5-19-2015

Characterization of Continuously Oscillating Neurons (CONs) of the Medial Septum of Rats

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by

Nadia N. Carreon

A Thesis Presented to the Faculty of the College of Biomedical Sciences and Health Professions

In Partial Fulfillment

of the Requirements for the Degree of

Master of Science

In the Field of Biology

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May 2015

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DEDICATION

To my mother.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Luis Colom, for providing me with this research opportunity and challenging me to take on such a project. I would also like to show my deepest gratitude to my lab supervisors, Dr. Luis Pacheco and Dr. Leandro Leite Antonio, who not only provided guidance in a time of obscurity, but a friendship as well. Thank you to the staff and students of Dr. Colom's lab for always offering a helping hand. Thank you, Dr. Michael Lehker, for stepping in as chair of my committee. I'd like to acknowledge my family and friends who cheer me on in my chosen career path. I would like to give recognition to my undergraduate mentor, Dr. Zen Faulkes, for continuously providing his mentorship and friendship. And last but not least, my professors at UTB, for kindly sharing their knowledge and encouraging our inquisitive minds.

ABSTRACT

Theta oscillation is the largest extracellular synchronous signal that can be recorded from the mammalian brain. It is known to influence information retention in the hippocampus, which plays a key role in declarative memory, recognition memory, working memory, and spatial memory. The theta oscillation field frequency is between 3 and 12 Hz and is present during exploratory behavior and sleep in rodents. Theta rhythm in the hippocampus is postulated to be produced by the rhythmical activity of pacemaking cells in the medial septum-vertical limb of the diagonal band of Broca (MS-vDBB). Previous work in our laboratory demonstrated the existence of continuously oscillatory neurons (CONs), the pacemaking cells, and sporadically oscillatory neurons (SONs) in the MS-DB. CONs were found to fire rhythmical action potential bursts within the duration range of a theta wave. The frequency at which they fire correlates with the simultaneously recorded hippocampal theta rhythm. It is believed that inputs from CONs and other ascending neurons are necessary to recruit non-rhythmic neurons to fire along a theta oscillation pattern. Altogether, this initiates a propitious environment for hippocampal theta frequency, which becomes the foundation for memory formation important in neurodegenerative diseases such as Alzheimer's disease (AD). The MS oscillatory mechanism is believed to lead and recruit theta rhythm generation in the hippocampus. However, the state-dependent alterations of the septo-hippocampal connection and the possible imbalance leading to septal or hippocampal dominance are poorly understood. In our investigations, we report that our CON cell recording was immuno-reactive to

a GABAergic marker, supporting our hypothesis that MS GABAergic neurons are key cells in pacing hippocampal theta. Additionally, we report our findings for one SON cell and one NON-NC cell recorded in the MS.

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LIST OF ABBREVIATIONS

Aβ – Amyloid Beta

AC – Auto Correlation

AD – Alzheimer's Disease

AP – Action Potential

CA – *cornu Ammonis*

CC – Cross Correlation

CNS – Central Nervous System

CON – Continuously Oscillating Neuron

CR – Calretinin

EC – Entorhinal Cortex

EEG – Eletroencephalogram

GABA – Gamma Aminobutyric Acid

HCN – Hyperpolarization-activated Cyclic Nucleotide-gated channels

ISI – Interspike Interval

LIA – Large Irregular Activity

LTP – Long Term Potentiation

MPO – Membrane Potential Oscillations

MS – Medial Septum

MS-DB – Medial Septum Diagonal Band

MS-DBB – Medial Septum Diagonal Band of Broca

MS-vDBB – Medial Septum vertical limb of the Diagonal Band of Broca

NON-C – Non oscillating correlated to theta

NON-NC – Non-oscillating non-correlated to theta

PBS – Phosphate Buffered Saline

PV – Parvalbumin

REM – Rapid Eye Movement

RPO – Reticularis Pontis Oralis

SON – Sporadically Oscillating Neuron

Vm – Membrane Potential; $V_m = Q/C_{in}$ (measured in volts)

CHAPTER 1 – INTRODUCTION

Alzheimer's disease (AD) is the most common senile dementia that affects about one in eight people over 65 years old (Kandel 2013). The first sign of the illness is failure to remember simple things and is followed by gradual memory deficits and progressive loss of cognitive abilities. These behavioral symptoms are attributed to much more complex physical alterations. The neurodegenerative alterations of cortical networks that take place affect specific regions of the brain such as the neocortex, entorhinal cortex (EC), and the hippocampus. The memory loss that accompanies AD is specifically due to alterations in the medial temporal cortex and the hippocampus, which are characterized by a thinning cortex, enlarged ventricles, amyloid beta (Aβ) plaques and fibrillary tangles (Morris 2003). In order to address the topics of learning and memory jeopardized in Alzheimer's disease, studies have placed special importance on the hippocampus, hippocampal cells and their activity. Focus is also placed on the connections it makes and receives with other structures of the brain such as the medial septum (MS), the cortical networks affected due to the progression of the disease, and how this alters the patterns of synchronous activity of the cells involved. Our focus is centered on the connections between the MS and hippocampus, which forms the septo-hippocampal pathway.

Hippocampus

The hippocampus is a structure in the medial temporal lobe of the brain, located on each side of the cerebral hemispheres. The hippocampus, along with structures such as the dentate gyrus and fimbria, form the hippocampal formation (Figure 2). The hippocampal formation, mammillary bodies, and fornix form part of the limbic system in the forebrain, which is crucial for memory formation and retrieval (Figure 1). The hippocampus makes connections with the EC and the MS. It has been associated with working memory, long term memory, episodic memory and spatial navigation. Damage to the hippocampus is known to affect the formation of episodic memory and semantic memory as well as cause pathologies such as epilepsy, anterograde amnesia, and the memory problems found in AD.

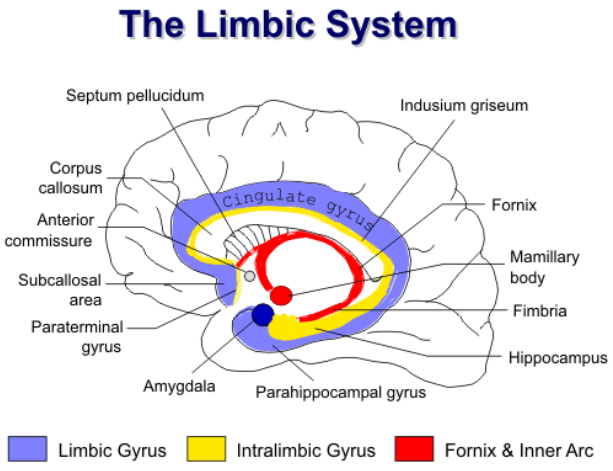

Figure 1

The limbic system is comprised of several structures (labeled above), and is located in the medial temporal lobe of the forebrain. The hippocampus, the mammillary bodies, and the fornix form connections with the MS. The septal region and the hippocampus share their connectivity through the fimbria-fornix system. This connection is jeopardized in AD, evident in patient learning, memory, and spatial navigation problems.
http://thebrainlabs.com/brain.shtml

Structure of the Hippocampus

The hippocampus is composed of several structures that are arranged in a peculiar seahorse shape. The structures begin with the dentate gyrus, the *cornu Ammonis* (CA) regions labeled 1-4, followed by the subiculum, and at the top layer of the CA regions is the alveus, which makes a connection with the fimbria-fornix system (Figure 2). The hippocampal formation generally refers to the CA regions and the dentate gyrus, which are composed of pyramidal cells and granule cells respectively. Studies have focused on the different populations of neurons residing in these structures and the network connections that result in the electroencephalogram (EEG) patterns commonly produced. It has been established that GABAergic septohippocampal afferents disinhibit GABA-hippocampal interneurons, affecting large numbers of principal cells through the dentate gyrus, CA3, and CA1 regions of hippocampal formation process (Freund and Antal 1988; Toth and Freund 1992; Toth, Borhegyi et al. 1993). The hippocampampal CA3 and CA1 regions, in turn, have been shown to project to the MS-DBB from GABAergic interneurons (Alonso and Kohler 1982; Toth, Borhegyi et al. 1993). Rhythmical slow activity has been confirmed to be generated in the CA1 region and dentate gyrus of the hippocampus (Bland and Whishaw 1976). It was also shown that the maximum discharge of hippocampal sharp wave activity that inhibited MS-DBB neurons was consistent with the maximum discharge of CA1 interneurons during theta activity (Dragoi, Carpi et al. 1999).

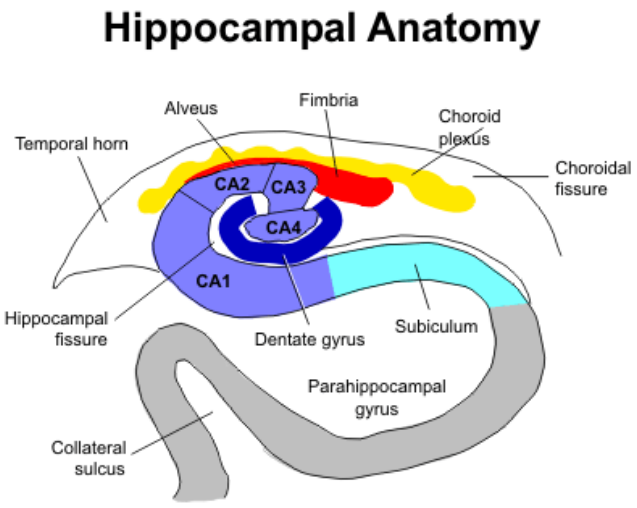

Figure 2

Coronal view demonstrating the different structures of the hippocampus in addition to the areas surrounding the hippocampal formation in grey and white. The hippocampal formation is comprised of the hippocampus, dentate gyrus, and subiculum. The hippocampus is important for encoding events and places, affected in AD.
http://spinwarp.ucsd.edu/NeuroWeb/Text/br-800epi.htm

Neuron Activity in the Hippocampus

Extracellular studies performed in the hippocampus have demonstrated different cellular discharge patterns that contribute to the generation of theta rhythm. Bland and Colom were among the researchers that described rhythmically bursting cells in the hippocampal formation. Their 1987 paper describes the two distinct populations of theta related cells they found. Theta-on cells were described as those that increase their firing rate with hippocampal theta wave activity. There are two subgroups: phasic theta-on and tonic theta-on cells. Phasic theta-on cells increase their firing patterns in a rhythmical and linear fashion in relation to theta rhythm and have a consistent phase relation to each wave. Tonic theta-on cells also increase their firing rate but do not show

rhythmicity or change in relation to theta; only constant discharges. They speculated that tonic theta-on cells signaled change from large irregular activity (LIA) to theta; phasic theta-on cells also signaled change from LIA to theta, along with shifts within the theta state. Conversely, theta-off cells are silent during theta activity, but fire during LIA. Subgroups within this type include phasic theta-off and tonic theta-off cells. Phasic theta-off cells do not fire during LIA, but begin to fire as theta slowly declines and are reciprocally related to phasic theta-on cells. Tonic-theta off cells only fire during LIA at a low constant rate and are reciprocally related to tonic theta-on cells. It was concluded that phasic theta-off cells signal the decline of theta frequency and change from theta to LIA, while tonic theta-off cells signal change from theta to LIA. In short, hippocampal phasic and tonic theta-on cell firing accompanies theta field activity, while hippocampal phasic and tonic theta-off cell firing accompanies LIA (Smythe, Cristie et al. 1991; Bland, Oddie et al. 1999). Their follow up research suggested that theta-on cells were projection cells and theta-off cells were inhibitiory interneurons (Bland and Colom 1993). Further, McNaughton postulated that phasic hippocampal activity in combination to similar frequency activity in other structures was necessary for effective cognitive processing (McNaughton, Ruan et al. 2006).

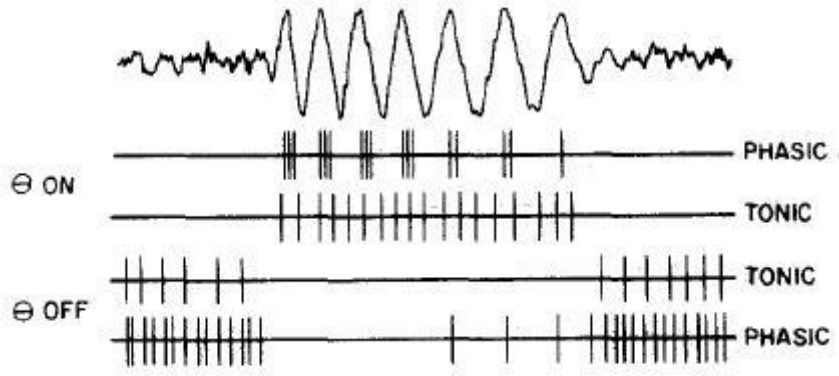

Figure 3

As hippocampal theta wave emerges, theta-on cells begin to fire while tonic theta off become silent (Bland and Colom 1993).

Theta Rhythm

There are several brain waves such as alpha, beta, delta, theta, and gamma that are produced by the electrical communication between neurons. EEGs allow us to detect subcortical structures producing such activity. Hippocampal EEG activity is at the center of many learning and memory studies. Two activity patterns that this study is concerned with is LIA and theta rhythm in rodents. LIA is found in the frequency band ranging from 0.5 – 25.0 Hz and has been linked with slow wave sleep, waking immobility, resting and eating (Leung 1982). Theta rhythm is widely accepted as a sinusoidal waveform with frequencies ranging from 3 – 12 Hz. It is associated with rapid eye movement (REM) sleep, alert and active behavior, and strongly correlated to learning and memory.

The hippocampal field activity's importance lies in its oscillation and synchrony function within the central nervous system (CNS). Specifically, hippocampal theta

field activity has been discovered to be a crucial phenomenon in hippocampal synaptic plasticity and larger brain processes such as sensory motor behavior, registration and retrieval of information, and spatial navigation in rats (Bland and Whishaw 1976; Bland, Oddie et al. 1999; Klimesch 1999; McNaughton, Ruan et al. 2006). Hippocampal theta rhythm facilitates the production of neuron long-term potentiation (LTP), a synaptic long lasting increase in effectiveness of synaptic transmission. LTP is an activity known to be one of the bases for learning and memory, neuron plasticity, and is related to cell synchronization (Kiss, Patel et al. 1990). Additionally, theta rhythm has been linked to declarative memory, recognition memory, working memory, and spatial memory in humans and has even been proposed to be a representative tag for short term memory processing (Klimesch 1999; Tesche and Karhu 2000; Vertes 2005). Theta rhythm frequency, as seen in rodents and other mammals, is mostly present in exploratory behavior and sleep. Further, two types of theta activities have been described in laboratory animals. Type 1 theta is correlated with voluntary motor activity and type 2 theta is correlated with motor activity in relation to processing sensory information (Colom, Ford et al. 1987). Rats that are anesthetized with urethane display only type 2 theta and the frequency lies at the lower end of the range.

Hippocampal pyramidal and granular cells in the hippocampus were first discovered to fire in bursts correlated with theta waves while others did not fire at all, giving rise to the speculation that rhythmicity originated prior to this structure

(Petsche, Stumpf et al. 1962). Since then, theta rhythm has been a focal point in many rising theories attempting to explain its origin.

The longstanding, widely accepted classic septal pacemaker hypothesis suggests that MS neurons precede hippocampal theta field activity and therefore serve as the pacemaker units. Because there are projections from the hippocampus back to the MS-DB, which originate mainly from GABAergic neurons in the *st.oriens* of CA1 and CA3, the classic septal pacemaker hypothesis was challenged by the hippocampal pacing theta hypothesis (Buzsaki 2002). This hypothesis suggested an intrahippocampal theta genesis, which projects back to the MS-DBB, phase locking GABAergic neurons with hippocampal theta (Manseau, Goutagny et al. 2008). However, many studies point in the direction of the classic view and in studies where MS activity is abolished, results show a termination in hippocampal theta, not allowing much popularity to be gained by the latter (Green and Arduini 1954; Winson 1978; Vinogradova 1995).

Theta rhythm has long been hypothesized to be a result of the communication that runs from the MS to the dentate gyrus, CA3, and CA1 of the hippocampus (Figure 4). Hippocampal theta frequency has been produced by stimulating the MS-vDBB and is directly correlated with the amount of electrical stimulation thereby concluding that there is a one-to-one relationship (Bland and Colom 1993).

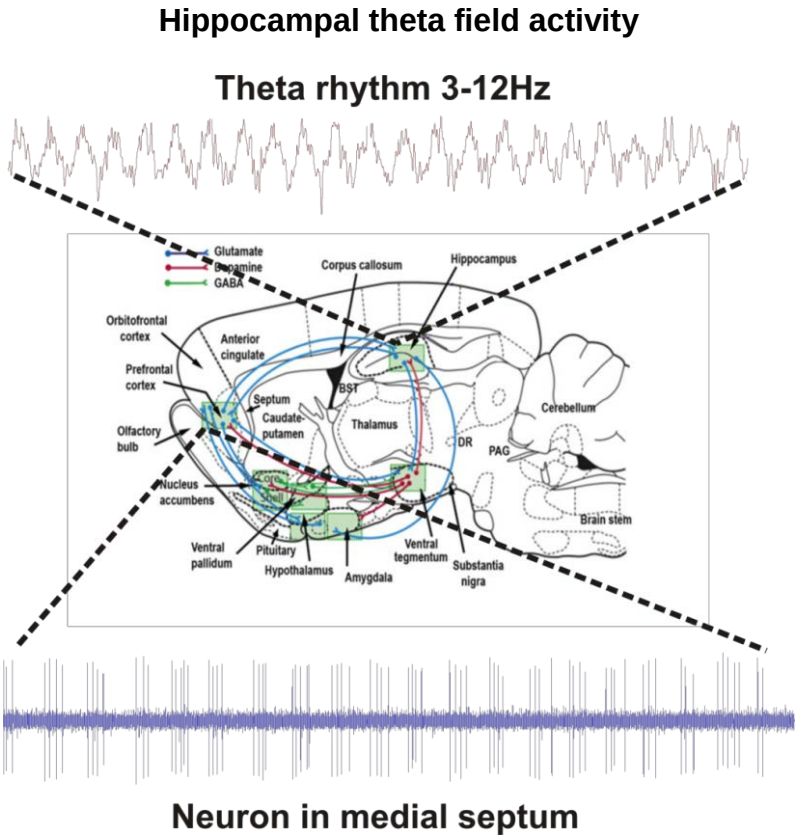

Figure 4

Saggital view of brain illustrating the connectivity between structures formed by 3 neuron populations. The top EEG shows an expanded view of theta rhythm produced in the hippocampus. Speculations are that the preceding structure, the MS, produces rhythmicity via neurons that discharge in rhythmic bursts into the hippocampus. The lower part of the illustration shows an expanded view of a single MS-DBB neuron firing rhythmically.
http://sites.sinauer.com/animalcommunication2e/chapter10.04.html

The Medial Septum

The medial septum is a medium of stimulations directed between the hippocampus and the diencephalon and mesencephalon (Petsche, Stumpf et al. 1962). Investigations on this structure have shown that the connection from the medial septum to the hippocampus is crucial for the production of theta rhythm. Experiments performed on the medial septum such as lesions, local anesthesia, and high frequency electrical stimulations have proved to abolish hippocampal

theta rhythm completely (Petsche, Stumpf et al. 1962; Lee, Chrobak et al. 1994; McNaughton, Ruan et al. 2006). It has been widely accepted to conclude that MS-DBB cells work as the pacemakers of theta rhythm in the hippocampus because many lines of evidence have supported this idea for decades. Specifically, because studies had documented the continued activity of rhythmically bursting cells in the MS after theta activity in the hippocampal formation was disrupted, the septal pacemaker hypothesis for hippocampal theta generation was formed (Ford, Colom et al. 1989). Moreover, theta rhythm is flawed in aged rodents, further supporting the hypothesis that changes in the MS-DBB complex are taking place and affecting the learning and memory functions of the hippocampus (Colom 2006). However, the precise mechanism on how the MS-DBB paces hippocampal theta is still not completely understood.

CHAPTER 2 – REVIEW OF THE LITERATURE

Neurons of the Medial Septum

Three neuronal populations compose the medial septum—cholinergic, glutamatergic, and GABAergic neurons (Simon, Poindessous-Jazat et al. 2006). Septo-hippocampal cholinergic neurons synapse with hippocampal principal cells and hippocampal interneurons. Studies have shown that age related loss and atrophy of cholinergic septal neurons contribute to alterations of rhythmic activity in the hippocampus (Rubio, Vega-Flores et al.). Further, neurons using the neurotransmitter acetylcholine are especially vulnerable in AD (Morris 2003). Currently, drugs that boost the amount of acetylcholine are used for treatment.

Septo-hippocampal glutamatergic neurons are known to project to the CA1, CA3, and the dentate gyrus. Septo-hippocampal GABAergic neurons synapse only with hippocampal interneurons (Freund and Antal 1988). Medial septal cholinergic and GABAergic neurons that project to the hippocampus have been speculated to influence hippocampal theta genesis (Rubio, Vega-Flores et al.; Serafin, Williams et al. 1996). Experiments in septally deafferented rats have determined that there exists an important balance between septal cholinergic and GABAergic contribution for modulating theta field activity in the hippocampus (Bland and Colom 1993). One key characteristic of AD is the degeneration or dysfunction in septal cholinergic neurons (Henke and Lang 1983).

Furthermore, non-cholinergic, presumed to be GABAergic neurons have shown the ability to discharge in rhythmic bursts of action potentials, implicating that

they may transmit rhythmic frequency to the hippocampus (Serafin, Williams et al. 1996). Since the development of the septal pacemaker hypothesis, these rhythmically bursting cells have been investigated in more detail. Studies have shown that voltage-gated sodium channels modulate synaptic activity, thus controlling cellular and network excitability (Wang 2002; Meisler and Kearney 2005). Experiments eliminating GABAergic neurons in the MS showed a termination of theta oscillation in the hippocampus along with memory impairment (Varga, Hangya et al. 2008). Accordingly, it was discovered that MS GABAergic neurons disinhibit GABAergic interneurons in the hippocampus, making it a critical component in neural synchronization, which is greatly affected when there is a reduction in the number and complexity of GABAergic septo-hippocampal axon terminals (Rubio, Vega-Flores et al.).

Bland and Colom studied cells in the MS and found that the majority were theta-related and followed the same classification scheme as mentioned above for the hippocampal theta cells but discovered a group that were rhythmic during both theta and LIA (Ford, Colom et al. 1989). The same categories were applied and followed to describe MS theta-on and theta-off cells with subpopulations of phasic and tonic in each. A portion of the phasic cells reported continued to discharge in a rhythmic pattern during LIA activity. The cells were seen to retain their rhythmicity but had greater variability in interburst and intraburst intervals compared to theta state. Their studies found that MS tonic theta-on cells are involved in the control and synchrony of the hippocampal formation by tonically depolarizing hippocampal phasic theta-on cells (Colom, Ford et al. 1987). MS

phasic theta-on cells synchronize the membrane potential oscillations (MPOs) of hippocampal phasic theta on cells, as well as, synchronize the discharges of hippocampal tonic theta-on cells (Bland, Oddie et al. 1999). Therefore, theta rhythm is produced when the medial septum inhibits hippocampal theta-off cells while at the same time initiating MPOs that recruit and synchronize both hippocampal phasic and tonic theta-on cells (Bland, Oddie et al. 1999). Recently, it was demonstrated that “candidate pacemaker neurons” in the MS precede putative hippocampal interneuron activity in hippocampal field state (Hangya, Borhegyi et al. 2009).

The majority of GABAergic septohippocampal neurons have been found to contain the calcium binding protein parvalbumin (PV; Figure 5) (Kiss, Magloczky et al. 1997; Simon, Poindessous-Jazat et al. 2006; Hangya, Borhegyi et al. 2009). Dysfunction in PV cells contributes to abnormalities in oscillatory rhythms, network synchrony and has an effect on cognitive alterations (Verret, Mann et al.). Moreover, HCN channels, hypothesized to be the pacemaker channels, were thought to be expressed in the membranes of MS-DBB GABAergic neurons (Robinson and Siegelbaum 2003; Varga, Hangya et al. 2008). Vargas et al. (2008) conducted a study investigating the HCN-expressing cells in the septohippocampal pathway and confirmed them to be GABAergic, including the subpopulation of parvalbumin and GAD67. These results strengthened previous studies speculating that MS GABAergic neurons are the contributors in the production of theta rhythm oscillations in the hippocampus.

Previous work performed in our laboratory demonstrated the existence of continuously oscillatory neurons (CONs), speculated to be pacemaking cells, and sporadically oscillatory neurons (SONs) in the MS-DBB. CONs were found to fire rhythmical action potential bursts in the duration range of a theta wave. The frequency at which they fire correlates with theta rhythm recorded simultaneously in the hippocampus. It is believed that inputs from CONs and other ascending neurons are necessary to recruit non-rhythmic neurons into theta oscillation pattern. Altogether, this initiates a propitious environment for hippocampal theta frequencies, which becomes the foundation of memory formation important in neurodegenerative diseases such as AD.

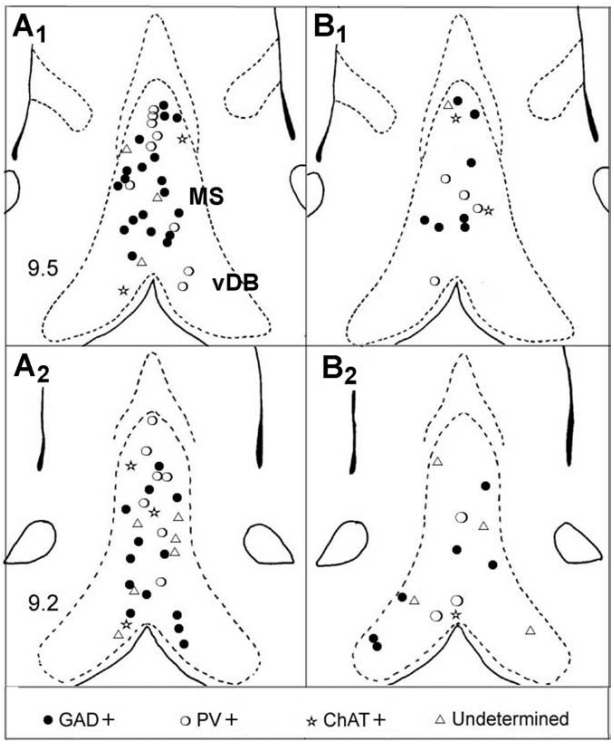

Figure 5

Simon labeled a total of 90 MS-DBB neurons with neurobiotin and examined them by immunohistochemistry for GAD, PV, and Chat and identified the firing patterns of each (Simon, Poindessous-Jazat et al. 2006).

The Septohippocampal Pathway

Studies on Alzheimer's disease have centered on the effects that aging has on the firing rates of neurons of the septohippocampal pathway. Extracellular recordings have shown that rhythmically bursting neurons, the frequency at which they fire, and the amplitude are significantly lowered in aged rats (Apartis, Poindessous-Jazat et al. 2000). The changes in cognitive learning and memory in aged rats may be attributed to alterations in the MS-DBB and in the nucleus basalis (Colom 2006).

Various brainstem inputs to the hippocampal system give rise to different EEG states and electrical stimulation has been shown to produce slow wave theta activity (Colom, Ford et al. 1987). The reticulari-spontisoralis nucleus (RPO) is a brainstem site that has been found to elicit hippocampal theta rhythm by affecting the membrane potential (Vm) levels of MS-DBB cells, which induce the rhythmic activity (Oddie, Bland et al. 1994; Barrenechea, Pedemonte et al. 1995). A study carried out by (Daitz and Powell 1954) demonstrated that the medial septum projects to the hippocampus via the fimbria, the most orally situated part of the hippocampus (Petsche, Stumpf et al. 1962; Stumpf, Petsche et al. 1962). Therefore, it can be assumed that the MS-DBB receives afferent inputs from the brainstem and then cholinergic and GABAergic projections ascend to the hippocampus (Freund and Antal 1988; Bland, Oddie et al. 1999). Additionally, stimulus to the hypothalamus has shown to increase cell discharge rate and theta frequency (Colom, Ford et al. 1987; Bland, Colom et al. 1990). Conversely, a transition from theta to LIA consists of the medial septum's disinhibition of

hippocampal theta-off cells through the GABAergic septohippocampal pathway (Smythe, Cristie et al. 1991; Bland, Oddie et al. 1999). Hypothalamic cells effects axons that terminate in the MS-DBB, which have been reported to be cholinergic or parvalbumin-containing GABAergic, within the septohippocampal pathway (Bland, Colom et al. 1990; Smythe, Cristie et al. 1991; Kiss, Magloczky et al. 1997). Other medial septal neurons have been found to be immuno-positive for Calretinin (CR). The functionality of these MS-DBB neurons is not clearly understood to date. In the septohippocampal pathway (Figure 6), GABA connections have been suggested to influence NMDA mediated functions, which if disturbed can result in pathological excitability in the hippocampal formation (Freund and Antal 1988).

In summary, the synchronized signaling pathway travels from the ascending brainstem to the medial septum, which recruit and initiate phasic theta-on cell MPOs, which then recruit phasic theta-on cell MPOs in the hippocampus (Smythe, Cristie et al. 1991; Konopacki, Bland et al. 1992; Bland, Konopacki et al. 1995; Bland, Oddie et al. 1999). The hippocampus then projects back to the medial septum in a feedback connection discovered to have a GABA transmitter (Toth, Borhegyi et al. 1993).

Septo-hippocampal pathway

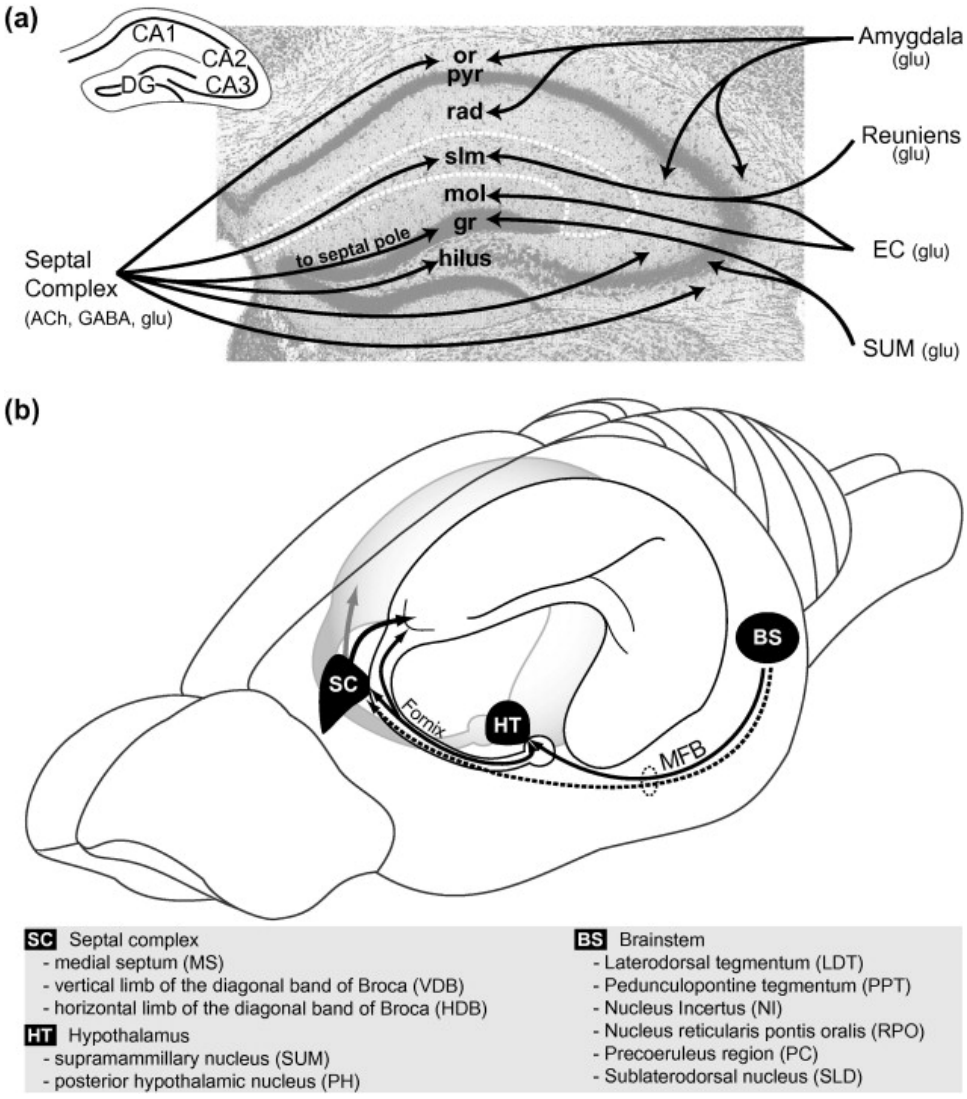

Figure 6

a) Coronal slice containing the hippocampal formation. Shown over the structure is a model of the neuron populations projecting to the hippocampus from their perspective locations. b) An overview of the connections between the brainstem, hypothalamus, and septal complex, forming the septo-hippocampal pathway.

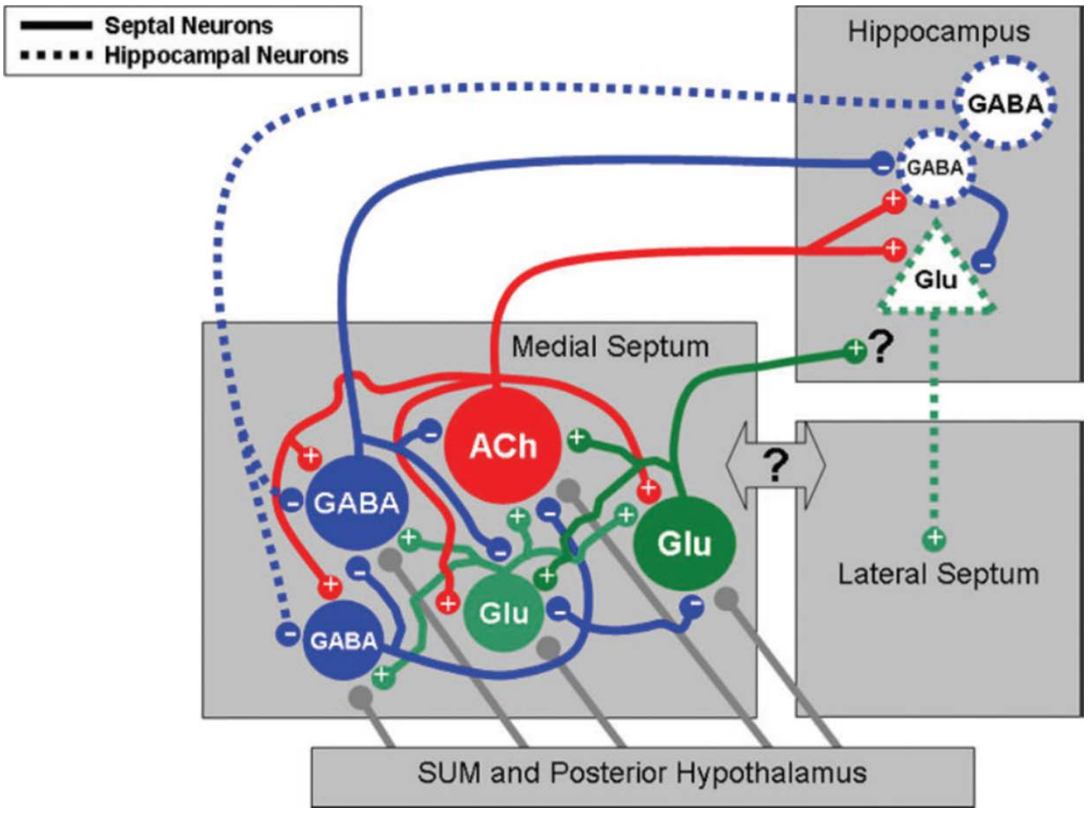

Figure 7

A hypothetical model proposed by Colom (2005) of the MS circuits including the three main neuronal populations and the connections they form with other structures. Solid lines indicate septal neurons and dashed lines indicate hippocampal neurons.

Genetic mutations can be a contributing factor to early onset Alzheimer's disease. However, the most common type is late onset AD. How can we address a disease in which the major risk factor is age? Genetic research and the use of MRI's to view structural changes have provided methods of early diagnosis, but a deeper understanding of cellular and structural relationships is important for finding better treatments. This study focuses on the cellular mechanisms of the MS cells previously recorded in our lab thought to underlie the genesis of hippocampal theta rhythm implicated in learning and memory.

Limitations

Extensive research applicable to humans has been performed using rodents for investigations on learning and memory and even using transgenic animals to mimic pathologies such as those seen in Alzheimer's disease. However, human epileptic patients with neural implants provide us with the best type of data to cross reference laboratory findings concerning brain activity. These studies have demonstrated a similar activity related to memory and navigation that resembles theta oscillations seen in rodents but at 1-4 Hz, a much slower range (Jacobs 2014). In spatial and temporal memory tasks, hippocampal oscillations ranged at 2-8 Hz (Jacobs 2014). Inevitably, a limitation in reporting our discoveries is the application of cellular activity at distinct frequencies that may vary across species. Moreover, our chances of encountering a CON cell in this study were about (8%), limiting the amount of cells we are able to report at this time. Additionally, our immunohistochemistry proved to have a limitation regarding how many GABAergic markers we could test at a time. Therefore, this study only investigated either calbindin (CB), GAD67, or parvalbumin (PV) positive neurons.

PURPOSE OF STUDY

This study aimed to confirm the electrophysiological nature and morphology of previously discovered continuously oscillating neuron (CON) cells in our laboratory. CONs are described as those that show rhythmical firing in the presence and absence of hippocampal theta activity. Our approach to investigate the firing properties was accomplished by using a juxtacellular recording labeling technique. In order to investigate morphological characteristics of the recorded cells, we used various primary antibodies to identify GABAergic neurons, detailed in our report. Lastly, we used a computerized tracing technique for visualizing the morphology of the immuno-reactive cells.

HYPOTHESIS

Our hypothesis predicts that the medial septum (MS) provides rhythmicity to the hippocampus, through the rhythmical burst discharges of continuously oscillating neurons (CONs) expressing GABA neurotransmission.

CHAPTER 3 – MATERIALS AND METHODS

Animals

A total of 20 adult male Sprague Dawley rats, weighing between 250–350g, were used for the purpose of this project. The animals were housed and maintained on a 12h-12h light-dark cycle and provided with food and water *ad libitum*. The animal protocols used for this study are in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of UTB to minimize pain and suffering while reducing the number of animals used.

Anesthetic Procedure

Animals were initially anesthetized using Isoflurane (The Butler Company, Dublin, OH) in a chamber and then transferred to a surgical setting where anesthesia was continued using a Matrix VIP 3000 Isoflurane Vaporizer (MidMark Co., Versailles, OH), while surgically placing an external cannula through the jugular vein. Cannulation allows direct administration of Urethane anesthesia (Sigma-Aldrich Co., St. Louis, MO) for immediate control of anesthetic level, which allows us to obtain an optimal hippocampal theta state. Once the jugular cannula was secured, Isoflurane was gradually discontinued and 0.5 g/ml of Urethane was progressively administered in order to maintain the level of anesthesia for the remainder of the surgical and experimental procedures.

Electrophysiology

Rats were placed in a stereotaxic instrument (David Kopf Instruments, Tujunga, CA) with Bregma and lambda leveled horizontally. A self-regulating heating pad (Fine Science Tools Inc., Foster City, CA) was used to maintain a stable body temperature of 37°C. Trephine holes were drilled above the hippocampi using the following coordinates for the CA1 region: 3.8 mm posterior to Bregma, 0.2 mm lateral to the midline, and 2 mm ventral to the dural surface. An additional hole was drilled posterior to one hippocampus to place an uninsulated silver wire in the cortex to serve as an indifferent electrode. A tungsten electrode (0.1 MΩ resistance) was used to record the electroencephalogram (EEG) activity of the CA1 region of one hippocampus at a time. Electrodes were inserted at the coordinates listed above using a micro-positioner (David KOPF Instruments, Tujunga, CA). The electrodes were then left in place for 5-10 minutes to normalize EEG activity and then bound in position using dental cement (A-M Systems, Inc., Sequim, WA).

A small window was drilled above the medial septum using the following coordinates: 0.5 mm anterior to Bregma, 0.5 mm lateral to the superior sagittal sinus, and 5.2–7.2 mm ventrally. The dural matter was removed and the sinus was often cauterized for direct accessibility to the midline of the medial septal structure. Cells in the medial septum were recorded using glass microelectrodes constructed from 1.5 mm thin wall glass capillaries (World Precision Instruments, Inc., Sarasota, FL) and shaped using a Vertical Pipette Puller Model 720 (David KOPF Instruments, Tujunga, CA) with a tip resistance of 15–30 MΩ. Glass

electrodes were filled with 0.5 M sodium acetate and 5% Neurobiotin (Vector Laboratories, Inc., Burlingame, CA) before use and lowered to the medial septum coordinates using the micro-positioner mentioned above. A trephine hole was drilled above the medial septum window to place an uninsulated silver wire for grounding purposes.

Data Acquisition

All electrophysiological data was acquired using Spike 2 (Cambridge Electronic Design, Cambridge, England), which displayed, digitized, and recorded both cell firings in the medial septum and EEG activity simultaneously for off-line analysis. Medial septum cell firings were monitored by using a speaker (A-M Systems, Inc., Sequim, WA) and recordings were amplified and filtered (low-pass at 300 Hz) using a Cyber Amp 320 (Axon Instruments, Union City, CA). The EEG signal was amplified and filtered using a Microelectrode AC Amplifier Model 1800 (A-M Systems Inc., Carlsborg, WA).

Cells in the MS-DBB were monitored for approximately 5 minutes preceding recording of firing patterns during four hippocampal field conditions: (1) LIA only (baseline control), (2) transitioning from LIA to theta, (3) theta only (experimental condition), and (4) transitioning from theta to LIA. In the instances where theta was not spontaneously produced, a tail pinch was performed for 30 second intervals to induce activity. Recordings averaged 355 seconds in duration. Following the recording of a rhythmically behaving cell, a current injection was applied for cell labeling using the juxtacellular technique described by (Pinault

1996). The labeled cell was monitored using the Spike 2 display for 5-10 minutes succeeding the current injection to ensure cell survival.

Data Analysis

Analyses of cell recordings were performed using Clampfit 9.2 software (Molecular Devices, LLC., Sunnyvale, CA) to determine theta phase preference, burst frequency (Hz), interspike interval (ISI), and amplitude (mV). Cell firing patterns and hippocampal activity were individually examined by autocorrelation analysis. Correlations between cell behavior and hippocampal activity was determined using cross-correlograms.

LIA was defined as a large amplitude irregular activity with a frequency band ranging from 0.5 – 25.0 Hz as described by (Leung 1982). Hippocampal theta activity was defined as a sinusoidal waveform with a frequency ranging from 3 – 12 Hz. Firing rates were examined against both hippocampal activities to evaluate the electrophysiological properties of each cell. Neurons were then classified according to their rhythmical firing, or lack thereof, and its correlation, if any, with the simultaneously ongoing hippocampal activity, specifically, with hippocampal theta. The classification is as follows:

1. *Continuously oscillating neurons (CON):* Neurons which fired rhythmic bursts of action potentials in the presence or absence of hippocampal theta and were highly correlated with theta rhythm.

2. *Sporadically oscillating neurons (SON):* Neurons which fired rhythmic bursts of action potentials only in the presence of hippocampal theta and were highly correlated with theta rhythm.
3. *Non-oscillating neurons correlated to theta rhythm (NON-C):* Neurons which fired arrhythmically but were correlated with hippocampal theta rhythm.
4. *Non-oscillating neurons non-correlated to theta rhythm (NON-NC):* Neurons which fired arrhythmically and did not correlate with hippocampal theta rhythm.

Statistical analyses were performed using OriginPro 8.5.1 (Origin Lab. Corp., Northampton, MA) and Microsoft Excel (Microsoft Windows Vista, 32-bit Operating System). A Kruskal-Wallis one-way analysis of variance was used to determine electrophysiological differences among neurons. Significant differences were set at $p < 0.05$.

Histological Analysis

After successfully recording and labeling MS cells with neurobiotin, animals were perfused transcardially. A perfusion wash was first administered using phosphate buffered saline (PBS: 0.1M, pH=7.4), followed by a fixative containing 4% paraformaldehyde prepared in 0.1 M PBS. The brain was extracted and incubated in the 4% paraformaldehyde fixative overnight and then transferred into a 30% sucrose solution for dehydration over a 3 day period. Brains were then placed in frozen section medium (Richard-Allan Scientific Neg-50, Thermo

Fisher Scientific, Inc., Waltham, MA) to cut coronal serial sections 40 μm thick using a vibratome (Microm HM 550, Thermo Fisher Scientific, Inc., Waltham, MA). Once slices containing the medial septum were collected, three tissue washes, each lasting 10 minutes, were performed using 0.1 M PBS. We then incubated the tissue in 0.3% H_2O_2 prepared in PBS for 1 hour followed by a washing process. Brain tissues were then incubated in 5%NDS-1%BSA-0.1%PBS-Tween for a 1 hour blocking process to improve antibody penetration. Next, tissue was incubated overnight in streptavidin-conjugated Alexa 568 (diluted in 1.25%NDS-0.25%BSA-0.025%Tween at 1:200) in a dark environment at room temperature. The washing process was repeated and slices were mounted on a slide using chemical permount mounting medium (Thermo Fisher Scientific, Inc., Waltham, MA) to be viewed under fluorescent microscopy in order to locate the Neurobiotin labeled neuron.

After acquiring the section with the neurobiotin labeled cell, the anatomical position was confirmed and the former and subsequent sections were collected for further preparation. The blocking process, detailed above, was repeated and followed by incubation in primary antibodies, calbindin 28 (CB) and GAD67 (rabbit anti-calbindin28, rabbit anti-GAD67, diluted in 1.25%NDS-0.25%BSA-0.025%Tween at 1:250), over a 3 day period. Sections were once again washed and incubated in the secondary antibody, Alexa488 conjugated goat (anti-rabbit diluted in 1.25%NDS-0.25%BSA-0.025% Tween at 1:200), for 2 hours at room temperature. A subsequent wash was performed and sections were mounted on a slide for colocalization analysis using fluorescent microscopy.

Next, sections were treated overnight with Vectastain ABC Kit (diluted in 1.25%NDS-0.25%BSA-0.025%Tween; Vector Laboratories Inc., Burlingame, CA). Sections were then stained using DAB Substrate Kit for Peroxidase (SK-4100; Vector Laboratories, Inc., Burlingame, CA) for 10 minutes and placed on a slide to dry overnight. A serial dehydration process was performed using 70%, 90%, 100% ethanol and two successive submersions in xylene, coverslipped and allowed to dry overnight.

Cell Reconstruction

The DAB results were then viewed under a microscope using Neurolucida software (MicroBrightField, Inc., Williston, VT) for sectional mapping and neuron tracing. A branched analysis was performed using Neurolucida Explorer, to describe the anatomical properties detailed by tracing (Table 1).

CHAPTER 4 – RESULTS AND DISCUSSION

Studies on the medial septum have reported that this structure is of great importance in hippocampal theta generation. Medial septal neurons send cholinergic, GABAergic and glutamatergic projections to the hippocampus (Figure 7) (Colom 2006). The hippocampus also sends projections back to the medial and lateral septal structures, in which a small portion appears to be GABAergic (Toth, Borhegyi et al. 1993). The mutual connection between the septum and the hippocampus has been shown to be mediated by the MS-DBB, which has been referred to as the pacemaker structure in providing a rhythmic drive to the hippocampus (Dragoi, Carpi et al. 1999; Apartis, Poindessous-Jazat et al. 2000; Wang 2002). The medial septum rhythmically bursting neurons that are theta-related have been found to be GABAergic (Figure 5) (Simon, Poindessous-Jazat et al. 2006). MS GABAergic neurons are known to connect to GABAergic interneurons in the hippocampus, exerting excitibitly control on pyramidal cells, and thus producing hippocampal theta rhythm (Garcia-Hernandez, Bland et al.). This was settled by testing immunoreactivity of the calcium binding protein, calbindin, present in the medial septum (Kiss, Magloczky et al. 1997; Simon, Poindessous-Jazat et al. 2006). Similarly, the hippocampal neurons that project to the medial septum have also been found to contain calbindin (Toth and Freund 1992; Toth, Borhegyi et al. 1993).

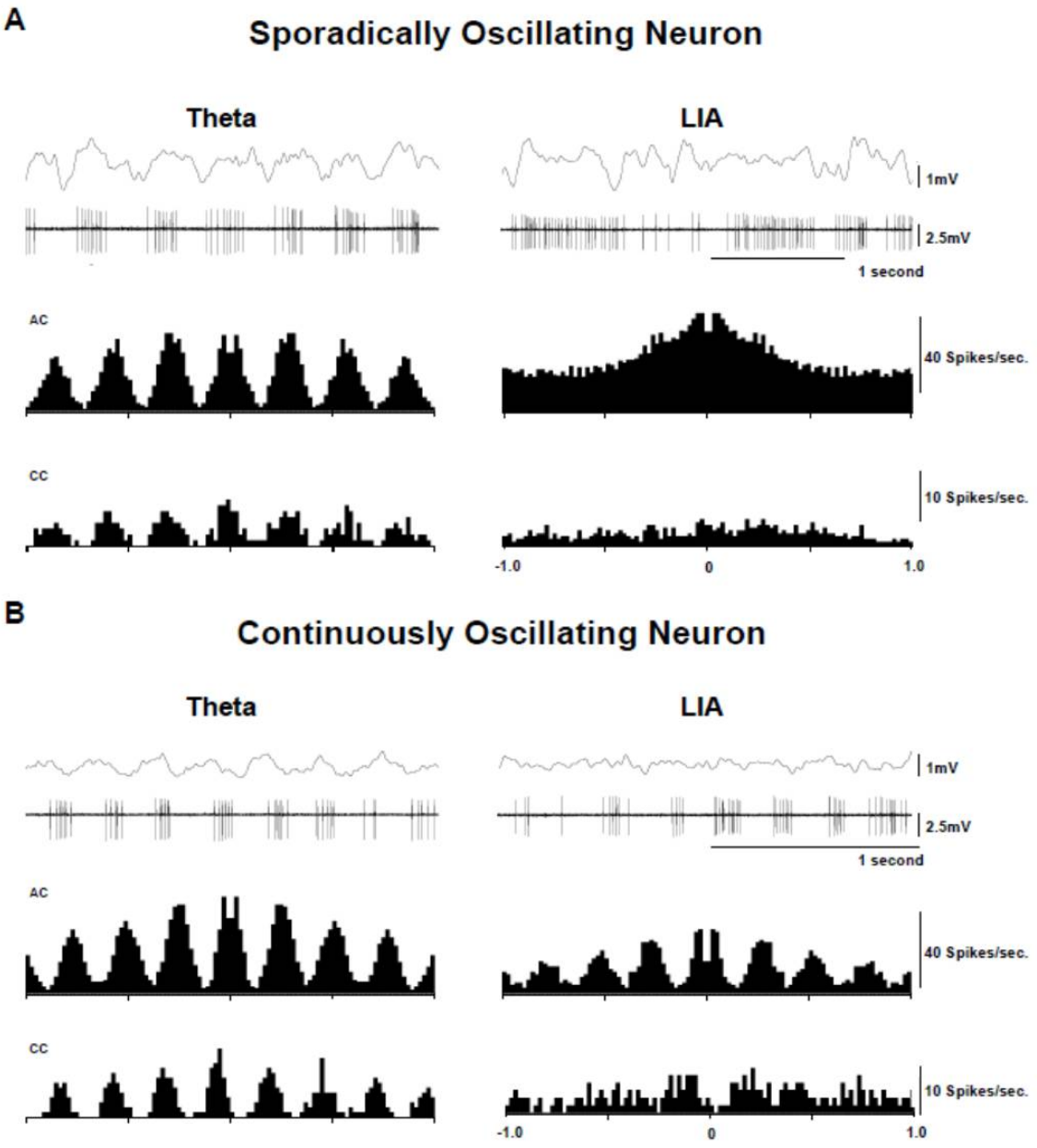

Figure 8

Uppermost part of illustration shows theta and LIA field activity as recorded in our laboratory. The firing patterns of one neuron are shown under each EEG condition. A) Shows the activity of one neuron classified as SON. Under is the AC of the action potentials of the cell and the CC between the cell activity and the EEG. B) Shows the activity of one neuron described as CON. The histograms that follow show the AC of the AP, which do not show a drastic change under each field activity condition. The CC of the CON activity against the EEG, continues to show rhythmic patterns.

Electrophysiology

Seventy nine neurons were recorded. Seventeen of those neurons (21.52%) presented rhythmic bursts of action potentials. Out of those 17 neurons, 10 (12.65%) displayed rhythmic bursts only during the occurrence of hippocampal theta rhythm (SON) (Figure 8A). The remaining 7 neurons (8.86%), presented continuous rhythmic oscillations during the entire recording period regardless of the EEG activity (CON) (Figure 8B). Sixty two neurons (78.5%) fired action potentials in non-rhythmic patterns. Only 4 (5%) of those neurons fired action potentials that were correlated to hippocampal theta rhythm (NON-C).

Rhythmical MS-DBB neurons

CON and SON cells (n = 7 and 10, respectively) displayed high firing frequencies during theta oscillations (16.9±3.4 Hz and 19.06±3.9 Hz, respectively). During LIA, firing frequencies were slightly, but not significantly reduced (15.1±3.45 Hz and 13.65±3.4 Hz, respectively). Firing frequencies were not statistically different between CON and SON cells (Kruskal-Wallis ANOVA, P=0.084). In addition, firing phases could not separate CONs from SONs. Both types of cells fired in particular phases of theta wave, demonstrating an average firing phase of 120.34±10.6 degrees for CONs and 156±15.6 degrees for SONs in relation to theta (Figure 9) (one-way ANOVA, F=2.94, P=0.106).

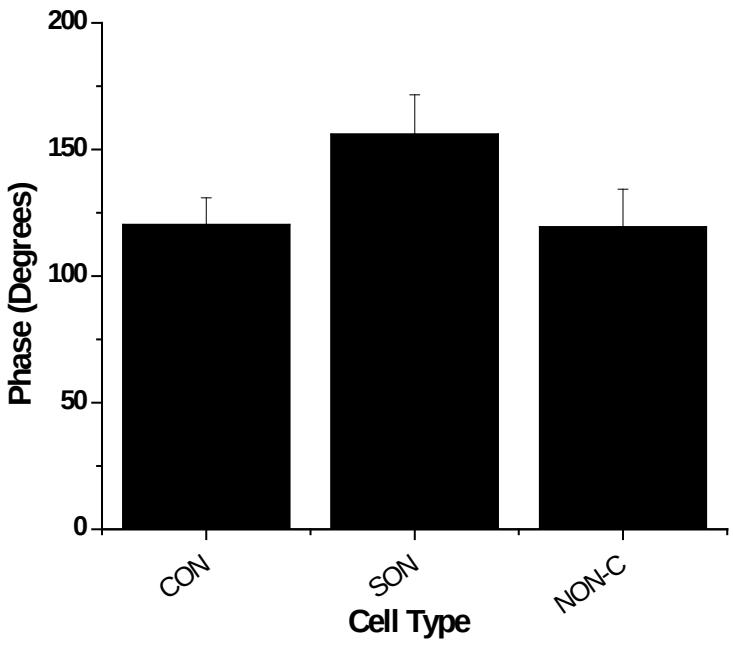

Figure 9

MS-DBB neurons fire at a particular phase of theta when simultaneously recorded with hippocampal theta activity.

However, the burst firing frequencies of CON and SON cells were different during the occurrence of the hippocampal theta rhythm. CON cells fired bursts of action potentials at higher frequencies than SON cells (63.8±8.5 Hz and 39.05±4.3 Hz, respectively; one-way ANOVA, F=8.03 and P=0.012) (Figure 10). Moreover, CON's average burst duration was shorter than SON (78.9±12 ms and 132.78±17 ms, respectively; one-way ANOVA, F=5.45, P=0.033). Thus, during theta rhythm CON cells showed shorter ISI (23.6±3 ms), when compared to SON cells (43.63±5 ms; one-way ANOVA, F=38.79, P=0.00156). During LIA, only CON cells continued displaying rhythmic bursts.

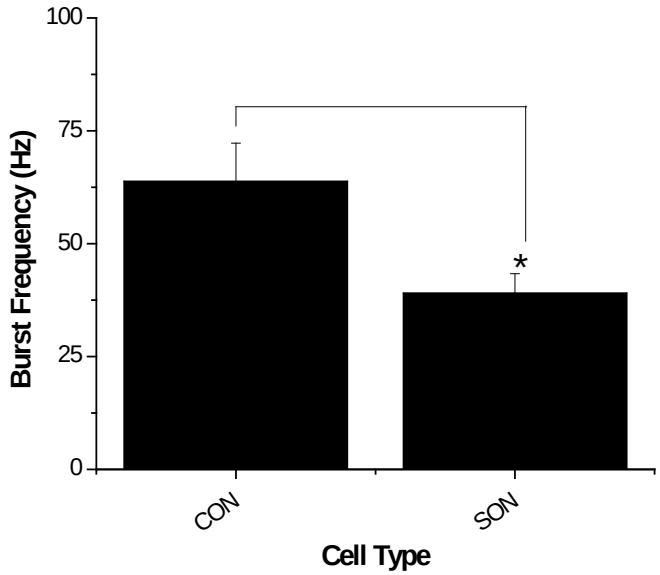

Figure 10

Rhythmical neurons in the MS-vDBB, labeled CON and SON, influence hippocampal theta activity through their burst firing frequency.

Non-rhythmical MS-DBB neurons

The average firing frequency of NON-C cells observed during theta was 9.25±4.6 Hz and 4.82±1.6 Hz during LIA. Two out of four neurons (50%) were considered slow firing neurons (i.e. firing frequencies <12 Hz) (Colom 2006). The remaining two were considered fast firing neurons (i.e. firing frequencies >12 Hz) (Colom, 2006). The theta phase in which they fired was 119.4±14.9 degrees and do not statistically differ from the rhythmical neurons ($p>0.05$). The remaining 58 neurons displayed non-rhythmical firing patterns that were non-correlated to theta (NON-NC). The average firing frequency of a NON-NC cell was 17.36±2.4 Hz during theta and 15.68±2.7 during LIA. Twenty five of those neurons (42%)

were considered slow firing neurons and the remaining 33 neurons were considered fast firing neurons (Figure 11).

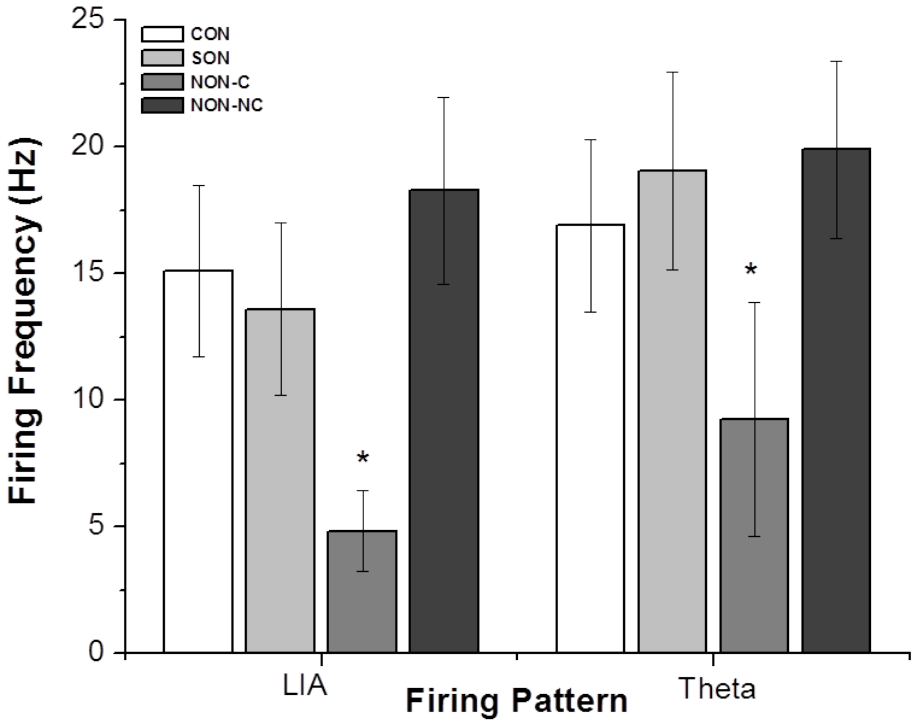

Figure 11

The graph represents the different firing frequencies of the four classified neurons in the MS-DBB. CONs and SONs display high firing frequencies with theta oscillations. The firing frequencies of a NON-C cell significantly change with the presence of theta activity. The average firing of a NON-NC cell change only slightly with theta rhythm.

CON

Out of our recorded cells, we selected a total of eleven cells to further analyze for rhythmical patterns. Five out of the eleven cells were considered potentially rhythmical and continued with immunohistochemistry. One of the five cells

proved to have CON characteristics, as reported in our preliminary data. It showed an increase in firing frequency by 31.9% (X^2 = 13.00295, df: 1, p<0.00001), when stimulated by a tail pinch to produce theta rhythm (Figure 12A). A Kruskal-Wallis ANOVA (LIA= 11 and theta = 18; Table 2) demonstrates a significant difference in the duration of bursts, a 23.45% change when compared to the LIA state (X^2 = 5.50056 DF=1, p< 0.01901). The rhythmical pattern, as demonstrated by the auto-correlation (AC) and cross-correlation (CC) histograms, show no significant changes in action potentials (X^2 = 0.33506, df: 1, p=0.56269).

Following juxtacellular recordings, we utilized GABAergic markers to test for immuno-reactivity. We chose CB, GAD 67, and PV to test our hypothesis that CON is of GABAergic nature, in line with research suggesting this characteristic of pacemaker MS cells. Our results show CB immunoreactivity overlayed with our recorded neuron (Figure 12B), which presented high rhythmic firing patterns after the tail pinch stimulation (Figure 12A). This confirms that GABAergic neurons are related to theta rhythm generation in the hippocampus. A CC analysis shows that our recorded neuron, classified a CON, had a firing rate correlated to the concurrent hippocampal activity, shown in the rhythmic bursts of action potentials that are present under both LIA and theta conditions (Figure 12). However, it is clearly more pronounced under the hippocampal theta state.

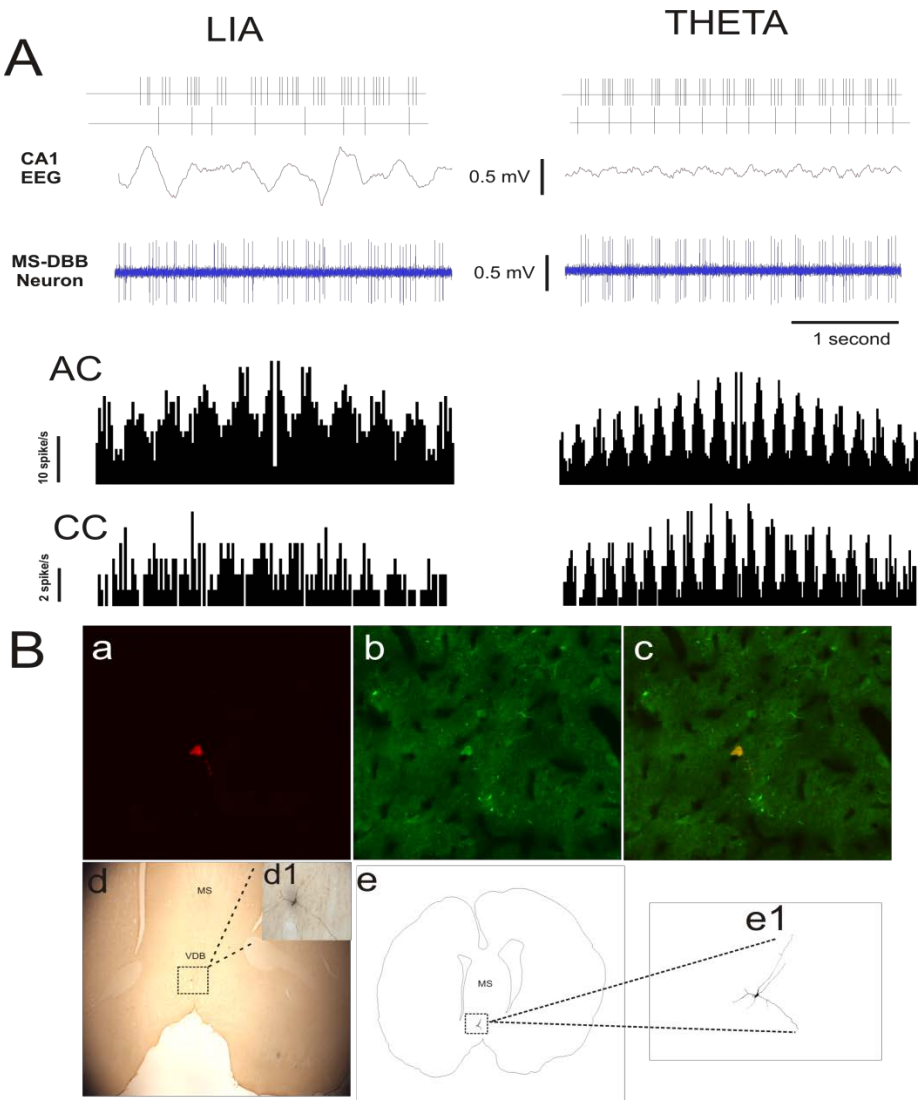

Figure 12

Squared sections of a juxta-cellular recording (blue) in the MS with simultaneous hippocampal EEG activity (red) were analyzed during LIA and theta (tail pinch) conditions. Auto AC and CC histograms show the activity of one CON labeled neuron (A). The immuno-reactivity for the recorded cell B-a (Streptavidin red) and Calbindin B-b (FITC green) overlapped in B-c. Immunohistochemistry illustrating the recorded cell in the MS-vDBB (B-d). Square is amplified to 40x (B-d1). Contour map and neuron tracing is shown in B-e and B-e1, respectively.

Another important characteristic about CON cells is their tendency to fire in a consistent manner in alignment to a particular degree of theta wave. Results for our CON cell recording demonstrate a tendency to fire at a phase of 202° on

theta wave (Figure 13). A prevalent theta wave phase suggests synchrony, in which the cell bursts play a role in sending oscillatory cues at a specific moment to the hippocampus to generating theta rhythm.

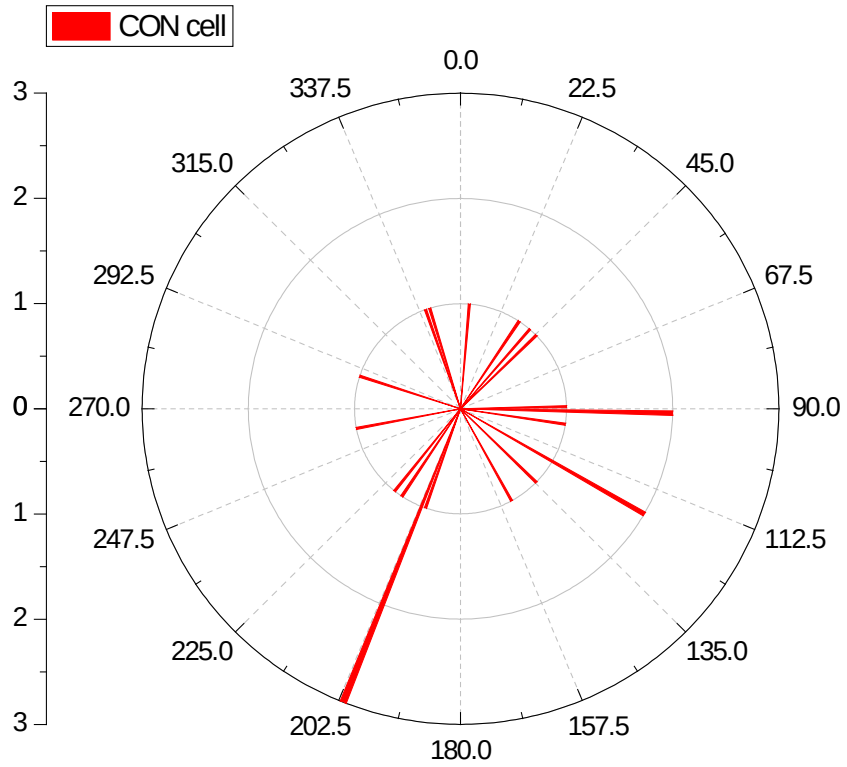

Figure 13

Circular distribution showing phases, in degrees, of hippocampal theta wave and the average MS-vDBB rhythmical burst firing prevalent at each value. In red are the average firing action potentials of the labeled CON cell.

SON

Our second recorded neuron fit the classification characteristics for SON. This neuron proved to have arrhythmical firing during the LIA condition, as shown in the AC and CC histograms (Figure 14A). A Kruskal-Wallis ANOVA (LIA= 14 and

theta = 10; Table 3) demonstrates a significant difference in the duration of bursts, a 29.05% change when compared to the LIA state (X^2 = 5.09529, df: 1, p< 0.02399). The rhythmical pattern is accompanied with an increase in action potentials by 66.6% (X^2 = 12.84627, df: 1, p<0.0001). However, the frequency and ISI had no significant difference.

Here, we used a GAD67 GABAergic marker to continue testing our hypothesis. Our results indicated that this type of cell was immuno-negative to GAD67, evident by its lack to overlap (Figure 14B). This does not eliminate the possibility of it being a GABAergic cell. It could potentially be another subclass, which was not tested for in this study (see limitations).

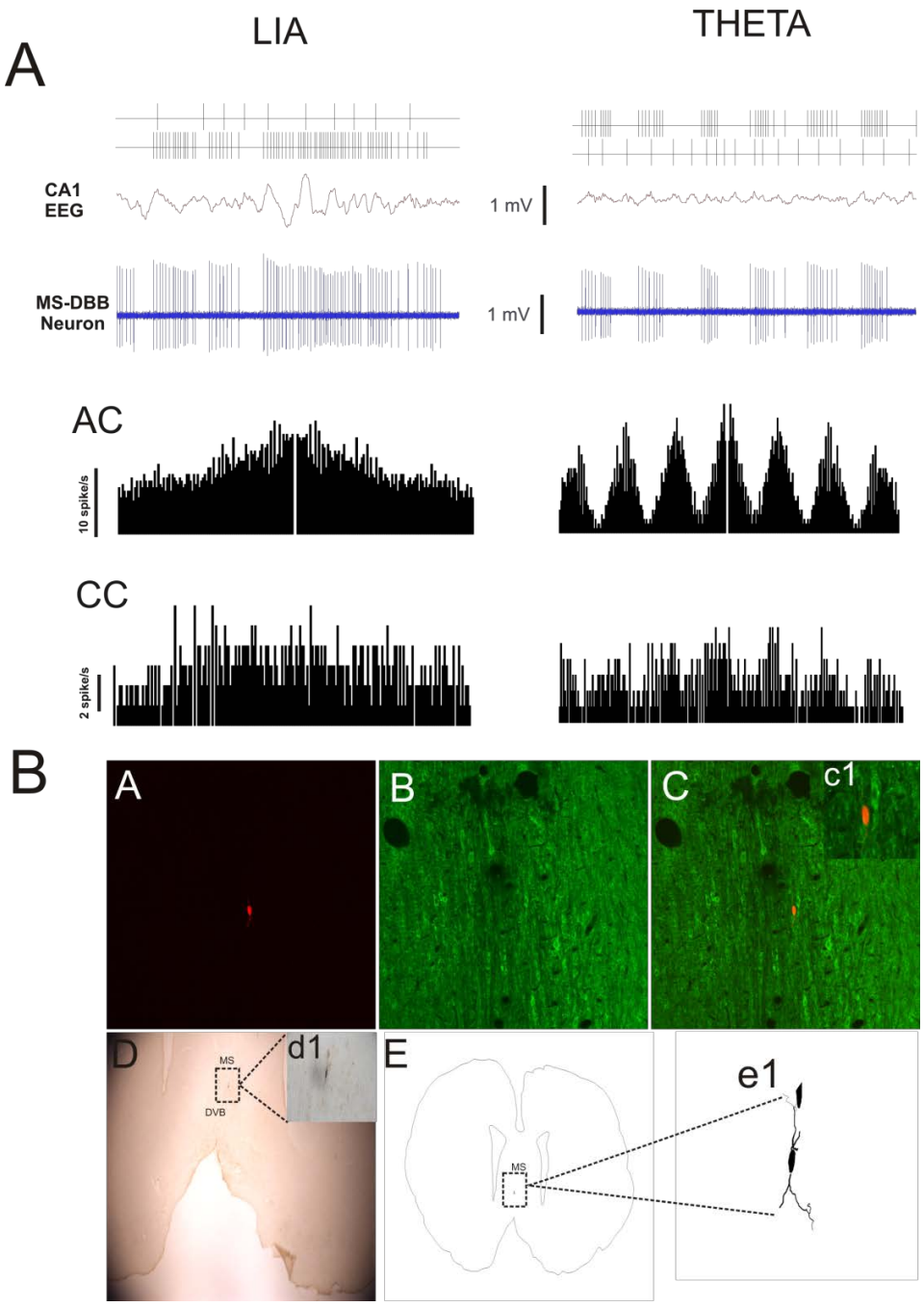

Figure 14

Squared sections of a juxta-cellular recording in the MS (blue) with simultaneously recorded hippocampal EEG activity (red) were analyzed during LIA and theta conditions. AC and CC histograms show the activity of one SON labeled neuron (A). The immuno-reactivity for the recorded cell B-A (Streptavidin red) and GAD 67 B-B (FITC green) does not overlap in B-C. The tissue was treated with DAB in order to visualize the location of the recorded cell in the MS-vDBB (B-D). Square is amplified to 40x (B-d1). Contour map of the slice containing the cell of interest and neuron tracing of the labeled cell is shown in B-E and B-e1, respectively.

The firing characteristic of SON cells, as previously mentioned, is to fire rhythmically only under hippocampal theta rhythm (Figure 14A). They do not follow consistent alignment with a particular phase of theta as CON cells do, but show common occurrences. Figure 15 shows the results for our recorded SON cell, whose mean firing was at 127° of theta wave.

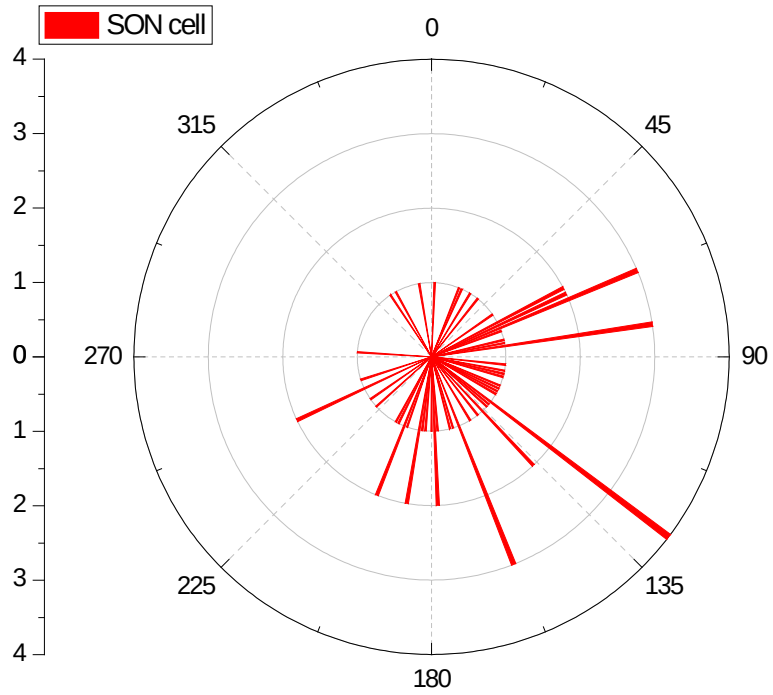

Figure 15

Circular distribution showing theta phase values, in degrees, of an MS-vDBB cell which only fired rhythmical bursts while hippocampal theta activity was present. In red are the average firing action potentials of the labeled SON cell. Note this cell's rhythmicity lies at several phases of the theta wave, but has a phase preference at 127°.

NON-NC

Our third recorded neuron fits the category of a NON-NC cell. The recording shows sporadic firing under LIA condition but correlated with hippocampal theta as seen in the AC and CC (Figure 16A). This cell fired arrhythmically and increased its firing frequency after inducing hippocampal theta. A Kruskal-Wallis ANOVA (LIA= 8 and theta = 10; Table 4) did not show a significant difference in duration, AP, burst frequency, or ISI. This cell was immuno-negative when tested with the parvalbumin GABAergic marker as demonstrated in Figure 16BA.

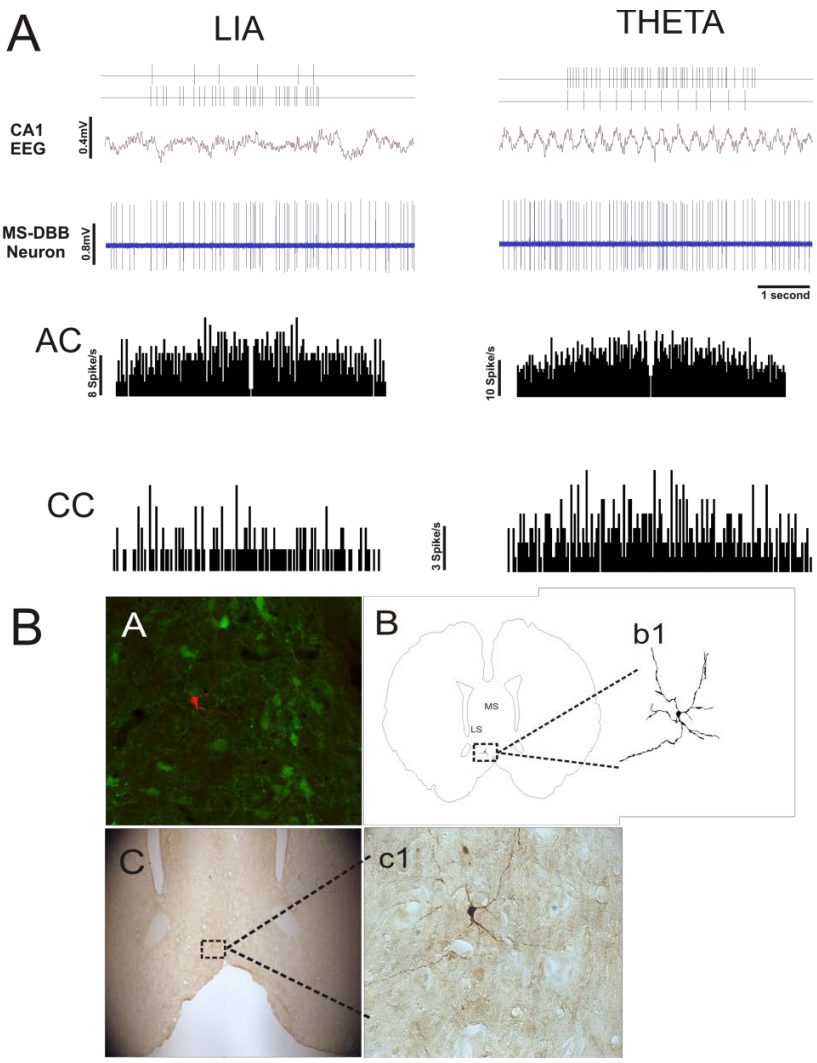

Figure 16

Juxta-cellular recording in the MS (blue) with simultaneously recorded hippocampal EEG activity (red) were analyzed during LIA and theta conditions. AC and CC histograms show the activity of one NON-NC labeled neuron. The immuno-reactivity for the recorded cell with Streptavidin red and PV (FITC green) are shown in B-A, which do not overlap. The contour map of the slice containing the labeled cell and neuron tracing is shown in B-B and B-b1, respectively. Tissue containing the recorded cell in the MS-vDBB was stained with DAB (B-C). The dashed square is amplified to 40x and shown in B-c1.

The firing tendency that characterizes a NON-NC cell in relation to theta phase, as opposed to the CON and SON, is that there is no particular inclination for a certain phase degree of theta wave (Figure 17).

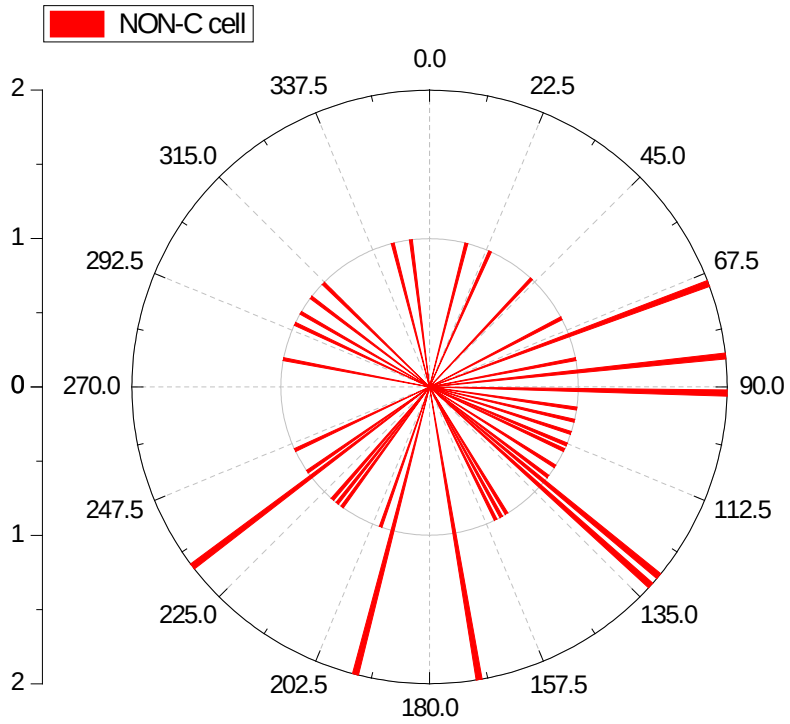

Figure 17

Circular distribution showing phase (degree) values of hippocampal theta activity in relation to an MS-vDBB NON-C cell. In red are the average firing action potentials of the labeled cell, which does not have a consistent phase within the theta wave.

Table 1

Branched Analysis of neurons stained and investigated

		Average Branched Analysis					
Samples	Category	Area $(\mu m)^2$	Perimeter (µm)	Max Trees	Avg Nodes	Avg Mean Length (µm)	Avg Total Length (µm)
Cell 1	**CON**	**103.183**	**38.9**	**9**	**1.4**	**111.26**	**227.46**
Cell 2	**SON**	153.254	49.7	7	0.588	46.612	64.33
Cell 3	**NON-C**	**286.256**	**109.9**	**17**	**0.666**	**163.75**	**214.733**

CHAPTER 5 – SUMMARY AND RECOMMENDATIONS

The goal of this study was to reveal the characteristics of neurons presumed to precede hippocampal theta activity in the MS-DBB, specifically the CON. Our results for the CON recorded cell in the MS-DBB supports our hypothesis that this cell is of GABAergic nature and whose activity influences the generation of hippocampal theta. We recommend expanding the number of CON cells recorded and testing more subclasses of GABAergic markers. It would be interesting to see if a CON cell is immunoreactive to more than one GABAergic marker.

The SON and NON-NC reported cells provide additional data to what was previously described in our lab, extending our knowledge of some of the properties of these categorized cells. More specifically, the SON, which we suggest plays a recruiting role to further encourage theta activation, confirming the existence of the reciprocal loop between the MS and hippocampal structures.

Unfortunately, our staining attempt to identify this as a GABAergic neuron was restricted by our use of GAD67. Perhaps another GABA marker will prove to be successful; therefore, we recommend additional testing on this particular cell. To broaden our knowledge of those cells previously described in our lab, and given that our NON-NC cell was immuno-negative to a GABA marker, we are interested in testing this particular cell with glutamatergic or cholinergic markers.

Bursting cells, such as CONs, are key for neural synchrony that essentially forms a population of neurons that have the capacity to encode memory. Knowing the order of events in which regions of the brain containing these cells send or receive messages, the type of cells that precede and influence others and the kind of cellular firing patterns that are capable of modifying certain outcomes allow us to recognize functional and dysfunctional behaviors and interactions. Optogenetics, a method in which specific neurons are reactivated in a light induced manner, is a recently used method that has been successful in mapping cellular populations bearing memory engrams. This is a more innovative technique in which cellular activity at the molecular level is manipulated to control memory expression. Being able to target specific neurons is an encouraging alternative in preventing or delaying pathology. Overall, detecting activity at the micro-level of pathology is a promising therapeutic approach in which electrical modulation can set a dysfunctional neural circuit back to a functional tempo. Our results contribute a minute gain in a vast amount of knowledge that lays ahead in preventative treatments for neurodegenerative disease such as AD.

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APPENDICES

Analysis of CON's Burst Frequency with simultaneous theta field activity

Table 2

Descriptive statistics

	LIA					
	N total	**Mean**	**SE of mean**	**Minimum**	**Median**	**Maximum**
Duration	11	0.17182	0.01704	0.098	0.162	0.277
AP	11	4.36364	0.38783	3	4	6
Frequency	11	25.8641	1.14478	19.055	24.439	30.824
ISI	11	0.04228	0.00195	0.03387	0.04268	0.05172

	Theta					
	N total	**Mean**	**SE of mean**	**Minimum**	**Median**	**Maximum**
Duration	18	0.12267	0.00467	0.095	0.124	0.163
AP	18	4	0.14003	3	4	5
Frequency	18	32.9838	1.0281	24.659	32.246	41.92
ISI	18	0.03026	0.00132	0.02302	0.03109	0.0422

Kruskal-Wallis ANOVA:

		N	**Min**	**Q1**	**Median**	**Q3**	**Max**
Duration	LIA	11	0.098	0.124	**0.162**	0.21	0.277
	Theta	18	0.095	0.105	**0.124**	0.134	0.163
AP	LIA	11	3	3	4	6	6
	Theta	18	3	4	4	4	5
Frequency	LIA	11	19.055	24.185	**24.439**	29.943	30.824
	Theta	18	24.659	29.943	**32.246**	37.429	41.92
ISI	LIA	11	0.03387	0.03557	**0.04268**	0.04855	0.05172
	Theta	18	0.02302	0.02398	**0.03109**	0.03448	0.0422

Test Statistics:

	Chi-Square	**DF**	**Prob>Chi-Square**
Duration	5.50056	1	0.01901
AP	0.33506	1	0.56269
Frequency	13.00295	1	3.11E-04
ISI	14.95984	1	1.10E-04

Analysis of SON's Burst Frequency with simultaneous theta field activity

Table 3

Descriptive statistics

LIA

	N total	Mean	SE of mean	Sum	Minimum	Median	Maximum
Duration	14	0.15007	0.00887	2.101	0.095	0.148	0.209
AP	14	3.35714	0.13289	47	3	3	4
Frequency	14	23.2915	1.52993	326.081	14.291	22.26	33.092
ISI	14	0.04542	0.00424	0.63585	0.02321	0.04381	0.08424

Theta

	N total	Mean	SE of mean	Sum	Minimum	Median	Maximum
Duration	10	0.2308	0.03213	2.308	0.134	0.191	0.42
AP	10	5	0.33333	50	3	5	7
Frequency	10	24.4774	2.86106	244.774	13.1	22.8035	37.428
ISI	10	0.04663	0.00646	0.46632	0.02014	0.0444	0.08239

Kruskal-Wallis ANOVA

		N	Min	Q1	Median	Q3	Max
Duration	LIA	14	0.095	0.12175	**0.148**	0.1735	0.209
	Theta	10	0.134	0.1535	**0.191**	0.303	0.42
AP	LIA	14	3	3	**3**	4	4
	Theta	10	3	4.75	**5**	5.25	7
Frequency	LIA	14	14.291	18.494	22.26	28.446	33.092
	Theta	10	13.1	16.4348	22.8035	32.6815	37.428
ISI	LIA	14	0.02321	0.03465	0.04381	0.05271	0.08424
	Theta	10	0.02014	0.0303	0.0444	0.06362	0.08239

Test Statistics

	Chi-Square	DF	Prob>Chi-Square
Duration	5.09529	1	0.02399
AP	12.84627	1	3.38E-04
Frequency	0.00343	1	0.95328
ISI	0	1	1

Analysis of NON-NC's Burst Frequency with simultaneous theta field activity

Table 4

Descriptive statistics

LIA

	N total	Mean	SE of mean	Minimum	Median	Maximum
Duration	8	0.294	0.03981	0.144	0.286	0.487
AP	8	4.375	0.625	2	4	7
Frequency	8	15.17738	1.33491	11.031	14.4195	20.96
ISI	8	0.06995	0.0077	0.04282	0.06689	0.10466

Theta

	N total	Mean	SE of mean	Minimum	Median	Maximum
Duration	10	0.2949	0.02542	0.143	0.2905	0.405
AP	10	5.2	0.41633	3	5	7
Frequency	10	18.0424	1.0868	13.789	17.944	25.152
ISI	10	0.05927	0.00447	0.03496	0.05897	0.07768

Kruskal-Wallis ANOVA:

		N	Min	Q1	Median	Q3	Max
Duration	LIA	8	0.144	0.186	0.286	0.36575	0.487
	Theta	10	0.143	0.23575	0.2905	0.3665	0.405
AP	LIA	8	2	3	4	6	7
	Theta	10	3	4	5	6.25	7
Frequency	LIA	8	11.031	11.81525	14.4195	19.296	20.96
	Theta	10	13.789	14.53	17.944	19.9775	25.152
ISI	LIA	8	0.04282	0.05127	0.06689	0.09118	0.10466
	Theta	10	0.03496	0.04958	0.05897	0.07445	0.07768

Test Statistics

	Chi-Square	DF	Prob>Chi-Square
Duration	0.00198	1	0.96455
AP	1.17567	1	0.27824
Frequency	2.56319	1	0.10938
ISI	0.78947	1	0.37426