CHOLINERGIC NEUROMODULATION OF ACTIVITY-DEPENDENT DISINHIBITION-MEDIATED PLASTICITY

by

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Abstract

Activation of muscarinic acetylcholine receptors (mAChRs) has pronounced effects on GABAergic interneurons, including depolarization of their resting membrane potential, and increasing their action potential and vesicular release frequency. Moreover, postsynaptic mAChR activation in hippocampal pyramidal neurons reduces the expression of the K⁺-Cl⁻ cotransporter (KCC2). However, whether mAChR activation modulates the expression of disinhibition-mediated synaptic plasticity has not been examined.

I induced inhibitory long-term potentiation (LTP) by applying coincident pre/postsynaptic stimulation in the hippocampus. This plasticity was characterized by an increase in the postsynaptic potential (PSP) amplitude and a depolarization in the inhibitory postsynaptic potential (IPSP) reversal potential; characteristics of disinhibition-mediated LTP (dmLTP). Activation of mAChRs during this plasticity induction protocol prevented the expression of dmLTP via a presynaptic downregulation of transmitter release. This was concluded from evidence that the PSP amplitude and IPSP reversal potential were unaltered, and paired-pulse depression occurred following plasticity induction in the presence of mAChR activation.
Acknowledgements

This thesis could not be completed without the motivating and inspirational support of Dr. John Yeomans. His enthusiasm for asking questions and proposing ideas related to my research project have allowed me to appreciate the defining ethos of a successful neuroscientist. His words of wisdom have never failed to provoke reflection and inspire maturity in my pursuit of my goals.

In making preparations and conducting the experiments for this thesis, I must thank the undergraduate work-study students Adwitia Dey and Vyshnavy Balendra for their tireless help. They each provided much needed assistance when I first started to make whole-cell patch clamp recordings, allowing me to devote my time to learning about the nuances of this recording setup and to climb the steep learning curve towards proficiency.

The production of this thesis has depended greatly on the academic and scholarly support of my supervisory committee members, without whom my research project endeavours would be misguided and lacking a coherent synthesis. Dr. Martin Wojtowicz has been a great source of knowledge about hippocampal physiology and electrophysiological techniques. He has provided invaluable guidance in proper scientific writing at the graduate level, as well as challenged me to escape the misconception of looking at the hippocampus as an isolated structure, but rather to appreciate the complexity with which it interacts with other brain areas. Dr. Vincent Tropepe has always managed to constructively question my research objectives in order to encourage me to think more clearly about the rationale of my project and how this thesis would eventually come together. Lastly, Dr. Melanie Woodin has been an immense help in all aspects of this thesis, and has provided encouragement and support throughout my graduate degree. She has provided the resources to complete this thesis, and allowed me to work independently to approach the goals of my research objectives, while always being available to answer questions and keep me focused on those objectives.
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<th>Definition</th>
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<tbody>
<tr>
<td>ACh</td>
<td>acetylcholine</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial cerebral spinal fluid</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>CA1/2/3</td>
<td><em>cornu ammonis</em> 1/2/3</td>
</tr>
<tr>
<td>CCh</td>
<td>carbamylcholine chloride, or carbachol</td>
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<tr>
<td>ChAT</td>
<td>choline acetyltransferase</td>
</tr>
<tr>
<td>CNQX</td>
<td>6-cyano-7-nitroquinoxaline-2,3-dione</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DF</td>
<td>driving force</td>
</tr>
<tr>
<td>DG</td>
<td>dentate gyrus</td>
</tr>
<tr>
<td>EC</td>
<td>entorhinal cortex</td>
</tr>
<tr>
<td>E_GABA</td>
<td>GABA reversal potential</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>E_PSP</td>
<td>PSP reversal potential</td>
</tr>
<tr>
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<td>Fisher’s F ratio</td>
</tr>
<tr>
<td>fEPSP</td>
<td>field excitatory postsynaptic potential</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide-binding protein</td>
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<tr>
<td>GTP</td>
<td>guanosine 5’-triphosphate</td>
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<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid</td>
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<tr>
<td>IPSP</td>
<td>inhibitory postsynaptic potential</td>
</tr>
<tr>
<td>KCC2</td>
<td>K⁺-C⁻ cotransporter 2</td>
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<tr>
<td>LTD</td>
<td>long-term depression</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>----------</td>
<td>------------------------------------------------</td>
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<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
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<td>LTPm</td>
<td>muscarinic receptor dependent long-term potentiation</td>
</tr>
<tr>
<td>$M_1$</td>
<td>muscarinic acetylcholine receptor 1</td>
</tr>
<tr>
<td>$M_2$</td>
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<td>$M_5$</td>
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</tr>
<tr>
<td>mAChR</td>
<td>muscarinic acetylcholine receptor</td>
</tr>
<tr>
<td>$n$</td>
<td>number of samples</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>NMDA</td>
<td>$N$-methyl-$D$-aspartate</td>
</tr>
<tr>
<td>$p$</td>
<td>probability of test statistic occurring by chance</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PPD</td>
<td>paired-pulse depression</td>
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<tr>
<td>PPR</td>
<td>paired-pulse ratio</td>
</tr>
<tr>
<td>PSC</td>
<td>postsynaptic current</td>
</tr>
<tr>
<td>PSP</td>
<td>postsynaptic potential</td>
</tr>
<tr>
<td>RMP</td>
<td>resting membrane potential</td>
</tr>
<tr>
<td>s.l-m.</td>
<td>stratum lacunosum-moleculare</td>
</tr>
<tr>
<td>s.or.</td>
<td>stratum oriens</td>
</tr>
<tr>
<td>s.pyr.</td>
<td>stratum pyramidale</td>
</tr>
<tr>
<td>s.rad.</td>
<td>stratum radiatum</td>
</tr>
<tr>
<td>STDP</td>
<td>spike timing-dependent plasticity</td>
</tr>
<tr>
<td>$t$</td>
<td>Student’s test statistic</td>
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<tr>
<td>$U$</td>
<td>unbiased statistic</td>
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<tr>
<td>$V_m$</td>
<td>membrane potential</td>
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Chapter 1
Introduction

1 Overview

Neuronal synaptic connections are locations at which signaling occurs between neurons, providing a distinction between pre and post cellular roles and their combined functional relevance in information transfer. The structures forming the synapse and participating in the information transfer across the synapse are dynamic, and in some cases respond to the previous level of activity across the synapse (Bliss and Lømo, 1973; Zucker, 1989). They can also be modulated by distinct classes of neuromodulators, which can act to either augment or attenuate the information transfer between the pre and post synaptic neurons. This neuromodulation is a regulatory mechanism by which synaptic molecules, either from the primary synaptic partners or from other cellular sources in the vicinity, act to maintain, or facilitate change in synaptic transmission (Bliss et al., 1983; Segal and Auerbach, 1997). Activity dependent synaptic plasticity can be enhanced or diminished in the presence of specific neuromodulators as they act to fine tune information transfer (Bear and Singer, 1986; Thomas et al., 1996; Seol et al., 2007), and are thought to be important for learning and memory (Morris et al., 1986; Huerta and Lisman, 1993; Hasselmo, 2006). Neuromodulators include the classes of diffuse modulatory neurotransmitter systems of the mammalian brain, including the noradrenergic, dopaminergic, serotonergic, and cholinergic systems, and are classified based on the neurotransmitter type they predominantly utilize (Hasselmo, 1995).

Acetylcholine (ACh) modulates excitatory glutamatergic synapses in the mammalian brain, and can facilitate LTP in the CA1 region of the hippocampus (Blitzer, et al, 1990; Auerbach and Segal, 1994), in the dentate gyrus (Natsume and Kometani, 1997), and in piriform cortex (Patil, et al., 1998). However, although neuromodulators, including ACh, have been shown to regulate GABAergic inhibitory interneuron activity and synaptic functions, particularly by altering the resting membrane potential and transmitter release probability (Pitler and Alger, 1992; Behrends and ten Bruggencate, 1993; McQuiston, and Madison, 1999; Patil and Hasselmo, 1999), the influence of ACh on the plasticity of inhibitory synapses is unclear. There is no evidence for the role of ACh in the modulation of dmLTP. Therefore, the general objective of this thesis was to elucidate the contribution of ACh, acting via muscarinic receptors, to dmLTP.
1.1 The hippocampus as a model system

For centuries the hippocampus has been an ideal structure for studying neurobiology in the mammalian brain. Santiago Ramón y Cajal and Rafael Lorente de Nó are credited for distinguishing the various subregions of the hippocampus as we know them today, and outlining the direction in which neurons communicate with each other across subregions within this structure (Andersen et al., 2007).

The hippocampal formation is a medial temporal lobe structure which is made up of the entorhinal cortex (EC), the dentate gyrus (DG), the hippocampus proper (or the subdivisions of the cornu ammonis [CA], CA3, 2, and 1), subiculum, presubiculum, and parasubiculum (Amaral and Lavenex, 2007). It forms a bilateral structure, which is largely conserved in organization in rodents, primates, and humans (Amaral and Lavenex, 2007). Within this structure are the allocortical regions, the DG, hippocampus proper, and subiculum, of which the DG, CA3, and CA1 form the synaptic termination points of the trisynaptic circuit (figure 1.1). Although reciprocal connections are a common feature in the neocortex, the apparent lack of direct reciprocal connections between subdivisions of the hippocampal formation give this area of cortex a unique organization (Amaral and Lavenex, 2007).

The perforant pathway forms the first connection in the processing loop of the hippocampal formation. It forms between the superficial cell layers of the EC and the DG granule cell layer. These DG granule cells subsequently form the mossy fiber connections to CA3 pyramidal neurons, which in turn form the Schaffer collateral pathway from CA3 to CA1 pyramidal neurons. Similarly, CA1 pyramidal neurons project their axons predominantly to subiculum pyramidal neurons, which close the processing loop of the hippocampal formation by subsequently forming axon terminations in the deep cell layers of the EC. Each of these connections utilizes excitatory glutamatergic neurotransmission, and forms the main thoroughfare information processing circuit of the hippocampal formation (figure 1.2) (Andersen et al., 1966, 1971; Malthé-Sörenssen et al. 1979; Hablitz and Langmoen, 1982). However, in the mature brain, the excitatory synapses are balanced by feed-forward and feed-back inhibitory GABAergic interneuron synapses, adding further complexity to the network activity (Sloviter, 1991). Furthermore, the ramification and termination of diffuse projection systems into the cellular layers of the intrinsic circuitry of the hippocampal formation provide extrinsic sources of
neurotransmission acting to regulate intercellular signaling, signal transduction, and oscillatory rhythm generation (Cobb and Davies, 2005).

The extensive electrophysiological data collected from studies in rodent hippocampi reflects the well organized and clearly demarcated of regions within the hippocampal formation, and their unidirectional neurotransmission allowing for the study of the causal mechanisms of altered synaptic activity. In particular, each area of the hippocampal formation has a clearly distinguishable layered structure. In the CA1, these layers, from the most superficial to deep, consist of the stratum lacunosum-moleculare bordering the hippocampal fissure, stratum radiatum, stratum pyramidale, and the stratum oriens (figure 1.1) (Amaral and Lavenex, 2007). The stratum lacunosum-moleculare consists of the afferent fibers of the Schaffer collateral pathway and their synapses onto CA1 pyramidal neuron apical dendrites (Zimmer and Gähwiler, 1984). In addition to the constituents of stratum lacunosum-moleculare, the stratum radiatum is the suprapyramidal region which includes interneurons and commissural fibers. The principal cell layer of the CA1 is the stratum pyramidale, consisting of relatively small pyramidal neurons receiving afferent input from Schaffer collateral axons, as well as interspersed populations of interneurons. Lastly, the stratum oriens, the deepest layer of the CA1, adjacent to the alveus, contains the basal dendrites of pyramidal neurons, the cell bodies of interneurons, and commissural fibers, making it similar in composition to the stratum radiatum.

The organization of cells into particular layers, and their projections within the transverse plane offer remarkable advantages to studying neuronal connections in the hippocampus in an in vitro preparation. In this way, a target neuron can be readily visualized, and its afferent fibers can be stimulated with the presumption that at least a subset of those fibers synapse onto that particular target neuron, or onto an intermediate interneuron.

1.1.1 Advantages

The mouse hippocampus was used as a model system to address the objectives of this thesis. This brain structure possesses a defined architecture, outlined above, by which putative identification of target neurons can be made within the acute slice preparation by their location within the laminate structure of the hippocampal subregions, despite the absence of biomarkers or immunolabeling. Thus, for the following electrophysiology experiments, CA1 pyramidal neurons were identified solely by their location within the lamina of the stratum pyramidale.
Electrophysiological evidence for the mechanisms underlying various forms of synaptic plasticity has utilized the rodent hippocampus primarily due to the ease of dissection and identification of the hippocampal formation. The hippocampus proper has clear axonal projections and sites of termination within the trisynaptic circuit, which can be used to identify the pre/post synaptic regions of interest for studying synaptic plasticity. As such, stimulation of the stratum radiatum between CA3 and CA1, during pharmacological blockade of excitatory transmission provides a basis for studying the inhibitory component to synaptic transmission, and *vice versa* for studying excitatory transmission. This reflects the presence of both excitatory synapses between principal cells and inhibitory synapses from GABAergic interneurons in the mature mammalian hippocampus. Each of these contribute to the integration of an excitation/inhibition balance which may be maintained or altered following plasticity induction (Pettit and Augustine, 2000; Liu, 2004; Gatto and Broadie, 2010). Furthermore, the accessibility and resilience of the rodent hippocampal slice preparation has made it an ideal method by which to examine plasticity mechanisms while maintaining tissue architecture and connectivity in an *in vitro* setting. This *in vitro* preparation further provides the advantage of studying changes in transmission in response to applied changes in the extracellular environment; i.e. by altering the extracellular dissolved salt composition, or by applying pharmacological manipulations.

Lastly, hippocampi are largely conserved in structure, and presumably function, between mammalian species (Chiaia and Teyler, 1982, Amaral and Lavenex, 2007). Therefore knowledge of how synaptic plasticity is modulated in the rodent hippocampus will provide a general basis for understanding how mechanisms of synaptic plasticity are modulated in neuronal networks.
Figure 1.1 Transverse connectively of the rodent hippocampus.
A) Depicts excised hippocampal neuroanatomical features from the left hemisphere of the rodent brain, and its septotemporal (S→T) orientation. B) Expanded transverse section highlights unidirectional connections of perforant pathway (PP) to dentate gyrus (DG), mossy fibers (mf) from DG granule neurons to CA3 pyramidal neurons, Schaffer collateral (sc) axons from CA3 to CA1 pyramidal neurons, and CA1 pyramidal axon termination on subiculum (S) pyramidal neurons. C) Expanded CA1 region depicts laminate allocortex: stratum oriens (s. or.), stratum pyramidale (s. pyr.), stratum radiatum (s. rad.), and stratum lacunosum-moleculare (s. l-m.). Illustration adapted from Amaral and Witter (1989).
Figure 1.2 Connectivity of the hippocampal formation and its output pathways.
Schematic of unidirectional connections between neurons in each subregion of the hippocampal formation, beginning with the medial and lateral entorhinal areas (MEA and LEA, respectively), and the subsequent outputs to various brain regions are listed. Connections reflect topographically conserved organization of information processing in the transverse plane of the hippocampal formation. Omitted are varicosity terminations of diffuse modulatory neurotransmitter systems. From Amaral and Lavenex (2007).
1.2 Hippocampal neuron populations and interneuron networks

The hippocampal formation is made up of principal cells and interneurons which form various connections with each other to make up the hippocampal network. In the CA1 regions, the principal cell type is the pyramidal neuron, which has its soma located in the stratum pyramidale. It is the most easily distinguishable neuron in the CA1 based on its size and orderly arrangement in the principal cell layer. Pyramidal neurons use glutamate as their neurotransmitter, and from the CA1, have axons which terminate on other pyramidal neuron in the subiculum (Amaral and Lavenex, 2007). They receive their main glutamatergic input from CA3 pyramidal neurons, forming the final synaptic connection of the trisynaptic circuit (Andersen et al., 1971).

The interneurons of the CA1 hippocampus are diverse in structure and function. Based on morphological characteristics, they can broadly be categorized as: oriens-lacunosum moleculare (O-LM), basket, or bistratified cells (figure 1.3). Their structure and axon termination sites on pyramidal neurons highlight a presumed distinction in their function in regulating hippocampal excitability (Lawrence, 2008). In particular, basket cells target perisomatic regions, and thus are involved in regulating the action potential production in pyramidal neurons. In contrast, bistratified interneurons have axons which are spatially arranged to allow it to influence the integration of excitatory inputs from the stratum oriens and radiatum.

Interneurons can also be classified by the positive expression of a wide variety of neurochemicals, such as: parvalbumin (PV+), cholecystokinin (CCK+), and somatostatin (SOM+). These interneurons have distinct roles in the hippocampal network, such as the regulation of fast rhythmic network oscillations by the fast spiking PV+ basket cells (Sohal et al. 2009; Cardin et al., 2009).

Furthermore, interneurons also create inhibitory synaptic networks in the hippocampus, and can function to provide either feedforward or feedback inhibition through disynaptic circuits (Kullmann, 2011). Feedback inhibition occurs via the innervation of an interneuron by a CA1 pyramidal neuron, and the subsequent re-innervation of the pyramidal neuron by that interneuron. This form of auto-inhibition may play a role in the generation of pacemaker rhythms, and contribute to network oscillatory activity (Andersen et al., 1963; Economo and White, 2012). Feedforward inhibition in the CA1 occurs by Schaffer collateral innervation of interneurons targeting CA1 pyramidal neurons. This process is involved in the expression of
disinhibition-mediated plasticity, as it has been suggested that a reduced feedforward inhibitory drive facilitates excitation at the CA1 pyramidal neuron (Ormond and Woodin, 2009).

1.2.1 GABAergic signaling and Cl⁻ regulation

The primary inhibitory neurotransmitter of the mature central nervous system is γ-aminobutyric acid (GABA) (Hayashi, 1959; Roberts et al., 1960), which generally acts on GABA receptors to promote membrane hyperpolarization (Iversen et al., 1971; Connors et al., 1988; McCormick, 1989). GABA is synthesized from glutamate (Olsen and DeLorey, 1999) in interneuron terminals and released in response to interneuron action potential firing. GABA receptors include the GABA_A and GABA_B subtypes, which are differentially expressed on either the pre- and/or postsynaptic membrane (Dutar and Nicoll, 1988), allowing GABA to influence the synaptic activity at either location.

GABA_A receptors are pentameric ligand-gated Cl⁻ channels which are generally expressed postsynaptically on CA1 pyramidal neurons (Sperk et al., 1997; Essrich et al., 1998; Pettit and Augustine, 2000), and are the primary receptor subtype mediating fast synaptic inhibition of CA1 pyramidal neurons (Isaacson et al., 1993). GABA_A receptors are made up of 5 subunits in either a homomeric or heteromeric composition (Maric et al., 1999). GABA binding to GABA_A receptors allows Cl⁻ to flow across the membrane (Dunn et al., 1989; Kardos, 1993). The magnitude and direction of the Cl⁻ current is determined by the relationship between the membrane potential (V_m) and the equilibrium potential for Cl⁻ (E_Cl⁻) (Thompson and Gähwiler, 1989; Ling and Benardo, 1995). The ionic driving force (DF) for Cl⁻ is the difference between V_m and E_Cl⁻. At a V_m hyperpolarized to E_Cl⁻ the Cl⁻ current is depolarizing, whereas the Cl⁻ current is hyperpolarizing when V_m is depolarized to E_Cl⁻ (Fiumelli and Woodin, 2007). GABA_A receptor activation can also shunt neighbouring excitatory currents, a phenomenon termed shunting inhibition (Fiumelli and Woodin, 2007). When E_Cl⁻ is equal to V_m, no current is directly evoked by GABA_A receptor activation and inhibition is entirely achieved through shunting (Ben-Ari, 2002). In mature neurons, the electrochemical gradient governing the Cl⁻ current results in a hyperpolarization of V_m (Rivera et al., 1999; Ben-Ari et al., 2012). However, the magnitude and direction of the Cl⁻ current may change in response to changes in the [Cl⁻] or the membrane potential, which can be invoked following excessive synaptic firing during seizure-like activity (Kaila et al., 1997; Zhang et al., 2012). Since Cl⁻ is the primary permeable ion species of the
GABA<sub>A</sub> receptor channel, the value of $E_{\text{Cl}^-}$ is often taken as being equivalent to the reversal potential of the GABA mediated PSP ($E_{\text{GABA}}$) (Collingridge et al., 1984). GABA<sub>A</sub> receptors are also permeable to bicarbonate ($\text{HCO}_3^-$), however, $\text{HCO}_3^-$ is 70-80% less permeable than Cl<sup>-</sup>, and therefore contributes considerably less to the GABA<sub>A</sub> mediated postsynaptic activity (Kaila et al., 1993).

In mature neurons with low a $[\text{Cl}^-]_i$ the Cl<sup>-</sup> concentration gradient arises from membrane transporters that predominantly extrude Cl<sup>-</sup> from the neuron (Rivera et al., 1999). In particular, KCC2 is a K<sup>+</sup>/Cl<sup>-</sup> symporter, which moves Cl<sup>-</sup> out by utilizing the K<sup>+</sup> gradient (Payne, 1997; Willams and Payne, 2004). In contrast, the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> symporter 1 (NKCC1) moves Cl<sup>-</sup> into the cell along with Na<sup>+</sup> and K<sup>+</sup>. However, during development, KCC2 expression is upregulated, while NKCC1 expression is downregulated, thereby favoring Cl<sup>-</sup> extrusion, and a more hyperpolarized $E_{\text{Cl}^-}$ (Rivera et al., 2004; Blaesse et al., 2009; Hyde et al., 2011). Therefore, KCC2 functions to predominately regulate [Cl<sup>-</sup>] homeostasis in mature neurons.

GABA<sub>B</sub> receptors are G protein-coupled receptors which activate GIRK channels (Sodickson and Bean, 1996) and inhibit voltage-gated Ca<sup>2+</sup> channels (Wang and Lambert, 2000), hyperpolarizing and suppressing transmitter release, respectively, when expressed on presynaptic interneuron terminals (Wang and Lambert, 2000). During plasticity induction, excessive GABA release from interneuron terminals results in feedback inhibition by the synaptic GABA activating presynaptic GABA<sub>B</sub> receptors, thereby suppressing further release (Davies et al., 1991; Jarolimek and Misgeld, 1997). This disinhibits the postsynaptic neuron, shifting the excitation/inhibition balance towards promoting neuronal excitation, and is a mechanism for the expression of short-term plasticity (STP) (Zucker and Regehr, 2002).
Figure 1.3 Pyramidal neuron, and the morphological diversity of interneuron subtypes in the CA1 hippocampus.

Morphological reconstructions depict various interneuron types present in the CA1 hippocampus. A pyramidal neuron representation is used to distinguish the approximate locations of dendrites and somata within the hippocampal layers. The interneurons of the CA1 region of the hippocampus can be distinguished by their morphology, which is specialized to innervate particular regions of pyramidal neurons. Oriens-lacunosum moleculare (O-LM) cells have their soma within the pyramidal cell layer, and project their axon into the stratum lacunosum-moleculare to synapse on the distal dendrites of pyramidal neurons. Basket cells reside in the stratum oriens and have axons that terminate in the stratum pyramidale layer, and thus mainly synapse on the pyramidal neuron perikarya and proximal dendrites. Bistratified stratum oriens interneurons project their axons to both the stratum radiatum and stratum oriens to innervate apical and basal dendrites of the pyramidal neurons, as well as Schaffer collateral axon terminals. From Lawrence, 2008.
1.3 Cholinergic signaling and its cognitive relevance

ACh as a chemical neurotransmitter was first identified by Dale et al. (1914, 1936) and Loewi (1921), and holds the special distinction of being the first identified neurotransmitter. Since being identified as a vagal nerve neurotransmitter involved in regulating cardiac muscle contraction (Dale, 1914), the number of roles for the use of ACh as a transmitter has burgeoned. ACh is also a neuromodulator in both the peripheral and central nervous systems (PNS and CNS, respectively), and it is widely used at the skeletal neuromuscular junction (NMJ) across many phyla of the animal kingdom (Karczmar, 2006). This demonstrates its ancient phylogenetic development and evolutionary conservation as an information transfer molecule.

In the mammalian brain, neurons utilizing ACh as their primary neurotransmitter are generally localized to subcortical nuclei within the midbrain and forebrain, and have diffuse axonal projections to both cortical and subcortical structures (Yeomans, 2012). Anatomical evidence from rodents suggests that there are a total of eight distinguishable nuclei that predominantly express choline acetyltransferase (ChAT); an indicator of the synthesis and utilization of ACh in neurotransmission (Mesulam, 1988). These nuclei, cholinergic cell group 1-8 (Ch1-8) as defined by Mesulam et al. (1983), have unique and diffuse projections to other brain areas, and thus fundamentally underlie a diverse collection of cognitive and behavioural processes. In particular, the Ch3 group of cells, located bilaterally within the medial septal nuclei, project along the fimbriae-fornix pathway to ramify and terminate within the principal cell layers of the hippocampus proper (Yeomans, 2012). This forms the septohippocampal pathway, made up of cholinergic and GABAergic afferent fibers (Dutar et al. 1995). In the hippocampus, ACh neurotransmission is critical in learning and memory (Hasselmo, 2006), and physiologically in entraining the underlying theta band oscillatory rhythm of these and their related processes (Chapman and Lacaille, 1999). In addition, the ascending diffuse cholinergic projection system has fundamental roles in the sleep/wake cycle (Marrosu et al., 1995), circadian rhythms (Liu and Gillette, 1996), and in promoting arousal and attention (Dutar et al., 1995). Taken together, the roles of cholinergic signaling within the mammalian brain demonstrate it to be a vital component in cognition and behavior. This is further evident from the pathophysiological implications of cholinergic signaling dysregulation and/or degeneration, and in the resulting cognitive deficits and associated disease states, such as in pathological addictive behaviour, and underlying various symptoms of schizophrenia and Alzheimer’s disease (Bowen et al., 1983).
The diversity of cholinergic receptor types, nicotinic (nAChR) and mAChR, and the various combinations of subunits of the former and subtypes of the latter, allow ACh to have a diverse range of effects on cellular activity. Generally, nAChRs are ligand-gated ion channel ionotrophic receptors, formed from 5 transmembrane subunits arranged to form a cation channel (figure 1.4B) (Unwin, 2005). By contrast, mAChRs are ligand-dependent G protein coupled metabotropic receptors, whose cellular effects are dependent on the downstream intracellular effector cascade (figure 1.4A) (Hulme et al. 1990).

1.4 Muscarinic receptor-dependent signaling

The main synaptic effects of mAChR activation consist of postsynaptic excitation, postsynaptic inhibition, or presynaptic inhibition (Qian and Saggau, 1997; Kremin et al., 2006; Brown, 2010). These effects are mediated by separate subtypes of mAChRs and their localization on either pre- or postsynaptic sites. All mAChRs are coupled to intracellular G proteins through which signal transduction occurs (Brown, 2010). The class of G protein coupled to each mAChR determines the intracellular effectors which propagate the signal transduction cascade (figure 1.3A) (Hulme et al., 1990). M1, M3, and M5 receptor subtypes are coupled to Gq-type G proteins, by which membrane-bound phospholipase C β (PLCβ) activation mediates cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) (Berstein et al., 1992; Felder, 1995; Brown, 201). Increased cytosolic [IP3] leads to an increase in intracellular [Ca2+] via opening of IP3-dependent calcium channels on the endoplasmic reticulum (ER) (de Sevilla et al., 2008). Intracellular Ca2+ and DAG activate protein kinase C, by which various protein phosphorylation events lead to postsynaptic excitation (Felder, 1995). Postsynaptic excitation by M1, M3, and/or M5 receptor activation occurs through intracellular cascade coupling to the modulation of various membrane channels, depending on which channels are co-expressed on the postsynaptic membrane. Neuronal depolarization and/or increased excitability responses to M1, M3, and/or M5 activation are mediated by Kv7 inhibition, KsAHP inhibition, Kleak inhibition, or cation channel activation (Brown et al., 2007). In hippocampal pyramidal neurons, a K+ current mediated by Kv7 channels, the M-current, is inhibited by the PLCβ mediated decrease in PIP2, thus causing an increase in intracellular K+ and membrane depolarization (Brown and Adams, 1980; Halliwell and Adams, 1982; Shah et al., 2008). This leads to an increase in spontaneous action potential firing (Brown et al, 2007). Furthermore, in hippocampal pyramidal neuron, mAChRs also inhibit Ca2+-dependent K+
channels, which also prevents K\(^+\) from repolarizing the neuron during action potential firing, and enhancing neuronal excitability (Nicoll, 1985).

Muscarinic receptor dependent postsynaptic inhibition is mediated by M\(_2\) receptor activation coupled to the G\(_i\)-type G protein, and the subsequent activation of G protein-gated inward rectifier K\(^+\) channels (GIRK channels; e.g. K\(_{ir}\)3.1 and K\(_{ir}\)3.2) by coupling to G\(_{\beta\gamma}\)-type G-proteins (Egan and North, 1986; Fernandez-Fernandez et al., 1999, 2001). By contrast, presynaptic inhibition is mediated by M\(_4\) receptor activation coupled to the G\(_o\)-type G proteins (Zhang et al., 2002). However, although the G\(_o\)-type G proteins also directly couple with G\(_{\beta\gamma}\)-type G-proteins, in the presynaptic terminal this leads to inhibition of Ca\(_{v}\)2 channels (Blackmer, et al., 2001). This prevents Ca\(^{2+}\) entry into the presynaptic neuron, and inhibits presynaptic transmitter release (D’Agostino et al., 1997; Trendelenburg et al., 2003).

The response of CA1 neurons to mAChR activation depends on the receptor subtypes which they express. In particular, based on an immunoprecipitation analysis of mAChR expression in rat, the M\(_1\) receptor is highly expressed on hippocampal pyramidal neuron dendrites and somata, but low in various GABAergic interneuron subtypes (Levey et al., 1995; Yamasaki et al., 2010; Dasari and Gulledge, 2011). Also, M\(_1\) expression represents approximately 60% of the total hippocampal mAChR content, while M\(_3\) represents <10% and M\(_5\) expression is sparse (Dasari and Gulledge, 2011). M\(_3\) is mostly expressed on pyramidal neurons and in stratum lacunosum-moleculare (Levey et al., 1995). M\(_2\) and M\(_4\) receptors are mostly expressed on non-pyramidal neurons, and presynaptically on non-cholinergic axons of the septohippocampal pathway (Levey et al., 1995). Thus, in hippocampal pyramidal neurons, the predominant mediator of the muscarinic-receptor dependent postsynaptic excitation is the M\(_1\) receptor subtype. Therefore, through blockade of M- and afterhyperpolarization-currents, ACh activation of M\(_1\) receptors facilitates pyramidal neuron firing (Gulledge et al., 2009).

Furthermore, the effect of mAChR activation on synaptic plasticity also depends on the localization of mAChR subtypes and their relative expression pattern. In CA3 pyramidal neurons, muscarine depresses LTP via blockade of voltage-gated Ca\(^{2+}\) channels (Williams and Johnston, 1988). By contrast, in the DG and CA1, LTP is enhanced by muscarine application (Blitzer et al., 1990; Burgard and Sarvey, 1990), and is blocked by scopolamine, a muscarinic receptor antagonist (Hirotsu et al., 1989).
Figure 1.4 Structural conformations of acetylcholine receptor subtypes, and their intracellular effectors.

A) Diagrammatic depiction of the generic structure of mAChRs, showing orthosteric and allosteric binding sites. Muscarinic receptors are 7-transmembrane spanning G protein coupled receptors. G protein coupled intracellular signal transduction pathways highlight key effectors mediating the neuronal response to receptor ligand binding. M1, M3, and M5 receptor subtypes, through coupling with Gq/G11-type G proteins, share similar intracellular effectors, listed on the left. M2 and M4 receptor subtypes share key effectors, listed on the right, via coupling to Gi/o-type G proteins. B) The signaling pathway and structure of two example subtypes of nAChRs consisting of pentamers of α and β subunits. Transmembrane pore formation and selective cation currents allow nAChRs to trigger rapid intracellular signaling pathways. Homomeric α7 subtype nAChRs are more permeable to Ca\(^{2+}\) than are the heteromeric α4β2 subtype. However, through coupling with intracellular mechanisms, distinct patterns of Ca\(^{2+}\) signaling can provide a broader regulation of synaptic plasticity, transmitter release, and gene transcription. Key intracellular signaling effects are listed below. Adapted from Jones et al. 2012.
1.5 Synaptic plasticity

Activity-dependent synaptic plasticity is a property of synaptic connections by which the magnitude of the postsynaptic response is altered in response to a specific pattern of synaptic activity. This is the cellular mechanism underlying Hebb’s postulate of how particular connections are strengthened by repeated or persistent firing (Hebb, 1949), and by which neuronal networks encode learning and memory (Shapiro and Eichenbaum, 1999).

LTP was first demonstrated by Bliss and Lømo (1973) in the hippocampi of anesthetized rabbits. Several studies since then have shown that providing high frequency stimulation at a presynaptic locus leads to a potentiation of synaptic efficacy which can last for hours in an in vitro preparation (Neves et al., 2008), or up to a year in vivo (Abraham et al., 2002). Furthermore, the plasticity expressed is input-specific, and thus confined to the synapses which were innervated by the stimulated pathway (Andersen et al., 1977). This LTP, evoked by high frequency, or tetanic stimulation, is the classical description of synaptic plasticity at excitatory glutamatergic synapses (Bliss and Collingridge, 1993). During low frequency stimulation, or basal synaptic activity, glutamate triggers the opening of the Na\(^+/K^+\) permeable AMPA receptors on the postsynaptic neuron, which allow Na\(^+\) current into the postsynaptic neuron, thereby depolarizing the membrane potential (Malenka and Nicoll, 1999). However, although the Ca\(^{2+}/Na^+\) permeable NMDA glutamate receptor is expressed on the postsynaptic membrane as well, a Mg\(^{2+}\) ion blockade of the receptor pore prevents Ca\(^{2+}\) entry into the postsynaptic neuron, despite ligand-receptor binding (Otmakhova et al., 2002). The AMPA receptor mediated depolarization represents the excitatory postsynaptic potential (EPSP) evoked in the absence of a high frequency stimulus (Bliss and Collingridge, 1993). During high frequency stimulation, however, the magnitude and duration of postsynaptic depolarization via Na\(^+\) entry by AMPA receptor opening leads to the electrostatic repulsion and removal of the Mg\(^{2+}\) block on NMDA receptors (Ault et al., 1980; Mayer et al., 1984; Nowak et al., 1984). This allows Ca\(^{2+}\) entry into the postsynaptic neuron, in which it acts as a second messenger to trigger the activation of Ca\(^{2+}\)-dependent protein kinases and alters the gene expression (Bliss and Collingridge, 1993). Ultimately, the expression of LTP following induction is dependent on the phosphorylation and insertion of AMPA receptors on the postsynaptic membrane, thus facilitating the AMPA receptor mediated Na\(^+\) conductance, and potentiating the magnitude of the EPSP (Bliss and Collingridge, 1993).
A novel form of LTP, dmLTP, has been demonstrated at CA1 pyramidal neurons (Ormond and Woodin, 2009). During dmLTP, the postsynaptic response to Schaffer collateral mediated glutamateric transmission is enhanced by the attenuation of the disynaptic GABAergic feed-forward inhibition (Ormond and Woodin, 2011). This disinhibition results from the accumulation of Cl\textsuperscript{-} in the postsynaptic neuron, thereby diminishing its DF during subsequent GABAergic transmission (Ormond and Woodin, 2009). In particular, plasticity induced at the GABAergic synapse leads to Ca\textsuperscript{2+} entry into the postsynaptic neuron via L-type Ca\textsuperscript{2+} channels, leading to the downregulation of the K\textsuperscript{+}-Cl\textsuperscript{-} cotransporter 2 (Woodin et al., 2003). This results in an increase in the intracellular [Cl\textsuperscript{-}] causes a depolarizing shift in the Cl\textsuperscript{-} current reversal potential. This inhibitory synaptic plasticity was evoked by low frequency (5 Hz), and short duration (30 s) pairing of pre- and postsynaptic stimulation. This protocol utilizing coincident stimulation at pre- and postsynaptic neurons is known as spike timing-dependent plasticity (STDP), and is thought to reflect a more physiologically relevant model of synaptic plasticity (Bi and Poo, 1998).

**1.5.1 Spike timing-dependent plasticity**

The coincident stimulation of pre- and postsynaptic neurons is the fundamental cellular mechanism by which changes in synaptic efficacy occur. This reflects the Hebbian theory of learning and memory, by which coincident activity leads to a facilitation of the synaptic strength, and the cellular mechanism of NMDA receptor-dependent coincident detection. Therefore, STDP results from the relative timing of pre- and postsynaptic stimulation, and does not require a crude high frequency stimulation protocol in order to modify the synaptic efficacy.

However, the relative timing of pre- and postsynaptic stimulation is vital to the expression of LTP. Bi and Poo (1998) showed that the precision of spike timing required that both the presynaptically evoked EPSP and the postsynaptic action potential be correlated within an 80 ms time window or order to alter the synaptic strength (figure 1.5). Furthermore, the polarity of the synaptic plasticity depends on the relative order of pre- and postsynaptic stimulation. Presynaptic followed by postsynaptic stimulation results in LTP, whereas the converse order of pairing results in long-term depression (LTD) (Bi and Poo, 1998).

The spike timing effects of plasticity induction have also been demonstrated at GABAergic synapses. Woodin et al., (2003) showed that GABAergic STDP is unique, in that the order of pre- and postsynaptic activity does not alter the polarity of the plasticity expression (figure 1.6).
Furthermore, a more narrow 40 ms time window between correlated pre- and postsynaptic activity is necessary for a change in the GABAergic postsynaptic current (GPSC) amplitude. Inhibitory synaptic activity has been suggested to be mediated by a postsynaptic GABA\textsubscript{B} receptor mediated IP\textsubscript{3}-dependent Ca\textsuperscript{2+} signal (Komatsu, 1996).

Since an increase in the intracellular [Ca\textsuperscript{2+}] underlies the signal transduction pathways leading to the postsynaptic expression of synaptic plasticity, activation of Ca\textsuperscript{2+} signaling by other receptors during plasticity induction may further enhance the plasticity expression.

### 1.6 Modulation of synaptic plasticity

Synaptic plasticity can be enhanced or suppressed by the action of neuromodulatory transmitters (Hawkins et al., 1993). In particular, cholinergic innervation by muscarinic receptors has previously been shown to form an integral component of hippocampal LTP (Blitzer et al., 1990; Leung et al., 2003). Muscarinic receptor antagonists, such as scopolamine, block LTP (Ito et al., 1988), while mAChR agonists, such as carbachol (CCh), lower the threshold for LTP at glutamatergic synapses (Segal and Auerbach, 1997). Cholinergic innervation of the CA1 hippocampus, and the expression of several mAChR subtypes, allow ACh to modulate the expression of muscarinic-dependent LTP (LTPm) (Auerbach and Segal, 1996). Muscarinic-dependent LTP involves a G protein mediated block of I\textsubscript{AHP} and I\textsubscript{M}, while enhancing NMDA receptor function (Segal and Auerbach, 1997; Marino et al., 1998). Furthermore, cholinergic activity is necessary for the generation of the hippocampal theta rhythm (Buzsáki, 2002), which is thought to be involved in learning and memory formation (Hasselmo and Giocomo, 2006). Therefore, ACh is thought to have a crucial role in modulating the expression of synaptic plasticity in the hippocampus.
Figure 1.5 Spike timing-dependent effects on the expression of synaptic plasticity.
Coincident pre- and postsynaptic activity forms the basis of STDP induction at glutamatergic synapses. Each open circle represents an individual synapse recorded 20-30 min following repetitive correlated activity. Synaptic plasticity is represented as a percentage change in the excitatory postsynaptic current (EPSC) relative to the measured pre-induction baseline amplitude. A critical window of ±40 ms between EPSC and action potential firing (Δt) is present, in which STDP is expressed as either LTD (−40 < Δt < 0) or LTP (0 < Δt < 40). Figure adapted from Bi and Poo (1998).
Figure 1.6 Spike timing-dependence of inhibitory synaptic plasticity expression. Coincident pre- and postsynaptic activity also forms the basis of STDP induction at GABAergic synapses. Each open circle/square represents an individual synapse recorded 10-20 min following repetitive correlated activity. Synaptic plasticity is represented as a percentage change in the GABAergic postsynaptic current (GPSC) relative to the measured pre-induction baseline amplitude. A critical window of ±20 ms between GPSC and action potential firing is present, in which LTP is expressed. Figure adapted from Woodin et al., 2003.
1.7 Evidence for a mAChR role in modulating STDP at inhibitory synapses

The presence of mAChRs on interneurons and pyramidal neurons in the CA1 (Fukudome et al., 2004), and the projection of cholinergic septal nuclei varicosities to the hippocampus suggest a role for mAChR activation in regulating hippocampal network activity and neuron populations (Auerbach and Segal, 1994; Cobb and Davies, 2005; Giocomo and Hasselmo, 2007).

ACh, acting on M1 mAChRs potentiates Ca\(^{2+}\) transients in CA1 pyramidal neurons, and through second messengers is known to increase the intracellular calcium concentration from intracellular stores (Nakamura et al., 1999; Cho et al., 2008). This response has been shown to modulate plasticity induction at glutamatergic synapses invoked by high frequency stimulation (Giessel et al., 2010; Rathouz et al., 1995; Fisher et al., 1990).

Furthermore, the reversal potential for GABAergic currents, which depends on the concentration gradient of the main GABA\(_A\)-receptor permeable ion species, Cl\(^—\), is altered following the induction of dmLTP (Ormond and Woodin, 2009). This concentration gradient is established during neuronal development, and critically depends on the K\(^+\)-Cl\(^—\) cotransport activity of KCC2 (Rivera et al., 1999). CCh, a muscarinic receptor agonist, has been shown to reduce the total cellular expression of KCC2 (Lee et al., 2010), and thereby presumably alter the Cl\(^—\) gradient. Therefore, I examined whether mAChR activation can facilitate, or alter the expression of dmLTP.

1.8 Objectives and Hypothesis

The hippocampus is of critical importance in the formation of long-term declarative memories through the modulation of synaptic connections via LTP and LTD (Hasselmo and Giocomo, 2006). GABAergic inhibitory synapses regulate the glutamatergic synaptic circuitry of the hippocampus and the dynamics of its output signal characteristics, and thereby presumably mediate the processes of learning and memory in the cerebral cortex (Hasselmo, 2006). Therefore, this intrinsic inhibitory synaptic architecture is a vital component of signal processing among neuronal cells in the hippocampus. However, although plasticity has been extensively examined at the excitatory synaptic connections in the hippocampus (Bliss and Lømo, 1973),
only recently has dmLTP been documented as a mechanism by which neuronal activity may be potentiated (Ormond and Woodin, 2009).

The role played by neuromodulators in regulating dmLTP is currently unknown. Anatomical evidence implicates cholinergic inputs from the medial septum as being involved in signal processing in the hippocampus (Dutar et al., 1995), and their loss contributing to memory and cognitive deficits, such as in Alzheimer’s disease (Terry Jr. and Buccafusco, 2003). This mechanism is supported by pharmacological data using mAChR antagonists, which block memory encoding *in vivo* (Vannuchhi et al., 1997), suggesting that mAChRs are critical to memory formation. Furthermore, *in vitro* cholinergic agonists and mAChR activation have profound effects on GABAergic interneurons in the CA1: they depolarize their membrane potentials (McQuiston and Madison, 1999), increase their spiking activity (McQuiston and Madison, 1999; Martin et al., 2001), and increase the postsynaptic IPSC frequency (Pitler and Alger, 1992). Moreover, there are also neuromodulatory cholinergic effects on CA1 pyramidal neurons: mAChR activation increases pyramidal neuron excitability, and causes their depolarization (Benardo and Prince, 1982). Also, muscarinic agonists enhance NMDA currents (Marino et al., 1998), and attenuate the activity of the Ca\(^{2+}\)-dependent K\(^+\)-channel (Gulledge and Stuart, 2005) and M-currents (Kirkwood et al., 1991) that contribute to pyramidal neuron adaptation. Thus, the effects of ACh on both interneurons and pyramidal neurons enhance neuronal excitability and strengthen inhibition.

ACh-induced modifications of intrinsic neuronal properties, coupled with the effects of ACh on synaptic transmission and plasticity within the hippocampal circuitry, led to my proposal of the following hypothesis: ACh facilitates CA1 pyramidal neuron activity by facilitating dmLTP expression. Based on the evidence that mAChR activation increases \([\text{Ca}^{2+}]_i\) (Nakamura et al., 1999; Cho et al., 2008), as well as decreases KCC2 expression (Lee et al., 2010), I intended to address whether mAChR activation by CCh during plasticity induction would change the expression of inhibitory synaptic plasticity, and thus alter disinhibition-mediated plasticity in CA1 pyramidal neurons.
Chapter 2
Materials and Methods

2 Mouse Hippocampus

In order to collect data to address the objectives of this thesis, all experiments were done using mouse hippocampal preparations made in accordance with procedures approved by the University of Toronto Animal Care Committee.

2.1 Hippocampal slice preparation

All slice experiments were conducted using brain tissue from 14-40 day old male C57BL/6 mice housed under standard conditions in a 12 h light/dark cycle. Mice were housed with male littermates and provided food and water *ad libitum*. Slice preparation was consistently done at the same time of day in order to avoid the confounding influence of the circadian rhythms of glucocorticoids/mineralocorticoids and their impact on synaptic plasticity, Ca$^{2+}$ currents, and muscarinic and GABAergic receptor binding (Harris and Teyler, 1982; Kendall et al., 1982; Finkelstein et al., 1985; Hesen and Joëls, 1993; Joëls and de Kloet, 1994; Karst et al., 1994; Pavlides et al., 1996; Chaudhury et al., 2005; Maggio and Segal, 2007; Gerstner and Yin, 2010).

Prior to dissection, one mouse was isolated from its littermates and anesthetized using isoflurane (Halocarbon Products Corporation, River Edge, NJ, USA), followed by decapitation and rapid removal of the brain, which was then placed into chilled modified artificial cerebral spinal fluid (aCSF). This modified aCSF, henceforth termed “cutting solution” (CS), consisted of (mM) sucrose (216), KCl (2.5), NaH$_2$PO$_4$ (1.25), NaHCO$_3$ (25), glucose (25), ascorbic acid (0.4), CaCl$_2$ (1), MgCl$_2$ (2), and sodium pyruvate (3), in double distilled water (Millipore Corporation, Billerica, MA, USA); pH ≈ 7.4, osmolality ≈ 300 mOsm/kg. After 1 min in chilled CS, the brain was placed onto filter paper, on which the rostral $\frac{1}{3}$ of the brain, corresponding approximately to the forebrain anterior of the corpus callosum, and the cerebellum were removed using a sterile surgical blade (Feather, Kita-ku, Osaka, JP). The remaining tissue portion was secured to a vibratome chuck by its ventral surface, and stabilized with a 4% agar block set to abut the caudal surface of the brain. Both the tissue and agar block were adhered to the chuck via the application of Krazy Glue adhesive (Toagosei Co., Ltd., West Jefferson, OH, USA) onto the chuck beforehand, and submerged in chilled CS bubbled with carbogen (95% O$_2$/5% CO$_2$). The chuck
was surrounded with ice for the duration of the slicing process. Horizontal slices were cut using a Vibratome 1000 Plus tissue sectioning system (Vibratome Company, St. Louis, MO, USA). Slices were made to a thickness of 400 μm for field recording experiments, and 375 μm for patch-clamp experiments in order to facilitate the visualization of neuronal surface contours. The CS was continuously bubbled with 95% O₂/5% CO₂ carbogen during slicing, and was renewed from a separately chilled and carbogenated source following the completion of each slice. Tissue slices were moved into a separate chamber, submerged, and allowed to equilibrate in a 50:50 aCSF/CS solution at room temperature, superfused with carbogen, for at least 60 min for field recording experiments. In patch-clamp experiments, in order to foster long-term cellular viability, slices were maintained in an interface chamber for at least 60 min at room temperature, in which the equilibration solution was replaced with Earle’s Balanced Salt Solution (EBSS) (Gibco-Life Technologies, Grand Island, NY, USA) supplemented with (mM) CaCl₂ (1) and MgCl₂ (3), and bubbled with 95% O₂/5% CO₂ carbogen. Slice experiments were conducted with tissue maintained in artificial cerebral spinal fluid (aCSF) which was made fresh daily, and consisted of (mM) NaCl (125), KCl (2.5), NaH₂PO₄ (1.25), NaHCO₃ (25), glucose (25), CaCl₂ (2), and MgCl₂ (1), in double distilled water (Millipore Corporation, Billerica, MA, USA); pH ≈ 7.4, osmolality ≈ 300 mOsm/kg. This aCSF was bubbled with 95% O₂/5% CO₂ carbogen and maintained at 38 °C prior to each experiment. Brain slices were longitudinally cut into hemisections; one hemisection was submerged and anchored in a slice chamber during each recording.

### 2.2 Chemicals

Patch pipette internal solution was made to mimic the neuronal intracellular solution dissolved salt composition, supplemented with buffer and energy substrates, and was prepared and kept frozen until needed. This intracellular solution (ICS), consisted of (mM) potassium gluconate (130), KCl (10), HEPES (10), ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA) (0.2), adenosine 5’-triphosphate (ATP) (4), guanosine 5’-triphosphate (GTP) (0.3), and phosphocreatine (10), in double distilled water (Millipore Corporation, Billerica, MA, USA); pH ≈ 7.4, osmolality ≈ 300 mOsm/kg.

In some experiments, glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and kainate receptor transmission were blocked by the competitive antagonist
6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), which was prepared in advance in double distilled water, and frozen in 1 ml aliquots until needed. Also, the muscarinic receptor agonist carbamylcholine chloride (carbachol [CCh]) was prepared in advance in a similar manner. Drugs were thawed and diluted to the appropriate concentration in aCSF before adding them to the slice perfusion.

Drugs and chemicals were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA) and Tocris Bioscience (Tocris Bioscience, Bristol, UK) unless otherwise specified.

2.3 Electrophysiology

Experiments were conducted using an Olympus BX51WI upright microscope (Olympus Canada Inc., Richmond Hill, ON, Canada) equipped with a perfusion system set to supply a constant aCSF perfusion at 2 ml/min, and vacuum pump fluid removable system. Tissue slices were visualized with a low magnification objective (5x magnification, with a 0.1 numerical aperture (NA)) in order to position micropipette/bipolar electrodes in approximately the desired locations, followed by switching to a high-power water immersion objective (40x 0.8 NA) for fine adjustments as these elements were lowered into the appropriate tissue layers. Using high-power magnification, visualization of the tissue architecture was accomplished using a combination of differential interference contrast (DIC) (i.e. Nomarski microscopy) and infrared (IR) video microscopy via an OLY-150IR video camera (Olympus Canada Inc., Richmond Hill, ON, Canada) and television monitor. The positions of micropipette/bipolar electrodes were controlled using MC1000e controller micromanipulators (Siskiyou Design Instruments Inc., Grants Pass, OR, USA). Bath aCSF perfusion temperature was monitored and maintained at 37.4 °C using an in-line solution heater (Warner Instruments, Hamden, CT, USA) controlled by a TC-344B Dual Automatic Temperature Controller (Warner Instruments, Hamden, CT, USA).

Micropipettes were made from thin-walled borosilicate glass capillaries - 1.12 mm inner diameter (ID), 1.5 mm outer diameter (OD) (World Precision Instruments Inc., Sarasota, FL, USA) using a P-87 Flaming/Brown Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). Micropipettes, containing a Ag/AgCl electrode and back-filled with aCSF were made to have a resistance of 7-10 MΩ for field recording microelectrodes, and to have resistances of 2-3 MΩ for unipolar stimulus electrodes, and back-filled with ICS to have resistances of 4-6 MΩ.
for patch-clamp recording electrodes. A reference electrode was also constructed and consisted of a Ag/AgCl wire which was immersed in the aCSF perfusion within the slice chamber.

Signals were recorded using a Digidata 1322A Data Acquisition System, and Multiclamp 700B Microelectrode Amplifier controlled with Multiclamp 700B Commander software and pClamp 9.2 software (Axon Instruments Inc., Union City, CA, USA). Signal sampling was done at 200 μs, and all signals were low-pass filtered to 10 kHz.

2.3.1 Field recording

Extracellular recording provides a measure of the population response of neurons within a tissue sample during basal activity or in response to an applied stimulus. Based on the nature of the trisynaptic circuit of the hippocampus, the property of neuronal recruitment can be determined based on recordings of the population spike amplitude in response to evoked orthodromic stimulation. The population spike amplitude represents the probability by which neurons discharge action potentials in response to the applied stimulus, and is thus a compound action potential representing the synchronous discharge of a population of pyramidal neurons. This provides an indication of the excitability of neurons to a given stimulus, and may be used to detect changes in excitability under changing circumstances or following the induction of synaptic plasticity.

2.3.1.1 Stimulation and recording

Schaffer collateral axons were stimulated using an insulated bipolar tungsten electrode (World Precision Instruments Inc., Sarasota, FL, USA) placed within the stratum radiatum, approximately at the distal terminus of the CA3. Stimulus intensity ranging from 0.00–0.40 mA pulses was manually controlled using an A.M.P.I. ISO-FLEX stimulus isolator (IBIS Instrumentation Canada Inc., Ottawa, ON, CA), whereas stimulus duration and frequency were set using Multiclamp 700B Commander software and pClamp 9.2 software (Axon Instruments Inc., Union City, CA, USA). The field recording protocol involved extracellular stimulation stepped by 0.02 mA, and consisted of 0.4 ms pulses at 0.033 Hz. In order to record the population spike responses to Schaffer collateral stimulation, a micropipette electrode was placed within the CA1 stratum pyramidale (figure 2.1). All field recordings were made in current clamp in order to measure voltage changes in response to the specified current injection stimulation.
2.3.1.2 Experimental protocol

Hippocampal slice field recording experiments were done while the slice was perfused with aCSF in the presence and absence of dissolved drugs; 10 μM CCh, 100 μM CCh, 1 μM CNQX, or 1 μM CNQX + 10 μM CCh. Each experiment was done in a separate slice to avoid confounding effects from a previously applied stimulation protocol. Each drug was diluted in aCSF and the solution was allowed to perfuse through the slice chamber for 10 minutes before the start of each experiment. Experiments consisted of generating input-output curves as per the field recording protocol outlined above. Data were aggregated per stimulus intensity, and the population spike amplitude was recorded. Population spike amplitude was measured as the maximum negative voltage deflection from the mean voltage change corresponding to the current source (figure 2.2). Data were presented as the mean ± standard error of the mean (SEM), unless otherwise stated. The analysis of population spike amplitude utilized the combined data from experiments in 10 μM and 100 μM CCh in order to demonstrate the effect of muscarinic receptor activation. The stimulus intensity and population spike amplitude were described by a sigmoidal relationship, which was used to determine the stimulus intensity for a maximal and half-maximal response. Data analysis was performed using Clampfit 9.2 software (Axon Instruments Inc., Union City, CA, USA), and Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Sigmoid curve fitting was done using SigmaPlot 12.3.0 (Systat Software Inc., San Jose, CA, USA). Statistical analysis, using SigmaStat (Systat Software Inc., San Jose, CA, USA), consisted of a two-way analysis of variance (ANOVA), followed by a Student’s t-test, from which $p < 0.05$ was considered to be statistically significant.
Figure 2.1 Field recording protocol to record the population spike at increasing stimulus intensities in the CA1 stratum pyramidale.

Sample traces of field recordings in CA1 stratum pyramidale in response to stimulation of Schaffer collateral axons in stratum radiatum. Stimulus intensity ranged from 0.00-0.40 mA, varying by 0.02 mA per step, and was applied as 0.4 ms pulses at 0.033 Hz, resulting in 21 sweeps per recording. Visible in each trace are the i) stimulus artifact and initial presynaptic ii) fiber volley. A stimulus intensity-dependent voltage change, corresponding to the iii) voltage change of current source, as well as the iv) population spike deflection are also depicted.
Figure 2.2 Measurement of the population spike response in the CA1 stratum pyramidale to Schaffer collateral axon stimulation.
Sample trace of a field recording in stratum pyramidale due to a 300 μA stimulus applied in the Schaffer collateral pathway, depicting a voltage change corresponding to a current source with a population spike. Population spike amplitude was measured from the mean amplitude of the current source.
2.3.2 Whole-cell patch clamp recording

Patch clamp recordings provide a method to measure a neuron’s electrophysiological properties, such as the action potential firing characteristics, membrane currents, and the biophysical properties of the cell membrane, while minimizing the damage caused to the neuron (Hamill et al., 1981; Coleman and Miller, 1989). Thus, long-term recordings can be made from neurons in order to determine how they respond to an applied stimulus or pharmacological agent, and how their response changes over time.

Patch clamp recordings were made by positioning a micropipette electrode on the membrane surface of a CA1 pyramidal neuron, and forming a gigaohm seal in order to minimize current flow between the micropipette tip and the extracellular environment. A whole-cell patch clamp recording configuration was accomplished by using a membrane perforating agent, gramicidin, in order to acquire a perforated patch recording, or by rupturing the membrane directly in contact with the tip of the micropipette, in order to gain cell access while maintaining a seal between the micropipette and the membrane. In the perforated patch clamp configuration, the use of gramicidin, which formed inorganic monovalent cation selective channels in the membrane, prevented the $\left[\text{Cl}^-\right]$ within the micropipette from reaching an equilibrium with the $\left[\text{Cl}^-\right]_i$ (Kyrozis and Reichling, 1995). Therefore, this method provided an accurate measurement of the intrinsic membrane $\text{Cl}^-$ current, and thus the strength of GABAergic inhibition in mature CA1 pyramidal neurons. In contrast, during ruptured whole-cell patch clamp recording the contents of the micropipette internal solution were allowed to reach equilibrium with the neuronal intracellular solution (Marty and Neher, 1983). As such, subtle variations in neuronal $\left[\text{Cl}^-\right]$ between neurons were presumed to be altered as the substantially greater volume of the micropipette diluted the neuron’s $\text{Cl}^-$ contribution to the equilibrium. Therefore, the strength of GABAergic inhibition was normalized prior to the application of any manipulations, and any subsequent changes in inhibition were quantified.

During patch clamp recordings, the strength of synaptic transmission was determined by the amplitude of the postsynaptic potential (PSP). Also, the PSP reversal potential recorded in current clamp was measured, which corresponded to the current reversal potential in voltage clamp. The $\text{Cl}^-$ reversal potential was taken to be the ensuing postsynaptic reversal potential recorded in the presence of AMPA and kainate receptor blockade. In order to establish changes
in Cl\textsuperscript{−} conductance and the magnitude of the inhibitory transmission while factoring in any concurrent changes in the resting membrane potential, the DF for Cl\textsuperscript{−} was calculated as the difference between the resting membrane potential and the Cl\textsuperscript{−} reversal potential.

2.3.2.1 Stimulation and recording

A micropipette stimulating electrode was positioned within the stratum radiatum between the CA3 and CA1 subregions of the hippocampus, at a depth of approximately 2-3 cell layers in order to stimulate Schaffer collateral axons upon command. Subsequently, a patch-clamp micropipette electrode was positioned above the tissue slice, the micropipette resistance was confirmed to be approximately 4–6 MΩ, the pipette resistance and capacitance were compensated, and a gentle positive pressure was applied by insufflation to prevent debris from entering the pipette tip. Ideal CA1 neurons for patching were selected based on their depth within the tissue (approximately 2–3 cell layers from the surface), morphological characteristics, including the presence of a clear soma surface and edges, and without displaying any obvious signs of changes in membrane surface tension. Also, neurons were putatively identified as hippocampal pyramidal neurons based on their shape and position within the lamina of the CA1 stratum pyramidale. Series resistance was monitored while achieving a stable gigaohm seal and subsequent whole-cell patch by controlled aspiration. Membrane capacitance was compensated in order to remove both transient fast and slow capacitive currents. The seal leak current (I\textsubscript{leak}) was monitored throughout each experiment; patch-clamp recordings were abandoned if the I\textsubscript{leak} exceeded 100 pA.

Stimulus intensity was controlled using an A.M.P.I. ISO-FLEX stimulus isolator (IBIS Instrumentation Canada Inc., Ottawa, ON, CA), whereas stimulus duration and frequency was controlled using Multiclamp 700B Commander software and pClamp 9.2 software (Axon Instruments Inc., Union City, CA, USA).

2.3.2.2 Experimental protocol: input resistance and E\textsubscript{GABA}

In order to measure the whole-cell patch input resistance an episodic stimulation protocol was used during which a step current protocol was applied via the patch electrode, and changes in membrane potential were recorded. Step current stimulus intensity varied from -50.0 to 50.0 pA, with a 10 pA current interval, and was applied for 500 ms while recordings were low-pass
filtered at 10 kHz during 100 μs sampling (figure 2.3). The membrane potential at each current step was measured 375 ms after the current step was initiated, allowing for the membrane potential to stabilize and to avoid confounding effects from variations in the membrane time constant. A current-voltage (I-V) curve was generated using Microsoft Excel (Microsoft Corp., Redmond, WA, USA), from which the input-resistance was calculated as the inverse of the slope of the linear relationship.

As the current injection was stepped to higher values at which the membrane potential was above the action potential threshold, a number of action potentials were recorded, as shown in figure 2.3. Changes in cell membrane properties were assessed throughout each experiment based on changes in action potential duration, amplitude, and threshold. A post hoc analysis of these properties was used to determine whether data from a neuron was to be included within the experimental analysis.

The PSP reversal potential was recorded in current clamp using a similar episodic stimulation protocol, however, step current \( (I_{\text{holding}}) \) stimulus intensity varied from -100 to 50 pA, with a 25 pA current interval, and was applied for 600 ms while recordings were low-pass filtered at 10 kHz during 100 μs sampling (figure 2.4, top). 200 ms after the step current was initiated, a 150 μA, 2 ms stimulation of the Schaffer collateral axons was triggered, and the subsequent PSP was recorded. The PSP amplitude was recorded at each current step and was plotted relative to the preceding membrane potential (figure 2.5). The intersection with the abscissa was taken to be PSP reversal potential (i.e. at which point the PSP amplitude reversed from depolarizing to hyperpolarizing), and was calculated from the linear relationship. In plasticity experiments, the change in the reversal potential was calculated as the difference in reversal potential relative to the recorded value prior to plasticity induction.

The resting membrane potential was recorded before each current step was applied in both the protocol used to record the input resistance and that used to record the PSP reversal potential.

The postsynaptic current (PSC) reversal potential was recorded in voltage clamp using an episodic stimulation protocol. A voltage step \( (V_{\text{holding}}) \) protocol was applied in which the membrane potential was initially stepped to -20 mV below the resting membrane potential, to 50 mV above the resting membrane potential, with a 10 mV voltage step interval, and was applied for 540 ms while recordings were low-pass filtered at 10 kHz during 90 μs sampling.
(figure 2.4, bottom). The Schaffer collateral pathway was stimulated 90 ms after each voltage step at 150 μA for 2 ms. The resulting PSC amplitude was recorded, and an I-V curve was generated from which the reversal potential was calculated from the linear relationship, as described above (figure 2.5). The reversal potential value was found to be similar whether it was recorded in current clamp or voltage clamp. Therefore, the reversal potential recording protocol in voltage clamp was omitted in later experiments.
Figure 2.3 Current step protocol to record the input resistance during whole-cell current clamp recording.

Sample step protocol recording for measurement of input resistance during whole-cell patch clamp recording in current clamp. Current was stepped from -50 to 50 pA; input-resistance was measured from the membrane potential 375 ms following the initiation of each current step relative to the magnitude of the current step (i.e. \( R = V/I \)). Also, the resting membrane potential was measured prior to each current step. Action potential firing was apparent when the membrane potential reached the action potential threshold.
Figure 2.4 Step protocols for the recording reversal potential during whole-cell patch clamp recording.

Sample step protocol recordings for measurement of PSP (top) and PSC (bottom) reversal potential during whole-cell patch clamp recording in current clamp and voltage clamp, respectively. During the current clamp step protocol, the $I_{\text{holding}}$ was stepped from -100 pA to 75 pA for 600 ms, and a 50 μA stimulus was triggered 200 ms after the initiation of each current step. Voltage clamp involved stepping the $V_{\text{holding}}$ from -20 mV to 20 mV for 525 ms; a 50 μA stimulus was triggered 100 ms after the initiation of each voltage step. Visible in each trace is a i) stimulus artifact, and the subsequent ii) PSP, or iii) PSC.
Figure 2.5 Plot of sample PSP and PSC reversal potential quantification of $E_{\text{GABA}}$. Sample of plotted PSP (■) and PSC (○) amplitude measurements from step protocols during current clamp and voltage clamp recordings, respectively. Amplitude was plotted relative to membrane potential in response to $I_{\text{holding}}$, or to $V_{\text{holding}}$. The reversal potential ($E_{\text{GABA}}$) was measured in the presence of 10 μM CNQX, and was taken to be the voltage at which the PSP and PSC amplitudes were zero in response to a 50 μA stimulus.
2.3.2.3 Experimental protocol: plasticity induction and paired-pulse ratio

STDP was induced by pairing presynaptic stimulation with postsynaptic excitation, in which the time interval between the evoked PSP from the former and the action potential evoked by the latter was no more than 20 ms. 100 ms recordings were made with an episodic protocol in which pairings were repeated at 5 Hz for 60 s; recordings were low-pass filtered to 10 kHz during 100 μs sampling. During each sweep, a 20 ms, 350 pA depolarizing step current was triggered 14 ms after a 2 ms, 0.1 mA presynaptic stimulus (figure 2.6). The postsynaptic response was continuously monitored to ensure that spike firing occurred. The PSP-spike interval was measured from the peak of the PSP to the beginning of the first action potential. Also, the STDP induction trace from each brain slice was categorized as containing either 1 action potential, or multiple action potentials; the latter of which has previously been shown to lead to a more robust expression of LTP (Meredith, et al., 2003).

Plasticity expression was measured as a change in the magnitude of a PSP in a CA1 pyramidal neuron in response to orthodromic Schaffer collateral stimulation. Concurrently, the locus of plasticity expression was examined based on the analysis of a paired-pulse ratio (PPR) stimulation protocol. The PPR was calculated as the ratio of the amplitude of the second PSP, triggered 180 ms after the first, to the amplitude of the first PSP (i.e. $\text{PPR} = \frac{\text{PSP}_2}{\text{PSP}_1}$). A change in the PPR suggests a presynaptic locus of plasticity expression, whereas no change in the PPR following plasticity induction would suggest that presynaptic mechanisms do not contribute to the synaptic plasticity. In order to record the PSP amplitude and subsequently calculate the PPR, an episodic recording protocol was used in which 930 ms recordings were made at 0.05 Hz while being low-pass filtered to 10 kHz during 90 μs sampling. A 100 μA, 2 ms stimulus pulse was applied at 25 ms and 205 ms during each sweep, and the resulting PSP amplitude was recorded from the preceding baseline membrane potential (figure 2.7).
Figure 2.6 Plasticity induction protocol sample trace of a single pairing of pre/postsynaptic stimulation.

Sample STDP induction protocol trace. Plasticity was induced by pairing the subsequent EPSP evoked by Schaffer collateral stimulation with postsynaptic spike firing at 5 Hz for 60 s. Visible are the i) stimulus artifact, ii) EPSP, and iii) postsynaptic action potential firing.
Figure 2.7 Paired pulse protocol and recording of PSP amplitude.
Sample trace of PSP amplitude measurement from whole-cell patch clamp recordings in a CA1 pyramidal neuron in response to stimulation of Schaffer collateral axons in the stratum radiatum. Stimulus intensity was 0.10 mA, and was applied as 2 ms pulses 25 ms and 205 ms from the beginning of each sweep. Sweeps were repeated at 0.05 Hz for 5 min, resulting in 16 sweeps per recording. PSP amplitude was measured as the maximum PSP magnitude measured from the baseline, as depicted for the 1st PSP in one sweep. The PPR was calculated as the ratio between the 2nd and 1st PSP amplitudes (PPR = PSP₂/PSP₁).
2.4 Analysis and Statistics

Data analysis was performed using Clampfit 9.2 software (Axon Instruments Inc., Union City, CA, USA), and all figures were prepared using Microsoft Excel (Microsoft Corp., Redmond, WA, USA) or SigmaPlot 12.3.0 (Systat Software Inc., San Jose, CA, USA). Statistical analyses were done using SigmaStat (Systat Software Inc., San Jose, CA, USA). For each test \( p < 0.05 \) represented significance. Data were accepted as marginally significant for \( 0.05 \leq p < 0.065 \).

For extracellular recordings, the absolute population spike amplitude (PSA) data per stimulus intensity were quantified as the mean ± one standard error of the mean (SEM). The data were plotted and fit with a 3-parameter sigmoid function using SigmaPlot 12.3.0 (Systat Software Inc., San Jose, CA, USA) \[ \text{equation (1)} \]. \( PSA_{\text{max}} \) is the maximum PSA, \( x \) is the stimulus intensity, \( x_{50} \) is the stimulus intensity to reach 50% of the maximal response, and \( b \) is inversely proportional to the slope of the function. Maximum PSA data were analysed using a Mann-Whitney rank sum test, or a two-tailed \( t \)-test, in which normality was assessed by a Shapiro-Wilk test.

\[
PSA = \frac{PSA_{\text{max}}}{\left[1 + \exp\left(-\frac{(x - x_{50})}{b}\right)\right]}
\]  

(1)

For whole-cell patch clamp recordings, discrete reversal potential data were analyzed by one-way analysis of variance (ANOVA), followed by analysis by the post hoc Holm-Sidak method, or a Kruskal-Wallis one-way ANOVA on ranks test. Pharmacological effects on resting membrane potential (RMP) and DF were assessed using a two-tailed \( t \)-test. Time course experiments were analyzed by one-way repeated measures (RM) ANOVA for individual pharmacological treatments, or two-way RM ANOVA for comparing pharmacological effects over time. A post hoc Holm-Sidak analysis was performed in order to determine the level of significance in either case. The long-term effect of plasticity induction experiments was assessed 30 min following the induction protocol, and data at this time point were analyzed by a two-tailed \( t \)-test, or using the Mann-Whitney ranked sum test. Whole-cell patch clamp data values presented herein were not corrected for the liquid junction potential.
In all cases, outliers were identified in normally distributed data as data points exceeding 1.5 times the interquartile range (IQR) below the first quartile and above the third quartile. Outliers were removed prior to data analysis.

In time course figures, sample sizes represent the number of neurons whose data were used for the full duration of the experiment. Occasionally, sample sizes decreased as the experiment progressed due to an inability to accurately quantify the necessary measurement, or as a result of removal of data in accordance with the identification of outliers outlined above.
Chapter 3  
Results

3  Results

3.1  Aim #1: Record the change in the field population response of CA1 pyramidal neurons due to CCh and CNQX

The synchronous discharge of CA1 pyramidal neurons evoked in response to Schaffer collateral axon stimulation is manifested as a population spike during extracellular field recording (figure 2.1). A change in the amplitude of this population spike is an indication of the propensity of the postsynaptic pyramidal neurons to reach action potential firing threshold as a result of either an intrinsic change in the neuronal excitability, or of the summation of EPSPs and IPSPs in each neuron at a given stimulus intensity (Andersen et al. 1970, 1980; Haas et al., 1996). Changes in this latter excitation/inhibition profile of CA1 pyramidal neurons would provide an indication of the magnitude of the response of pyramidal neurons to glutamatergic/GABAergic presynaptic transmission, respectively, from which plasticity in either factor could then be further characterized.

It has been shown previously that CCh decreases the population spike amplitude (PSA) in the CA1 stratum pyramidale, reflecting a decrease in the ability of a given presynaptic stimulus to evoke postsynaptic excitation (Hesen et al. 1998; Auerbach and Segal, 1994; and Konopacki et al., 1987). In order to confirm this result in a mouse ventral hippocampal transverse slice preparation, extracellular recordings were made in the CA1 stratum pyramidale during Schaffer collateral axon stimulation in the stratum radiatum. Specifically, the following hypothesis was addressed:

**Hypothesis #1:** The acute application of CCh to hippocampal slices decreases the population spike amplitude in CA1 pyramidal neurons in response to Schaffer collateral stimulation.

The CA1 stratum pyramidale population spike response to orthodromic stimulation of stratum radiatum Schaffer collateral axons was recorded as the stimulus intensity was progressively increased to 400 μA. A sigmoid relationship defined the interaction between the PSA and the
stimulus intensity, whereby incremental (20 μA) increases in the stimulus intensity above 100 μA produced only modest increases in the PSA (figure 3.1A).

During aCSF perfusion, increase in the stimulus intensity increased the PSA to an average maximum of $2.48 \pm 0.45$ mV ($n = 10$; figure 3.1B). Relative to aCSF perfusion, muscarinic receptor activation, via CCh, attenuated the maximum PSA. In the presence of CCh (10-100 μM), the stimulus intensity increased the PSA to an average maximum of $0.82 \pm 0.17$ mV ($n = 4$; figure 3.1B). The maximum PSA during aCSF and CCh had median values of 2.41 mV and 0.95 mV, respectively, representing a significant reduction in the maximum PSA by 60.43% in the presence of CCh (Mann-Whitney rank sum test, $U = 5.0$; $n_{aCSF} = 10$, $n_{CCh} = 4$; $p = 0.040$; figure 3.1B). A substantial increase in the PSA, taken to be >20% for a 20 μA change in the stimulus current intensity, was calculated at a maximum stimulus intensity of 80 μA for aCSF, and 140 μA for CCh. A stimulus current intensity of 100 μA was calculated as a moderate level during both aCSF and CCh perfusion, and was thus used in subsequent experiments to supply sufficient presynaptic activity to evoke an approximately half-maximal postsynaptic response.

The presence of an AMPA and kainate receptor competitive antagonist, 1 μM CNQX, further diminished the PSA during 10 μM CCh perfusion (figure 3.2A). During aCSF perfusion, the maximum PSA evoked by a 0-300 μA stimulus intensity step protocol, utilizing 20 μA increments, was $2.72 \pm 0.42$ mV ($n = 13$; figure 3.2B). The inclusion of 1 μM CNQX and 10 μM CCh during the step protocol lead to an average maximum PSA of $0.13 \pm 0.03$ mV ($n = 4$; figure 3.2B). This represented a significant reduction by 95.23% in the average maximum population spike response due to 1 μM CNQX + 10 μM CCh relative to aCSF, in which the median of the maximum amplitudes were 2.55 mV and 0.15 mV, respectively (Mann-Whitney rank sum test, $U = 0.000$; $n_{aCSF} = 13$, $n_{1 \mu M \text{ CNQX + 10 } \mu M \text{ CCh}} = 4$; $p = 0.004$). Notably, 1 μM CNQX further reduced the already attenuated response caused by CCh alone, as is depicted in figure 3.1B. From figure 3.1A, the maximum PSA recorded between 0-300 μA during CCh perfusion was $0.69 \pm 0.12$ mV ($n = 4$), which was significantly different from $0.13 \pm 0.03$ mV when 1 μM CNQX was present (two-tailed $t$-test, $t = 4.471$; d.f. = 6; $p = 0.002$; figure 3.2C).
Figure 3.1 Acute application of the mAChR agonist, CCh, diminishes the PSA in CA1 pyramidal neurons.
A) PSA recorded in CA1 s. pyramidale following s. radiatum Schaffer collateral stimulation at 0-400 μA. Mean PSA during aCSF (O; n = 10) and CCh (■; 10-100 μM; n = 4) perfusion at various stimulus intensities, depicting the effect of mAChR activation on the PSA. Inset: sample recordings from s. pyramidale during aCSF (upper) and CCh (lower) perfusion. Bars represent 0.5 mV and 5 ms. B) Mean maximum population spike amplitude during two conditions: aCSF and CCh. Each bar represents the average of the maximum population spike amplitude recorded during aCSF perfusion (n = 10), or in the presence of CCh (n = 4). Error bars represent ± SEM. * indicates significance (p = 0.040, Mann-Whitney ranked sum test).
Figure 3.2 Decrease in the PSA due to CCh cannot be attributed to a change in inhibition via field recordings due to the attenuation of a measurable population response by CNQX.

A) Sample recordings from CA1 s. pyramidale due to 300 μA stimulation applied in Schaffer collateral s. radiatum during aCSF (upper), and 1 μM CNQX + 10 μM CCh (lower) perfusion. B) Mean maximum PSA during aCSF (n = 13) and 1 μM CNQX + 10 μM CCh (n = 4). C) Mean maximum PSA during CCh (n = 4) and 1 μM CNQX + 10 μM CCh (n = 4). Error bars represent ± SEM. Significance indicated by * (p = 0.004, Mann-Whitney ranked sum test) and † (p = 0.004, two-tailed t-test).
3.2 Aim #2: Examination of the response of individual neurons to GABAergic inhibitory transmission in the presence of CCh

Alterations in hippocampal network activity can be attributed to changes in either excitatory or inhibitory synaptic transmission. It has previously been established that the postsynaptic response to glutamateric transmission can be modified by the level of synaptic activity, and that plasticity at excitatory synapses can be modulated by other neurotransmitters (Bliss and Lømo, 1973; Shinoe, et al., 2005). Particularly, mAChR dependent modulation of LTP, termed LTPm, potentiates excitatory transmission between CA3 and CA1 pyramidal neurons (Auerbach, J.M., and Segal, M., 1996). Whether inhibitory synaptic transmission can be modulated by mAChRs as well, however, has remained unclear. Previously it has been shown that repeated coincident pre/post synaptic activity can depolarize the postsynaptic $E_{\text{GABA}}$, resulting in a long-term reduction in the magnitude of inhibitory Cl⁻ currents at particular GABAergic synapses (Woodin, MA, et al., 2003). This dmLTP demonstrates that inhibitory synapses are plastic, and respond just as readily as their excitatory counterparts. Furthermore, since this plasticity depends on changes in the magnitude of synaptic Cl⁻ currents, and ACh has previously been shown to alter KCC2 expression (Lee, HHC, et al., 2010), for this thesis it was therefore hypothesized that ACh has a regulatory role in the expression of inhibitory synaptic plasticity. Since the cellular mechanism underlying inhibitory synaptic plasticity is a depolarization of $E_{\text{GABA}}$, and dmLTP is defined as a change in the excitation/inhibition balance in response to presynaptic activity, this will be observed as a depolarization of the $E_{\text{PSP}}$ (Woodin, MA, et al., 2003; Ormond and Woodin, 2009). The main objective of this thesis was to determine whether ACh regulates the induction of dmLTP, presumably through a regulation of $E_{\text{GABA}}$ and $E_{\text{PSP}}$. Alternatively, a mAChR dependent regulation of $E_{\text{GABA}}$ and $E_{\text{PSP}}$ may have been independent of synaptic activity. Therefore, whether $E_{\text{GABA}}$ and $E_{\text{PSP}}$ responded directly to acute CCh application had to be determined.

3.2.1 Is there an effect of CCh on $E_{\text{GABA}}$ in transverse hippocampal slices during basal synaptic activity?

The main mechanism underlying STDP at inhibitory synapses in the hippocampus is a depolarization of $E_{\text{GABA}}$. This parameter was recorded in putatively identified CA1 pyramidal neurons in transverse hippocampal slices in order to determine whether 10 μM CCh was sufficient to alter $E_{\text{GABA}}$ during basal synaptic activity. Due to the evidence that the mAChR
modulation of LTP at glutamatergic synapses is activity dependent, it was expected that the modulation of dmLTP should also be dependent on synaptic activity.

**Hypothesis #2:** The acute application of CCh will not directly alter \( E_{\text{GABA}} \) or \( E_{\text{PSP}} \) in CA1 pyramidal neurons during basal synaptic activity.

In the absence of pharmacological receptor inhibition, stimulation of the Schaffer collateral axons produced a postsynaptic potential that was a combination of a monosynaptic EPSP and a disynaptic IPSP; hereafter referred to as a mixed PSP (figure 3.3A). Sample calculations of the reversal potential for each pharmacological treatment are depicted in figure 3.3A, B, and C. During aCSF perfusion, the mean reversal potential of this mixed PSP (\( E_{\text{PSP}} \)) was found to be \(-44.59 \pm 4.13 \text{ mV (} n = 13; \text{ figure 3.3D □)}\). The inclusion of an AMPA and kainate receptor antagonist, 1 \( \mu \text{M} \) CNQX, inhibited the excitatory glutamatergic postsynaptic component of the PSP, resulting in the significant hyperpolarization of the mean reversal potential to \(-71.67 \pm 3.07 \text{ mV (one-way ANOVA; } \( F = 14.561; \text{ d.f. } = 2; n_{\text{aCSF}} = 13, n_{\text{CNQX}} = 7; p < 0.001; \text{ post hoc Holm-Sidak method}; p < 0.001; \text{ figure 3.3D □)}\). This value was taken to be \( E_{\text{GABA}} \) during basal synaptic activity. The activation of mAChRs by 10 \( \mu \text{M} \) CCh for 10 min prior to and during the recording of \( E_{\text{GABA}} \) did not alter the mean reversal potential, measured as \(-69.74 \pm 4.21 \text{ mV (one-way ANOVA; } n = 7; \text{ post hoc Holm-Sidak method}; p = 0.78; \text{ figure 3.3D □)}\).

The ionic DF is the difference between the \( V_m \) and the ionic reversal potential (\( E_{\text{rev}} \)), and is specific for each ion (i.e. DF = \( V_m - E_{\text{rev}} \)). This DF is related to the ionic current (\( I_{\text{ion}} \)) and its conductance (\( g_{\text{ion}} \)) across the cell membrane; DF = \( I_{\text{ion}}/g_{\text{ion}} \); such that a change in the DF by way of a change in the \( V_m \) is sufficient to alter the \( I_{\text{ion}} \) of a membrane permeable ion. Thus the magnitude of postsynaptic inhibition may be altered by a change in the CI- DF. Both the \( V_m \) and \( E_{\text{GABA}} \) were recorded during 1 \( \mu \text{M} \) CNQX and 1 \( \mu \text{M} \) CNQX + 10 \( \mu \text{M} \) CCh perfusion. During 1 \( \mu \text{M} \) CNQX perfusion, the average \( V_m \) in the absence and presence of 10 \( \mu \text{M} \) CCh was \(-58.05 \pm 3.13 \text{ mV (} n = 7) \) and \(-55.96 \pm 2.81 \text{ mV (} n = 8) \), respectively (figure 3.4A). Therefore, mAChR activation alone did not alter the \( V_m \) during basal synaptic activity (two-tailed \( t \)-test, \( t = -0.498; \text{ d.f. } = 13; p = 0.627; \text{ figure 3.4A} \)). The average DF calculated during 1 \( \mu \text{M} \) CNQX perfusion was \( 15.45 \pm 5.16 \text{ mV (} n = 7; \text{ figure 3.4B □)} \), and perfusion with 1 \( \mu \text{M} \) CNQX + 10 \( \mu \text{M} \) CCh lead to an average calculated DF of \( 9.06 \pm 4.97 \text{ mV (} n = 8; \text{ figure 3.4B □)} \). The average
calculated DF did not differ by the inclusion of 10 μM CCh (two-tailed \( t \)-test, \( t = 0.891; \) d.f. = 13; \( p = 0.389 \)).
Figure 3.3 Activation of mAChRs did not alter $E_{\text{GABA}}$ during basal synaptic activity.

The postsynaptic potential (PSP) reversal potential was measured during A) aCSF, B) 1 μM CNQX, and C) 1 μM CNQX + 10 μM CCh perfusion. The PSP reversal potential was taken to be the membrane potential at which the PSP amplitude was zero (i.e. the value at which the linear trend line intersects with the abscissa). D) Summary of mean PSP reversal potential during aCSF (□; $n = 13$), 1 μM CNQX (■; $n = 7$), and 1 μM CNQX + 10 μM CCh (▲; $n = 7$) perfusion. Error bars represent ± SEM. * indicates significance ($p < 0.001$, one-way ANOVA; $p < 0.001$, post hoc Holm-Sidak method). Insets: sample whole-cell current clamp PSP reversal potential protocol recordings from CA1 pyramidal neurons. Bars represent 10 mV and 75 ms.
Figure 3.4 Activation of mAChRs did not alter the postsynaptic current DF through a change in the membrane potential.

A) Resting membrane potential ($V_m$) measured during whole cell current clamp recording in CA1 pyramidal neurons. Data represent average resting membrane potential during 1 μM CNQX (■; $n = 7$), and 1 μM CNQX + 10 μM CCh (■; $n = 8$) perfusion. B) Average DF ($V_m - E_{rev}$) calculated from measurements made during whole cell current clamp recording in CA1 pyramidal neurons. Data represent average DF during 1 μM CNQX (■; $n = 7$), and 1 μM CNQX + 10 μM CCh (■; $n = 8$) perfusion. Error bars represent ± SEM.
3.2.2 Is the effect of CCh on $E_{GABA}$ during basal synaptic activity concentration-dependent?

Although 10 μM CCh did not alter $E_{GABA}$ during basal synaptic activity, it was possible that the concentration of CCh was too low to alter the neuronal Cl⁻ gradient. Previously it was shown that 100 μM CCh for 120 min decreased the total neuronal expression of KCC2 in cultured hippocampal neurons (Lee et al., 2010). Also, a decrease in the expression of KCC2 has previously been shown to cause a depolarization of $E_{GABA}$ (Rivera et al., 1999). Therefore, whether an increase in the concentration of CCh to 100 μM regulated $E_{GABA}$ during basal synaptic activity was examined. However, it was found that increasing the concentration of CCh in the intact transverse hippocampal slice to 100 μM increased the neuronal excitability and network seizure-like activity such that achieving stable and accurate recordings of $E_{GABA}$ were not feasible. Therefore, cultured hippocampal neurons were used as an alternative in vitro preparation in which the propensity for seizure-like activity was diminished, presumably by the lack of an intact hippocampal cytoarchitecture and decreased population density of neurons.

Hypothesis #3: The acute application of 100 μM CCh will depolarize $E_{GABA}$ in cultured hippocampal neurons during basal synaptic activity.

Perforated-patch voltage clamp recordings were made from cultured hippocampal neurons during which GABA-dependent PSPs were elicited, at various holding potentials, by direct administration of GABA onto the patched neuron. Perforation was achieved by the use of gramicidin D, a membrane anion-impermeable channel forming bactericide, thus preventing neuronal Cl⁻ loading from the patch micropipette ICS. Furthermore, the direct administration of GABA to elicit PSPs was necessary due to the lack of an intact hippocampal cytoarchitecture through which GABAergic interneurons could be stimulated to release GABA onto the target neuron. Since the GABA-dependent PSPs were neither mixed PSP nor dependent on presynaptic stimulation, the pharmacological blockade by CNQX of AMPA and kainate receptors was not needed.

During a voltage step protocol, $E_{GABA}$ was calculated as the value at which the evoked GABA current was zero. Sample $E_{GABA}$ recordings and calculations are depicted in figure 3.5, during A) extracellular solution (XCS), B) 10 μM CCh, and C) 100 μM CCh perfusions. The perfusion of
XCS lead to a mean $E_{\text{GABA}}$ of -62.61 ± 4.96 mV ($n = 9$; figure 3.5D □). The inclusion of 10 μM CCh lead to a mean $E_{\text{GABA}}$ of -56.61 ± 1.53 mV ($n = 5$; figure 3.5D ■). Also, increasing the concentration of CCh to 100 μM lead to a mean $E_{\text{GABA}}$ of -56.69 ± 2.50 mV ($n = 6$). Neither 10 nor 100 μM CCh significantly altered $E_{\text{GABA}}$ from the value recorded during XCS perfusion (Kruskal-Wallis one-way ANOVA on ranks, $H = 0.370$, d.f. = 2, $p = 0.831$). This suggested that the lack of modulation of $E_{\text{GABA}}$ by micromolar concentrations of CCh during basal synaptic activity was not concentration-dependent.

However, as was suggested previously, mAChR activation could alter the level of GABAergic inhibition at hippocampal neurons by way of altering the Cl⁻ DF. In the absence of a change in $E_{\text{GABA}}$, a change in the resting membrane potential will change the Cl⁻ DF. Furthermore, since both the resting membrane potential and $E_{\text{GABA}}$ contribute to the Cl⁻ DF, the DF was calculated for each pharmacological condition in order to determine whether small, non-significant changes in either parameter, when combined, would reveal a difference in the Cl⁻ DF between conditions.

The mean resting membrane potentials recorded in XCS and 10 μM CCh were -58.62 ± 2.45 mV ($n = 9$), and -58.52 ± 1.09 mV ($n = 5$), respectively (figure 3.6A). 10 μM CCh did not significantly change the mean resting membrane potential in cultured hippocampal neurons (one-way ANOVA, $F = 4.965$, d.f. = 2, $p = 0.021$, post hoc Holm-Sidak method, $p = 0.977$). However, relative to XCS, 100 μM CCh significantly hyperpolarized the mean resting membrane potential to -68.06 ± 2.20 mV (one-way ANOVA, $F = 4.965$, d.f. = 2, $p = 0.021$, post hoc Holm-Sidak method, $n = 6$, $p = 0.029$; figure 3.6A). The 16.30% hyperpolarization of the resting membrane potential by 100 μM CCh relative to 10 μM CCh was also marginally significant (one-way ANOVA, post hoc Holm-Sidak method, $p = 0.054$).

Lastly, the mean Cl⁻ DF calculated for cultured hippocampal neurons in XCS was 3.99 ± 6.17 mV ($n = 9$; figure 3.6B). The mean Cl⁻ DF in 10 μM CCh and 100 μM CCh perfusions were -3.12 ± 1.54 mV ($n = 3$) and -11.37 ± 4.38 mV ($n = 6$), respectively (figure 3.6B). Therefore, although 100 μM CCh altered the resting membrane potential in cultured hippocampal neurons, neither 10 μM CCh nor 100 μM CCh had a significant impact on the GABA-dependent Cl⁻ DF (one-way ANOVA, $F = 1.918$, d.f. = 2, $p = 0.181$).
Figure 3.5 Activation of mAChRs did not alter $E_{\text{GABA}}$ in cultured hippocampal neurons.

$E_{\text{GABA}}$ in cultured hippocampal neurons was recorded using a gramicidin perforated patch voltage clamp recording protocol. Sample $E_{\text{GABA}}$ measurement during A) XCS, B) 10 μM CCh, and C) 100 μM CCh. D) Summary of data representing mean $E_{\text{GABA}}$ in the presence of XCS (□; $n$ =9), 10 μM CCh (■; $n$ =5), and 100 μM CCh (▲; $n$ =6). Error bars represent ± SEM. Insets: sample perforated-patch voltage clamp postsynaptic potential (PSP) reversal protocol recordings from cultured hippocampal neurons. Bars represent 100 pA and 75 ms.
Figure 3.6 The effect of CCh on $V_m$, but not DF, in cultured hippocampal neurons was dose dependent.

A) Resting membrane potential ($V_m$) of cultured hippocampal neurons as recorded using a gramicidin perforated patch voltage clamp recording protocol. Data represent mean $V_m$ in the presence of XCS ($\square; n = 9$), 10 μM CCh ($\blacksquare; n = 4$), and 100 μM CCh ($\blacksquare; n = 6$). 

B) GABA-induced Cl$^-$ DF ($\text{DF}_{\text{Cl}} = V_m - E_{\text{GABA}}$) in cultured hippocampal neurons as recorded using a gramicidin perforated patch voltage clamp recording protocol. Data represent mean Cl$^-$ DF in the presence of XCS ($\square; n = 9$), 10 μM CCh ($\blacksquare; n = 3$), and 100 μM CCh ($\blacksquare; n = 6$). Error bars represent ± SEM. * is significant ($p = 0.029$, one-way ANOVA, *post hoc* Holm-Sidak method). † is marginally significant ($p = 0.054$, one-way ANOVA, *post hoc* Holm-Sidak method).
3.3 Aim #3: Examination of the neuromodulatory effect of CCh on the expression of dmLTP

Since CCh did not regulate E_{GABA} in the preceding hippocampal slice and culture preparations during basal synaptic activity, its regulation of E_{GABA} and disinhibition-mediated plasticity expression following STDP were addressed. Disinhibition-mediated LTP of CA1 pyramidal neurons has previously been shown to be induced following correlated pre/postsynaptic activity at 0.5 Hz for 60 s (Ormond and Woodin, 2009). The expression of disinhibition-mediated plasticity is NMDA receptor dependent, relies on an increased intracellular [Ca^{2+}], and is manifested as a depolarization of E_{GABA}, thus reducing the inhibitory DF, and in turn facilitating the amplitude of Schaffer collateral evoked PSPs in CA1 pyramidal neurons. A STDP protocol utilizing repeated coincident pre/postsynaptic stimulation (0.5 Hz for 60 s) was used in the presence of aCSF and CCh in order to determine the contribution of mAChR activation by CCh on the expression of dmLTP. Changes in E_{GABA} were examined by recording the E_{rev} of the mixed PSP due to the need for intact glutamatergic transmission in order to determine the postsynaptic pyramidal neuron response to disinhibition.

3.3.1 Does the presence of CCh alter the expression of dmLTP?

The activation of mAChRs in the hippocampus has previously been shown to promote spike firing and depolarize the resting membrane potential of both CA1 pyramidal cells and GABAergic interneurons (Fraser and MacVicar, 1996; Chapman and Lacaille, 1999; McQuiston and Madison, 1999). This contributes to ability of mAChR agonists to induce status epilepticus (SE) at high doses (Turski et al., 1984; Berkeley et al., 2002). Furthermore, a mAChR dependent mechanism underlies the production of transient intracellular [Ca^{2+}] spikes, and the modulation of glutamatergic plasticity at CA3→CA1 synapses. Coupled with the evidence of the activity dependent phosphorylation of KCC2 and its degradation (Lee et al., 2010), this suggests that a mAChR dependent mechanism may contribute to the expression of inhibitory synaptic plasticity as well, possibly by way of modulating the expression of dmLTP.

**Hypothesis #4:** The presence of CCh facilitates the expression of dmLTP by potentiating the depolarization of E_{GABA}.
Whole-cell patch clamp recordings were made from CA1 pyramidal neurons in order to record the plasticity expression in individual neurons. During the perfusion of CCh, continuous whole-cell patch recordings were made in order to visualize the mAChR dependent depolarization of the resting membrane potential, and the facilitation of spike production. Figure 3.7 represents a sample recording of the membrane potential of a CA1 pyramidal neuron as the tissue slice chamber perfusion system was switched from 2 ml/min aCSF to deliver an equal flow rate of 1 μM CCh. Following the confirmation of the membrane potential depolarization and spike facilitation, the PSP amplitude and $E_{rev}$ were monitored for 10 min prior to STDP induction in order to allow for each parameter to stabilize at an approximate baseline value.

Disinhibition-mediated LTP was induced by pairing presynaptic stimulation with postsynaptic spiking at a frequency of 5 Hz for 60 s. The PSP amplitude was recorded in CA1 pyramidal neurons following Schaffer collateral axon stimulation at 100 μA for 2 ms, during a 0.05 Hz, 5 min sampling interval. Between PSP amplitude recordings, the $E_{rev}$ was calculated by way of a current-step protocol, and was taken to be the $E_{rev}$ for the subsequent 5 min interval. The PSP amplitude was divided by the DF, and normalized to the average of the baseline value (i.e. the mean PSP amplitude/DF in the 5 min preceding STDP induction). These values were averaged per 5 min intervals. In the presence of aCSF perfusion, the mean normalized PSP amplitude/DF significantly increased in the 5 min post STDP-induction to $2.22 \pm 2.16 \ (n = 9)$, and again at 25 min post-induction to $2.10 \pm 0.69 \ (two-way \ ANOVA, \ n = 9, \ p < 0.001, \ post \ hoc \ Holm-Sidak \ method, \ p < 0.001; \ figure \ 3.8A \ □)$. The potentiation of the PSP/DF ratio remained steady following 25 min post-induction for the duration of the recording. However, tissues exposed to 1 μM CCh perfusion for the duration of the experiment failed to express a long-term increase in the PSP amplitude/DF ratio. During 1 μM CCh, the mean normalized PSP amplitude/DF ratio significantly increased to $2.09 \pm 0.41 \ (n = 4)$ in the 5 min post-induction, and again transiently increased to $1.99 \pm 0.92 \ (n = 4)$ at 20 min post-induction (two-way ANOVA, $p < 0.001$, post hoc Holm-Sidak method, $p < 0.001$; figure 3.8B □). Furthermore, from 10 min post-induction until the end of each recording, the postsynaptic response in aCSF relative to 1 μM CCh significantly differed at each 5 min interval (two-way ANOVA, $p < 0.001$, post hoc Holm-Sidak method, $p \leq 0.022$; figure 3.8C). Notably, at 35 min post-induction the mean normalized PSP amplitude/DF in aCSF had increased to $2.08 \pm 0.73 \ (n = 9)$, whereas during 1 μM CCh, the ratio at this time bin was $1.16 \pm 0.32 \ (n = 4)$, representing a significant difference in the long-term plasticity.
expression (Mann-Whitney rank sum test, $U = 14,000$, $M_{aCSF} = 1.984$, $M_{1 \mu M CCh} = 1.125$, $p < 0.001$; figure 3.8D). Thus, as opposed to potentiating the long-term postsynaptic response of pyramidal neurons to Schaffer collateral stimulation, this aspect of dmLTP appeared to be prevented by CCh.

However, as described previously, dmLTP results from a depolarization of the PSP reversal potential ($E_{\text{rev}}$) in pyramidal neurons (Ormond and Woodin, 2009). If mAChR activation prevents dmLTP, then it was presumed that $E_{\text{rev}}$ would also fail to depolarize in the presence of CCh. During the perfusion of either aCSF or $1 \mu M$ CCh the $E_{\text{rev}}$ did not significantly change from its pre-induction value (figure 3.9A, C). Figure 3.9B, D depict sample $E_{\text{rev}}$ recordings before and after STDP induction in the presence of aCSF and $1 \mu M$ CCh. Furthermore, although a depolarization of the change in $E_{\text{rev}}$ has previously been demonstrated in response to paired pre/postsynaptic stimulation in rat hippocampal slices (Ormond and Woodin, 2009), neither aCSF nor $1 \mu M$ CCh induced a depolarization of $E_{\text{rev}}$ (two-way RM ANOVA, $F = 1.110$, d.f. = 6, $p = 0.361$, post hoc Holm-Sidak method, $p \leq 0.561$; figure 3.10A, B, C). Although neither the $E_{\text{rev}}$ nor the change in $E_{\text{rev}}$ following plasticity induction significantly differed during either aCSF or $1 \mu M$ CCh perfusion, the mean change in the $E_{\text{rev}}$ at 32.5 min post-induction was $4.66 \pm 1.41$ mV ($n_{aCSF} = 4$) and $-3.40 \pm 0.70$ mV, respectively ($n_{1 \mu M CCh} = 3$; figure 3.10). Thus, the long-term change in $E_{\text{rev}}$ was significantly hyperpolarized due to $1 \mu M$ CCh relative to aCSF (two-tailed $t$-test, $t = 4.554$; d.f. = 5; $p = 0.006$; figure 3.10D).
Figure 3.7 CCh perfusion of a hippocampal slice caused a depolarization of the resting membrane potential and facilitated spike production.
Sample whole-cell patch clamp recording of neuronal activity in CA1 pyramidal neuron during 1 μM CCh perfusion. An arrow indicates the start of the 1 μM CCh perfusion. The majority of the response delay may be attributed to the lag before the solution reached the tissue in the recording chamber. Depicted are the measurement of the resting membrane potential (V_m) depolarization, and the facilitation of spike production by CCh. The instantaneous membrane potential was measured to be -70.04 mV prior to 1 μM CCh perfusion, and -50.08 mV during the interspike interval in 1 μM CCh perfusion.
Figure 3.8 mAChR-dependent activation prevented the postsynaptic expression of dmLTP.

The effect of 1 μM CCh on the postsynaptic potential (PSP) amplitude per DF was recorded in CA1 pyramidal neurons. Data represent the average postsynaptic response per 5 min time bins. An arrow indicates the time at which plasticity was induced. A) Normalized PSP amplitude per DF during aCSF perfusion (n = 9). * indicates significant change in postsynaptic response relative to pre-induction value (i.e. time = 2.5 min; p < 0.001). B) Normalized PSP amplitude per DF during 1 μM CCh perfusion (■; n = 4). * indicates significant change in postsynaptic response relative to pre-induction value (i.e. time = 2.5 min; p < 0.001). C) Normalized PSP amplitude per DF during both aCSF perfusion (n = 9) and 1 μM CCh perfusion (■; n = 4). ‡ indicates significant change in postsynaptic response at time point between aCSF and 1 μM CCh (p ≤ 0.022). D) Summary of postsynaptic response 32.5 min following STDP induction during aCSF (□; n = 9), and 1 μM CCh (■; n = 4) perfusion. Error bars represent ± SEM. * indicates significance (p < 0.001). Dashed line indicates normalized baseline amplitude.
Figure 3.9 CCh prevents the postsynaptic depolarization of $E_{rev}$.

$E_{rev}$ recorded before and after STDP induction during aCSF and 1 μM CCh perfusion. An arrow indicates the time at which STDP was induced. Points represent average $E_{rev}$ recorded within a range of 2.5 min of each data point. **A)** $E_{rev}$ during aCSF perfusion (○; $n=29$). **B)** Sample $E_{rev}$ recording before (above) and 30 min after (below) STDP induction during aCSF perfusion. **C)** $E_{rev}$ during 1 μM CCh perfusion (■; $n=16$). **D)** Sample $E_{rev}$ recording before (above) and 30 min after (below) STDP induction during 1 μM CCh perfusion. Error bars represent ± SEM. Dashed line indicates normalized baseline $E_{rev}$. 
Figure 3.10 CCh prevents the long-term postsynaptic depolarization of $E_{\text{rev}}$.
Change in $E_{\text{rev}}$ calculated before and after STDP induction during aCSF and 1 μM CCh perfusion. An arrow indicates the time at which STDP was induced. Points represent average change in $E_{\text{rev}}$ calculated within a range of 2.5 min of each data point. A) Average $E_{\text{rev}}$ change relative to pre-STDP induction during aCSF perfusion (○; $n = 29$). B) Average $E_{\text{rev}}$ change relative to pre-STDP induction (i.e. time = 2.5 min) during 1 μM CCh perfusion (■; $n = 16$). C) Summary average $E_{\text{rev}}$ change during aCSF and 1 μM CCh perfusion. D) Summary average $E_{\text{rev}}$ change at 28.5 min post-induction during aCSF (□; $n = 4$) and 1 μM CCh (■; $n = 3$) perfusion. Error bars represent ± SEM. * indicates significance ($p < 0.05$). Dashed line indicates normalized baseline $E_{\text{rev}}$. 
### 3.3.2 Is the prevention of dmLTP by CCh mediated by a presynaptic locus of modulation?

The PSP amplitude potentiation and $E_{rev}$ depolarization are aspects of dmLTP which are expressed as postsynaptic responses to repeated coincident pre/postsynaptic activity of the Schaffer collateral axons. However, mAChRs are present on both presynaptic axon terminals and on the postsynaptic membrane, and thus can modulate synaptic transmission at either or both locations (Shimoshige et al., 1997; Fukudome et al., 2004; Kremin et al., 2006; Kamsler et al., 2010). The activation of mAChRs on both interneuron terminals and pyramidal cells has also been associated with a suppression of GABA release from presynaptic terminals (Fukudome et al., 2004). Therefore, the lack of change in $E_{rev}$ may be accounted for by a suppression of GABA release. In order to address this possible mechanism of mAChR modulation, the paired pulse ratio (PPR) was recorded before and after the STDP induction protocol. The PPR reflects the propensity of synaptic terminals to release more neurotransmitter vesicles if stimulated rapidly following a previous stimulation. This has been attributed to an increase in the $[Ca^{2+}]$ in the synaptic terminal, which facilitates vesicle docking and transmitter release (Manabe et al., 1993; Debanne et al., 1996).

**Hypothesis #5:** The prevention of dmLTP expression by CCh, via presynaptic modulation, will be observed as a depression of the PPR.

The PPR was recorded by stimulating the Schaffer collateral axons twice, with a delay of 180 ms before the second stimulus, and calculating the ratio of the amplitude of the second PSP to that of the first. The PPR have previously been correlated with changes in the probability of transmitter release, and thus this procedure was used to provide an indication of whether the mechanism by which CCh prevented dmLTP was presynaptic (Schulz et al., 1994, 1995). During aCSF perfusion the mean PPR before plasticity induction was $1.13 \pm 0.09$ mV ($n = 9$). It was found that following the induction of dmLTP in aCSF, there was no significant change in the mean PPR (one-way ANOVA, $F = 1.327$, d.f. = 7, $p = 0.233$, *post hoc* Holm-Sidak method, $p \leq 0.479$; figure 3.11A ☐). The addition of 1 μM CCh lead to a mean PPR before plasticity induction of $1.27 \pm 0.04$ mV ($n = 4$). Paired pre/postsynaptic stimulation lead a depression of the PPR such that at 32.5 min the mean PPR was $0.86 \pm 0.04$ mV ($n = 4$). Thus, STDP induction in the presence of 1 μM CCh lead to a significant long-term paired pulse depression (PPD) (one-way ANOVA, $F = 34.987$, d.f. = 1, $p < 0.001$, *post hoc* Holm-Sidak method, $p = 0.034$; figure 3.11B ☐).
Notably, STDP induction lead to a significant depression of the mean PPR immediately following the induction protocol during 1 μM CCh perfusion relative to aCSF, and for the duration of the recording protocol (two-way ANOVA, $F = 3.195$, d.f. = 7, $p = 0.002$, post hoc Holm-Sidak method, $p \leq 0.017$; figure 3.11C$^1$). At 32.5 min, the mean PPR was reduced by 30.23% in 1 μM CCh perfusion relative to aCSF (figure 3.11D). This represented a significant reduction in the long-term mean PPR due to 1 μM CCh (Mann-Whitney rank sum test, $U = 1728.000$; $n_{aCSF} = 9$, $n_{1 \mu M \text{CNQX} + 10 \mu M \text{CCh}} = 4$; $p \leq 0.001$).

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$^1$ The difference in the mean PPR between aCSF and 1 μM CCh at 27.5 min was marginally significant (post hoc Holm-Sidak method, $p = 0.064$).
Figure 3.11 CCh prevents dmLTP by causing a long-term depression of the PPR.

Presynaptic modulation of synaptic activity by 1 μM CCh was tested via the PPR. An arrow indicates the time at which STDP was induced. Data represent the average PPR recorded within 2.5 min of each point. PPR during A) aCSF (○; n = 9) and B) 1 μM CCh (square; n = 4) perfusion. * represents significant difference at specific time bin relative to pre-induction PPR at time = 2.5 min (p < 0.05). C) PPR during aCSF (○; n = 9) and 1 μM CCh (square; n = 4) perfusion. * represents significant difference in PPR between aCSF and 1 μM CCh at specified time point; p < 0.05. D) Average PPR recorded at time 32.5 min during aCSF (n = 9) and 1 μM CCh (n = 4) perfusion. All values represent mean per 5 min 0.05 Hz sampling interval ± SEM. Insets: sample paired-pulse protocol traces from whole-cell patch clamped neurons before (above) and after (below) STDP induction during A) aCSF and B) 1 μM CCh perfusion. Bars represent 1 mV and 20 ms. Each trace was averaged from 16 individual sweeps. Dashed line indicates normalized baseline PPR.
4 General Conclusions and Future Directions

4.1 Effect of mAChR activation on hippocampal CA1 network activity

Muscarinic receptor activation decreased the PSA in CA1 pyramidal neurons. The decrease in PSA by CCh was consistent with the dose-dependent reduction published previously (Hesen et al. 1998). In particular, Hesen et al. (1998) showed that the reduction in PSA was also accompanied by a reduction of the fEPSP slope, suggesting that the former resulted, at least in part, from a decreased excitatory drive at the Schaffer collateral to CA1 pyramidal neuron synapse. However, a decrease in the fEPSP could also be due to either a pre- or postsynaptic mechanism, such as an attenuation of neurotransmitter release, or receptor desensitization, respectively. This is consistent with the localization of mAChRs to both pre- and postsynaptic neurons (Giocomo and Hasselmo, 2007), but does not address whether either mechanism predominantly accounts for the reduced synaptic efficacy. Furthermore, whether this was attributable to a change in inhibitory synaptic transmission by a feed-forward disynaptic circuit was not addressed.

The activation of mAChRs on O-LM interneurons has been shown to accelerate their firing and induce a suprathreshold afterdepolarization, increasing GABA release onto pyramidal neurons (Lawrence et al., 2006). mAChR control of GABA release demonstrates that the modulation of network activity by mAChR activation also involves changes in interneuron activity. Valentino and Dingledine (1981) showed that ACh has a presynaptic inhibitory effect on both excitatory and inhibitory afferents to CA1 pyramidal neurons. This inhibition had a dose-dependent relationship to the application of atropine, suggesting that it was mediated by mAChRs activation (Valentino and Dingledine, 1981). Therefore, taken together, this suggests that changes in glutamatergic/GABAergic synaptic transmission onto pyramidal neurons account for the change in the excitation/inhibition profile of pyramidal neurons to synaptic transmission. This is complicated, however, by the differences in mAChR subtype expression in separate interneurons, thus evoking separate effects in separate groups. For instance, the expression of M2
receptors on PV+ interneurons in the CA1 hippocampus would be expected to lead to a reduction in GABA transmission by cholinergic transmission (Hájos et al., 1998). This would facilitate the fEPSP magnitude, but is in contrast to what has been observed.

For this thesis, in order to address whether dmLTP can be modulated by mAChR activation, it was necessary to directly measure a change in the strength of inhibition at pyramidal neurons in the presence of an AMPA and kainite receptor antagonist. Since the population spike response was nearly completely abolished by the blockade of this non-NMDA receptor dependent glutamatergic transmission, it was clear that recording of the postsynaptic potential response at individual neurons would be necessary.

4.2 Effect of mAChR activation on inhibitory synaptic strength in CA1 pyramidal neurons during basal network activity

It was observed that CCh does not affect $E_{\text{GABA}}$ during basal synaptic activity, despite depolarizing the resting membrane potential. This latter observation is consistent with published reports of the response of CA1 pyramidal neurons to CCh (Fraser and MacVicar, 1996; Chapman and Lacaille, 1999). The lack of an effect of mAChR activation on $E_{\text{GABA}}$, however, would suggest that the postsynaptic mAChR signal transduction cascade is not coupled to the establishment of $E_{\text{Cl}}^-$. This finding is in partial contrast to a previous report that CCh can regulate KCC2 expression (Lee et al., 2010). However, in that study, 100 $\mu$M CCh was applied for 2 h. Therefore, whether a high concentration and longer exposure to CCh was required for modulation of $E_{\text{GABA}}$ was considered. To address this, cultured hippocampal neurons were used which could be incubated with pharmacological agents for longer periods of time. Using cultured hippocampal neurons, gramicidin perforated patch recordings were made to determine the value of $E_{\text{GABA}}$ after 2 h exposure to CCh. However, even following a long-term exposure to CCh, $E_{\text{GABA}}$ remained unchanged in both 10 $\mu$M and 100 $\mu$M CCh, suggesting that mAChR activation alone does not alter the neuronal $E_{\text{Cl}}^-$. This would suggest that a reduction in KCC2 expression, as shown by Lee et al. (2010), might be compensated for by other Cl$^-\,$ extrusion mechanisms (e.g. the chloride-dependent chloride channel [CIC-2] [Rinke et al., 2010]) in order to maintain the strength of inhibitory synaptic activity. In particular, a downregulation of KCC2 expression may be compensated for by a concomitant downregulation of NKCC1 activity in order to maintain a baseline intracellular [Cl$^-\,$]. Furthermore, the study by Lee et al. (2010) was performed in human
embryonic kidney (HEK) 293 cells, which may possess a different expression pattern of mAChRs relative to CA1 pyramidal neurons, and thus respond in a unique way to excess CCh.

The response of hippocampal neurons to a high dose of 100 µM CCh was found to hyperpolarize the resting membrane potential. This was in contrast to what was expected, as CCh has previously been shown to cause a membrane potential depolarization (Fraser and MacVicar, 1996; Chapman and Lacaille, 1999). This may have been due to the activation of G protein-coupled inwardly-rectifying K⁺ (GIRK) channels (Fernandez-Fernandez et al., 1999). GIRK channels opening results in an outward K⁺ current at membrane potentials above -90 mV, and thus function to hyperpolarize the membrane (Ehrengruber et al., 1997). M₂ receptor coupling to GIRK channel activation via Gᵢ/ᵦγ₅-type G-proteins has previously been shown to suppress cellular excitability (Egan and North, 1986; Kunkel, and Peralta, 1995; Fernandez-Fernandez, et al., 1999, 2001).

4.3 Effect of mAChR activation on the expression of dmLTP in CA1 pyramidal neurons

As explained previously, disinhibition-mediated LTP results from inhibitory synaptic plasticity of feedforward inhibitory inputs onto pyramidal neurons (Ormond and Woodin, 2009, 2011). This depends on GABAergic interneuron-mediated GABA release, and results in a postsynaptic Cl⁻ accumulation (figure 4.1A). The mechanism underlying inhibitory synaptic plasticity is a postsynaptic Ca²⁺-mediated decrease in KCC2 function (Fiumelli et al., 2005), which depolarizes E_{GABA}, essentially weakening synaptic inhibition (Woodin et al., 2003; Saraga et al., 2008; Balena et al., 2010; Lamsa et al., 2010). There is small but growing evidence in the literature that neuromodulators may regulate E_{GABA}. For example, Lee et al. (2010) demonstrated that prolonged activation of mAChRs on cultured hippocampal neurons enhances lysozomal degradation of KCC2, which would presumably depolarize E_{GABA}. However, in the present study we found that mAChR activation did not regulate E_{GABA}. The discrepancy in these results likely arises from the high concentration of CCh (100 µM) and prolonged use (2 h) of CCh in the Lee et al study, compared to the present procedures (1 µM, 15-30 min).

Also, the role of mAChR modulation of synaptic plasticity has previously to been shown to be dependent on the amount of cholinergic input during STDP induction (Sugisaki et al., 2011). Sugisaki et al. (2011) showed that during a positive timing protocol, eserine, a cholinesterase
inhibitor, increased the EPSP magnitude in CA1 pyramidal neurons, but only at a low dosage. Excess ACh was shown to suppress STDP, LTP, and LTD (Sugisaki et al., 2011). This may be attributed to an inhibition of presynaptic neurotransmitter release, as was suggested by the pair-pulse ratio experiment in this thesis. Furthermore, Sugisaki et al. (2011) showed that STDP was suppressed by the addition of atropine, a mAChR competitive antagonist. This further highlights the role of mAChR modulation of plasticity induction, as mAChRs appear to be necessary during STDP. This is in contrast to LTP induced by tetanic stimulation, and may be due to the increased sensitivity of STDP to a disruption of a mAChR-dependent hippocampal network oscillation. Also, the enhancement of LTP by mAChR activation may reach a plateau, after which further synaptic facilitation is not possible. This may be due to a saturation of Ca\(^{2+}\) transporter activity by M\(_1\) and M\(_3\) activation.

### 4.4 Presynaptic mAChR modulation of synaptic efficacy in the suppression of dmLTP

A rapid PPD immediately followed the STDP induction protocol when CCh was present. PPD has been attributed to a decrease in presynaptic neurotransmitter release, and may be due to a depletion of releasable vesicles (Liley and North, 1953, Dobrunz and Stevens, 1997, Bellingham and Walmsley, 1999). If this was the case, it would suggest that despite the ability of mAChR-activation to increase interneuron spiking (Pitler and Alger, 1992), there was a reduction in GABA release which could account for the inability of dmLTP to be induced. The possible reduction in GABA release could result from a direct action of mAChR signaling pathways on the presynaptic terminal release of GABA containing synaptic vesicles (Behrends and ten Bruggencate, 1993) (figure 4.1B), or via retrograde transmitter suppression of GABA release (Ohno-Shosaku et al., 2003) (figure 4.1C). These have previously been suggested to be endocannabinoid-independent and -dependent mechanisms, respectively (Hájos et al., 2000; Kim et al., 2002; Fukudome et al., 2004). Notably, a muscarinic receptor dependent suppression of CA1 stratum radiatum synaptic potentials is dependent on presynaptic M\(_1\) and M\(_2\) receptors (Kremin, et al., 2006). Also, M\(_2\) receptors localized to presynaptic interneuron terminals have been shown to directly suppress GABA release (Fukudome et al., 2004). In contrast, M\(_1\) and M\(_3\) receptor activation on postsynaptic pyramidal neurons result in endocannabinoid (eCB) production which subsequently suppresses GABA release by activation of presynaptic cannabinoid receptor type 1 (CB\(_1\)) (Fukudome et al., 2004) (figure 4.1C). Whether either the
direct inhibition of GABA release by presynaptic M₂ activation, or an eCB-mediated retrograde suppression of GABA release by postsynaptic M₁/M₃ receptor activation predominantly contributed to the prevention of dmLTP would need to be examined in future experiments (figure 4.1D). Also, it is unclear as to whether disinhibition-mediated plasticity induction failed due to a lack of GABA release by direct M₂ mediated suppression, or by a disruption in the ability of GABA release to lead to a substantial increase in the postsynaptic [Cl⁻]ᵢ.

Future experiments can explore whether a specific mAChR subtype underlies the suppression of dmLTP via genetic knockout of specific receptor subtypes, and repeating the STDP induction experiment. Alternatively the use of specific pharmacological agonists/antagonists may more precisely determine the contribution of specific mAChRs to dmLTP expression. Also, an analysis of postsynaptic Cl⁻ accumulation can be determined using optophysiology in order to determine how the [Cl⁻]ᵢ changes during dmLTP in the presence of mAChR activation.

### 4.5 Final Remarks

The present study provides the first evidence that dmLTP is modulated by the cholinergic neuromodulatory system. Specifically, it was found that mAChR-activation prevents the induction of dmLTP in CA1 pyramidal neurons. Disinhibition-mediated LTP is expressed postsynaptically by a depolarization of E_{rev} (Ormond and Woodin, 2009). While mAChR-activation prevented the depolarization of E_{rev} postsynaptically, recording of the PPR, and the observed rapid PPD suggested a presynaptic locus of modulation by which disinhibition-mediated plasticity was prevented. In addition to providing novel evidence for the neuromodulation of dmLTP, this is the first study to demonstrate that dmLTP can be induced in the mouse hippocampus.

Similar to classic glutamatergic LTP, dmLTP also requires the activation of NMDA receptors (Ormond and Woodin, 2009), which suggests that, like classic glutamatergic LTP, dmLTP may play an important role in hippocampal-dependent learning and memory (Bliss and Collingridge, 1993). Thus, dmLTP may be a previously unrecognized target for therapeutic intervention in disorders related to memory deficiencies. By demonstrating that dmLTP is neuromodulated by the cholinergic system, which plays a well-known role in learning and memory functions of the brain (Hasselmo, 2006), it is plausible to consider designing therapeutic strategies based on the cholinergic neuromodulation of dmLTP to treat pathologies linked to learning and memory.
Figure 4.1 Pre- and/or postsynaptic mAChRs may be involved in the suppression of GABA release, preventing dmLTP.

Stimulation of Schaffer collateral axons (sc.) from CA3 pyramidal neurons to CA1 pyramidal neurons results in excitation of a GABAergic interneuron-mediated disynaptic feed-forward inhibition. A) Repetitive coincident stimulation of the sc. and the CA1 pyramidal neuron results in presynaptic Ca\(^{2+}\)-dependent GABA release, and postsynaptic Cl\(^{-}\) accumulation. B) Presynaptic M\(_{2/4}\) receptor-dependent inhibition of voltage-dependent Ca\(^{2+}\) channels (VDCC) prevents GABA release, which prevents postsynaptic Cl\(^{-}\) accumulation. C) Postsynaptic M\(_{1/3}\) receptors facilitate the production of eCBs, which act as a retrograde signal, binding to presynaptic CB\(_{1}\) receptors. CB\(_{1}\) receptor-mediated inhibition of VDCCs suppresses GABA release, which prevents postsynaptic Cl\(^{-}\) accumulation. D) mAChR-dependent suppression of GABA release may depend on pre- and/or postsynaptic mAChRs activation, by which dmLTP is prevented.
References


