THE DEVELOPMENT OF BURSTING NETWORKS FOLLOWING CHEMICAL LONG TERM POTENTIATION

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By

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ABSTRACT

Collective rhythmic dynamics from neurons are vital for cognitive functions but how neurons self-organize to produce such activity is not well understood. Attractor-based computational models are a theoretical framework for memory storage in networks of neurons. Additionally, activity-dependent modification of synaptic transmission is thought to be the physiological basis of learning and memory referred to as long term potentiation (LTP). The goal of this study is to demonstrate that using a pharmacological treatment, which has been shown to increase synaptic strength, on in vitro networks of hippocampal neurons, follows the dynamical postulates theorized by attractor models. I use a grid of extracellular electrodes to study changes in network activity during this perturbation (induction phase of LTP) and then monitor the evolving network activity following the removal of the treatment (maintenance phase of LTP). Following a chemically-induced LTP (cLTP) there was an initial increase in activity. Phase plots indicated a conserved activity pattern suggesting that the network was initially operating in a stable dynamical state. This attractor state was not preserved during later time points, with an induction of superbursting and an incidence of theta frequency activity. Finally, activity 5 days post-treatment suggested a homeostatic mechanism to prevent runaway excitation.

Next I hypothesized that matrix metalloproteinases (MMPs), zinc-dependent endopeptidases that are released from neurons in an activity dependent manner, drive these changes in the attractor state. These changes may be associated with increased synaptic glutamate receptor incorporation, and an increased amplitude and/or frequency of α-amino-3-
hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) mini excitatory post-synaptic currents (EPSCs). While the mechanism(s) by which MMPs may influence synaptic structure and function are not completely understood, MMP dependent shedding of specific cell adhesion molecules (CAMs) could play an important role. In this study, cLTP evoked changes in activity and spatiotemporal dynamics were altered by MMP inhibition. These changes were also dependent on β1 integrin. I also showed that the ectodomain of ICAM-5 can stimulate β1 integrin dependent increases in spike counts and burst number. These results support the possibility that MMP dependent shedding of specific synaptic CAMs can contribute to these effects.
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<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>General Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Specific Aim 1</td>
<td>13</td>
</tr>
<tr>
<td>Introduction to Specific Aim 1</td>
<td>13</td>
</tr>
<tr>
<td>Material and Methods for Specific Aim 1</td>
<td>16</td>
</tr>
<tr>
<td>Results of Specific Aim 1</td>
<td>21</td>
</tr>
<tr>
<td>Figures for Specific Aim 1</td>
<td>26</td>
</tr>
<tr>
<td>Discussion of Specific Aim 1</td>
<td>39</td>
</tr>
<tr>
<td>Specific Aim 2</td>
<td>43</td>
</tr>
<tr>
<td>Introduction to Specific Aim 2</td>
<td>43</td>
</tr>
<tr>
<td>Material and Methods for Specific Aim 2</td>
<td>44</td>
</tr>
<tr>
<td>Results of Specific Aim 2</td>
<td>48</td>
</tr>
<tr>
<td>Figures for Specific Aim 2</td>
<td>52</td>
</tr>
<tr>
<td>Discussion of Specific Aim 2</td>
<td>60</td>
</tr>
<tr>
<td>Specific Aim 3</td>
<td>63</td>
</tr>
<tr>
<td>Introduction to Specific Aim 3</td>
<td>63</td>
</tr>
<tr>
<td>Material and Methods for Specific Aim 3</td>
<td>65</td>
</tr>
<tr>
<td>Results of Specific Aim 3</td>
<td>68</td>
</tr>
<tr>
<td>Figures for Specific Aim 3</td>
<td>71</td>
</tr>
<tr>
<td>Discussion of Specific Aim 3</td>
<td>79</td>
</tr>
<tr>
<td>Specific Aim 4</td>
<td>82</td>
</tr>
<tr>
<td>Introduction to Specific Aim 4</td>
<td>82</td>
</tr>
<tr>
<td>Material and Methods for Specific Aim 4</td>
<td>85</td>
</tr>
<tr>
<td>Results of Specific Aim 4</td>
<td>89</td>
</tr>
<tr>
<td>Figures for Specific Aim 4</td>
<td>95</td>
</tr>
<tr>
<td>Discussion of Specific Aim 4</td>
<td>101</td>
</tr>
<tr>
<td>General Discussion</td>
<td>106</td>
</tr>
<tr>
<td>References</td>
<td>113</td>
</tr>
</tbody>
</table>
List of Figures

1-1......................................................................................................................... 26
1-2......................................................................................................................... 27
1-3......................................................................................................................... 28
1-4......................................................................................................................... 29
1-5......................................................................................................................... 30
1-6......................................................................................................................... 31
1-7......................................................................................................................... 32
1-8......................................................................................................................... 33
1-9......................................................................................................................... 34
1-10......................................................................................................................... 35
1-11......................................................................................................................... 36
1-12......................................................................................................................... 38
2-1.......................................................................................................................... 52
2-2.......................................................................................................................... 53
2-3.......................................................................................................................... 54
2-4.......................................................................................................................... 55
2-5.......................................................................................................................... 56
2-6.......................................................................................................................... 57
2-7.......................................................................................................................... 58
2-8.......................................................................................................................... 59
3-1.......................................................................................................................... 71
3-2.......................................................................................................................... 72
3-3.......................................................................................................................... 73
3-4.......................................................................................................................... 74
3-5.......................................................................................................................... 75
3-6.......................................................................................................................... 76
3-7.......................................................................................................................... 77
3-8.......................................................................................................................... 78
4-1.......................................................................................................................... 95
4-2.......................................................................................................................... 96
4-3.......................................................................................................................... 97
4-4.......................................................................................................................... 98
4-5.......................................................................................................................... 99
4-6.......................................................................................................................... 100
List of Tables

1-1 ......................................................................................................................... 37
**General Introduction:**

**Memory and the Hippocampus:**

On September 1, 1953, Henry Gustav Molaison (who would become known to the world as H.M.) underwent a bilateral medial temporal lobe (MTL) resection. This experimental procedure was carried out in an attempt to alleviate a progressively worsening epilepsy that had not responded to anticonvulsant medication available at the time and had left the patient unable to hold a job. The resulting lesion would encompass a region "extending posteriorly for a distance of 8 cm from the midpoints of the tips of the temporal lobes, with the temporal horns constituting the lateral edges of the resection" (Scoville and Milner, 1957). During recovery from surgery, there was a documented, severe loss of memory. This disruption in memory would last until the patient's death.

On April 26, 1955, H.M. was first examined by William Scoville and Brenda Milner. Per their findings: "The memory defect was immediately apparent. The patient gave the date as March, 1953, and his age as 27. In conversation, he reverted constantly to boyhood events and seemed scarcely to realize that he had had an operation." During their examination, Scoville and Milner noted a good general intelligence, and reported an IQ of 112 on the Wechsler-Bellevue Intelligence Scale (compared to a preoperative IQ of 104 obtained in 1953). Additional testing also revealed no deficits in perception, abstract thinking, reasoning, personality or motivation. In contrast, H.M. scored below average on the Wechsler Memory scale and failed to improve with practice. In addition, they reported that "the patient appear[ed] to have a complete loss of memory for events subsequent to [his operation]... together with retrograde amnesia for the three years leading up to his operation." His earlier memories, however, appeared normal.

From these initial examinations, Scoville and Milner concluded that the anterior hippocampus and/or hippocampal gyrus were critical for the "retention of current experiences," although they could not rule out contributions from surrounding areas including the amygdala (Scoville and Milner, 1957).
During the ensuing decades leading up to his death, hundreds of studies were performed on H.M. From the findings of such studies several important principles of how memory is stored were developed, and extensively tested. For example, it was discovered that despite his impairment in declarative (facts/events) memory, H.M. could still learn new motor skills which led to an acceptance of multiple types of memory. In addition, H.M. demonstrated the capacity for sustained attention and retention of information for a brief period of time, which suggested that the MTL was not required for 'immediate memory,' which would later be termed 'working memory' (Squire and Wixted, 2011). Instead, H.M. struggled with synthesizing the immediate memory in a way that could become long term memory.

Beyond H.M., additional patient lesions studies have also provided evidence that bilateral injury to the medial temporal lobe is associated with impairments in memory function particularly declarative memory, but not implicit memory (Scoville and Milner, 1957; Duzel et al., 2001). Since the discovery of H.M.'s impaired memory, scientists have tried to determine which structural components of the MTL are necessary and sufficient to contribute to memory. Studies with ischemic patients who suffer lesions limited to the hippocampus have been used to specifically target the functionality of the hippocampus in memory. One such patient, identified as R.B., was found to have intact cognitive ability with the exception of impairment in declarative memory (Zola-Morgan et al., 1986) although to a lesser degree than that of patients who, like H.M., sustained a bilateral lesion of the MTL (Scoville and Milner, 1957). Other patients with mild hypoxia that led to severe hippocampal injury, but in whom the surrounding brain regions remained intact, also demonstrated a disruption in declarative memory (Duzel et al., 2001; Yonelinas et al., 2002; Aggleton et al., 2005) supporting the previous work.

While such human studies provided the groundwork for the study of memory, their inability to control the localization and extent of lesions limited how effective the approach is to elucidate the exact role of specific neural substrates. Accordingly, the use of animal models has
provided additional, important insights into the neural circuitry guiding memory function. However, as items traditionally associated with declarative memory (names, dates, etc.) would not be testable in an animal model, measures of 'recognition' were developed as an equivalent. For example, these tasks were designed to measure an animal's ability to remember or recall specific stimuli such as objects, odors, tones, or light cues and their responses scored using measures such as preference towards novelty.

The role of the hippocampus in memory has been extensively tested in the non-human primate. The traditional non-human primate model to test recognition memory is the Delay Non Match to Sample (DNMS) paradigm. During testing, the subject would be presented with a sample object then undergo a set delay time after which, it would be presented with the same object and a novel object. The subject would then be trained to choose the novel object to receive a reward taking advantage of the monkey's natural inclination to select novelty over familiarity. Following ablations of the MTL, subjects do not exhibit impairments in performance with short delays on DNMS task (Mishkin, 1978), however, there was a progressively impaired performance on DNMS with increased delays.

Because the MTL is comprised of many structures, several additional studies attempted to determine which specific structures within the MTL were necessary for recognition memory in monkeys. Early studies involving ablations of the hippocampus and the amygdala concluded that the ablations led to impairment in recognition memory (Mishkin, 1978; Zola-Morgan et al., 1982; Murray and Mishkin, 1984; Saunders et al., 1984). However, in these studies, regions located ventral to the hippocampus (i.e. perirhinal and parahippocampal cortical regions) were also ablated. It was later discovered that excitotoxic lesions specific to the hippocampus and amygdala leave recognition memory intact, while specific lesions to (and inactivation of) the perirhinal cortex impair recognition memory suggesting that the rhinal cortex is sufficient and necessary to maintain recognition (Murray and Mishkin, 1998). To date, while
the human studies suggest a role for the hippocampus in recognition memory, the animal literature is still inconclusive about the role of the hippocampus in recognition memory.

While hippocampal lesions without damage of surrounding cortices have been ineffective in inducing memory impairment using DMNS as a probe in monkeys, other memory tasks have shown deficits with hippocampal lesions. For example, the Visual Paired Comparison (VPC) task has also been tested on hippocampal lesioned monkeys. During the task, subjects would be presented a sample picture and then after a delay shown the same picture paired with a novel one. Because primates are naturally drawn toward novelty, recognition memory would be assumed when the subject looks at the novel picture longer than the sample picture (Baxter and Murray, 2001; Bachevalier et al., 2002; Nemanic et al., 2004). A possible explanation for the observed differences of hippocampal ablation on performance on VPC and DMNS paradigms could be due to the reward component in DMNS, i.e., the reinforcement is sufficient to engage other neural circuits to guide appropriate behavior such as the amygdala or prefrontal cortex.

Studies of the role of the hippocampus in memory have been carried out in rodents as well. In rodents, the hippocampus has been demonstrated to be necessary for learning spatial navigation tasks such as the Morris Water Maze and Radial Arm Maze (Jarrard and Elmes, 1982; Morris et al., 1982). In addition, the hippocampus has been shown to be involved in recognition memory (Bunsey and Eichenbaum, 1996) similar to what has been demonstrated in humans. Furthermore, in rodents, manipulations of some receptors or proteins necessary for synaptic signals of learning and memory (described below) in the hippocampus disrupt performance on hippocampal-dependent memory tasks (Meighan et al., 2006). While considerable progress was made in in vivo models of learning and memory, advancing methodological techniques allowed researchers to complement these approaches and study learning and memory at the cellular level.
Molecular Substrates of Memory:

In the late 1800s, Santiago Ramon y Cajal, utilizing the recently developed Golgi staining technique, suggested that neurons did not directly contact each other, but rather communicated via specialized junctions. It was changes within these junctions, Ramon y Cajal further speculated, where the cellular level of learning and memory took place (Gluck et al., 2008). Over the next 60 years, however, these speculations were not widely accepted and few experiments were directed toward studying and expounding upon this concept (Bailey et al., 2000).

With the advent of new intracellular recording techniques by the 1940s and '50s, there was a renewed interest in and a refinement of the concepts of learning and memory proposed by Ramon y Cajal. This would eventually lead to the development of the field of synaptic plasticity, which would become one of the most widely studied subjects in neuroscience. Initially, the theories of synaptic plasticity were divided into two broad categories: homosynaptic plasticity and heterosynaptic plasticity (Bailey et al., 2000). Homosynaptic plasticity refers to the concept that a synapse could change efficacy as a direct result of previous stimulating activity, originating from the pre- or post-synaptic neuron. Examples of homosynaptic plasticity include synaptic potentiation, depression and facilitation. In contrast, heterosynaptic plasticity involves changes in synapses as a result of changes in other neuronal pathways, more remote than those of homosynaptic plasticity (Emmert-Streib, 2008).

A significant milestone in the study of plasticity would come in 1949, when Donald Hebb published the seminal book, "The organization of behavior: a neuropsychological theory." In this book, Hebb proposed a homosynaptic rule for memory based on the concept that "neurons that fire together, wire together" (Hebb, 1949) Put another way, Hebb postulated that given two neurons, A and B, should A and B fire often within a short period of time, the synapse between them would become strengthened, increasing the probability of A and B firing together in the future. Hebb also proposed that this increased probability could last for a long period of time.

5
(Gluck et al., 2008). In addition, Hebb hypothesized that synaptic plasticity was input-specific, meaning that when a pair of neurons fire together within a short period of time, only the synapse or synapses between them, but not other synapses on either neuron, would strengthen (Hebb, 1949; Bailey et al., 2000).

In the 1960s, Lomo reported experimental evidence supporting Hebb's theories (Lomo, 1966). In his experiments, Lomo first identified a pair of connected neurons, then stimulated the presynaptic neuron and recorded the response from the postsynaptic neuron. There was a closely correlated response of the postsynaptic neuron relative to the frequency of the applied stimulation to the presynaptic neuron. Lomo noted, however, that after high-frequency stimulation of the presynaptic neuron, there was a change in the postsynaptic neuron such that it would "over-respond" to a low-frequency stimulation and that this change could last for hours or days (Lomo, 1966; Bliss and Lomo, 1970; Bliss and Gardner-Medwin, 1971, 1973). Although the earliest studies were carried out using neurons from the hippocampi of rabbits, later studies would demonstrate these principles applied to synapses between neurons from many brain regions and species (Shors and Matzel, 1997).

In the 1960s and '70s, a second, complementary, heterosynaptic model for synaptic plasticity was proposed based on the observation that a synapse could be strengthened without activity in either the pre- or post-synaptic neuron. This suggested that such a given synapse could be strengthened due to the activity of a third, modulatory neuron. Subsequent studies in the Aplysia and crayfish, as well as in the mammalian spinal cord and hippocampus would corroborate this concept [see the review of (Bailey et al., 2000)]. The relative contributions for both homosynaptic and heterosynaptic plasticity in learning and memory, however, remains a subject of study.

Since the initial observations and proposals of Hebb, synaptic plasticity continues to be considered vital for the segregation and integration of cognitive processes such as learning and memory. Neurons and the intricate circuitry networks of neurons form must have the capacity to
respond and adapt to external perturbations. This capacity would allow circuits to modulate their activity, a process essential for efficient transmission of information. However, the ability of networks to tune their outputs to respond to a wide range of sensory inputs does not negate the constraint that they must maintain a homeostatic state. Excessive network activity may lead to epileptiform activity, whereas insufficient activity may inhibit the network from performing necessary functions and depress potentiation.

This ability of neural systems to modulate their response is influenced by a variety of mechanisms including changes in intra- and extracellular ionic concentrations as well as time-dependent fluctuations in the concentrations of neurotransmitter(s) released at the synaptic cleft. Responses are also modulated by changes in synaptic structure that can allow for, or even promote, an increase in the abundance of post-synaptic neurotransmitter receptors. This type of change has been observed in association with learning and memory as well as the phenomenon of long-term potentiation [LTP; (Bliss and Lomo, 1970; Madison et al., 1991; Bliss and Collingridge, 1993; Bi and Poo, 1998)].

LTP is characterized by the measurable increase in synaptic efficacy between neurons. It can be induced via electrical or chemical stimulation and has been shown to last from several hours to many days (Bolshakov et al., 1997; Payne, 2008). While LTP is dependent on N-methyl-D-aspartate (NMDA) receptor activity, mechanisms that have been shown to underlie LTP involve increases in dendritic spine size and an associated number of 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptors (Nicoll, 2003; Kerchner and Nicoll, 2008). This phenomenon has been well studied between pairs of neurons within the hippocampus, specifically on synapses between the Schaffer collateral axons and apical dendrites of the CA1 pyramidal neurons (Frey et al., 1993; Remondes and Schuman, 2002; Enoki et al., 2009). This is a common neural region of LTP investigation as LTP is most reliably evoked in brain areas known to play a role in memory and learning.
While LTP is well documented to occur in brain regions associated with learning and memory, a direct association between the two has not been proven. To date, the link between LTP and learning and memory is most supported by data that pharmacological agents, as well as genetic manipulations that disrupt LTP processes *in vitro* will also impair measures of learning and memory (Gluck et al., 2008). Furthermore, in different animal model, genetic manipulations that increased LTP score higher on memory tasks compared to control subjects (Gluck et al., 2008).

In addition to studying the role of excitatory, glutamatergic (AMPA and NMDA) transmission, other factors necessary for LTP have been studied. For example, there is a correlation between the activity of a family of zinc dependent endoproteases (matrix metalloproteinase; MMP) and an increase in both LTP and hippocampal-dependent memory (Fragkouli et al.; Meighan et al., 2006; Nagy et al., 2006; Brown et al., 2007; Wang et al., 2008). While the exact mechanism(s) of actions of MMPs that would lead to these results is unclear, at least one MMP has been shown to stimulate increases in the size of dendritic spines (Wang et al., 2008). There is a strong correlation between size of the spine head and strength of the synapse, presumably in part because a larger spine allows for insertion of more glutamate receptors (Tada and Sheng, 2006). In addition to MMPs, learning-related plasticity is thought to be dependent on some postsynaptic integrin receptors (Kramar et al., 2006; Meighan et al., 2006; Nagy et al., 2006). While exact roles for MMPs and integrins in LTP are not well defined, recent experiments suggest that soluble ICAM-5, which is released into the synapse with MMP activity, can stimulate integrin-dependent phosphorylation of cofilin, and induce dendritic actin polymerization (Conant et al., 2011).

In contrast, the impact on network dynamics via the synaptic modifications modulated by LTP has not been well studied. Elucidating network effects of LTP is important as it is widely believed that the collective activity from neural assemblies contributes to cognition and attention (Buzsaki and Draguhn, 2004; Tiesinga et al., 2004b, a; Robbe et al., 2006; Montgomery et al.,
This therefore leads to the hypothesis that synaptic strengthening that occurs with LTP would have significant network-wide effects.

**Studying Neuronal Networks *In Vitro***:

While *in vivo* measurements provide a direct approach to studying physiological dynamics, it is difficult to visualize individual neurons and record single unit electrical activity from *in vivo* three-dimensional networks of neurons. Networks of cultured neurons, in contrast, are a reduced, two-dimensional experimental system that may provide insights into basic dynamical network interactions not currently achievable in complex *in vivo* brain preparations. In addition, cultured networks grant the ability to ask basic questions on a reduced system while preserving features of an *in vivo* model as it uses real neurons with their rich connectivity and complex patterns of activity.

The multi-electrode array (MEA) is an 8x8 grid of extracellular electrodes that measures changes in electrical potential, and allows for the recording of action potentials from nearby units. In addition, MEAs allow for simultaneous recordings to be obtained from many cells. Recordings can be acquired from cultures that are maintained for long periods of time, as well as cultures treated with stimuli that are difficult to administer *in vivo*. In addition, the MEA can be used to record electrical activity from slice preparations. Accordingly, MEAs are widely used to characterize dynamical activity from *in vitro* networks of neurons (Segev et al., 2004; Wagenaar et al., 2006b; Vajda et al., 2008; Chen and Dzakpasu, 2010; Stegenga et al., 2010). In addition, MEA studies that implement electrical stimulation protocols on *in vitro* networks of either hippocampal or cortical neurons have been established and demonstrate precedence of an *in vitro* learning paradigm (Jimbo et al., 1999; Shahaf and Marom, 2001; Madhavan et al., 2007; Chiappalone et al., 2008; Ide et al., 2010).

Chemical LTP is a commonly used method to induce the potentiation in a large fraction of synapses within *in vitro* preparations of neuronal networks without direct synaptic stimulation
(Otmakhov et al., 2004), and, when applied to networks of cultured neurons, the need for electrical stimulation is eliminated. Chemical LTP treatments have been shown to activate various biochemical pathways that in turn activate NMDA receptors (Cho et al., 2008) and are a useful technique to target large neural populations. This paradigm provides an advantage over inducing potentiation via high frequency electrical stimulation between two neurons as it can increase the probability of neuronal spike generation in a larger number of neurons within the cultured neuronal network.

There exists multiple pharmacological treatments that are utilized to study LTP such as treatments with glutamate, glycine, or potassium chloride. In the experiments described in this study, however, chemically-induced LTP occurred due to increases in intracellular cyclic adenosine monophosphate (cAMP) levels following an application of the adenylyl cyclase activator, forskolin and the phosphodiesterase inhibitor, rolipram. This protocol has been utilized previously in in vivo preparations (Grey and Burrell, 2008) and in slices (Otmakhov et al., 2004) and is believed to lead to the activation of protein kinase A (Cho et al., 2008) and, in turn, increase AMPA receptor density (Barad et al., 1998; Otmakhov et al., 2004; Oh et al., 2006).

Manipulating levels of cAMP to study LTP has merit in the context of understanding molecular processes underlying learning and memory. A role for cAMP signaling in regulating synaptic plasticity and memory formation has been established in multiple animal models (Nguyen and Woo, 2003; Abel and Nguyen, 2008; Lee et al., 2008; Zhang et al., 2011). In addition, double knockout mice lacking the genes for adenylyl cyclase (AC) 1 and 8, the major calcium-stimulated ACs of the brain, demonstrated significant impairment in LTP in vitro and deficiencies in hippocampus-dependent memory tasks in vivo (Shan et al., 2008; Zhang et al., 2011). In contrast, mice with increased AC activity demonstrated enhancements in LTP and memory (Wang et al., 2004).
The Modeling of Memory Dynamics

While in vitro examinations of neuronal networks preserve elements of in vivo models since they utilize living, biological units, i.e., neurons, the reduced model also makes such studies very relatable to computational experiments. Indeed, concurrent experiments in both in vitro cultures and computational models provide a useful tandem of techniques. Therefore, an important goal of this study was to combine the approaches of biological experimentation and computational modeling, to assess whether a synaptic perturbation that is thought to underlie the physiological basis of memory, i.e., LTP, is characterized on the network level by a theoretical postulate of memory. For example, it is proposed that memory storage in multiple brain regions including the hippocampus, operate under the principles of attractor dynamics. An accepted definition of an attractor is a closed set, A, of a dynamic system where any trajectory that starts in A remains within A (Strogatz, 2001). Importantly, there exists a magnitude of an external perturbation that would be capable of taking the system out of an attractor state (Samsonovich and McNaughton, 1997).

Within the field of computational neuroscience, attractors are comprised of highly interconnected networks of neurons. Previous experimental results support the presence of attractors in vivo using hippocampal-dependent memory tasks (Wills et al., 2005; Jezek et al., 2011). As it is believed that the dynamical correlate of working memory is the attractor state (Hopfield, 1982; Amit et al., 1987; Amit and Brunel, 1997; Durstewitz et al., 2000; Wang, 2001; Mongillo et al., 2003), attractor dynamics have been incorporated into studies using computational models of memory. Modifications to the connections between neurons of these networks resulted in lasting increases in firing rates, reminiscent of experimental results of LTP. These results support the strategy of studying effects of LTP computationally within the constraints of attractor dynamics.

Introduction to Thesis Project:
In the present project I have used MEAs to record activity of neuronal networks derived from hippocampal cultures with the goal of better understanding how LTP influences neuronal network dynamics. Accordingly, I split this overall theme into several specific aims. The first specific aim is to characterize the short-term, dynamical effects of cLTP during a short period of continuous exposure to cLTP reagents on measures of network activity described below. Next, I sought to examine long-term, dynamical effects of cLTP by characterizing changes in network activity hours and days following treatment. To avoid potential excitotoxicity issues while characterizing the long timescale effects of cLTP, I removed the cLTP reagents after a brief treatment period. Therefore, an additional investigation of the effect of the removal of reagents was warranted. Results obtained in the pursuit of these specific aims suggested a significant shift in network activity patterns. To investigate this change in activity, I added a final specific aim attempting to determine a mechanism behind these results, including examining the potential roles for both MMPs and β1 integrin. In addition, through scientific collaborations, I sought to corroborate and expound upon certain experimental results with computational simulations throughout this project.
**Specific Aim 1:**

Determine the acute effects on network activity induced by chemical LTP treatment.

**Introduction to Specific Aim 1:**

A major focus in dynamical neuroscience is identifying neural patterns of activity that might characterize human behavior. For example, it is believed that organized network activity that would be characterized by highly synchronized depolarization is critical to cognitive processes such as attention and memory consolidation (Buzsaki and Draguhn, 2004; Tiesinga et al., 2004b, a; Robbe et al., 2006; Montgomery et al., 2008; Benchenane et al., 2011). How neurons encode and store information regarding the diversity of features in the environment and how the resulting range of dynamical responses within the temporal lobe that was discussed in the General Introduction can be interpreted in response to external stimuli are some of the fundamental questions currently under investigation. However, an equally important question is how are neurons able to organize into clusters and/or assemblies of coherent activity. Such organized clusters of neuronal activity are believed to represent patterns that define different features of the external environment. In addition, the activity of units within such clusters might change to reflect different environmental elements.

While exactly how neural signals cluster or self-organize within a circuit when presented with a given stimulus is largely unknown, it is believed that the timing of activity between neurons and/or neuronal assemblies is involved. When network activity is modulated in response to a given stimulus, this new activity pattern may need to be stored, and the 'memory' of this activity pattern may need to be created for future use. This paradigm of a stimulus-dependent persistence in a neural activity pattern is believed to underlie working memory (Hebb, 1949; Wang, 2001; McNaughton et al., 2006). This learning rule, first postulated by Hebb, stipulates that persistent activity is characterized by strong recurrent activity of excitatory
connections between neurons (Hebb, 1949; Durstewitz et al., 2000). In addition, the self-organization of activity displayed by such pairs of neurons could also account for, the "delay between stimulation and response, that seems so characteristic of thought" (Hebb, 1949). This neural correlate of memory has been incorporated into computational models of information storage.

In addition, it is believed that the dynamical correlate of working memory is the attractor state (Hopfield, 1982; Amit et al., 1987; Amit and Brunel, 1997; Durstewitz et al., 2000; Wang, 2001; Mongillo et al., 2003). Attractor models consist of highly interconnected networks of neurons and are believed to be capable of information storage. Increasing the efficacy of the connections between units, which represent synapses within simulated networks, results in a persistent increase in firing rate. In addition, experimental studies support the presence of attractors during hippocampal-dependent memory tasks in vivo (Wills et al., 2005; Jezek et al., 2011). These data led me to ask whether similar patterns of activity might be retained in networks of hippocampal neurons in the absence of an intact anatomical architecture. In my experiments to investigate this question, I assessed the impact on network dynamics of applying a pharmacological treatment that modulates the strength of excitatory synapses.

Changes in network activity can induce changes in the density of the 2-amino-3-(5-methyl-3-oxo-1, 2-oxazol-4-yl) propanoic acid (AMPA) glutamate receptor subunits that are present on the spines of excitatory synapses (Nicoll, 2003; Kerchner and Nicoll, 2008). Such perturbations can influence action potential probability and the resulting firing rate within a network of neurons. These types of synaptic modulations have been observed in association with learning and memory and are thought to underlie the neural substrate of memory known as long-term potentiation [LTP; (Bliss and Lomo, 1970; Madison et al., 1991; Bliss and Collingridge, 1993; Bi and Poo, 1998)]. LTP results from an increase in synaptic efficacy between neurons and can be induced via high frequency electrical stimulation between pairs of neurons, or via chemical stimulation. Effects of LTP have been shown to last from several
hours to many days (Bolshakov et al., 1997; Payne, 2008). Importantly, if a population of neurons is subjected to this modification, they will self-organize and cluster into active assemblies of elevated activity. If such organization of activity persists, these assemblies might exhibit attractor dynamics.

LTP has been well studied between pairs of neurons within the hippocampus, including in synapses between the Schaffer collateral axons and apical dendrites of the CA1 pyramidal neurons (Frey et al., 1993; Remondes and Schuman, 2002; Enoki et al., 2009). However, the impact on network dynamics due to the synaptic modifications resulting from LTP induction has not been widely studied in experimental networks. In addition, computational models have successfully incorporated the attractor paradigm as a mechanism through which information storage can be reliable invoked. For these reasons, the goal of my experiments and our computational modeling was to assess whether a synaptic perturbation that is thought to underlie the physiological basis of memory is characterized on the network level by the theoretical postulates of memory.

Consequently, this chapter reports on the temporal network activity that arises when a pharmacological paradigm of LTP, chemical LTP (cLTP), is introduced in cultured hippocampal neurons. Specifically, following treatment with the pharmacological agents forskolin and rolipram, a large fraction of synapses in the network (Otmakhov et al., 2004) are potentiated via a detailed biochemical pathway that is believed to increase the AMPA receptor density (Barad et al., 1998; Otmakhov et al., 2004; Oh et al., 2006). The high percentage of synapses affected by this paradigm provides an advantage to potentiation via high frequency electrical stimulation between two neurons and is therefore a useful technique to facilitate synaptic potentiation in a large population of neurons such as that of a cultured neural network. We also constructed a computational model consisting of biologically plausible neurons found in the hippocampus at similar ratios, to assess whether manipulation of AMPA receptor density can account for the dynamical effects recorded in the experiments.
Experimentally, I used an array of extracellular electrodes, a multi-electrode array (MEA), to record spontaneous electrical activity of networks of hippocampal neurons prior to, and following LTP. MEAs have been widely used to characterize similar changes in dynamical activity from in vitro networks of neurons (Segev et al., 2004; Wagenaar et al., 2006b; Vajda et al., 2008; Chen and Dzakpasu, 2010; Stegenga et al., 2010). In addition, previous MEA studies have implemented electrical stimulation protocols on in vitro networks of either hippocampal or cortical neurons and results demonstrate precedence for an in vitro learning paradigm (Jimbo et al., 1999; Shahaf and Marom, 2001; Madhavan et al., 2007; Chiappalone et al., 2008; Ide et al., 2010). Lastly, an important temporal activity pattern found within developing in vivo circuits is the widespread prevalence of bursting activity (O'Donovan, 1999; Ben-Ari, 2001; Blankenship and Feller, 2010). Bursting activity is characterized by repeating motifs of relatively high firing rates and has been demonstrated to have importance during development as bursts can facilitate the normal function of developing neurons that, in turn, facilitates the formation of viable connections.

**Materials and Methods for Specific Aim 1:**

**A. Cell Cultures**

All experimental procedures were carried out in accordance with the Georgetown University Animal Care and Use Committee (GUACUC). Hippocampal tissue was extracted from embryonic day 18 (E18) Sprague-Dawley rats using a protocol modified from (Pak et al., 2001). Briefly, the neural tissue was finely chopped and digested with 0.1% trypsin followed by mechanical trituration. Upon reaching a single cell suspension, 200,000 cells were added to multi-electrode arrays (MEA, Multi Channel Systems MCS GmbH, Reutlingen, Germany) that were previously treated with poly-d-lysine and laminin (Sigma, St. Louis, MO) resulting in an approximate density of 600 cells/mm². Cultures were maintained in Neuralbasal A medium with
B27 (Invitrogen, Carlsbad, CA) with bi-weekly changes and kept in a humidified 5% CO$_2$ and 95% O$_2$ incubator at 37°C.

**B. Electrophysiological recordings**

I recorded all spontaneous electrical activity using a multi-electrode array. This MEA is composed of 59 titanium nitride electrodes, one reference electrode and four auxiliary analog channels each of which is 30 µm in diameter and arranged on an 8x8 square array with an inter-electrode spacing of 200 µm. Upon plating, the cells in suspension adhered to the silicon nitride substrate of the MEA and after seven days spontaneous electrical activity was detectable.

The MEA1060 preamplifier takes the analog electrical signals at a 10kHz-sampling rate in order to allow the detection of multi-unit spikes. The data was digitized and stored on a Dell personal computer (Round Rock, TX) for offline analysis. Figure 1-1 (left panel), shows a differential interference contrast (DIC) micrograph of a 14DIV culture of hippocampal neurons plated on the multi-electrode array and an accompanying screen shot of network electrical activity from the culture (right panel). The width of each box corresponds to one-second of activity and network activity consisted of a rich mix of bursts and spikes. Potential exposure to contaminants and fluctuations in osmolality and pH were significantly reduced during the data acquisition period by the use of an MEA cover made of a hydrophobic membrane (Potter and DeMarse, 2001). This membrane provides a tight seal, is semi-permeable to CO$_2$ and O$_2$ and is largely impermeable to water vapor.

Experiments from at least three MEAs for each condition, including controls, were performed on a heated stage at 37°C at 14 days *in vitro* (14DIV), a time point during development in which the network displayed vigorous spontaneous electrical activity and for which network connectivity is well-established (Wagenaar et al., 2006a). To ensure
reproducibility of results across animals, all reported experimental groups were comprised of multiple cultures derived from multiple experimental preparations. Results obtained from cultures within and across different preparations within experimental groups were not significantly different.

C. Pharmacological Induction of LTP

In this project, I used the pharmacological agents forskolin (50µM) and rolipram (100nM) to induce cLTP. Forskolin was first dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 50mM. Rolipram was dissolved in DMSO to a stock concentration of 100µM. Both chemicals and DMSO were acquired from Sigma-Aldrich (St. Louis, MO).

At the time of experimentation, baseline electrical activity was recorded for 20 minutes on a heated stage at 37°C. Next, to induce cLTP, 100µL of conditioned media was first removed from the MEA. Into this conditioned media, 1µL of each stock solution of forskolin and rolipram was diluted to their respective, working concentrations. This treated media was then slowly added back into the reservoir of the MEA. MEAs were returned to the stage and recordings resumed immediately and lasted for at least 30 minutes. Results for this specific aim represent network dynamics of the period 20 minutes after treatment.

To control for possible solvent effects as well as mechanical artifacts arising from the exchange of solutions, an additional series of MEA recordings were performed on cultures in which 1µL of DMSO was diluted into the conditioned media of another set of cultures prior to returning it to the MEA. Neither forskolin nor rolipram were added to these MEAs (vehicle experiments).

D. Data Analysis

I first removed low frequency components by high-pass filtering all traces at 200 Hz. It was necessary to filter the raw data in order to set the ground to 0 mV to allow for the
application of a threshold to extract spike time stamps. Accordingly, extracellularly recorded spikes were detected using a threshold algorithm in Offline Sorter (Plexon Inc., Dallas TX), which was calculated as a multiple of the standard deviation (-5σ) of the biological noise. I made no attempt to discriminate and sort spikes by electrode since the shape of a spike changes significantly during a burst due to changes in membrane excitability. In addition, for this study we concentrate on network activity and the signal from each electrode suitably reflected these dynamics.

I used custom software written in MATLAB (The MathWorks, Natick, MA) to analyze dynamical activity in the cultured hippocampal networks. To investigate changes in overall network activity, I first calculated the average firing rate, FR, over a binned (10-second binsize), five-minute window for each electrode within an MEA. Values were reported as means ± SEM. I then calculated the ratio of firing rates after treatment with respect to baseline for both the chemical LTP experiments and the vehicle. Next, to obtain a measure of spiking regularity, I calculated the coefficient of variation, CV, defined as the following equation:

\[ CV = \frac{\sigma(ISI)}{\langle ISI \rangle} \]

where \( \sigma \) is the standard deviation of the inter-spike interval (ISI) distribution.

Next, I investigated changes in a common temporal feature found in cultured networks, the burst, as it represents a collective network response. In my experiments, I analyzed bursts from each individual electrode. After the spike detection process described above, each electrode had a resulting spike train, \( \tau_{st}(t) \), expressed as:

\[ \tau_{st}(t) = \sum_{n=1}^{N} \delta(t - t_n) \]

where \( N \) was defined to be the total number of spikes, \( t_n \) was the time of the \( n \)th spike and \( \delta(t) \) was a delta function that indicates a spike taking place at time \( t = t_n \). The inter-spike interval between spike \( n \) and spike \( n-1 \) (\( n > 1 \)) was:
For both the control and chemical LTP experiments, I defined a burst from each electrode to consist of no less than four spikes with a maximum interspike interval (ISI) of 100 ms. Log histograms of the ISIs indicated that this corresponded to the cutoff of the first peak (see figure 1-3) in both conditions. Lastly, the burst durations, $\Delta_i$, were defined to be:

$$\Delta_i = t_{\text{spike\_final}} - t_{\text{spike\_initial}}$$

The final result of the burst identification process resulted in an $M \times N$ matrix where $M$ corresponded to the electrode number and $N$ represented the time stamps of the spikes within the bursts.

Lastly, I generated return maps of voltage activity to investigate the presence of nonlinear dynamical structures in the envelope of each bursting episode before and after chemical LTP treatment. The objective was to investigate the presence of a preserved structuring of the burst profile after chemical LTP. As with the initial assessment of the bursts, i.e., number of bursts and durations of bursts, I started with the high-pass filtered voltage signal from each electrode. This ensured that I was not including low-frequency components that might be non-physiological. Next, I low-pass filtered (10 Hz) each electrode to obtain a representative of the envelope of a given burst. Finally, I plotted $V_{i,t}$ vs. $V_{i,t+1}$ where $V_i$ is the voltage corresponding to electrode $i$ at time, $t$. A regularly repeating motif would suggest the presence of a conserved activity pattern.

**E. Computational Model**

To investigate whether the trafficking of AMPA receptors to the synapse can account for my observed network-wide effect, my collaborators and I used the simulator NEURON (Carnevale and Hines, 2006) to model the dynamics of a two-dimensional network consisting of 1000 biologically plausible neurons. We incorporated three cell types into the model that are
believed to reflect the dynamics of hippocampal neurons (numbers of cells are parenthetically indicated): an excitatory pyramidal cell with simplified dendritic morphology (800), and two types of GABAergic interneurons: oriens-lacunosum moleculare (OLM; 100) and basket cells (BAS; 100) (Wang and Buzsaki, 1996; Wang, 2002; Tort et al., 2007; Neymotin et al., 2011). The pyramidal cell consisted of five compartments: three apical dendrites, one basal dendrite coupled to the soma and both the basket and OLM cells were single compartment models. Cells are randomly connected within each type and these clustered homogeneous populations are connected to each other according to the diagram and connectivity schema in figure 1-2. There were a total of 124,000 synapses and they were randomly activated using a Poisson distribution. Synaptic and background activity parameters were taken from (Neymotin et al., 2011).

**Results of Specific Aim 1:**

I. **CLTP induced changes in network activity**

Figure 1-3 consists of representative raster plots of spiking activity over a 20-second time window from the control hippocampal networks (figure 1-3A) and the hippocampal networks 20 minutes after the application of chemical LTP (figure 1-3B). One row in each panel corresponds to one electrode and each small vertical tick mark is a detected spike. Below each raster plot is an expanded view of activity that shows a mix of bursts and single spikes. The raw voltage trace from the selected electrode is presented at the bottom. The control network exhibits bursts of a longer duration than bursts following cLTP, which appear to cluster into tightly organized episodes of shortened duration and higher frequency.

I began my analysis by investigating changes in overall network activity. Figure 1-4 is a log histogram of the inter-spike intervals from the chemical LTP and vehicle experiments showing that there is considerably more activity after chemical LTP (a larger total number of intervals, and thus spikes). In addition to the large increase in activity, there is a leftward shift
(towards shorter intervals) in the overall distribution. Within the left peak, containing relatively short intervals usually corresponding to the spike intervals within bursts, there is a well-defined peak around 50 ms embedded within a log normal-like distribution. In the longer interval regime, likely made up of intervals between bursts there is a pronounced peak near 10 seconds. The average ratio of firing rates (firing rate ratio after treatment relative to baseline) across the vehicle MEAs was 1.96 ±0.73 whereas the average ratio for the chemical LTP MEAs was 6.19 ±2.25 (one-way ANOVA, p<10^{-9}). Figure 1-5 highlights these differences in a spike count histogram using representative electrodes from the vehicle (A, B) and cLTP (C, D) treatments. There is an increase in spiking activity in the cLTP electrode while the activity in the electrode from the vehicle culture remains largely unchanged.

Next, I looked at the relationship between the aggregate number of spikes within a five-minute window before and 20 minutes after chemical LTP or vehicle treatment. Electrodes from all MEAs within each treatment were pooled and their spike counts are displayed on a log scale (figure 1-5). The diagonal line represents y=x and therefore points falling on this line have no change in activity. Nearly all of the electrodes from the chemical LTP MEAs are above this line indicating an increase in activity, with a majority showing an increase of more than two orders of magnitude (figure 1-5A). MEA vehicle experiments showed negligible change in the number of spikes (figure 1-5B).

The profile of the time evolution of spiking activity in figure 1-5 suggests that there is a change in the variability of inter-spike intervals (ISI) after chemical LTP. To address this, I calculated the coefficient of variation, CV, for all MEAs (figure 1-7). There is a uniform decrease in the CV across all electrodes that experienced the chemical LTP treatment indicating that the variability in network activity was reduced. The change in the CV for the vehicle MEAs was not significant.

The increase in the firing rate and the decrease in variability of inter-spike intervals led me to ask how cLTP affected bursts, a subset of network activity. The burst, which is a tight
barrage of spikes, is a dominant temporal motif in cultured networks, is present in developing in vivo systems, and is believed to represent coordinated activity from neural assemblies [44-46]. It has been suggested that a burst may be more efficient to modulate information leaving a diminished role in information transmission for individual spikes [55-59]. If the bursts were positively impacted by the chemical LTP treatment, this would contribute to the increase in network regularity as seen in the reduction of the CV.

Figure 1-8 quantifies the number of bursts and burst durations from the cLTP and vehicle MEAs. There is a significant increase in the number of bursts after chemical LTP and this increase contributes to the increase in the overall firing rate within the network as seen in the raster plots of figure 1-3. In the vehicle and pre-chemical LTP networks, the average number of bursts was approximately 2058±148 and 1564±429, respectively. However, the post-vehicle treatment increased the average number of bursts to approximately 2438±208 whereas 20 minutes after cLTP the average number of bursts increased to 10,300±2363 (one-way ANOVA, p=0.0003). In addition, the burst durations decreased considerably after cLTP (figure 1-8B). The average burst duration for the pre-chemical LTP MEAs was 140±18 ms and after treatment, 81±12 ms whereas the vehicle treatment the average was 130±3 before and 133±5 after treatment (one-way ANOVA, p<0.001). This decrease in event duration suggests that the collective network activity contracted and experienced a re-organization into short episodes.

Bursts represent the collective network response to our pharmacological perturbation and only those spikes that participate within a burst are considered in the burst analyses. The raster plots of figure 1-3 suggest that there may be a reduced number of spikes in between the bursts and therefore, I calculated the fraction of spikes not in bursts as a percent change from baseline. In the baselines of both the vehicle and chemical LTP experiment, approximately 20% of the spikes were not in bursts (data not shown). However, there was a marked change after chemical LTP; this fraction decreased nearly 50% while the fraction in the vehicle fluctuated
minimally (data not shown). Chemical LTP appears to incorporate more of the “errant” spikes into bursts, leaving the inter-burst regions quiescent.

Lastly, figure 1-9 presents frequency counts of spiking activity per 200 ms from untreated, baseline cultures (A) and 20 minutes following cLTP (D). Next the low-pass filtered, 'envelopes' of the bursts are depicted for baseline (B) and post cLTP (D) MEAs. Lastly, a representative return map of the low-pass filtered voltage for 10 seconds of activity from an electrode before (figure 1-9C) and after (figure 1-9F) cLTP. These filtered traces represent the envelope of each bursting epoch of activity. Whenever there is a peak or trough in the envelope, the return plot will cross the identity line. Figure 1-9 shows that baseline bursting activity pattern appears to be stable – each envelope shares a similar shape. This shape is preserved after the chemical LTP treatment suggesting that synaptic potentiation conserves a spatiotemporal pattern of activity.

II. Increasing synaptic efficacy in computational models

While it has been shown that the trafficking of AMPA receptors to the synapse accounts for the biological mechanism underlying LTP on a small spatial scale (Frey et al., 1993; Remondes and Schuman, 2002; Payne, 2008), collective neural activity is not linear and we investigated whether the manipulation of AMPA receptors might account for our observed network-wide dynamical effects. Increasing AMPA receptor density can conceivably take two forms: i) the synaptic inputs could increase their strength or weight by increasing the number or density of AMPA receptor sites, or ii) the number of synaptic connections onto a given postsynaptic cell could increase while the overall density of synaptic inputs remains unchanged. Figure 1-10 is a raster plot of baseline network activity before we manipulated the presence of the AMPA receptors. The pyramidal cells are very sparsely active and only the inhibitory OLM cells have a large degree of spiking activity.
Next, we increased the AMPA synaptic weights at different cell sites that contain AMPA synapses according to figure 1-2. Figure 1-11 is a raster plot of network activity after a 30% increase in the synaptic weights of the pyramidal, basket and OLM cells’ somas as well as at the apical dendrite of the pyramidal cell. Each cell population displays an increase in spiking activity and the activity is organized into bursting epochs. This persistent spiking activity was very similar when we increased the AMPA strength by 30% at both the pyramidal cell soma and apical dendrite or only at the pyramidal cell soma while leaving the AMPA weights at control levels on the OLM and basket cell soma sites (Table 1-1). However, when only the apical dendrite AMPA weights were increased or when the soma sites of both basket and OLM cells were increased, leaving the pyramidal cell soma parameters unchanged, spiking activity did not increase (Table 1-1). Lastly, we varied the total number of AMPA connections at each cell population (figure 1-12). Spiking activity did not appreciably increase above the baseline.
**Figures for Specific Aim 1:**

**FIGURE 1-1:** Hippocampal neurons cultured on multi-electrode arrays. Left: A DIC image of cells plated on the MEA. Scale bar = 200 µm Right: Screen shot of raw, unfiltered data of spontaneous activity from the networks. Each box represents one second of activity.
**FIGURE 1-2: Schematic for computation model.** The triangle represents the population of excitatory pyramidal cells (PYR); circles are the inhibitory basket (BAS) cell population and oriens-lacunosum moleculare (OLM) cell population. Cells are randomly connected within each cell population. Truncated lines represent sites in which the synaptic weights were modified: filled circles – AMPA, open circles – NMDA and filled stars – GABA\(_A\). Numbers of AMPA, NMDA and GABA synaptic connections are listed in panel B.
FIGURE 1-3: **Network spiking activity is increased after chemical LTP treatment.** A) Raster plots of 20 seconds of spontaneous activity at 14 days *in vitro* from untreated cultured hippocampal networks. There is a large degree of activity with each electrode displaying bursting and spiking dynamics. B) Raster plots 20 minutes after application of chemical LTP. The 1-second, expanded views show that the bursts increase in frequency and shorten in duration. Scale bar=500 ms
FIGURE 1-4: There is a bimodal distribution of inter-spike intervals (ISI) after chemical LTP (grey bars). The first peak is clustered around short ISIs – this defines the intervals within the bursts whereas the second peak is near 10 second and corresponds to the interval between bursts.
FIGURE 1-5: **Variability in spiking activity is reduced after chemical LTP.** A,B) Spike count histograms from a representative electrode in the vehicle networks. There is robust but highly variable spiking activity. C) Spike count histogram in an electrode before chemical LTP. D) While the initial baseline is low in this example (C), the spike rate increases dramatically, and with low variability, following chemical LTP treatment (one-way ANOVA, p<10^{-9}).
FIGURE 1-6: **There is a persistent increase in spiking activity after chemical LTP.** A) Spike counts from all electrodes before and after chemical LTP. Most electrodes have an increase in activity with a large cluster displaying an increase of at least two orders of magnitude. (one-way ANOVA, $p<10^{-9}$) B) Spike counts from the DMSO-treated MEAs show no increase in activity. (one-way ANOVA, $p<10^{-7}$). Each symbol corresponds to a different MEA. The diagonal line denotes $y=x$. 
FIGURE 1-7: The coefficient of variation (CV) of inter-spike intervals is reduced after chemical LTP. A) CV from the chemical LTP MEAs. There is an overall reduction in the CV indicating that variability in activity has been reduced. (one-way ANOVA, \( p<10^{-5} \)) B) MEAs treated with only DMSO show no change in the CV. (one-way ANOVA, \( p<10^{-8} \)) Each symbol corresponds to a different MEA. The diagonal line denotes \( y=x \). Note: different symbols represent different cultures (\( n=5-6 \))
FIGURE 1-8: Number of bursts is increased and burst durations are decreased 20 minutes following cLTP treatment. A) The bursting activity significantly increases (one-way ANOVA, p=0.0003) after the application of chemical LTP, contributing to the overall increase in network firing rates as seen in fig. 2. B) The durations of the bursts decreases after chemical LTP (one-way ANOVA, p=0.0004).
FIGURE 1-9: The burst activity pattern is maintained 20 minutes following chemical LTP treatment. Representative frequency counts of spiking activity per 200 ms from untreated, baseline cultures (A) and 20 minutes following cLTP (D) demonstrate increases in number of spikes and bursts. Activity traces from baseline (B) and cLTP-treated (E) were lowpass filtered to generate phase plots. Phase plot of bursts during 10 seconds of baseline activity (C) and during 10 second of activity 20 minutes after chemical LTP (F) show conserved, repeating motifs suggesting the preservation of an attractor state.
FIGURE 1-10: Basal network activity is sparse with only inhibitory OLM cells displaying high frequency spiking activity. Raster plot of simulated activity in which the tick marks in rows 0-800 represent the excitatory pyramidal cells; tick marks in rows 801-900 are inhibitory basket (BAS) cells and the tick marks in rows 901-1000 are the inhibitory OLM cells.

Note: Data presented in this figure were generated by Dr. Xin Chen and presented with permission.
FIGURE 1-11: Synchronized bursting activity appears after synaptic weights have been increased. Raster plot of simulated network activity after AMPA synaptic weights were increased throughout the network. Tick marks in rows 0-800 represent the excitatory pyramidal cells (PYR); tick marks in rows 801-900 are inhibitory basket cells (BAS) and the tick marks in rows 901-1000 are the inhibitory OLM cells.

Note: Data presented in this figure were generated by Dr. Xin Chen and presented with permission.
Table 1-1: Summary of effects of increasing AMPA synaptic input into different locations of the experimental model presented in Figure 1. Of note is that all trials where AMPA synaptic input is increased to the soma of the excitatory pyramidal cells exhibited long term potentiation-like activity. PYR: Pyramidal cells; BAS: Basket cells; OLM: oriens lacunosum moleculare neurons; LTP?: Long Term Potentiation-like activity pattern.

Note: Data presented in this figure were generated by Dr. Xin Chen and presented with permission.
FIGURE 1-12: Increasing the number of AMPA connections does not result in synchronized bursting activity. Raster plot of network activity after AMPA connections were increased throughout the network is sparse. Tick marks in rows 0-800 represents the excitatory pyramidal cells; tick marks in rows 801-900 are inhibitory basket cells and the tick marks in rows 901-1000 are the inhibitory OLM cells.

Note: Data presented in this figure were generated by Dr. Xin Chen and presented with permission.
**Discussion of Specific Aim 1:**

In these studies, I performed a global, biological manipulation that is believed to preferentially target a subset of structures residing on a small spatial scale—excitatory synapses on postsynaptic spines. I investigated the resulting dynamical effects on a large spatial scale—the network of cultured hippocampal neurons. The synapse was treated with a pharmacological paradigm that is known to increase the probability of action potential firing and quantified changes in spiking activity that reflect the response from the network. This increased likelihood of firing is due to the Hebbian-like strengthening of synapses that might occur during the creation of a memory. In addition, using a computational network model, we show that AMPA receptor trafficking results in the generation of persistent bursting activity within the network for at least 20 minutes.

While the application of this drug cocktail may affect changes on the microscopic level other than synaptic modification, my results strongly indicate that synaptic perturbations can account for the observed modifications on the macroscopic level, i.e. overall network spiking activity. Thus, there may be two phenomena that could explain these changes. The cLTP treatment elevates network activity but the state remains stable. The increase in overall network activity, as seen in the network firing rates, is due to the increase in potentiation of a large fraction of synapses. This persistent activity due to an increase in connection strength has been theoretically described using attractor models.

In addition, there is a reduction in the coefficient of variation, CV, after chemical LTP. This reduction in the CV implies that the variability in the inter-spike intervals from the electrodes is reduced. The firing pattern becomes relatively constant with no large fluctuations of high activity. Regulation of neural activity must be preserved to prevent extremes in neural output—either hyperexcitability, which can lead to neurotoxic or neuropathological conditions, or insufficient excitation, which can cause the neuron to cease firing altogether. These regulatory mechanisms on the cellular level must also propagate to the network level in the form of circuit-
stabilizing mechanisms and it has been suggested that appropriately modulated activity within a neural circuit could be maintained via the modulation of firing rates (Turrigiano and Nelson, 2004; Marder and Goaillard, 2006). There may be a tuning range of firing rates over which the neural circuit operates most effectively. While it is too early after the treatment to assess long-term regulation of activity, my results suggest that the process of chemical LTP may facilitate the reduced variability of firing rates in the short term.

All of the firing rates from the electrodes increased dramatically after the cLTP treatment. However, the relative increase was not uniform across all electrodes and may be indicative of the different developmental stages of the neurons. These differences may also affect the ability of each neuron to respond to a synapse-strengthening perturbation. There is a small fraction of electrodes that displayed at least an eight-fold increase in multi-unit firing rate activity after treatment. This effect is further emphasized by the log scale presentation of spike counts produced by each electrode. As previously stated, I did not spike sort the data from these experiments. With my relatively low plating density, I rarely saw more than one unit per electrode (analysis not shown). I therefore introduce a possible scenario with the understanding that targeted biochemical assays are necessary to confirm our hypothesis. Chemical LTP modulates the neuron via several mechanisms and it will be the integrated effect that produces an increase in network-wide spiking activity. I focus, in this case, on one of these mechanisms and suggest that some of the neurons with this large firing rate increase are glutamatergic, i.e., excitatory, neurons with immature spines that responded with a vigorous spine expansion under cLTP induction. The spine expansion caused the firing rates of those cells to 'catch up' to those of glutamatergic neurons with presumably more developed spines. This brought the previously immature cells within the range of the firing rates of the rest of the network. As a result, it appears from the dynamics within the network that all of the neurons, regardless of their initial developmental phase, had similar firing rates after treatment. Therefore, a striking network dynamical effect has materialized after cLTP in the reduced spiking variability. Chemical LTP
has a differential effect on the increase in firing rates on clusters of neural assemblies, and these clusters may represent different information storing units.

Bursting activity in the network also displayed dramatic changes after synaptic potentiation. There is an increase in burst frequency, and the individual bursts are of a shorter duration. However, the decreased burst duration did not alter the shape of the attractor. The additional spikes generated by strengthening of the synapses need not have contributed to bursting activity and could have simply raised the background level of single spikes. Interestingly, not only did the burst frequency increase but there also was a large reduction in the fraction of spikes that are not participating in bursts accompanied by the preservation of the attractor profile. The large increase in the inter-spike interval histogram combined with the reduction of the number of spikes that do not participate in bursts suggest that the previously “errant” spikes were either recruited into existing bursts or, more likely, created new bursts with a shortened duration. It has been speculated that bursts may be more efficient at information processing within a neural circuit (Lisman, 1997; Buzsaki et al., 2002; Izhikevich et al., 2003). In these experiments, processing efficiency may represent information storage. There was a repeating spatiotemporal pattern in the burst envelope return maps before and after treatment. The peaks and troughs of the envelope determined where the return maps crossed the identity line and the resulting similarity in shape before and after cLTP suggests that the system maintains a stable state of activity despite the persistent increase in activity. Lastly, the reduction in the coefficient of variation of inter-spike intervals suggests a more “regular” network temporal structure. These combined results demonstrate that synaptic potentiation evokes physiological events that restructure the burst profile. These restructured bursts represent the creation of a new functional entity that appears to facilitate information storage within the network.

Our computational model illustrates the importance of the contribution of AMPA receptor trafficking to the persistent state of increased activity. Increasing the synaptic strength appears
to have the largest effect on the pyramidal cell rather than on the basket or OLM cells. This suggests that there may be a differential impact of AMPA receptor trafficking and will require further experimental investigations. Lastly, we show that increasing the AMPA synaptic strength rather than the number of AMPA connections plays the predominant role in the generation of our observed tightly compacted epochs of persistent activity. Given that we are assessing changes to overall network activity a short time after application of the treatment, it would not be expected that entirely new connections would be created within this time interval.
Specific Aim 2:

Investigate later dynamical and spatiotemporal effects during continuous, and following acute chemical long term potentiation

Introduction to Specific Aim 2:

Results presented in specific aim 1 suggest that chemical Long Term Potentiation (cLTP) increased neuroexcitability in hippocampal networks without modulating their attractor state. It may be that the experimental time point (20 minutes post cLTP exposure) used in specific aim 1 might not be the optimal time to observe spatiotemporal changes in cLTP-induced network activity. Our unpublished data suggest that cLTP-induced activity patterns at timepoints earlier than 20 minutes are also characterized by increasing network excitability with no change in spatiotemporal network dynamics (data not shown). Therefore, for this specific aim, I studied cLTP-induced changes in network dynamics 30 minutes after treatment.

Thus, the second major goal of this project was to study long-term effects of cLTP, and ultimately long-lasting maintenance. Because continuous stimulation over many hours or days could produce potential problems (e.g., excitotoxicity) over many hours or days it was first necessary to devise an experimental paradigm with a finite exposure to cLTP reagents. Thus, in this specific aim, I aimed to determine how such a protocol affected network activity.

Previous studies using the same cocktail of forskolin and rolipram utilized a treatment period of around 15 minutes [Otmathov 2004]. In addition, unpublished data from another laboratory suggested that intracellular cAMP levels would reduce to near-baseline levels within “minutes” of removal of (John DiRaddo, personal communication). Due to this duration of exposure and possible period of lingering effects of the treatment, network activity was recorded and analyzed at 30 minutes post exposure to cLTP for this specific aim, rather than 20 minutes for specific aim 1.
Finally, similar physical perturbations that I previously studied resulted in decreases in overall network activity (data not shown). For these reasons, it was important to study any effects of the removal of cLTP-reagents and the physical perturbations alone and compare them to activity from pre-treatment conditions as well as from continuous exposure to cLTP reagents at the same time point.

**Materials and Methods for Specific Aim 2:**

**A. Cell Cultures**

All experimental procedures were carried out in accordance with the Georgetown University Animal Care and Use Committee (GUACUC). Hippocampal tissue was extracted from embryonic day 18 (E18) Sprague-Dawley rats using a protocol modified from (Pak et al., 2001). Briefly, the neural tissue was finely chopped and digested with 0.1% trypsin followed by mechanical trituration. Upon reaching a single cell suspension, 200,000 cells were added to multi-electrode arrays (MEA, Multi Channel Systems MCS GmbH, Reutlingen, Germany) that were previously treated with poly-d-lysine and laminin (Sigma, St. Louis, MO) resulting in an approximate density of 600 cells/mm². Cultures were maintained in Neuralbasal A medium with B27 (Invitrogen, Carlsbad, CA) with bi-weekly changes and kept in a humidified 5% CO₂ and 95% O₂ incubator at 37°C.

**B. Electrophysiological recordings**

I recorded all spontaneous electrical activity using a multi-electrode array. This MEA was composed of 59 titanium nitride electrodes, one reference electrode and four auxiliary analog channels each of which is 30 µm in diameter and arranged on an 8x8 square array with an inter-electrode spacing of 200 µm. Upon plating, the cells in suspension adhered to the silicon nitride substrate of the MEA and after seven days spontaneous electrical activity was
detectable. A MEA1060 preamplifier sampled the analog electrical signals at a 10kHz rate in order to allow the detection of multi-unit spikes. The data was digitized and stored on a Dell personal computer (Round Rock, TX) for offline analysis. Potential exposure to contaminants and fluctuations in osmolality and pH were significantly reduced during the data acquisition period by the use of an MEA cover made of a hydrophobic membrane (Potter and DeMarse, 2001). This membrane provides a tight seal, is semi-permeable to CO₂ and O₂ and is largely impermeable to water vapor. Experiments from at least three MEAs for each condition, including controls, were performed on a heated stage at 37°C at 14 days in vitro (14DIV), a time point during development in which the network displayed vigorous spontaneous electrical activity and for which network connectivity is well-established (Wagenaar et al., 2006a). To ensure reproducibility of results across animals, all reported experimental groups were comprised of multiple cultures derived from multiple experimental preparations. Results obtained from cultures within and across different preparations within experimental groups were not significantly different.

C. Pharmacological Induction of LTP

In this project, I used the pharmacological agents forskolin (50µM) and rolipram (100nM) to induce cLTP. Forskolin was first dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 50mM. Rolipram was dissolved in DMSO to a stock concentration of 100µM. Both chemicals and DMSO were acquired from Sigma-Aldrich (St. Louis, MO).

i. Protocol for continuous exposure to cLTP reagents.

At the time of experimentation, baseline electrical activity was recorded for 20 minutes on a heated stage at 37°C. Next, to induce cLTP, 100µL of conditioned media was first removed from the MEA. Into this conditioned media, 1µL of each stock solution of forskolin and rolipram was diluted to their respective, working concentrations. This treated media was then
slowly added back into the reservoir of the MEA. MEAs were returned to the stage and recordings resumed immediately and lasted for at least 40 minutes. Results for this specific aim represent network dynamics for the period 30 minutes after treatment.

To control for possible solvent effects as well as mechanical artifacts arising from the exchange of solutions, an additional series of MEA recordings were performed on cultures in which 1µL of DMSO was diluted into the conditioned media of another set of cultures prior to returning it to the MEA. Neither forskolin nor rolipram were added to these MEAs (vehicle experiments).

**ii. Protocol for acute exposure to cLTP reagents.**

Next, to test the effects of removing the cLTP-reagents, a new set of cultures were treated as follows. Initially, a 14 DIV (days in vitro) MEA was removed from the incubator and placed on the heated (37°C) recording stage. Baseline electrical activity was recorded for 20 minutes. To induce chemical LTP I first removed the conditioned media from the MEA. This was stored in the incubator until after the treatment process. The MEA was washed three times with 200 µL of artificial cerebrospinal fluid (ACSF; 140mM NaCl, 5 mM KCl, 1.5 mM CaCl$_2$, 0.75 mM MgCl$_2$, 1.25 mM NaH$_2$PO$_4$, 20 mM glucose, 15 mM HEPES/NaOH adjusted to 7.4pH). Next, I applied 400 µL of ACSF containing 50 µM forskolin and 0.1 µM rolipram to the MEA and incubated the MEA at 37°C for 15 minutes. After incubation, the ACSF was removed and the cells on the MEA were washed three times with 200 µL of unconditioned media. The conditioned media was returned to the MEA and returned to the recording stage. 20 minutes of activity were recorded and stored for offline analysis.

To control for possible solvent effects as well as mechanical artifacts arising from the exchange of solutions for these cultures, recordings were performed on hippocampal neuron-plated MEAs in which 1µL of DMSO was diluted into the ACSF (vehicle experiments). These
cultures then underwent an identical washing and recording schedule as the experimental group.

**D. Data Analysis**

I first removed low frequency components by high-pass filtering all traces at 200 Hz. It was necessary to filter the raw data in order to set the ground to 0 mV to allow for the application of a threshold to extract spike time stamps. Accordingly, extracellularly recorded spikes were detected using a threshold algorithm in Offline Sorter (Plexon Inc., Dallas TX), which was calculated as a multiple of the standard deviation (-5σ) of the biological noise. I made no attempt to discriminate and sort spikes by electrode since the shape of a spike changes significantly during a burst due to changes in membrane excitability. In addition, for this study we concentrate on network activity and the signal from each electrode suitably reflected these dynamics.

I used custom software written in MATLAB (The MathWorks, Natick, MA) to analyze dynamical activity in the cultured hippocampal networks. To investigate changes in a common temporal feature found in cultured networks, the burst, as it represents a collective network response. In my experiments, I analyzed bursts from each individual electrode. After the spike detection process described above each electrode had a resulting spike train, τsi(t), expressed as:

\[
\tau_{si}(t) = \sum_{n=1}^{N} \delta(t - t_n)
\]

where \(N\) was defined to be the total number of spikes, \(t_n\) was the time of the \(n\)th spike and \(\delta(t)\) was a delta function that indicates a spike taking place at time \(t = t_n\). The inter-spike interval between spike \(n\) and spike \(n-1\) (\(n > 1\)) was:

\[
\tau_{ISI}^{n} = t_n - t_{n-1}
\]
For both the control and chemical LTP experiments, I defined a burst from each electrode to consist of no less than four spikes with a maximum interspike interval (ISI) of 100 ms. Log histograms of the ISIs indicated that this corresponded to the cutoff of the first peak (see figure 1-3) in both conditions. Lastly, the burst durations, $\Delta_i$, were defined to be:

$$\Delta_i = t_{\text{spike, final}} - t_{\text{spike, initial}}$$

The final result of the burst identification process resulted in an $M \times N$ matrix where $M$ corresponded to the electrode number and $N$ represented the time stamps of the spikes within the bursts.

Lastly, I generated return maps of voltage activity to investigate the presence of nonlinear dynamical structures in the envelope of each bursting episode before and after chemical LTP treatment. The objective was to investigate the presence of a preserved structuring of the burst profile after chemical LTP. As with the initial assessment of the bursts, i.e., number of bursts and durations of bursts, I started with the high-pass filtered voltage signal from each electrode. This ensured that I was not including low-frequency components that might be non-physiological. Next, I low-pass filtered (10 Hz) each electrode to obtain a representative of the envelope of a given burst. Finally, I plotted $V_{i,t}$ vs. $V_{i,t+1}$ where $V_i$ is the voltage corresponding to electrode $i$ at time, $t$. A regularly repeating motif would suggest the presence of a conserved activity pattern.

**Results of Specific Aim 2:**

I. **Network activity is increased following 30 minutes of continuous cLTP-treatment**

First, I sought to investigate network activity following continuous cLTP-treatment at a time where it would be possible to study the effects of removing cLTP reagents. To allow adequate time to treat and wash reagents out of cultures, a timepoint of 30 minutes was chosen. Representative network activity following 30 minutes of cLTP treatment is depicted in...
One row in each panel corresponds to a single electrode and in each row a small vertical tick mark is a detected spike. As can be seen, there is a large increase in network excitability manifested as a large, induced increase in network firing rates (bottom, right panel) over baseline activity (bottom, left panel) when compared to vehicle-treated cultures (top panels). After 30 minutes of chemical LTP treatment, there is an increase in the number of bursts, which lengthen in duration. These are quantified across all cultures in figure 2-2 and changes in the ratio of post-treatment value to pre-treatment values (Post/Pre) is significant for both number of bursts and burst durations (n=5-6 per group; p<0.05, ANOVA).

These significant changes in burst duration suggest a change in burst structure. Zooms of individual, representative bursts are shown in figure 2-3 (middle panels). These representative burst are highlighted in rasterplots of overall, post-treatment activity for either vehicle (top, left panel) or cLTP (bottom, left panel). In addition, following 30 minutes of continuous treatment with cLTP, there is an induced period of repeating activity across all electrodes not present in vehicle-treated MEAs.

With the induction of the new period of time characterized by a repeating pattern of regular activity, there is an increase in the incidence of interspike intervals (ISIs) around 100ms long. Accordingly, a frequency plot of log[ISI] from representative MEAs that were treated with either cLTP or control conditions is presented in figure 2-3 (right panels). The increased incidence of 100ms ISIs results in a new, induced peak in the plot that is not observed in baseline conditions, time-matched (30 minutes post treatment) control conditions, or following 20 minutes of continuous exposure to cLTP reagents (see figure 1-3).

An overall increase in firing rate is evident in the differences in a spike count histogram using representative electrodes from the vehicle and chemical LTP treatments (figure 2-4, A, D). There is an increase in spiking activity in the chemical LTP electrode from baseline (A) while the activity in the electrode from the vehicle culture remains largely unchanged (D). In addition, there is an increase in the average spike number per unit time (data not shown).
traces depicting a representative burst were low-pass filtered (10Hz; figure 2-4, B,E) to generate phase plots. The phase plot of the representative burst of vehicle-treated (C) demonstrates a conserved motif suggesting the preservation of the attractor state. However, 30 minutes after chemical LTP induction (F) the motif is no longer conserved, suggesting a change in the attractor state. This new motif is also different from the representative phase plot obtained 20 minutes after the induction of cLTP (see figure 1-8), suggesting a possible time course for an unknown mechanism whereby the attractor state is not maintained.

II. Changes in network activity following brief exposure to- and removal of cLTP reagents are similar to activity following 30 minutes of continuous treatment

I removed cLTP reagents from a new set of MEAs to determine the effects of cLTP on network activity when cultures were not being continuously stimulated. Representative rasterplots of overall network activity for these cultures are depicted in figure 2-5. There is a decrease in overall network firing from baseline (A) in vehicle-treated (B) MEAs (cultures that were not treated with forskolin or rolipram, but otherwise underwent identical incubations and washes). In contrast, in the cLTP-treated culture, there is a change in organization of activity (C to D). In addition, there was not a significant change in overall network firing (data not shown).

Effects on bursting of these treatment regiments are quantified in figure 2-6. There was a significant decrease in the number of bursts for both vehicle- and cLTP-treated cultures (n=4 per group; left panel). However, there is a significant increase in burst duration similar to the change seen during continuous exposure to cLTP reagents (figures 2-6 and 2-2). Representative bursts from each cLTP treatment are compared in figure 2-7. As can be appreciated, despite an overall decrease in number of spikes following the removal of cLTP reagents (Washout, B) there is a similar overall motif as the representative burst following 30 minutes of continuous treatment (No Washout, A). Indeed, there is a similarly induced period of repeated and regular activity. Accordingly, there is also an increased incidence of ISIs of
around 100ms, and the related induction of a new peak in the frequency plot of the Log[ISIs] (figure 2-8) similar to continuous treatment (see figure 2-3).
Figures for Specific Aim 2:

**FIGURE 2-1:** *Network spiking activity is increased 30 minutes after chemical LTP treatment.* Rasterplots of activity 30 minutes following cLTP-treatment show increased spiking (right, bottom panel) over baseline (left panels) and over activity seen 30 minutes following control treatment with vehicle (right, top panel).
FIGURE 2-2: Both bursting numbers and burst durations are increased 30 minutes after chemical LTP treatment. CLTP treatment significantly increases both the number of bursts and the average burst duration 30 minutes following treatment (p<0.05, ANOVA).
FIGURE 2-3: 30 minutes following cLTP-treatment, there is a change in the burst profile compared to vehicle experiments. Expanded view of a representative burst of cLTP-treated MEAs (bottom, middle panel) highlighted in representative, overall activity (bottom, second panel), show the induction of a period of repeating activity not present in vehicle-treated MEAs. This new activity results in a new peak in the Log[interspike interval] graph centered near 100ms (right panels).
**FIGURE 2-4:** Burst phase profile is not maintained 30 minutes after chemical LTP. Representative frequency counts of spiking activity per 200 ms from untreated, baseline cultures and 30 minutes following vehicle or cLTP (A,D) demonstrate increases in number of spikes and bursts for cLTP over vehicle. Activity traces from vehicle- (B) and cLTP-treated (E) were filtered to generate phase plots. The Phase plot of a representative burst of a vehicle-treated MEA (C) demonstrates a conserved motif suggesting the preservation of the attractor state. However, 30 minutes following chemical LTP (F) the motif is no longer conserved, suggesting a change in the attractor state.
FIGURE 2-5: Network spiking activity is altered following 15 minutes of cLTP treatment. Rasterplots of activity of MEAs were treated with cLTP reagents for 15 minutes, which was then removed and cultures were returned to conditioned media. 30 minutes following the start of cLTP treatment (15 minutes following the removal of cLTP reagents), there is an overall decrease in spiking activity in vehicle-treated cultures (A-B). There is also an apparent change in the spiking pattern in cLTP-treated cultures (C-D).
FIGURE 2-6: Removing cLTP reagents causes a decrease in the number of bursts but does not prevent the increase in burst duration. MEAs were treated with cLTP reagents for 15 minutes, which was then removed and cultures were returned to conditioned media. 30 minutes following the start of cLTP treatment (15 minutes following the removal of cLTP reagents), there is an overall decrease in the number of bursts in both vehicle and cLTP-treated cultures (left panel). However, there is a significant increase in the average burst duration of cLTP-treat cultures over vehicle-treated cultures (p<0.05, ANOVA, right panel).
FIGURE 2-7: Removing cLTP reagents does not prevent the change in bursts seen following 30 minutes of continuous exposure to cLTP reagents. MEAs were treated with cLTP reagents for 15 minutes, which was then removed and cultures were returned to conditioned media. 30 minutes following the start of cLTP treatment (15 minutes following the removal of cLTP reagents), there is a similar period of repeating activity (B) to bursts following 30 minutes of continuous exposure to cLTP reagents (A).
FIGURE 2-8: Removing cLTP reagents does not prevent a new peak in the Log[ISI] distribution. MEAs were treated with cLTP reagents for 15 minutes, which was then removed and cultures were returned to conditioned media. 30 minutes following the start of cLTP treatment (15 minutes following the removal of cLTP reagents), there is an induction of a new peak in the Log[ISI] distribution (left to right, bottom panel) centered at about 100ms, and is similar to cultures treated continuously with cLTP reagents (see Figure 14). This is not seen in control, vehicle-treated cultures (left to right, top panel).
**Discussion of Specific Aim 2:**

My experimental results from this specific aim suggest that cLTP treatment induces changes in network activity secondary to those presented in specific aim 1. Previously, the effects of cLTP included an increase in bursting, a decrease in burst duration (two results indicative of an overall increase in network excitability), as well as a maintenance of the attractor state after 20 minutes of continuous LTP exposure. Network activity at this new time point (30 minutes) is, instead, characterized by an increase in the duration of bursts and an induction of periods of repeating activity.

Taken together, these changes could indicate that cLTP will induce the phenomenon of 'superbursting.' Superbursting has been previous described and defined as activity patterns in which a large number of small bursts occur within tight clusters. In addition, within superbursts, inter-cluster intervals are typically ten times longer than intracluster intervals (Wagenaar et al., 2006a). Superbursts can be induced by a strong stimulus efficacy such as spine enlargement. Interestingly, superbursts appear to be favored with increases in the number of synapses (Madhavan, 2007). Within my experimental time frame (30 minutes), the presence of superbursts might be indicative of a cLTP-induced maturation and subsequent signal transmission through previously silent synapses (Isaac et al., 1995).

This would contrast with modeling results obtained in specific aim 1, where increasing synapse numbers in computational models did not result in activity patterns similar to those obtained experimentally. This discrepancy could suggest that following exposure to cLTP reagents, network activity is predominately affected by an increase in synaptic efficacy after 20 minutes, but, by after 30 minutes, there is also an increase in the number of functional synapses. Accordingly, a potential future experiment would be to test the hypothesis that increasing both number of synapses and synaptic efficacy would result in superbursting in models of hippocampal networks.
Second, inter-cluster intervals within cLTP-induced superbursts were consistently 100ms in duration. Across all channels, there was a high enough incidence of these intervals to induce a new peak, significant in magnitude, in the frequency histogram of Log[ISIs]. A high incidence of interspike intervals near 100ms would indicate the potential for network firing rates of 10Hz, which is in the hippocampal theta range for rodents. This is potentially significant because theta range activity is detected in the hippocampus during learning in animals (Winson, 1978; Jones and Wilson, 2005) and humans (Lega et al., 2011). It is important to note, however, that while a high incidence of interspike intervals of 100ms does not directly demonstrate theta oscillatory activity, as the timing of spikes is not factored, it would be assumed that network activity without interspike intervals near 100ms do not exhibit theta oscillatory activity.

With the potential of an induction of theta activity following cLTP, further study of LTP-induced network activity at this time point could be an important follow up study. For example, as stimulation with theta frequencies can induce synaptic potentiation (Larson and Lynch, 1986; Otto et al., 1991; Huerta and Lisman, 1993), the confirmed presence of theta oscillations might suggest a positive feedback mechanism where an emergence of rhythmic activity further facilitates synaptic potentiation.

With the induction of superbursting, there is also a change in the spatiotemporal pattern of the burst envelope return maps from pretreatment bursts. This could suggest instability in the attractor state at this time point. This, again, is in contrast to results seen 10 minutes earlier. Accordingly, these data suggest a timeframe for effects of LTP where functional effects on the attractor state are not apparent until 20-30 minutes post treatment. Such instability could represent the ability of hippocampal networks to respond to extracellular stimuli and alter their dynamical properties in the absence of hippocampal anatomical features.

Finally, the time necessary for the detectable change in spatiotemporal dynamics, which could provide potential evidence of information storage in hippocampal networks (20-30 minutes), is well outside the expected time frame of ‘working memory’ in vivo, which this
paradigm is supposed to represent. This delay could be due to experimental conditions (e.g. low neuronal density), but also could represent effects of the lack of hippocampal, anatomical features.

Experiments designed to determine the effects of removing cLTP reagents (following a 15 minute exposure) suggest that the physical perturbations of the treatment caused a significant reduction in overall network activity. However, this reduction was presumably counterbalanced by the LTP-induced increase in network spiking demonstrated above and resulted in no significant change in overall firing rate. Interestingly, superbursting was still induced by this treatment regimen. This finding suggested that the mechanism through which cLTP induces this phenomenon is sufficiently activated within 15 minutes. Significantly, an increase in overall network activity at the time of superbursting was not required in these cultures, suggesting that basal activity levels were sufficient to drive cLTP-induced changes in network dynamics that appear 20-30 minutes following initial cLTP exposure.

Results obtained from this specific aim demonstrate that hippocampal network activity is capable of changing network dynamics following external stimulation. Whether these changes are long-lasting, an important component of information storage will be the central focus of Specific Aim 3.
Specific Aim 3:

Examine the evolution and maintenance of dynamical effects following chemical long term potentiation

Introduction to Specific Aim 3:

Long Term Potentiation (LTP) is characterized by an increase in synaptic efficacy, which, in turn, increases the likelihood of the production of an action potential. This synaptic potentiation is thought to form the cellular mechanism for learning and memory and is frequently induced via electrical stimulation. However, electrical stimulation is spatially restricted. As a result, chemical methods have been introduced to induce synaptic potentiation in a large population of synapses. Furthermore, it has been demonstrated that these methods produce similar biochemical mechanisms that have been elucidated in the electrical techniques.

In the previous two specific aims, I studied effects of cLTP on hippocampal cultures and presented changes in network activity over a relatively short time scale. Results from these specific aims demonstrated two, potentially independent, consequences of cLTP treatment. First, there is an increase in overall network activity during exposure to cLTP reagents (see specific aim 1), however, this increase does not result in any change in the spatiotemporal dynamics of the network (see specific aim 1) within a short time frame (20 minutes). Second, there was an induced reorganization of network activity into clusters of longer durations (superbursts; see specific aim 2) after 30 minutes. Only this latter effect remained following the removal of cLTP reagents (see specific aim 2). Therefore, for this specific aim, I sought to study the long-term effects and maintenance of this network activity reorganization.

In addition, increasing synaptic efficacy resulted in an increased probability of action potential firing leading to an increase in activity. However, if left unbounded, the network might evolve into a pathological state of epileptiform activity and/or excitotoxicity. Therefore, the
network, while still experiencing activity-dependent plasticity, must operate within a well-regulated regime to prevent an untenable outcome.

One of the major challenges for the brain is to maintain a stable operating state while retaining sufficient flexibility to grow and experience plasticity in response to external stimuli. This implies that in order for the brain to perform its normal functions, including synaptic changes that occur from experiential learning, neural mechanisms must operate such that activity is neither excessive nor insufficient. However, how these two opposing constraints reconcile is not well understood.

Thus, homeostatic mechanisms are needed to balance these effects of synaptic potentiation. Homeostatic synaptic plasticity is thought to involve mechanisms that modulate intrinsic cell excitability and the number and strength of synapses in order to stabilize neuronal firing rates. This type of plasticity has been observed in a wide range of experiments involving neurotransmitter or activity deprivation within several neural systems ranging from the neuromuscular junction to cortical circuits (Turrigiano and Nelson, 1998).

The major experimental focus on homeostatic synaptic plasticity has been on deprivation experiments in which activity has been reduced or suppressed. However, there exist studies involving a treatment-induced increase in network activity. For example, in cortical cultures, chronic inhibition of gamma-aminobutyric acid (GABA) mediated inhibition initially raised firing rates, but over a 48-hour period mESPC amplitudes and firing rates decreased to baseline levels (Turrigiano et al., 1998)). In addition, based on effects of LTP treatments in organotypic hippocampal slices, it has been suggested that multiple mechanisms exist and work together to maintain stable activity (Karmarkar and Buonomano, 2006).

For these reasons, an additional goal of this specific aim was to look for evidence of homeostatic mechanisms, and how they might affect the potential for information storage in my hippocampal networks.
Materials and Methods for Specific Aim 3:

A. Cell culture

All experimental procedures were carried out in accordance with the Georgetown University Animal Care and Use Committee (GUACUC). I used a protocol modified from [33] to extract hippocampal tissue from embryonic day 18 (E18) Sprague-Dawley rats. Briefly, the neural tissue was finely chopped and digested with 0.1% trypsin followed by mechanical trituration. Upon reaching a single cell suspension, approximately 200,000 cells were added to each multi-electrode array (Multi Channel Systems MCS GmbH, Reutlingen, Germany) that was previously treated with poly-d-lysine and laminin (Sigma, St. Louis, MO). This resulted in an approximate plating density of 600 cells/mm². Cultures were maintained in Neuralbasal medium with B27 (Invitrogen, Carlsbad, CA) and kept in a humidified 5% CO₂ and 95% O₂ incubator at 37°C. To provide a consistent supply of nutrients, one third of the media was changed on a bi-weekly basis.

B. Electrophysiological Recordings

I recorded all spontaneous electrical activity using a multi-electrode array (MEA). This MEA is composed of 59 titanium nitride electrodes, one reference electrode and four auxiliary analog channels each of which is 30 μm in diameter. These electrodes are arranged on an 8x8 square array with an inter-electrode spacing of 200 μm. Within a few hours after plating, the cells in suspension settled and adhered to the silicon nitride substrate of the MEA. After seven days spontaneous electrical activity was detectable. Activity sampled at a 10kHz acquisition rate to allow for the detection of multi-unit spikes. The data was digitized and stored on a Dell personal computer (Round Rock, TX). In addition, possible exposure to contaminants and fluctuations in osmolality and pH were significantly reduced during the data acquisition period by the use of an MEA cover made of a hydrophobic membrane [34]. This membrane provided a
seal that was semi-permeable to CO$_2$ and O$_2$ and largely impermeable to water vapor.

Experiments from at least four MEAs for each condition, including vehicle and control cultures, were performed on a heated stage at 37°C at 14 days in vitro (14DIV), a time point during development in which the network displayed vigorous spontaneous electrical activity and for which network connectivity is well-established [35]. To ensure reproducibility of results across animals, all reported experimental groups were comprised of multiple cultures derived from multiple experimental preparations. Results obtained from cultures within and across different preparations were not significantly different.

C. Pharmacological Induction of LTP

I used the pharmacological agents forskolin (50µM) and rolipram (100nM) to induce chemical LTP [Otmakhov 2004]. Forskolin was dissolved in cellular media to a stock concentration of 50mM. Rolipram was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 100µM. Both chemicals as well as the DMSO were purchased from Sigma-Aldrich (St. Louis, MO).

This drug cocktail was applied to cultured hippocampal neurons at 14DIV in the following manner. Initially, an MEA was removed from the incubator and placed on the heated (37°C) recording stage. Baseline electrical activity was recorded for 20 minutes. The MEA was removed from the recording stage and the existing, conditioned media was removed from the MEA and stored in the incubator until after the treatment process. The cells on the MEA were washed three times with 200 µL of artificial cerebrospinal fluid (ACSF; 140mM NaCl, 5 mM KCl, 1.5 mM CaCl$_2$, 0.75 mM MgCl$_2$, 1.25 mM NaH$_2$PO$_4$, 20 mM glucose, 15 mM HEPES/NaOH adjusted to 7.4pH). CLTP was induced by the application of 400 µL of ACSF containing 50 µM forskolin and 0.1 µM rolipram and the MEA was incubated at 37°C for 15 minutes. After incubation, the ACSF was removed and the cells on the MEA were washed three times with 200
μL of unconditioned media. The conditioned media was returned to the MEA and returned to the incubator. To investigate long-term dynamical affects, network activity in the absence of the drugs was recorded from the MEA two and five days after treatment.

To control for possible solvent effects as well as mechanical artifacts arising from the exchange of solutions, recordings were performed on hippocampal neuron-plated MEAs in which 1μL of DMSO was diluted into the conditioned media (‘vehicle’ experiments). Lastly, to control for changes in network activity due to network maturation, network activity was recorded from untreated, unperturbed hippocampal neuron-plated MEAs over the same time period (‘control’ experiments).

D. Data Analysis

To obtain a stable signal for the spike-threshold procedure, I removed low frequency fluctuations by high-pass filtering all voltage traces at 200 Hz. Extracellularly recorded spikes were detected using a threshold algorithm from Offline Sorter (Plexon Inc., Dallas TX). The threshold was calculated as a multiple of the standard deviation (5σ) of the biological noise. No attempt was made to discriminate and sort spikes within each electrode because the shape of a spike changes significantly during a burst due to changes in membrane excitability. In addition, for this study I concentrated on network spiking activity and the signal from each electrode suitably reflects these dynamics. I used proprietary software written in MATLAB (The MathWorks, Natick, MA) to analyze the dynamical activity recorded from the cultured hippocampal networks. I plotted the number of spikes from each electrode in the recordings before treatment against the spike counts two and five days after cLTP. The time evolution of these spike distributions was compared to those within the vehicle and control networks. I also generated log histograms of the inter-spike intervals (ISIs) from each MEA by computing ISIs for each electrode. This allowed me to distinguish between populations of ISIs that participate
within brief episodes of spiking activity (short time-scale ISIs) from those ISIs that represent intervals between periods of high activity (long time-scale ISIs). The log histograms were also used to characterize aspects of the high frequency activity in the chemical LTP networks.

**Results of Specific Aim 3:**

I. **Qualitative effects of cLTP treatment over 2 and 5 days**

Figure 3-1 displays representative raster plots of five minutes of activity from the chemical LTP and vehicle networks. Dynamics from vehicle experiments appeared to be uniform over the course of the five day period (figure 3-2 A-C). In contrast, the pattern of activity in the cLTP-treated networks continuously changes from baseline (figure 3-2 D-F). Activity from cLTP-treated MEAs was divided into epochs based on firing rates and each epoch is labeled (1-6) above the corresponding raster plot. As activity from vehicle-treated cultures was more uniform, it was not readily divided up into different epochs based on firing rate. 24 hours after the application of chemical LTP (day 2; figure 3-2E), the spiking activity organizes into long periods of intense activity (figure 3-2E; epochs 1, 3, 5). This becomes even more pronounced four days after the chemical LTP treatment (day 5, epochs 2,4; figure 3-2F). In addition, the spiking activity in the epochs between them decreases from day 2 to day 5.

When the activity is examined in a finer temporal detail, as shown in figure 3-2, the dynamics are not that of tonic firing of individual spikes. Preceding the high activity epochs are periods of activity characterized by shorter bursts with long inter-episode intervals (figure 3-2A, C). In addition, the epochs characterized by high activity still consist of assemblies of short bursts but with small inter-burst intervals (figures 3-2B and D). In comparison, the dynamics in the vehicle networks also display bursts of spikes but the intervals between bursts are large (figure 3-3).

Figure 3-4 depicts the average ratio of the total number of spikes per 5 minutes across all electrodes on a given time point (post) to the total number of spikes recorded pre-treatment.
from each MEA. As is shown, network activity increases gradually in untreated, control cultures (dark gray triangles). Cultures that underwent the vehicle treatment showed a slight but significant reduction in network activity on day 2, but recovered to baseline network activity levels by day 5 (light gray squares). Importantly, cLTP treatment induced a large, 2-fold increase in overall network activity by day 2 (black circles). However, by day 5, overall network activity had returned to control levels (activity expected after normal development).

Expounding on these changes in network activity, results are also graphed on a log scale in figure 3-6 as a function of each individual electrode. Baseline number of spikes from each electrode (x-axis) within each MEA is plotted against the number of spikes from each electrode two and five days (y-axis) after either chemical LTP (A, B) or the vehicle treatment (C, D). Different symbols in figure 3-5 represent different MEAs.

There is a distribution of channels within each MEA such that some electrodes experience an increase in activity and others, a decrease. As can be noted, the distribution for the change from baseline to day 2 and day 5 for cLTP-treated MEAs is consistently wider than that for control-treated MEAs. In addition, the distribution of changes from baseline to day 2 is also slightly skewed to the right for cLTP-treated MEAs only, consistent with the overall increase in network activity (see figure 3-5). Finally, figure 3-6 displays the spike counts from each electrode for the untreated, control networks over the same recording period (channels from all treatments are displayed by a symbol, different symbols represent different time points). These results are similar to vehicle-treatment, with a narrower distribution than that for cLTP (data not shown).

II. Quantitative effects of cLTP treatment over 2 and 5 days

As stated above, the chemical LTP treatment caused the spiking activity to cluster into periods of high activity as seen in the raster plots of figures 3-1, and, therefore I created epochs that corresponded to these distinct periods of activity (numbered in figure 3-1, and presented
again as reference in figures 3-7 and 3-8). Log histograms of the inter-spike intervals (ISI) from each epoch in these raster plots is compared to log histograms of ISIs from the raster plots of the vehicle networks is presented in figure 3-7. Because activity in the baseline networks was not differentiated over time, three 20 second periods of activity, spread out over the course of recording period, were used. Figure 3-7 displays the histograms of ISIs for the baseline chemical LTP activity (A-C) along with the ISIs for the high frequency activity on day 2 (epochs 1,3 and 5; D-F) and day 5 (epochs 2 and 4; G, H). After treatment, there was a reorganization of the distribution of inter-spike intervals and two notable features appear on each day. The population of long intervals, i.e., those greater than one second, decreased to near zero. This represented a shift the distribution from bi-modal to log-normal with most of the activity taking place within short intervals. In addition, there was an induced peak in the distribution at approximately 100ms, independent of the network-wide firing rate (black colored bins in D-H). This corresponded to an interval in the theta range, i.e., 7-9 Hz band, seen after cLTP induction within in vitro experiments (see specific aim 2). This theta peak is not present within the epochs before and after the high frequency activity (figure 3-9A).

Lastly, to compare these results to vehicle and control MEAs, I again used three 20 second periods of activity spread out over the entire recording as ‘epochs’. Following these treatments, the induction of the theta-range peak as well as the reorganization of inter-spike intervals to a log-normal distribution did not occur in the vehicle networks (figure 3-8B) or the control networks (data not shown).
Figures for Specific Aim 3:

Figure 3-4. Network activity changes following cLTP over 5 days. A) A 40-second window of activity 24 hours after chemical LTP. B) A 10-second window extracted from the epoch of high activity seen in A. C) A 40-second window of activity 24 hours after chemical LTP. D) A 10-second window extracted from the epoch of high activity seen in C.
Figure 3-2: Expanded view of activity from chemical LTP networks show clustering within bursts. A) A 40-second window of activity 24 hours after chemical LTP. B) A 10-second window extracted from the epoch of high activity seen in A. C) A 40-second window of activity four days after chemical LTP. D) A 10-second window extracted from the epoch of high activity seen in C.
Figure 3-3: Expanded view of activity from vehicle networks shows a different pattern of clustering than cLTP-treated cultures. A) A 30-second window of activity. B) A 10-second window of activity.
Figure 3-4: There is an increase in overall activity 2 days after cLTP, but not 5 days after cLTP. Time course of overall network activity following vehicle- and cLTP- treated cultures compared to untreated cultures. 2 days after cLTP there is a significant increase in overall activity (ANOVA, p<0.05), but by day 5, network activity returns to the level of untreated cultures.
Figure 3-5: CLTP treatment resulted in a differential effect on spiking activity across channels, which is higher than vehicle-treated channels. Between baseline and days 2 (A) or 5 (B), cLTP induced effects of different magnitude and direction on each channel to a greater degree than vehicle (C-D). In this figure, different symbols represent channels within different MEAs.
Figure 3-6: Activity changes across channels are not as high in untreated cultures as cLTP-treated cultures. Between baseline and days 2 (light gray) or 5 (dark gray), there is little change in the activity patterns of each channel, similar to vehicle-treatment.
No clear epochs were present therefore three 20 sec periods of activity spread out over the entire recording were used.

**Figure 3-7: High activity epochs are characterized by an induction of theta frequency activity.** There is a peak in Log(ISI) distributions within high-activity epochs on both day 2 and day 5 (labeled 1, 3, 5 2,5, respectively; above) after chemical LTP treatment, centered at ~100ms, and similar the peak seen 30 minutes following chemical LTP above.
No clear epochs so I used three 20 sec periods of activity spread out over the entire recording.

**Figure 3-8:** Periods of low activity are not characterized by an induction of theta frequency activity. There is no peak in Log[ISI] distributions within high-activity epochs on both day 2 and day 5 (labeled 2, 4, 6 and 1,3, respectively; above) after chemical LTP and all activity following vehicle treatment, centered at ~100ms.
**Discussion of Specific Aim 3:**

This specific aim reports the dynamical changes in a network of hippocampal neurons hours and days following a cLTP treatment. CLTP treatment induced networks to alternate between states of high, bursting epochs and periods of lower activity. In contrast, vehicle-treated and control untreated networks did not exhibit this activity pattern. Indeed, cLTP continued to modulate network dynamics after the removal of pharmacological agents, however, regulatory mechanisms appeared to prevent the network from transitioning into a state of unbridled excitation with a rampant increase in the number of overall spikes. Specifically, global spiking activity increased two days after treatment but returned to normal levels within five days after treatment.

Figure 3-4 demonstrates this significant increase in overall network activity. By day 5, however, the activity returns to a level comparable to the control networks. Indeed, there was a 24% increase in activity from baseline to day in the chemical LTP networks which is similar to the 28% increase seen in the control. The increase in the control networks represented network maturation due to the increase in connectivity during development (Potter; Grabrucker 2009). In comparison, there is a slight decrease in activity within the vehicle networks on day 2 and those dynamics return to within 10% of baseline activity by day 5 (figure 3-4). This offset of spiking activity is likely due to mechanical perturbations introduced by solution exchange. The differences in the initial spike counts are likely due to normal variation in culture density. However, these mechanical influences do not alter the distribution of spike counts as the network develops, as activity from the vehicle networks on both day 2 and day 5 with respect to baseline activity remains along the identity line (figures 3-5C, D).

In addition, there was a uniform expansion in the spike count distributions two and five days after treatment, as demonstrated in figure 3-5. This expansion appeared to take the shape of an ellipsoid with the principal axis of rotational symmetry on the identity line. Chemical LTP increased activity for a large number of electrodes but there were also a considerable number of
electrodes in which electrical activity decreases (as seen in figures 3-5 and 3-6). Indeed, some channels increased or decreased activity by a magnitude of 100.

Taken together, these data suggest that neuronal network exists in equilibrium and maintains a homeostatic or balanced state of activity after the chemical perturbation. Indeed, the effect of cLTP treatment was likely constrained by internal, regulating mechanisms of the network, such that any increase in activity in a subset of electrodes is offset by a decrease in activity in others. These results imply that the network is operating under some ‘conservation of spike’ principle, however further characterization would require additional study.

Combining an assumption that network activity is under the control of regulatory mechanisms to maintain activity levels with an examination of the raster plots in figure 3-1 suggested that network activity recorded from the chemical LTP networks on day 2 represented a period of transition between the baseline activity (figure 3-1D) and the activity from day 5 (figure 3-1F). Indeed, zooming in on the activity from each condition supported this notion and revealed that the activity of day 2 contained certain elements of each. First, high activity epochs were structurally similar on day 2 and day 5 (demonstrated in figure 3-2). On both days, these epochs consist of assemblies of short bursts but with small inter-burst intervals. Also within the periods of high activity, there is an increase in the incidence of interspike intervals around 100ms, as shown in figure 3-7. Presumably, this interval corresponded to the inter-burst intervals within the burstlets.

In contrast, in the periods of relatively lower activity between these epochs (visible to the left side of figure 3-2A, C), network dynamics are still characterized by bursts, but with a significantly longer interburst interval, the period of low or no activity between bursts. In the ISI distribution of low-activity periods of cLTP-treated MEAS (figure 3-8A) these intervals comprise a peak just above 1 second. This relative, high incidence of such intervals is maintained from baseline cultures and is also present in vehicle-treated (figure 3-8B) and control-treated (data not shown) cultures on all experimental timepoints. It is not present, however, in the high
activity epochs of cLTP-treated cultures (figure 3-7D-H), suggesting that activity patterns on this day are characterized by superbursting.

Finally, the chemical LTP networks display a strong, persistent theta peak in the log histograms centered near 100ms. This is intriguing because theta range activity is detected in the hippocampus during learning tasks in animals (Buzsaki, 2002) as well as in humans (Ekstrom et al., 2005; Ekstrom et al., 2007). In addition, this theta interval may be a resonant interval over which a subset of the network is responsive. It has been previously proposed that bursts having certain resonant inter-spike frequencies increase the likelihood of a postsynaptic response over bursts with higher or lower frequencies (Izhikevich et al., 2003). A resonance effect could therefore explain why some electrodes increase their activity while activity in other electrodes decrease, and could be an important component of a proposed mechanism that allows the network to operate under a spike conservation principle. Finally, despite the importance of homeostatic plasticity in maintaining stability in a neural circuit, the vast majority of computational effort has been in understanding the effects of activity dependent synaptic plasticity, most notably LTP and LTD. Results presented in this specific aim suggest that future computational studies, would benefit from intrinsic homeostatic capabilities.

Specific aims 2 and 3, together report long-lasting effects of cLTP treatment on hippocampal cultures. These results include the induction of superbursting, an increasing duration of these superbursts, and a decrease in activity outside these superbursts. These changes might reflect network dynamics involved in in vitro information storage and neuronal activity during in vivo learning and memory. Potential mechanisms driving these changes, however, remains a focus of investigation in specific aim 4.
Specific Aim 4:

Identify and investigate a possible mechanism by which hippocampal networks can change their spatiotemporal dynamics following chemical long term potentiation.

Introduction to Specific Aim 4:

Matrix metalloproteinases (MMPs) are a family of zinc dependent endoproteases that play a role in dynamic processes including cell migration and wound healing (McCawley and Matrisian, 2001). While studies of MMPs in the central nervous system (CNS) have generally focused on injury (Yong et al., 2007; Leonardo et al., 2008), accumulating evidence suggests an important role in normal CNS physiology for these enzymes (Milward et al., 2007; Rivera et al., 2010).

Neuronal activity has been shown to stimulate MMP release (Michaluk et al., 2007; Pauly et al., 2008; Conant et al., 2010), and rapid MMP dependent shedding of a neuronal substrate following treatment with N-methyl-D-aspartic acid (NMDA) has been observed in culture (Conant et al., 2010). Studies suggest that MMPs are stored in perisynaptic vesicles (Sbai et al., 2008), and that MMP release may be dependent on soluble NSF attachment protein receptors (SNAREs) (Kean et al., 2009). Taken together, these data suggest that MMP release will occur with select stimuli that increase intracellular calcium.

In several recent studies, MMP activity has also been shown to play a role in learning and memory (Fragkouli et al.; Meighan et al., 2006; Nagy et al., 2006; Brown et al., 2007; Wang et al., 2008; Fragkouli et al., 2012). While effects are likely influenced by several factors (e.g. dose/duration of MMP exposure and/or the developmental stage of neurons), these enzymes have the potential to increase glutamatergic transmission, long-term potentiation (LTP), and measures of hippocampal dependent memory (Nagy et al., 2007). For example, MMP-9 deficient mice showed defects in LTP (Nagy et al., 2006) and antisense oligonucleotides for MMPs reduced performance in the Morris water maze test (Meighan et al., 2006). In contrast,
mice that over-expressed MMP-9 have been shown to display enhanced performance in a spatial task (Fragkouli et al., 2012).

The mechanisms by which MMPs may contribute to changes that underlie learning and memory are likely multiple and not completely understood. Remodeling of the extracellular matrix has been suggested, as well as an MMP dependent increase in a matrix fragment that could stimulate integrin dependent phosphorylation of glutamate receptor subunits (Nagy et al., 2006). Consistent with a role for integrins are studies in which integrin antagonists blocked MMP-dependent changes in dendritic spine shape and LTP (Nagy et al., 2006; Meighan et al., 2007).

An additional mechanism by which MMPs might rapidly modulate synaptic structure and function would be to increase the size of dendritic spines, the post synaptic components of a majority of glutamatergic synapses. Indeed, at least one MMP has been shown to stimulate increases in the size of dendritic spines (Wang et al., 2008). There is a strong correlation between size of the spine head and strength of the synapse, presumably because a larger spine head allows for insertion of more glutamate receptors (Tada and Sheng, 2006).

In addition, a mechanism by which MMPs induced an increase in spine size could depend on MMP-dependent cleavage of certain, specific synaptic adhesion molecules. Of particular interest is MMP dependent shedding of ICAM-5, an adhesion molecule that is expressed on glutamatergic neurons of the telencephalon. A correlation between developmental shedding of ICAM-5 and spine maturation has been previously demonstrated (Matsuno et al., 2006). Importantly, long term NMDA treatment (16 h) of neurons has been associated with both spine enlargement and MMP dependent shedding of this molecule (Tian et al., 2007). ICAM-5 is well positioned to be targeted by synaptically released MMPs, and MMP dependent shedding of this CAM is observable within 5 minutes of NMDA application (Conant et al., 2010). ICAM-5 shedding could disrupt N and C terminal interactions of the full length molecule that are important to filopodial maintenance (Furutani et al., 2007), and thus, ICAM-5
shedding might be important for spine expansion. It is also possible is that the shed N terminal domain could interact with unengaged post synaptic integrins to stimulate actin polymerization within dendrites and thus spine expansion.

Integrin signaling plays a role in developmental changes in spine morphology (Shi and Ethell, 2006; Sfakianos et al., 2007). In addition, varied forms of learning related plasticity are thought to be integrin dependent (Kramar et al., 2006; Meighan et al., 2006; Nagy et al., 2006). Consistent with this, it has also been shown that soluble ICAM-5 can stimulate integrin dependent phosphorylation of cofilin, which would favor dendritic actin polymerization (Conant et al., 2011).

Increases in the size of dendritic spines and an associated increase in the number of glutamate receptors (Malinow and Malenka, 2002; Kerchner and Nicoll, 2008; Kessels and Malinow, 2009; Conant et al., 2011) could result in increases in both frequency and amplitude of post-synaptic mini excitatory post synaptic currents (mEPSCs). MEPSC Frequency could increase from an increase in the number of responsive units and amplitude increases could reflect an increased number of AMPARs within a given unit. In addition, synapses that are initially weak may be especially susceptible to change (Bi and Poo, 1998). Changes in frequency and amplitude could, in turn, influence action potential probability. Indeed, a link between increased dendritic AMPARs and action potential probability has recently been described (Savtchouk and Liu).

The multi-electrode array (MEA) extracellularly records changes in electrical potential, and more specifically action potentials from nearby units. It has been widely used to characterize dynamics from in vitro networks of neurons (Wagenaar et al., 2006b; Chiappalone et al., 2008; Bologna et al., 2010; Chen and Dzakpasu, 2010; Stegenga et al., 2010). MEAs allow for simultaneous recordings to be obtained from many cells, and activity can be measured from cultures that are maintained for long periods of time, as well as cultures treated with stimuli that are difficult to administer in vivo or to slices.
In the present specific aim, I have used MEAs to record from hippocampal cultures with the goal of better understanding specific mechanisms by which MMPs influence neuronal excitability. At the same time, MEA recordings described herein have allowed me to examine the question of whether MMP activity can modulate important aspects of network activity and, in particular, burst dynamics.

**Materials and Methods for Specific Aim 4:**

**A. Cell Culture**

All experimental procedures were carried out in accordance with the Georgetown University Animal Care and Use Committee (GUACUC). Hippocampal tissue was harvested from embryonic day 18 Sprague-Dawley rats using a protocol modified from (Pak et al., 2001). Briefly, neural tissue was finely chopped and digested with 0.1% trypsin and by mechanical trituration. Cells were plated onto multi-electrode arrays (MEA, Multi Channel Systems MCS GmbH, Reutlingen, Germany) that were previously treated with poly-d-lysine and laminin (Sigma, St. Louis, MO) at an approximate density of 600 cells/mm². Cultures were maintained in Neuralbasal A medium with B27 (Invitrogen, Carlsbad, CA) with bi-weekly changes and stored in a humidified 5% CO₂ and 95% O₂ incubator at 37°C. Experiments were performed on cultures at 14 days *in vitro* (DIV).

**B. MEA recordings**

Spontaneous electrical activity was recorded using a multi-electrode array (MEA). This MEA is composed of 59 titanium nitride electrodes, arranged on an 8x8 square array, and comprised of one reference electrode and four auxiliary analog channels each of which are 30 µm in diameter. The inter-electrode spacing is 200 µm. Upon plating, cells adhered to the silicon nitride substrate of the MEA and spontaneous electrical activity is detected after seven days. Electrical activity is amplified (MEA1060 preamplifier) and sampled at a 10 kHz
acquisition rate in order to allow the detection of spikes. Data were digitized and stored on a Dell personal computer (Round Rock, TX) for offline analysis. Possible exposure to contaminants and fluctuations in osmolality and pH were significantly reduced during the data acquisition period by covering the MEA with a hydrophobic membrane that is permeable to CO$_2$ and O$_2$ (Potter and DeMarse, 2001). Recordings were performed on a heated microscope stage at 37°C at 14 days in vitro (14DIV), because this is a time point during development where the network displayed vigorous spontaneous electrical activity and network connectivity is well-established (Wagenaar et al., 2006a). To ensure reproducibility of results across animals, all reported experimental groups were derived from multiple experimental preparations. Results obtained from cultures within and across different preparations were not significantly different.

C. Pharmacological Induction of LTP

I used the pharmacological agents forskolin (50µM) and rolipram (100nM) to induce chemical LTP. Forskolin was diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 50mM. Rolipram was dissolved in DMSO to a stock concentration of 100µM. Both chemicals and DMSO were acquired from Sigma-Aldrich (St. Louis, MO).

On the day of the experiment, baseline electrical activity was recorded for 20 minutes. Next, to induce chemical LTP, 100 µL of conditioned media were removed from the MEA. 1µL of each stock solution of forskolin and rolipram were added to the conditioned media and slowly added back into the MEA. 30 minutes following treatment, 6-10 minutes of continuous network activity was recorded for offline analysis.

To control for possible mechanical artifacts arising from the exchange of solutions, series of MEA recordings were performed on cultures in which conditioned media was removed and subsequently returned, but neither forskolin or rolipram were added. To control for solvent
effects (DMSO), 1 µL of DMSO was diluted into the conditioned media prior to returning it to the MEA.

D. Application of MMP inhibitors, anti-β1, and the soluble ICAM-5 ectodomain

The MMP inhibitors GM-6001 and (2R)-2-[(4-Biphenylylsulfonyl) amino]-3-phenylpropionic Acid (also known as MMP-2/9 inhibitor I or BiPS) (cat# 444241) were purchased from Calbiochem and used at a concentration of 2.5 µM. They were dissolved in DMSO to make a 2.5 mM stock solution. MMP inhibitors were added to cells 20 minutes before control of cLTP recordings. Anti-β1 integrin was purchased from BD Pharmingen. This antibody was used as described (Pasterkamp et al., 2003) and has previously been shown to be neutralizing (Pasterkamp et al., 2003) and to inhibit soluble ICAM-5 stimulated phosphorylation of cofilin (Conant et al., 2011). Recombinant ICAM-5 was purchased from R and D systems (Minneapolis, MN). This construct consists of leu31-arg828, followed by an IEGRMD linker and pro100-lys330 of human IgG1. An isotype matched immunoglobulin control (Santa Cruz Biotechnology) for the Ig portion of ICAM-5 was also used in select experiments. Cultures were treated with ICAM-5 or IgG control on an identical experimental timeline to cLTP, as described above.

E. Western blot

Western blot was performed using hippocampal lysates as previously described (Conant et al., 2011). Relative equivalency of protein loading and transfer across lanes was assessed by Ponceau staining prior to incubation of membranes with appropriate primary and secondary antibodies. Molecular weights were inferred by comparison to prestained markers (BioRad). Blots were probed with a commercially available polyclonal antibody to ICAM-5 (R & D Systems), which was raised against the major portion of the ectodomain (amino acids Leu 31 to
Arg 828 of recombinant mouse ICAM-5), or to a C terminal, intracellular domain specific antibody produced in collaboration with Dr. Seung Lim (Conant et al., 2010).

F. Data Analysis

All traces were high-pass filtered at 200 Hz to remove low frequency components. Next, extracellularly recorded spikes were detected using algorithm from Offline Sorter (Plexon Inc., Dallas TX), and thresholded at a multiple of the standard deviation (-5s) of the biological noise. Due to significant changes in the shape of a spike during a burst resulting from changes in membrane excitability, no attempts were made to discriminate and sort spikes by electrode. In addition, this study concentrated on network activity that is reflected in the overall, thresholded activity from each electrode without spike-sorting.

Network activity was then analyzed with proprietary software written in MATLAB (The MathWorks, Natick, MA). First, to investigate changes in overall network activity, I calculated the average firing rate, FR, over a binned (150ms bin size), five-minute window for each electrode within the MEA. Next, changes in a common temporal feature found in cultured networks, i.e. the burst, were investigated, as bursts can occur across the collective network. Following the spike detection process described above each electrode had a resulting spike train, \( \tau_{st}(t) \), expressed as:

\[
\tau_{st}(t) = \sum_{n=1}^{N} \delta(t - t_n)
\]

where \( N \) is the total number of spikes, \( t_n \) is the time of the \( n \)th spike and \( \delta(t) \) is a delta function that indicates a spike taking place at time \( t = t_n \). Next, the inter-spike interval (ISI) between spike \( n \) and spike \( n-1 \) (\( n > 1 \)) is:

\[
\tau_{ISI} = t_{n} - t_{n-1}
\]
For all experimental groups, a “burst” from each electrode was defined to consist of no fewer than three spikes with a maximum inter-spike interval (ISI) of 150 ms. 150 ms was selected because it represented a time between a distribution of ISIs presumed to be within bursts and the intervals between bursts (see Baseline ISI distributions in Figure 2B). Lastly, the burst durations, $\Delta_i$, were defined as:

$$\Delta_i = t_{\text{spike}_{\text{final}}} - t_{\text{spike}_{\text{initial}}}$$

Lastly, return maps of voltage activity were generated to investigate the presence of nonlinear dynamical structures in the envelope of each bursting episode before and after chemical LTP treatment. The objective was to investigate the presence of a preserved structuring of the burst profile after chemical LTP. As with the initial assessment of the bursts, i.e., number of bursts and durations of bursts, the high-pass filtered voltage signal from each electrode was used. This ensured that low-frequency components that might be non-physiological were not included in the analysis. Next, the signal from each electrode was low-pass filtered (10 Hz) to obtain a representative of the envelope of a given burst. Finally, $V_{i,t}$ vs. $V_{i,t+1}$ where $V_i$ is the voltage corresponding to electrode i at time, t were plotted. A regularly repeating motif would suggest the presence of a conserved activity pattern.

**Results of Specific Aim 4:**

I. **MMP activity and $\beta_1$ integrins contribute to cLTP evoked changes in spike counts detected by MEAs**

MMP inhibitors have been shown to block LTP measured in rat hippocampal slices in the CA1 region and evoked by stimulation of Schaffer collateral-commissural afferents through four trains of 100Hz, 1sec each and separated by 5 minutes (Nagy et al., 2006). MMP inhibitors also block cLTP associated changes in field EPSP slopes in rat hippocampal CA1 (Nagy et al.,
2006). Chemical LTP paradigms stimulate changes, including spine enlargement, that are observed with tetanic stimulation protocols (Kopec et al., 2006).

To determine whether inhibition of MMP activity could reduce cLTP associated changes in action potential probability, I used MEAs to record from cultured hippocampal neurons. Two MMP inhibitors were tested, including a broad spectrum inhibitor (MMPi) and one more selective for MMP-2 and -9 (MMPi_{2,9}). A neutralizing antibody to β₁ integrins was also examined. Representative raster plots are shown in figure 4-1A, while spike counts are shown in 4-1B. The average number of spikes per bin (unit time) in the cLTP paradigm was reduced by approximately 20% by the MMP inhibitors or the β₁ integrin neutralizing antibody (vehicle: 6.18 +/- 0.45; cLTP: 51.8 +/- 1.2; cLTP + MMPi: 39.4 +/- 1.76; cLTP + MMPi_{2,9}: 42.6 +/- 1.9; cLTP + anti-β₁: 38.1 +/- 1.4; MMPi alone: 5.83 +/- 0.82; MMPi_{2,9}: 8.21 +/- 2.67; anti-β₁ alone 5.29 +/- 1.36). The differences between average spikes per bin in the cLTP and cLTP with MMP inhibitors or anti-β₁ integrin groups were statistically significant (p< 0.01, ANOVA). The difference between average spikes per bin in the vehicle and cLTP groups was also significant, as was the difference between vehicle and cLTP plus either MMP inhibitor or anti-β₁, consistent with partial rather than complete inhibition (p < 0.01). Differences between vehicle and inhibitors alone were not significant. Results are from 4 to 6 replicate experiments in vehicle control and cLTP +/- inhibitor groups, and from 3 replicate experiments in the inhibitor alone groups.

Spike count data (figure 4-1B) show that cLTP induces the highest spiking activity after an initial period of relative quiescence. This is in line with work suggesting that action potential probability is influenced by an initial silent period as well as a sufficient level of excitation (Harris et al., 2001). In addition, in the cLTP paradigm, the period in which spike counts are largest is followed by several shorter epochs of lesser but, in comparison to other groups, relatively
increased spike activity. This activity may reflect a relatively increased level of excitability that exists following cLTP.

II. MMP activity and $\beta_1$ integrins contribute to cLTP associated changes in burst dynamics

Rhythmic bursting activity is important to information propagation and memory consolidation (Harris et al., 2001). To determine whether inhibition of MMP activity or $\beta_1$ integrin signaling can alter cLTP dependent effects on burst dynamics, bursts were examined as a function of treatment group. While a precise burst definition can vary, and is influenced by culture preparation and experimental conditions, bursts are generally defined as brief periods during which the spike rate detected in many electrodes is several fold greater than baseline (Wagenaar et al., 2006a). Representative raster plot data with activity shown in an approximate 10 sec interval, and also in an expanded 100 msec interval, is shown in figure 4-2A. Each tick mark corresponds to an action potential and bursts can be observed as patterns of high frequency spiking activity.

Representative raster plots show that cLTP is associated with “superbursts” in which a large number of small bursts occur within tight clusters (inter-cluster intervals are typically 10x longer than intracluster intervals) (Wagenaar et al., 2006a). A representative superburst is presented in the expanded window for the cLTP treatment (figure 4-2A). Superbursts occur with strong stimulus efficacy and may thus be favored following spine enlargement. Superbursts might also be favored when synapse number increases (Madhavan, 2007), as might occur with cLTP-induced activity transmitted through previously silent synapses (Isaac et al., 1995).

Superbursts were observed in the cLTP treatment group, but not following pretreatment of cultures with MMP inhibitors or a neutralizing antibody to $\beta_1$ integrins. Consistent with this, a log histogram of interspike intervals (figure 4-2B) shows a prominent ISI peak at approximately
100 msec (star) in the cLTP group (as presented in specific aim 2), and represents the ISI between spike clusters, or burstlets, occurring within the superbursts (figure 4-2A, arrows). In the other treatments of figure 4-2B, this increased incidence of ISIs in this range were absent. In the expanded log ISI histogram shown in 4-2C, in which normalized ISIs having a given time value are presented for the four treatment groups, the difference between cLTP and other groups was significant at the two time points indicated by an asterisk ($p< 0.05$, ANOVA).

In addition, the emergence of superbursts in the cLTP-treated cultures represented a significant increase in the duration of the epoch of increased action potential occurrence (the 'burst'), over vehicle treatment as shown in figure 4-2D ($p<0.001$; ANOVA). Alternatively, cLTP following pretreatment of cultures with MMP inhibitors or the $\beta_1$ neutralizing antibody significantly shortened burst durations compared to vehicle treatment (Figure 4-2D; $p<0.001$; ANOVA). On their own, the MMP inhibitors and anti-$\beta_1$ did not significantly affect on burst duration (Figure 4-2D).

In addition, phase plots of representative burst following vehicle treatment demonstrated a conserved motif from baseline suggesting the preservation of the attractor state (figure 4-3). In contrast, following cLTP treatment the motif was no longer conserved, suggesting a change in the attractor state. Pretreatment with both MMP inhibitors or the $\beta_1$ neutralizing antibody prevented a change in the attractor state following cLTP (figure 4-3). All represented phase plots are superimposed for direct comparison in figure 4-4.

**III. The MMP generated integrin binding ligand, soluble ICAM-5, stimulates a dose dependent increase in spike counts**

In previous work, it has been demonstrated that the ectodomain of ICAM-5 is shed in a neuronal activity dependent manner (Conant et al., 2010). NMDA stimulation of cultured hippocampal neurons, and high frequency stimulation of hippocampal slices, stimulate MMP
dependent shedding of this molecule (Conant et al., 2010). Cleavage at two sites in the ectodomain has been demonstrated, leading to generation of the near full length ectodomain as well as two smaller fragments which both contain Ig-like domains (Matsuno et al., 2006; Tian et al., 2007). Shown in figure 4-5A and B are Western blot results from hippocampal culture lysates prepared at the conclusion of MEA recordings (40-45 minutes post treatment). A cLTP associated reduction in full length ICAM-5 can be appreciated (figure 4-5A) and a previously described (Conant et al., 2011) MMP generated cleavage fragment can be observed at approximately 110 kDa (figure 4-5B).

It has also been previously shown that the ICAM-5 ectodomain can interact with integrins known to be expressed on dendritic spines, and that this ectodomain can stimulate phosphorylation of cofilin (Conant et al., 2011), a biochemical change that allows for actin polymerization. Herein I have tested the potential for the ICAM-5 ectodomain to increase neuronal excitability as detected by the MEA system. As shown in figure 4-5C, the ectodomain stimulated a dose dependent increase in firing rate. The difference between the 1 µg/ml and 5 µg/ml dose was statistically significant ($p < 0.05; \text{ANOVA}$). Finally, the spike number as a function of time for the 5 µg/ml dose is presented in figure 4-5D.

IV. Soluble ICAM-5 stimulates $\beta_1$ integrin dependent changes in network and burst activity

I also examined burst activity as affected by the ICAM-5 ectodomain. In addition, I tested the neutralizing antibody to $\beta_1$ on ICAM-5 stimulated effects on network and burst activity. Results are shown in Figure 4-6 and suggest that ICAM-5 associated changes in network excitability are abrogated by the neutralizing antibody, as observed in representative raster plots (Figure 4-6A) and in the number of bursts and their durations (Figure 4-6B). The difference between burst number in control and ICAM-5 treated cultures, as well as the difference between
burst duration in ICAM-5 versus ICAM-5 + anti-β1 treated cultures, was significant ($p < 0.05$; ANOVA).

Of interest is that despite the increased burst duration with ICAM-5, there was no observed superbursting in association with this stimulus. It is possible that cLTP provides a stronger stimulus for spine expansion and/or that cLTP stimulates additional effects, independent of those mediated by ICAM-5 or achieved by spine expansion, to increase neuronal excitability.
Figures for Specific Aim 4:

**FIGURE 4-1:** MMP activity and β1 integrins contribute to cLTP evoked changes in network firing rate (A) Representative raster plots of network activity before (Pre) and following (Post) vehicle, cLTP, cLTP + GM-6001 (MMPi), cLTP + BIPS (MMPI$_{2,9}$) or cLTP + anti-β1 treatment. The scale bar represents 10 seconds. (B) Representative, total spike number (Y axis) as a function of binned (150ms) intervals (X axis) for the four treatment groups. Differences in the average spike number per bin between the vehicle versus cLTP, as well as between cLTP and cLTP + MMPi or anti-β1 were significant ($p<0.05$, ANOVA, n= 4-6 per group).
FIGURE 4-2: MMP activity and β₁ integrins contribute to cLTP associated changes in interspike interval (ISI) distributions

(A) Raster plots for each of the treatment groups (gray box in left panel is expanded in right panel). (B) Log histograms showing ISI distributions for each MEA represented in (A). The peak at approximately -1 (cLTP, asterix) likely represents the ISI between burstlets within a superburst (illustrated by arrows in 2A). Data were normalized to the bin containing the largest number of ISIs for each culture. (C) Expansions of log histograms show 2 time points, denoted by an asterix, for which cLTP was significant increased (p < 0.05, ANOVA). (D) Burst duration is also significantly increased by cLTP treatment in an MMP or β₁ integrin dependent manner (*p < 0.001, ANOVA)
FIGURE 4-3: MMP activity and $\beta_1$ integrins contribute to cLTP associated changes in phase profiles of bursts. Representative bursts from all treatments were lowpass filtered (left panels) to generate phase plots (right panels). 30 minutes following cLTP, the phase profile of the burst is not conserved, suggesting the attractor state is not preserved. This effect is prevented by inhibiting MMP- and $\beta1$ integrin activity, suggesting that the attractor state is maintained in the absence of MMP and $\beta1$ integrin activity.
FIGURE 4-4: MMP activity and β₁ integrins contribute to cLTP associated changes in phase profiles of bursts (continued). Representative phase plots from all treatments superimposed to highlight similarities of bursts between baseline- and vehicle- treated, as well as cLTP plus inhibitors of MMP and β₁ integrin activity, and differences of bursts from cLTP-treated cultures.
FIGURE 4-5: Soluble ICAM-5 is generated with cLTP and stimulates a dose dependent increase in spike counts. A) and B) Western blot analysis of lysates from control, cLTP, and cLTP + MMPi treated hippocampal neurons. (A) cLTP stimulates a reduction in immunoreactivity for full length (FL) ICAM-5, as detected by an antibody to the ectodomain and inferred from apparent molecular weight (~140 kDa), that is abrogated by MMP inhibition (MMPi). Ponceau staining, showing equal transfer of total protein at approximately 50 kDa, is shown below. (B) A separate set of control and treated lysates, tested by Western blot and probed with a relatively sensitive C terminal specific antibody, shows a previously described 110 kDa ICAM-5 cleavage product in the cLTP lane (arrowhead). (C) Treatment with increasing amounts of soluble ICAM-5 result in a relative increase in spike number. Differences between the 1 and the 5 mg/ml doses were significant (n=4 per group, \( p < 0.05 \), ANOVA; data are presented as the mean and SEM of the ratios of pre to post treatment values). (D) Spike number as a function of time for the 5 mg dose.
significant (p < 0.05, ANOVA, n = 4 per group).

ICAM-5 groups, as well as the difference between burst duration in ICAM-5 and ICAM-5 + anti-Pi, was
number and duration (mean and SEM) are shown. The difference between burst number in control and
representative raster plot showing spike data for control (IG), ICAM-5 and ICAM-5 + anti-Pi. (b) Burst
Figure 4: ICAM-5 stimulates interleukin dependent changes in network and burst activity (A)

A.

B.

Post/Pre

10s 10s 10s

ICAM (5µg) + anti-Pi

ICAM (5µg)

IG (5µg)
Discussion of Specific Aim 4:

Emerging evidence suggests that MMPs play a role in hippocampal dependent learning and memory, addiction, and LTP (Meighan et al., 2006; Nagy et al., 2006; Mizoguchi et al., 2007; Brown et al., 2008). While the mechanisms by which MMPs influence learning and memory are not fully understood, one possibility is that these enzymes cleave synaptic adhesion molecules to release N terminal fragments that go on to interact with previously unengaged integrins. Synaptic CAMs are ideally positioned to be targeted by MMPs that are released and/or activated in a neuronal activity dependent manner. Consistent with this possibility, it has been shown that the N terminal fragment of at least one CAM can stimulate \( \beta_1 \) dependent phosphorylation of cofilin, an event that would favor an increase in the size of dendritic spines (Conant et al., 2011). In addition, an increase in spine size has been observed following treatment of hippocampal neurons with exogenous MMP-9 (Wang et al., 2008).

An increase in spine size has also been linked to an increased number of post-synaptic AMPA receptors (Malinow and Malenka, 2002). This change may in turn increase the amplitude and/or frequency of excitatory post synaptic potentials (ePSPs) (Savtchouk and Liu, 2011). An increase in frequency would be expected with AMPAR incorporation into synapses which were previously silent (Isaac et al., 1995; Kerchner and Nicoll, 2008). In addition, an increase in EPSP amplitude and/or frequency would increase the probability of action potential occurrence detected by the MEA system. While the regulation of burst probability and duration is a complex, inverse correlation (Cohen and Segal, 2011), increases in action potential probability lead to an increase in burst occurrence (Harris et al., 2001; Popescu et al., 2010; Cohen and Segal, 2011).

In the current specific aim, I examined cLTP evoked changes in spiking activity and burst dynamics as affected by inhibitors of MMPs or \( \beta_1 \) integrin signaling. I also examined a potential mechanism by which MMPs and \( \beta_1 \) integrins can influence network dynamics. In particular, I
examined the ectodomain of ICAM-5, a product of MMP proteolysis, for its ability to stimulate integrin dependent changes in network activity parameters. Inhibitors reduced, but did not block cLTP stimulated increases in spike activity, consistent with the possibility that the cLTP paradigm increased network activity through varied mechanisms only a subset of which are MMP and β₁ independent. Experiments with the ICAM-5 ectodomain also supported the hypothesis that MMPs and β₁ integrins can increase neuronal excitability. This product of MMP proteolysis significantly increased spike and burst number. Moreover, blocking β₁ integrins with neutralizing antibodies diminished ICAM-5 dependent effects on these parameters.

I also observed that inhibition of MMP activity or β₁ integrin signaling had a substantial effect on cLTP associated changes in burst dynamics. In addition, MMP and β₁ integrin activity also appears to be involved in the evolution of the attractor state following cLTP. Of particular interest is that both the MMP inhibitor and neutralizing antibody to β₁ integrins abolished cLTP associated superbursts, and an associated intraburst rhythmic activity in the theta range. Since stimulated theta activity can be used to induce synaptic potentiation (Larson and Lynch, 1986; Otto et al., 1991; Huerta and Lisman, 1993), changes, at least partially by evoked MMP activity might be part of a positive feedback mechanism in which widespread but synaptically localized changes favor the emergence of rhythmic activity that further facilitates synaptic potentiation. In addition, the lack of potential theta activity in cultures lacking MMP- or β₁ integrin activity could have significant implications. For example, in schizophrenia patients, there are changes with both theta (Lisman and Buzsaki, 2008) and MMP (Rybakowski et al., 2009) activity

ICAM-5 also stimulated an increase in firing rate and burst number, suggesting that MMP generated CAM fragments might contribute to MMP dependent effects on neuronal excitability. Although soluble ICAM-5 treatment was not associated with the emergence of superbursts, characteristic of cLTP, a phenomenon that may require a stronger stimulus for spine expansion and/or effects independent from those stimulated by ICAM-5, this CAM
fragment did increase burst duration suggesting that it too could increase the duration of periods of relatively increased excitability.

The potential for MMPs to influence not only spike but burst dynamics is significant. The downstream effects of a burst are considered to be stronger than those of a single spike (Harris et al., 2001). While I examined bursting at the single electrode detection level, from figure 4-2 it is apparent that bursting increased across the population of cells. Bursting on a single cell and/or population level may be important to varied processes including facilitated information propagation and memory consolidation (Ben-Ari, 2001; Pennartz et al., 2002; Israel et al., 2008; Blankenship and Feller, 2010). Indeed, sedatives or anaesthetic agents administered in amounts insufficient to promote unconsciousness, but sufficient to disrupt rhythmic brain activity, have been associated with amnesia (Perouansky et al., 2010). In addition, slow wave sleep, which is characterized by a particular rhythmic activity, has been linked to reactivation of neuronal activity patterns from preceding behavior in rat cortex and hippocampus (Kudrimoti et al., 1999). Bursting activity may strengthen synapses that have previously been strengthened through other means.

As compared to stimuli that target spines and other effectors of neuronal excitability in a more restricted or circuit specific manner, cLTP stimulation of cultured hippocampal neurons would be expected to have relatively more diffuse and widespread effects. This stimulus may nonetheless have in vivo relevance. For example, when modestly elevated, norepinephrine, which like cLTP can increase adenylate cyclase activity, can enhance memory. Levels of this neurotransmitter may be diffusely altered with anxiety or in association with specific stages of the sleep/wake cycle. Norepinephrine is concentrated in neurons of the locus coeruleus which project in a widespread manner to hippocampus and cortex (Swanson and Hartman, 1975; Loughlin et al., 1986; Snyder et al., 2012). Though highly speculative, it is possible that norepinephrine could increase rhythmic bursting to strengthen synapses that were previously strengthened through the learning of a specific task.
While previous studies have implicated localized and regulated proteolysis in the learning of specific tasks, such as the Morris water maze (Wright et al., 2007), my data suggest that enzymes may also play a role in facilitating rhythmic changes that are evoked by a widely applied/received stimulus, such as cLTP. The extent to which emergent widespread bursting can be engendered by stimuli that are important to formation of smaller units (e.g. synapses or discrete circuits) could warrant further investigation.

In addition, varied molecular mechanisms may contribute to both increased efficacy of neurotransmission at specific synapses as well as to widespread rhythmic activity. For example, pyramidal cell dendritic calcium spikes contribute to one form of in vitro theta activity (Gillies et al., 2002). Understanding the mechanisms by which MMPs, and MMP generated integrin binding ligands in particular, contribute to changes in burst dynamics, however, will require further study.

Nonetheless, effects observed herein are consistent with those expected to result from dendritic spine maturation. Additional support for the hypothesis that spine changes play a role is published data showing that EphA4 expression can promote both spine maturation and increased burst duration (Clifford et al., 2010). It should be noted, however, that like cLTP, MMPs may have relevant effects that are independent of spine size and/or AMPARs. For example, integrin binding ligands may increase NMDAR phosphorylation and function through a src kinase dependent mechanism (Bernard-Trifilo et al., 2005).

In terms of specific MMP generated integrin binding ligands, it is unlikely that ICAM-5 is the only CAM that is shed in a neuronal activity dependent manner to stimulate spine expansion. In addition, while GM-6001 inhibits the activity of MMP-2 and -9, which have been specifically implicated in ICAM-5 shedding (Tian et al., 2007), this inhibitor could act on other metalloproteinases that might contribute to neuronal activity dependent CAM cleavage. Though not yet tested for effects on neurotransmission, additional CAM ectodomains that are released with neuronal activity include nectin-1, L1 and NCAM (Hoffman et al., 1998; Tanaka et al., 2002;
Thelen et al., 2002; Hinkle et al., 2006; Kim et al., 2010; Lim et al., 2012). Of interest is that several CAM ectodomains, including that of L1, can interact with integrins (Thelen et al., 2002; Ogita et al., 2008). In conclusion, my data suggest that MMPs and soluble ICAM-5 have the potential to influence neuronal excitability and burst dynamics, with both stimulating an overall increase in excitability of the system as determined by an increase in overall spike counts, burst number, and/or burst duration.
**General Discussion:**

To summarize, the primary goal of my work was to gain a better understanding of cellular processes involved in *in vivo* learning and memory by studying how the induction of Long Term Potentiation (LTP) influenced neuronal network activity *in vitro*, and to investigate how stimulation-induced changes are maintained over time. To accomplish this goal, I used Multielectrode Arrays (MEAs) to record activity of networks derived from hippocampal cultures. Because effects of LTP are time-dependent, cultures were utilized rather than slices, and this allowed me to characterize effects of LTP across a wide timeframe including 5 days following treatment. However, unlike a slice preparation, hippocampal cultures do not preserve connections within hippocampus. Accordingly, results obtained in this study are limited to network dynamics not dependent on hippocampal anatomy.

Next, to induce LTP, I applied a cocktail of Forskolin and Rolipram (cLTP). This LTP paradigm acted to raise intracellular levels of cAMP which, in turn, increased synaptic efficacy. Such a pharmacological induction of LTP is advantageous to an electrical induction of LTP in my study because cLTP can increase synaptic efficacy network-wide, whereas electrical induction would be limited to the synapses of a small number of cells. Accordingly, the entire network represented a single neuronal assembly with a shared input. In addition, inducing LTP by increasing intracellular cAMP levels can be related to *in vivo* results, as experimentally altering its level has been shown to affect learning and memory in animal models (Nguyen and Woo, 2003; Abel and Nguyen, 2008; Lee et al., 2008; Zhang et al., 2011).

Initially, I sought to characterize network activity during an induction phase of plasticity represented by activity during ongoing pharmacological stimulation (i.e. in the presence of forskolin and rolipram). Within minutes of treatment, there was an increase in overall network activity which began to level off after around 15 minutes of cLTP treatment (data not shown). Data in specific aim 1 above, therefore, was derived from experimental traces of activity recorded following 20 minutes of exposure to cLTP reagents. Data collected from this time point
demonstrated large, significant increases in overall activity. In addition, there was an increase in the number of observed bursts, but a decrease in burst duration.

Through a collaboration with another member of my laboratory, I was able to further characterize the effects of the cLTP treatment with a computational model. We constructed a network of biologically plausible units representing the cell-types of the hippocampus (excitatory pyramidal cells and 2 types of inhibitory, GABA interneurons). In addition, pyramidal cells consisted of five compartments: three apical dendrites, one basal dendrite coupled to the soma. This allowed us to test a variety of potential sites of changes in both synaptic density and efficacy. Result suggested that the effects of forskolin and rolipram at this timepoint most reflected an increase in somatic AMPA-receptor efficacy (see table 1-1).

Finally, although bursts had a significantly shorter duration, their spatiotemporal dynamics were not affected by the cLTP treatment. Therefore, these data represented network activity at a time point where cLTP affected network excitability but not network plasticity. Activity patterns following 30 minutes of exposure to cLTP, however, did exhibit changes in spatiotemporal dynamics and suggest that cultures of hippocampal neurons are able to alter its attractor state. Related to this, there is a drive in network activity away from bursting and towards superbursting. The induction of superbursting, also resulted in a new, relative increase in the incidence of interspike intervals of around 100ms. This interval could be reflective of hippocampal theta activity. Finally, there was still a large increase in overall network activity.

Taken together, these results demonstrated that a cLTP-induced increase in intracellular cAMP might be representative of in vivo learning. Accordingly, changes in network activity from my cultures may represent changes in neural activity within the in vivo brain. In addition, computational data suggest that the increase in neuronal excitability is primarily due to increases in probability of synaptic transmission through somatic AMPA receptors. This might reflect an increase in AMPA-receptor channeling observed following LTP. While the earliest effects of increasing intracellular cAMP would occur in the soma, increasing synaptic efficacy in
the dendrites did not further affect network activity. In addition, increasing the number of connections to the pyramidal cell did not result in 'LTP-like' activity.

Future directions for studying this 'induction' phase of LTP could focus on the effects of certain neuromodulators known to affect learning and memory in vivo. One such pharmacological agent is nicotine [e.g. see recent review (Davis and Gould, 2008)]. Using nicotine pretreatment to produce the large, widespread network dynamics might provide valuable insight into how memory formation is enhanced by nicotine, and how this might be related to nicotine addiction. In addition, utilizing nicotine to enhance weaker, but more focal, electrical stimuli to induce LTP might allow for the study of network dynamics that reflect multiple attractor basins (representing multiple, functional in vivo brain assemblies). This could provide information about how multiple 'units of information' (e.g. patterns or memories) can be stored in the brain.

From a computational standpoint, it would be interesting to investigate how network activity evolves, with time, from bursting to superbursting. Following up on data presented here, models could be used to determine if a greater increase in synaptic efficacy than used in this study might induce superbursting. Else, while increasing the number of connections alone did not significantly alter network activity, combining it with increased synaptic efficacy might lead to superbursting.

In order to study the maintenance of network activity, and obtain potential insight into the molecular basis of memory, I first needed to remove the drive towards potentiation. I hypothesized that removing this drive would represent the shift from memory induction to memory consolidation and/or maintenance. In these studies this was achieved by removing the pharmacological agents from the culture. Data from such cultures were shown from 3 time points throughout this study, namely 15 minutes, 24 hours, and 4 days following a 15 minute cLTP treatment.
For the first time point studied, control experiments suggested that the mechanical perturbation of changing the media caused a significant reduction in overall network activity. This decrease in network activity effectively counterbalanced an expected increase in activity previously associated with cLTP (see above), and assumed to be present during the cLTP treatment. However, the spatiotemporal dynamics of the cLTP-treated cultures demonstrated the induction of superbursting 15 minutes following cLTP removal, which was similar to superbursting seen after 30 minutes of continuous, cLTP stimulation (see figure 2-7). These results could suggest that a large increase in network excitability is either not necessary, or not necessary beyond a certain time, for the network to alter its attractor state.

Network activity largely recovered from the mechanical treatment by the second time point studied, and, 24 hours following cLTP treatment, there was a new activity pattern in potentiated networks. This pattern was characterized by an alternation between high-activity epochs which contained a high incidence of 100ms interspike intervals (ISIs), and lower activity periods that did not have a relative increase in the incidence of 100ms ISIs. Finally, there was an increase in overall network activity from pretreatment levels.

By day 5 (4 days following cLTP treatment), however, the overall network activity had reduced to the level of untreated, control cultures. In addition, data suggested that network activity recorded from the chemical LTP networks on day 2 represented a period of transition between the baseline activity and the activity seen on day 5. However, while the overall activity was at control level, there were differential effects of the cLTP treatment. For example, cLTP increased activity for a large number of electrodes but there were also a considerable number of electrodes in which electrical activity decreases (as seen in figures 3-6 and 3-7). Indeed, some channels increased or decreased activity by a magnitude of 100.

Taken together, these data suggest that the neuronal network existed in equilibrium and maintained a homeostatic or balanced state of activity after the chemical perturbation. Indeed, the effects of cLTP treatment were likely constrained by internal, regulating mechanisms of the
network or intrinsic mechanisms within the neuron, such that any increase in activity in a subset of electrodes is offset by a decrease in activity in others. These results imply that the network is operating under some 'conservation of spike' principle, however further characterization would require additional study. Follow-up studies to demonstrate such a principle could be done computationally utilizing a model of LTP, and adding in potential mechanisms (e.g. LTD). A second, potential follow-up could be to look at other measures of network dynamics. One example could be to measure and characterize changes in functional connections [e.g. by utilizing the conditional firing properties described by (le Feber et al., 2007)] in order to study the efficacy of synapses into units with increased and decreased activity.

Finally, I attempted determine a potential molecular mechanism underlying the above-described changes in network spatiotemporal dynamics. Accordingly, I tested the hypothesis that MMPs could influence in vitro measures of learning and memory by cleaving synaptic adhesion molecules to release N terminal fragments that go on to interact with previously unengaged integrins. Results suggested that blocking MMP activity resulted in an inability of the network to appropriately alter its attractor state. Using another, more specific inhibitor further suggested that spatiotemporal effects of cLTP were dependent on MMP-2 and/or -9 activity. In addition, blocking β1-integrin receptors also prevented the emergence of network activity characterized by superbursting. Finally, while there was a significant decrease in cLTP-induced increases in overall network activity that were pretreated with a reduction in the activity of any of these molecules, it was still significantly above control levels.

Taken together, these results suggest that MMPs and β1-integrin play an important role in the mechanism driving changes in network attractor state following 30 minutes of cLTP exposure. Indeed, network activity from networks pretreated with any of these antagonists most closely resembled activity patterns from cultures following 20 minutes of cLTP.
Next, to further characterize a mechanism involving both MMPs and β1-inTEGRIN, I tested the hypothesis that the ectodomain of ICAM-5, a product of MMP proteolysis, stimulated integrin dependent changes in network activity parameters. Cultures treated with ICAM-5 exodomain significantly increased overall network activity and network bursting in a dose-dependent manner. In addition, while there was no induction of superbursting with the highest dose of ICAM-5 tested, there was an increase in burst durations. I hypothesize that this increase in burst durations could suggest the presence of a drive towards-, but insufficient to induce superbursting activity. Importantly all effects of ICAM-5 treatment in this study were prevented by blocking β1-inTEGRINS.

In order to more fully characterize the potential roles of MMPs, integrins and ICAM-5 in cLTP-induced changes in network dynamics, future experiments with enhanced synaptic release of MMPs as potentially mediated by viral vector delivery, are warranted. In addition, future studies with CAM cleavage resistant mutants would be necessary to determine the extent to which cleavage of specific CAMs per se plays a role. Finally, given that varied MMP and CAM polymorphisms have also been linked to altered learning and memory or propensity for addiction (Uhl et al., 2008; Docherty et al., 2010; Samochowiec et al., 2010), future experiments that examine network dynamics in cultures expressing these polymorphisms may also be of interest.

In conclusion, I have described a variety of dynamical effects of cLTP induction using MEA recordings of hippocampal network activity. These results demonstrate the potential of the MEA system to act as high throughput screening tool for potential cognitive enhancers or other drugs that can improve memory for disorders such as Alzheimer’s. In addition, dynamical effects of non-pharmacological treatments (e.g. electrical stimulation, shRNA-gene expression knockdown, etc) can be measured to test new hypothesis of neuronal network dynamics. Taken
together, data obtained through experiments utilizing MEAs could provide valuable insights into *in vivo* brain activity.
References:


