.

Colgin LL (2011) Oscillations and hippocampal-prefrontal synchrony. Curr Opin Neurobiol 21:467-474.

Colgin LL (2013) Mechanisms and functions of theta rhythms. Annu Rev Neurosci 36:295-312.

Colgin LL (2015) Theta-gamma coupling in the entorhinal-hippocampal system. Curr Opin Neurobiol 31:45-50.

Colgin LL, Denniger T, Fyhn M, Hafting T, Bonnevie T, Jensen O, Moser MB, Moser EI (2009) Frequency of gamma oscillations routes flow of information in the hippocampus. Nature 462: 353-358.

Craig MT, McBain CJ (2015) Navigating the circuitry of the brain's GPS system: Future challenges for neurophysiologists. Hippocampus 25:736-743.

Dasari S, Gulledge AT (2011) M1 and M4 receptors modulate hippocampal pyramidal neurons. J Neurophysiol 105:779-792.

Davies CH, Collingridge GL (1996) Regulation of EPSPs by the synaptic activation of GABAB autoreceptors in rat hippocampus. J Physiol 496:451–470.

Day M, Carr DB, Ulrich S, Ilijic E, Tkatch T, Surmeier DJ (2005) Dendritic excitability of mouse frontal cortex pyramidal neurons is shaped by the interaction among HCN, Kir2, and Kleak channels. J Neurosci 25:8776-8787.

Deng PY, Lei S. (2007) Long-term depression in identified stellate neurons of juvenile rat entorhinal cortex. J Neurophysiol, 97:727-737.

de Curtis M, Pare D (2004) The rhinal cortices: a wall of inhibition between the neocortex and the hippocampus. Prog Neurobiol 74:101–110.

Deisz RA, Prince DA (1989) Frequency-dependent depression of inhibition in guinea-pig neocortex *in vitro* by $GABA_B$ receptor feed-back on GABA release. J Physiol 412:513-541.

Dickson CT, Biella G, de Curtis M (2000a) Evidence for spatial modules mediated by temporal synchronization of carbachol-induced gamma rhythm in medial entorhinal cortex. J Neurosci 20:7846-7854.

Dickson CT, de Curtis M (2002) Enhancement of temporal and spatial synchronization of entorhinal gamma activity by phase reset. Hippocampus 12:447–456.

Dickson CT, Magistretti J, Shalinsky MH, Fransen E, Hasselmo ME, Alonso A (2000b) Properties and role of Ih in pacing of subthreshold oscillations in entorhinal cortex layer II neurons. J Neurophys 83:2562-2579.

Douchamps V, Jeewajee A, Blundell P, Burgess N, Lever C. (2013) Evidence for encoding versus retrieval scheduling in the hippocampus by theta phase and acetylcholine. J Neurosci, 33:8689-8704.

Douglas RJ (1967). The hippocampus and behavior. Psychol Bull 67:416-422.

Doyere V, Errington ML, Laroche S, Bliss TVP (1996) Low-frequency trains of paired stimuli induce long-term depression in area CA1 but not dentate gyrus of the intact rat. Hippocampus 6:52-57.

Drever BD, Riedel G, Platt R. (2011) The cholinergic system and hippocampal plasticity. Behav Brain Res 221:505-514.

Dutar P, Nicoll RA (1988) Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: Electrophysiological studies in vitro. J Neurosci 8:4214-4224.

Duzel E, Penny WD, Burgess N (2010) Brain oscillations and memory. Curr Opin Neurobiol 20:143-149.

Easton A, Douchamps V, Eacott M, Lever C (2013) A specific role for septohippocampal acetylcholine in memory? Neuropsychologica 50:3156-3168.

Economo MN, Martínez JJ, White JA (2014) Membrane potential-dependent integration of synaptic inputs in entorhinal stellate neurons. Hippocampus 24:1493-505.

Eichenbaum H, Sauvage M, Fortin N, Komorowski R, Lipton P (2012) Towards a functional organization of episodic memory in the medial temporal lobe. Neurosci Biobehav Rev 36:1597–1608.

Feldman DE (2012) The spike timing dependence of plasticity. Neuron 75: 556-571.

Fernandez FR, Malebra P, Bressloff PC, White JA (2013) Entorhinal stellate cells show preferred spike phase-locking to theta inputs that is enhanced by correlations in synaptic activity. J Neurosc 33:6027-6040.

Fiashn A, Yamada M, Duttaroy A, Gan JW, Deng CX, McBain CJ, Wess J (2002) Muscarinic induction of hippocampal gamma oscillations requires coupling of the M1 receptor to two mixed cation currents. Neuron 33:615-624.

Forster E, Zhao S, Frotscher M (2006) Laminating the hippocampus. Nat Rev Neurosci 7:259-267.

Funahashi M, Stewart M (1997a) Presubicular and parasubicular cortical neurons of the rat: electrophysiological and morphological properties. Hippocampus 7:117-129.

Funahashi M, Stewart M (1997b) Presubicular and parasubicular cortical neurons of the rat: functional separation of deep and superficial neurons in vitro. J Physiol 501:387-403.

Funahashi M, Stewart M (1998) GABA receptor-mediated post- synaptic potentials in the retrohippocampal cortices: regional, laminar and cellular comparisons. Brain Res 787:19–33.

Furtak SC, Wei SM, Agster KL, Burweel RD (2007) Functional neuroanatomy of the parahippocampal region in the rat: The perirhinal and postrhinal cortices. Hippocampus 17:709-722.

Fyhn M, Molden S, Witter MP, Moser EI, Moser MB (2004) Spatial representation in the entorhinal cortex. Science 305:1258-1264.

Garden DLF, Dodson PD, O'Donnel C, White MD, Nolan MF (2008) Tuning of synaptic integration in the medial entorhinal cortex to the organization of grid cell firing fields. Neuron 60:875–889.

Gaykema RPA, Luiten PGM, Nyakas C, Traber J (1990) Cortical projection patterns of the medial septum–diagonal band complex. J Comp Neurol 293:103–124.

George MS, Abbott LF, Siegelbaum SA (2009) HCN hyperpolarization-activated cation channels inhibit EPSPs by interactions with M-type K+ channels. Nat Neurosci 12:577-584.

Giocomo LM, Zilli EA, Fransen E, Hasselmo ME (2007) Temporal frequency of subthreshold oscillations scales with entorhinal grid cell field spacing. Science 315:1719–1722.

Glasgow SD, Chapman CA (2007) Local generation of theta-frequency EEG activity in the parasubiculum. J Neurophysiol 97:3868-3879.

Glasgow SD, Chapman CA (2008) Conductances mediating intrinsic theta-frequency membrane potential oscillations in layer II parasubicular neurons. J Neurophysiol 100:2746-2756.

Glasgow SD, Chapman CA (2013) Muscarinic depolarization of layer II neurons of the parasubiculum. PLoS One, 8, 1-14.

Glasgow SD, Glovaci I, Karpowicz LS, Chapman CA (2012) Cholinergic suppression of excitatory synaptic transmission in layers II/III of the parasubiculum. Neuroscience 201:1-11.

Gloveli T, Egorov AV, Schmitz D, Heinemann U, Muller W (1999) Carbachol-induced changes in excitability and [Ca2+]i signaling in projection cells of medial entorhinal cortex layers II and III. Eur J Neurosci 11:3626–3636.

Good M (2002) Spatial memory and hippocampal function: Where are we now? Psicologica 23:109-138.

Grover LM, Kim E, Cooke JD, Holmes WR (2009) LTP in hippocampal area CA1 is induced by burst stimulation over a broad frequency range centered around delta. Learn Mem 16:69-81.

Grover LM, Yan C (1999) Blockade of $GABA_A$ receptors facilitates induction of NMDA receptor-independent long-term potentiation. J Neurophysiol 81:2814-2822.

Haas JS, White JA (2002) Frequency selectivity of layer II stellate cells in medial entorhinal cortex. J Neurophysiol 88:2422–2429.

Habib D, Tsui CKY, Rosen LG, Dringenberg HC (2013) Occlusion of low-frequency-induced, heterosynaptic long-term potentiation in the rat hippocampus in vivo following spatial training. Cereb Cortex 24:3090-3096.

Hafting T, Fyhn M, Bonnevie T, Moser MB, Moser EI (2008) Hippocampus-independent phase procession in entorhinal grid cells. Nature Lett 453:1248-1253.

Hamam BN, Sinai M, Poirier G, Chapman CA (2007) Cholinergic suppression of excitatory synaptic responses in layer II of the medial entorhinal cortex. Hippocampus 17:103-113.

Han EB, Heinemann SF (2013) Distal dendritic inputs control neuronal activity by heterosynaptic potentiation of proximal inputs. J Neurosci 33:1314-1325.

Hargreaves EL, Yoganarasimha D, Knierim JJ (2007) Cohesiveness of spatial and directional representations recorded from neural ensembles in the anterior thalamus, parasubiculum, medial entorhinal cortex, and hippocampus. Hippocampus 1: 826-841.

Hasselmo ME (2006) The role of acetylcholine in learning and memory. Curr Opin Neurobio 16: 710-715.

Hasselmo ME (2008) Grid cell mechanisms and function: contributions of entorhinal persistent spiking and phase resetting. Hippocampus 18:1213–1229.

Hasselmo ME, Brandon MP (2012) A model combining oscillations and attractor dynamics for generation of grid cell firing. Front Neural Circuits 6:1–13.

Hasselmo ME, Giocomo LM, Zilli EA (2007) Grid cell firing may arise from interference of theta frequency membrane potential oscillations in single neurons. Hippocampus 17:1252–1271.

Hasselmo ME, Hay J, Ilyn M, Gorchetchnikov A (2002) Neuromodulation, theta rhythm and rat spatial navigation. Neural Netw 15:689-707.

Hasselmo ME, Sarter M (2011) Modes and models of forebrain cholinergic neuromodulation of cognition. Neuropyschopharmacology 36:52-73.

Hasselmo ME, Stern CE (2014) Theta rhythm and the encoding and retrieval of space and time. Neuroimage 85:656-666.

Hebb DO (1949) The Organization of Behavior. New York: Wiley & Sons.

Heys JG, Giocomo LM, Hasselmo ME (2010) Cholinergic modulation of the resonance properties of stellate cells in layer II of medial entorhinal cortex. J Neurophysiol 104:258-270.

Heys JG, Schultheiss NW, Shay CF, Tsuno Y, Hasselmo ME (2012) Effects of acetylcholine on neuronal properties in entorhinal cortex. Front Behav Neurosci 6:1-19.

Hioki H, Okamoto S, Konno M, Kameda H, Sohn J, Kuramoto E, Fujiyama F, Kaneko T (2013) Cell type-specific inhibitory inputs to dendritic and somatic compartments of parvalbumin-expressing neocortical interneuron. J Neurosci 33:544-555.

Hoffman DA, Magee JC, Colbert CM, Johnston D (1997) K+ channel regulation of signal propogation in dendrites of hippocampal pyramidal neurons. Nature 387:869-875.

Hoffman DA, Sprengel R, Sakmann B (2002) Molecular dissection of hippocampal theta-burst pairing potentiation. PNAS 99:7740-7745.

Hsieh LT, Ranganath C (2013) Frontal midline theta oscillations during working memory maintenance and episodic encoding and retrieval. Neuroimage 85:721-729.

Hu H, Martina M, Jonas P (2010) Dendritic mechanisms underlying rapid synaptic activation of fast-spiking hippocampal interneurons. Science 327:52-58.

Huang YY, Pittenger C, Kandel ER (2004) A form of long-lasting, learning-related synaptic plasticity in the hippocampus induced by heterosynaptic low-frequency pairing. PNAS, 101:859-864.

Huang Z, Lujan R, Kadurin I, Uebele VN, Renger JJ, Dolphin AC, Shah MM (2011) Presynaptic HCN1 channels regulate Cav3.2 activity and neurotransmission at select cortical synapses. Nat Neurosci 14:478–486.

Hulme SR, Jones OD, Raymond CR, Sah P, Abraham WC (2013) Mechanisms of heterosynaptic metaplasticity. Phil Trans Royal Soc B. 369:1-8.

Igarashi KM, Lu L, Colgin LL, Moser MB, Moser EI (2014) Coordination of entorhinal-hippocampal ensemble activity during associative learning. Nature: 510:143-147.

Ino T, Kaneko T, Mizuno N (2001) Projections from the hippocampal and parahippocampa; regions to the entorhinal cortex. An anterograde and retrograde tract-tracing study in the cat. Neuroscience Research 39:51-69.

Insausti R, Trinidad Herroero M, Witter MP (1997) Entorhinal cortex of the rat: Cytoarchitectonic subdivisions and the origin and distribution of cortical efferents. Hippocampus 7:146-183.

Ito M, Kano M (1992) Long-lasting depression of parallel fiber-Purkinje cell transmission by conjunctive stimulation of parallel fibers and climing fibers in the cerebellar cortex. Neurosci Lett 33:253-258.

Jerusalinsky D, Kornisiuk E, Izquierdo I (1997) Cholinergic neurotransmission and synaptic plasticity concerning memory processing. Neurochem Res 22:507-515.

Jarrard LE, Davidson TL, Bowring B (2004) Functional differentiation within the medial temporal lobe in the rat. Hippocampus 14:434-449.

Jezek K, Henriksen EJ, Treves A, Moser EI, Moser MB (2011). Theta-paced flickering between place-cell maps in the hippocampus. Nature 478:246-251.

Johnston D, Christie BR, Frick A, Gray R, Hoffman DA, Schexnayder LK, Watanabe S, Yuan LL (2003) Active dendrites, potassium channels and synaptic plasticity. Phil Trans R Soc Lond B 358:667-674.

Jones RSG (1990) Synaptic responses of neurons in layer II of the rat medial entorhinal cortex to stimulation of the para-subiculum in vitro. Proc Physiol Soc 426:48P.

Jones RSG, Buhl EH (1993) Basket-like interneurons in layer II of the entorhinal cortex exhibit a powerful NMDA-mediated synaptic excitation. Neurosci Lett 149:35-39.

Judge SJ, Hasselmo ME (2004) theta rhythmic stimulation of stratum lacunosum-moleculare in rat hippocampus contributes to associative LTP at a phase offset in stratum radiatum. J Neurophysiol 92:1615-1624.

Kawasaki H, Palmieri C, Avoli M (1999) Muscarinic receptor activation induces depolarizing plateau potentials in bursting neurons of the rat subiculum. J Neurophysiol 82:2590-2601.

Kerr KM, Agster KL, Furtak SC, Burwell RD (2007) Functional neuroanatomy of the parahippocampal region: the lateral and medial entorhinal areas. Hippocampus 17:697-708.

Kesner R. (1973) A neural system analysis of memory storage and retrieval. Psychol Bull 80:177-203.

Kesner RP, Giles R (1998) Neural circuit analysis of spatial working memory: Role of pre- and parasubiculum, medial and lateral entorhinal cortex. Hippocampus 8:416-423.

Khakpai F, Nasehi M, Haeri-Rohani A, Eidi A, Zarrindast MR (2013) Septo-hippocampal-septal loop and memory formation. Basic Clin Neurosc 4:5-23.

Khateb A, Fort P, Williams S, Serafin M, Jones BE, Muhlethaler M (1997) Modulation of cholinergic nucleus basalis neurons by acetylcholine and N-methyl-D-aspartate. Neuroscience 8:47–55.

Kitamura T, Pignatelli M, Suh J, Kohara K, Yoshiki A, Kuniya A, Tonegawa S (2014) Island cells control temporal association memory. Science 343:896-901.

Klink R, Alonso A (1993) Ionic mechanisms for the subthreshold oscillations and differential electroresponsiveness of medial entorhinal cortex layer II neurons. J Neurophysiol 70:144-157.

Klink R, Alonso A (1997a) Ionic mechanisms of muscarinic depolarization in entorhinal cortex layer II neurons. J Neurophysiol 77:1829-1843.

Klink R, Alonso A (1997b) Muscarinic modulation of the oscillatory and repetitive firing properties of entorhinal cortex layer II neurons. J Neurophysiol 77:1813-1828.

Knierim JJ (2015) From the GPS to HM: Place cells, grid cells, and memory. Hippocampus 25:719-725.

Knierim JJ, Lee I, Hargreaves EL (2006) Hippocampal place cells: parallel input streams, subregional processing, and implications for episodic memory. Hippocampus 16:755–764.

Knierim JJ, Neunuebel JP, Deshmukh SS (2013) Functional correlates of the lateral and medial entorhinal cortex: Objects, path integration and local-global references frames. Phil Trans Roy Roc Bio 369:20130369

Kohler C (1985) Intrinsic projections of the retrohippocampal region in the rat brain. I. The subicular complex. J Comp Neurol 236:504-522.

Kohler C (1986) Intrinsic connections of the retrohippocampal region in the rat brain. II. The medial entorhinal area. J Comp Neurol 246:149-169.

Kourrich S, Chapman CA (2003) NMDA receptor-dependent long-term synaptic depression in the entorhinal cortex in vitro. J Neurophysiol 89:2112-2119.

Kourrich S, Glasgow SD, Caruana DA, Chapman CA (2008) Postsynaptic signals mediating induction of long-term synaptic depression in the entorhinal cortex. Neural Plast 2008:1-9.

Kremin T, Gerber D, Giocomo LM, Huang SY, Tonegawa S, Hasselmo ME (2006) Muscarinic suppression in stratum radiatum of CA1 shows dependence on presynaptic M1 receptors and is not dependent on effects at GABAB receptors. Neurobiol Learn Mem 85:153–163.

Kunitake A, Kunitake T, Stewart M (2004) Differential modulation by carbachol of four separate excitatory afferent systems to the rat subiculum in vitro. Hippocampus 14:986-999.

Larson J, Munkacsy E (2014) Theta-burst LTP. Brain Res 1621:38-50

Lasztoczi B, Klausberger T (2014) Layer-specific GABAergic control of distinct gamma oscillations in the CA1 hippocampus. Neuron 81:1126-1139.

Lingenhohl K, Finch DM (1991) Morphological characterization of rat entorhinal neurons in vivo: Soma-dendritic structure and axonal domains. Exp Brain Res 84:57-74.

Levy WB, Steward O (1983) Temporal contiguity requirements for long-term associative potentiation/depression in the hippocampus. Neuroscience 8:791-797.

Lisman JE, Grace AA (2005) The hippocampal-VTA loop: Controlling the entry of information into long-term memory. Neuron 46:703-713.

Lisman JE, Jensen O (2013) The theta-gamma neural code. Neuron 77:1002-1016.

Liu P, Jarrard LE, Bilkey DK (2001) Excitotoxic lesions of the pre- and parasubiculum disrupt object recognition and spatial memory processes. Behav Neurosci 115:112-124.

Liu P, Jarrard LE, Bilkey DK (2004) Excitotoxic lesions of the pre- and parasubiculum disrupt the place fields of hippocampal pyramidal cells. Hippocampus 14:107-116.

Liu S, Shipley MT (2008) Intrinsic conductances actively shape excitatory and inhibitory postsynaptic responses in olfactory bulb external tufted cells. J Neurosci 28:10311–10322.

Lopes da Silva FH, Witter MP, Boeijinga PH, Lohman AHM (1990) Anatomic organization of physiology of the limbic cortex. Physiol Rev 70:453-511.

Luthi A, McCormick DA (1998) H-current: Properties of a neuronal and network pacemaker. Neuron 21:9-12.

Magee JC (1998) Dendritic hyperpolarization-activated currents modify the integrative properties of hippocampal CA1 pyramidal neurons. J Neurosci 18:7613-7624.

Magee JC (1999) Dendritic Ih normalizes temporal summation in hippocampal CA1 neurons. Nat Neurosci 2:508-514.

Magee JC (2000) Dendritic integration of excitatory synaptic input. Nat Rev Neurosci 1:181-190.

Magee JC (2001) Dendritic mechanisms of phase precession in hippocampal CA1 pyramidal neurons. J Neurophysiol 86:528-532.

Magistretti J, Ragsdale DS, Alonso A (1999) Direct demonstration of persistent Na+ channel activity in dendritic processes of mammalian cortical neurones. J Physiol 521:629-636.

Malenka RC, Bear MF (2004) LTP and LTD: An embarrassment of riches. Neuron 44:5-21.

Malenka RC, Nicoll RA (1993) NMDA-receptor-dependent synaptic plasticity: Multiple forms and mechanisms. TINS 16:521-527.

Markram H, Lubke J, Fortscher M, Sakmann B (1997) Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. Science 275:213-215.

McHugh TJ, Blum KI, Tsien JZ, Tonegawa S, Wilson MA (1996). Impaired hippocampal representation of space in CA1-specific NMDAR1 knockout mice. Cell 87:1339-1349.

McQuiston AR (2010) Cholinergic modulation of excitatory synaptic input integration in hippocampal CA1. J Physiol 588:3727-3742.

Medinilla V, Johnson O, Gasparini S (2013) Features of proximal and distal excitatory synaptic inputs to layer V neurons of the rat medial entorhinal cortex. J Physiol 591:169-183.

Metherate R, Ashe JH (1994) Facilitation of an NMDA receptor-mediated EPSP by paired-pulse stimulation in rat neocortex via depression of GABAergic IPSPs. J Physiol 481:331-348.

Meuth SG, Kanyshkova T, Meuth P, Landgraf P, Munsch T, Ludwig A, Hofmann F, Pape H, Budde T (2006) Membrane resting potential of thalamocortical relay neurons is shaped by the interaction among TASK3 and HCN2 channels. J Neurophys 96:1517–1529.

Mitchell SJ, Ranck JB (1980) Generation of theta rhythm in medial entorhinal cortex of freely moving rats. Brain Res 189:49-66.

Mitchell SJ, Rawlins JNP, Steward O, Olton DS (1982) Medial septal area lesions disrupt Θ rhythm and cholinergic staining in medial entorhinal cortex and produce impaired radial arm maze behavior in rats. J Neurosci 2:292-302.

Monteggia LM, Eisch AJ, Tang MD, Kaczmarek LK, Nestler EJ (2000). Cloning and localization of the hyperpolarization-activated cyclic nueclotide-gated channel family in rat brain. Molec Brain Res 81:129-139.

Morris NP, Fyffe REW, Robertson B (2004) Characterisation of hyperpolarization-activated current (Ih) in the medial septum/diagonal band complex in the mouse. Brain Res 1006:74-86.

Morris RGM, Anderson E, Lynch GS, Baudry M (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature 319:774-776.

Morris RGM, Garrud P, Rawlins JNP, O'Keefe J (1982) Place navigation impaired in rats with hippocampal lesions. Nature 297:681-683.

Moser EI, Kropff E, Moser MB (2008) Place cells, grid cells, and the brain's spatial representation system. Annu Rev Neurosci 31:69-89.

Moser MB, Rowland DC, Moser EI (2015) Place cells, grid cells, and memory. Cold Spring Harb Perspect Biol 7:1-15.

Mouly AM, Di Scala G (2006) Entorhinal cortex stimulation modulates amygdala and piriform cortex responses to olfactory bulb inputs in the rat. Neuroscience 137:1131-1141.

Naber PA, Witter MP, Lopez da Silva FH (1999) Perirhinal cortex input to the hippocampus in the rat: Evidence for parallel pathways, both direct and indirect. A combined physiological and anatomical study. Eur J Neurosci 11:4119-4133.

Naber PA, Witter MP, Lopes da Silva FH (2001) Evidence for a direct projection from the postrhinal cortex to the subiculum in the rat. Hippocampus 11:105-117.

Nevian T, Sakmann B (2004) Single spine Ca2+ signals evoked by coincident EPSPs and backpropogating action potentials in spiny stellate cells of layer 4 in the juvenile rat somatosensory cortex. J Neurosci 24:1689-1699.

Newman EL, Gillet SN, Climer JR, Hasselmo ME (2013) Cholinergic blockade reduces theta-gamma phase amplitude coupling and speed modulation of theta frequency consistent with behavioral effects of encoding. J Neurosci 33:19635-19646.

Nolan MF, Dudman JT, Dodson PD, Santoro B (2007) HCN1 channels control resting and active integrative properties of stellate cells from layer II of the entorhinal cortex. J Neurosci 27:12440-12451.

Nyhus E, Curran T (2010) Functional role of gamma and theta oscillations in episodic memory. Neurosci Biobehav Rev 34:1023-1035.

O'Keefe J, Nadel L (1978) The hippocampus as a cognitive map. Oxford University Press, London.

O'Keefe J, Recce ML (1993) Phase relationship between hippocampal place units and the EEG theta rhythm. Hippocampus 3:317–330.

O'Reilly KC, Alarcon JM, Ferbinteanu J (2014) Relative contributions of CA3 and medial entorhinal cortex to memory in rats. Front Behav Neurosci 8:1-17.

Palmer LM, Stuart GJ (2009) Membrane potential changes in dendritic spines during action potentials and synaptic input. J Neurosci 29:6897–6903.

Parron C, Poucet B, Save E (2006). Cooperation between the hippocampus and the entorhinal cortex in spatial memory: A disconnection study. Behav Brain Res 170:99-109.

Paxinos G, Watson C (1998) The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press Inc.

Penley SC, Hinman JR, Sabolek HR, Escabi MA, Markus EJ, Chrobak JJ (2012) Theta and gamma coherence across the septotemporal axis during distinct behavioral states. Hippocampus 22:1164-1175.

Pepeu G, Giovannini MG (2004) Changes in acetylcholine extracellular levels during cognitive processes. Learn Mem 11:21-27.

Pian P, Bucchi A, DeCostanzo A, Robinson RB, Siegelbaum SA (2007) Modulation of cyclic nucleotide-regulated HCN channels by PIP2 and receptors coupled to phospholipase C. Eur J Physiol 455:125-145.

Pian P, Bucchi A, Robinson RB, Siegelbaum SA (2006) Regulation of getting and rundown of HCN hyperpolarization-activated channels by exogenous and endogenous PIP_2. J Gen Physiol 128:593-604.

Picciotto MR, Higley MJ, Mineur YS (2012) Acetylcholine as a neuromodulator: Cholinergic signaling shapes nervous system function and behavior. Neuron 76:116-129.

Quilichini P, Sirota A, Buzsaki G (2010) Intrinsic circuit organization and theta-gamma oscillation dynamics in the entorhinal cortex of the rat. J Neurosci 30:11128-11142.

Raisman G, Cowan WM, Powell TP (1966) An experimental analysis of the efferent projection of the hippocampus. Brain 89:83-108.

Ray S, Naumann R, Burgalossi A, Tang Q, Schmidt H, Brecht M (2014) Grid-layout and thea-modulation of layer 2 pyramidal neurons in medial entorhinal cortex. Science 343:891-896.

Rempel-Clower NL, Zola SM, Squire LR, Amaral DG (1996) Three cases of enduring memory impairment after bilateral damage limited to the hippocampal formation. J Neurosci 16:5233-5255.

Richter M, Schilling T, Muller W (1999) Muscarinic control of intracortical connections to layer II in rat entorhinal cortex slice. Neurosci Lett 273:200-202.

Rolls ET (2013) The mechanisms for pattern completion and pattern separation in the hippocampus. Front Syst Neurosci 7:1-21.

Room P, Groenewegen HJ (1986) Connections of the parahippocampal cortex. I. Cortical afferents. J Comp Neurol 251:415-450.

Rosenkranz JA, Johnston D (2006) Dopaminergic regulation of neuronal excitability through modulation of Ih in layer V entorhinal cortex. J Neurosci 26:3229-3244.

Sanchez-Alonso JL, Halliwell JV, Colino A (2008) ZD 7288 inhibits T- type calcium current in rat hippocampal pyramidal cells. Neurosci Lett 439:275–280.

Santoro B, Chen S, Luthi A, Pavlidis P, Shumyatsky GP, Tibbs GR, Siegelbaum SA (2000). Molecular and functional heterogeneity of hyperpolarization-activated pacemaker channels in the mouse CNS. J Neurosci 20:5264-5275.

Saudargiene A, Cobb S, Graham BP (2015) A computational study on plasticity during theta cycles at schaffer collateral synapses on CA1 pyramidal cells in the hippocampus. Hippocampus 25:208-218.

Scharfman HE, Goodman JH, Du F, Schwarcz R (1998) Chronic changes in synaptic responses of entorhinal and hippocampal neurons after amino-oxyacetic acid (AOAA) –induced entorhinal cortical neuron loss. J Neurophysiol 80:3031–3046.

Schenk F, Morris RGM (1985) Dissociation between components of spatial memory in rats after recovery from the effects of retrohippocampal lesions. Exp Brain Res 58:11-28.

Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. J Neurol Neurosurg Psychiatry 20:11-21.

Shalinsky MH, Magistretti J, Ma L, Alonso AA (2002) Muscarinic activation of a cation current and associated current noise in entorhinal-cortex layer-II neurons. J Neurophysiol 88:1197–1211.

Shatz CJ (1992) The developing brain. Sci Am 267:60-67.

Shen W, Tian X, Day M, Ulrich S, Tkatch T, Nathanson NM, Surmeier, DJ (2007) Cholinergic modulation of Kir2 channels selectively elevates dendritic excitability in striatopallidal neurons. Nat Neurosci 11:1458-1466.

Shors TJ, Matzel LD (1997) Long-term potentiation: What's learning got to do with it? Behav Brain Sci 20:597-655.

Sjostrom PJ, Rancz EA, Roth A, Hausser M (2008) Dendritic excitability and synaptic plasticity. Physiol Rev 88:769-840.

Solger J, Wozny C, Manahan-Vaughan D, Behr J (2004) Distinct mechanisms of bidirectional activity-dependent synaptic plasticity in superficial and deep layers of rat entorhinal cortex. Eur J Neurosci 19:2003-2007.

Sorensen KE, Shipley MT (1979) Projections from the subiculum to the deep layers of the ipsilateral presubicular and entorhinal cortices in the guinea pig. J Comp Neurol 188:313-333.

Sparks DW, Chapman CA (2013) Cholinergic receptor activation induces a relative facilitation of synaptic responses in the entorhinal cortex during theta- and gamma-frequency stimulation of parasubicular inputs. Neuroscience 230:72-85.

Sparks DW, Chapman CA (2014) Contribution of I_h to the relative facilitation of synaptic responses induced by carbachol in the entorhinal cortex during repetitive stimulation of the parasubiculum. Neuroscience 278:81-92.

Spruston N (2008) Pyramidal neurons: Dendritic structure and synaptic integration. Nat Rev Neurosci 9:206-221.

Squire LR, Stark CEL, Clark RE (2004) The medial temporal lobe. Annu. Rev. Neurosci 27:279-306.

Squire LR, Zola-Morgan S (1991) The medial temporal lobe memory system. Science 253:1380-1386.

Staubli U, Otaky N (1994) Serotonin controls the magnitude of LTP induced by theta bursts via an action on NMDA-receptor- mediated responses. Brain Res 643:10–16.

Staubli U, Scafidi J, Chun D (1999) GABAB receptor antagonism: Facilitatory effects on memory parallels those on LTP induced by TBS but not HFS. J Neurosci 19:4609–4615.

Steffenach HA, Witter M, Moser MB, Moser EI (2005) Spatial memory in the rat requires the dorsolateral band of the entorhinal cortex. Neuron 45:301-313.

Sun C, Kitamura T, Yamamoto J, Martin J, Pignatelli M, Kitch LJ, Schnitzer MK, Tonegawa S (2015) Distinct speed dependence of entorhinal island and ocean cells, including respective grid cells. PNAS, 112:9466-9471.

Swanson LW, Cowan WM (1977) An autoradiographic study of the organization of the efferent connections of the hippocampal formation in the rat. J Comp Neurol 172:49084.

Takehara-Nishiuchi K (2014) Entorhinal cortex and consolidated memory. Neurosci Res 84:27-33.

Taube JS (1995) Place cells recorded in the parasubiculum of freely moving rats. Hippocampus 5:569–583.

Thompson RF (1976) The search for the engram. Am Psychol 31:209-227.

Thuault SJ, Malleret G, Constantinople CM, Nicholls R, Chen I, Zhu J, Panteleyev A, Vronskaya S, Nolan MF, Bruno R, Siegelbaum SA, Kandel ER (2013) Prefrontal cortex HCN1 channels enable intrinsic persistent neural firing and executive memory function. J Neurosci 33:13583-13599.

Tamas G, Szabadics J, Lorincz A, Somogyi P (2004) Input and frequency-specific entrainment of postsynaptic firing by IPSPs of perisomatic or dendritic origin. Eur J Neurosci 20:2681-2690.

Thiels E, Barrionuevo G, Berger T (1994) Excitatory stimulation during postsynaptic inhibition induces long-term depression in hippocampus in vivo. J Neurosci 15:3009-3116.

Tolner EA, Frahm C, Metzger R, Gorter JA, Witte OW, da Silva FHL, Heinemann U (2007) Synaptic responses in superficial layers of medial entorhinal cortex from rat with kainite-induced epilepsy. Neurobiol Dis 26:419–438.

Tsay D, Dudman JT, Siegelbaum SA (2007) HCN1 channels constrain synaptically evoked Ca2+ spikes in distal dendrites of CA1 pyramidal neurons. Neuron 56:1076–1089.

Tsuno Y, Schultheiss NW, Hasselmo ME (2013) In vivo cholinergic modulation of the cellular properties of medial entorhinal cortex neurons. J Physiol 591:2611-2627.

van Groen T, Wyss JM (1990a) The connections of presubiculum and parasubiculum in the rat. Brain Res 518:227-243.

van Groen T, Wyss JM (1990b) Extrinsic projections from area CA1 of the rat hippocampus: olfactory, cortical, subcortical, and bilateral hippocampal formation projections. J Comp Neurol 302:515-528.

van Hoesen GW (1995) Anatomy of the medial temporal lobe. Magn Reson Imaging 13:1047-1055.

van Welie I, Remme MW, van Hooft JA, Wadman WJ (2006) Different levels of Ih determine distinct temporal integration in bursting and regular-spiking neurons in rat subiculum. J Physiol 576:203-214.

Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. Science 313:1093-1097.

Witter MP (1993) Organization of the entorhinal-hippocampal system: a review of current anatomical data. Hippocampus 3:33-44.

Witter MP, Groenewegen HJ, Lopes da Silva FH, Lohman AH (1989) Functional organization of the extrinsic and intrinsic circuitry of the parahippocampal region. Prog Neurobiol 33:161-253.

Witter MP, Moser EI (2006) Spatial representation and the architecture of the entorhinal cortex. Trends Neurosci 29:671-678.

Witter MP, Naber PA, van Haeften T, Machielsen WC, Rombouts SA, Barkhof F, Scheltens P, Lopes da Silva FH (2000b) Cortico-hippocampal communication by way of parallel parahippocampal-subicular pathways. Hippocampus 10:398-410.

Witter MP, Wouterlood FG, Naber PA, van Haeften T (2000a) Anatomical organization of the parahippocampal-hippocampal network. Ann NY Acad Soci 911:1-24.

Woodhall G, Evans DI, Cunningham MO, Jones RS (2001) NR2B- containing NMDA autoreceptors at synapses on entorhinal cortical neurons. J Neurophysiol 86:1644–1651.

Yamada WM, Zucker RS (1992) Time course of transmitter release calculated from simulations of a calcium diffusion model. Biophys J 61:671-682.

Yamamoto J, Suh J, Takeuchi D, Tonegawa S (2014) Successful execution of working memory linked to synchronized high- frequency gamma oscillations. Cell 157:845-857.

Yang CR, Seamans JK, Gorelova N (1996) Electrophysiolgical and morphological properties of layers V-VI principal pyramidal cells in rat prefrontal cortex in vitro. J Neurosci 16:1904-1921.

Yejun H, Zorumski CF, Mennerick S (2002) Contribution of presynaptic Na+ channel inactivation to paired-pulse synaptic depression in cultured hippocampal neurons. J Neurophys 87:925-936.

Yi E, Roux I, Glowatzki E (2010) Dendritic HCN channels shape excitatory postsynaptic potentials in the inner hair cell afferent synapse in the mammalian cochlea. J Neurophysiol 103:2532-2543.

Ying SW, Jia F, Abbas SY, Hofmann F, Ludwig A, Goldstein PA (2007) Dendrtiic HCN2 channels constrain glutamate-driven excitability in reticular thalamic neurons. J Neurosci 27:7819-8732.

Yoshida M, Alonso A (2007) Cell-type-specific modulation of intrinsic firing properties and subthreshold membrane oscillations by the M(Kv7)-current in neurons of the entorhinal cortex. J Neurophysiol 98:2779–2794.

Yun SH, Cheong MY, Mook-Jung I, Huh K, Lee CJ, Jung MW (2000) Cholinergic modulation of synaptic transmission and plasticity in entorhinal cortex and hippocampus of the rat. Neuroscience 97:671-676.

Yun SH, Mook-Jung I, Jung MW (2002) Variation in effective stimulus patterns for induction of long-term potentiation across different layers of rat entorhinal cortex. J Neurosci 22:RC214.

Zhu Y, Wang R (2016) A comparative study of the impact of theta-burst and high-frequency stimulation on memory performance. Front Hum Neurosci 10:19.

Zola-Morgan S, Squire LR, Amaral DG (1986) Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. J Neurosci 6:2950-2967.

Zola-Morgan S, Squire LR (1990) The neuropsychology of memory: Parallel findings in humans and nonhuman primates. Ann NY Acad Sci 608:434-450.