PRESYNAPTIC REGULATION OF AFFERENT PROJECTIONS TO HILAR MOSSY CELLS

By

CHINKI BHATIA

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Dedicated to my grandmother and father

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By

Chinki Bhatia

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Hilar mossy cells form a major class of unusual local excitatory interneurons in the dentate gyrus. They receive strong glutamatergic, GABAergic, and likely cholinergic innervation from a variety of intrinsic and extrinsic sources. Under normal conditions, it has been proposed that mossy cells play a significant role in memory formation and information processing, while the loss and dysfunction of mossy cells has been implicated in different theories of epileptogenesis. In this study we used in vitro electrophysiological and optical techniques to expand our understanding of the neurophysiological mechanisms responsible for modulating the activity of synaptic inputs to hilar mossy cells. Results from my first project indicate that the activity of excitatory inputs to mossy cells can be modulated by cholinergics. Specifically, we find that both mossy fiber and non mossy fiber afferents express presynaptic γ-aminobutyric acid type B (GABAB) receptors that are subject to tonic inhibition by ambient GABA. Further, cholinergic agonists produce action potential-dependent increases in ambient GABA that can indirectly inhibit mossy fiber inputs. By contrast, non mossy fibers inputs are directly inhibited by activation of presynaptic muscarinic acetylcholine receptors on their terminals. Finally, we demonstrate that mossy cells express high-affinity postsynaptic GABAA receptors that are also capable of detecting changes in ambient GABA produced by cholinergic agonists. Our results

are the first to demonstrate the key neurophysiological mechanisms that modulate glutamatergic transmission to hilar mossy cells. This work also identifies clear differences between two primary types of excitatory afferents to mossy cells. In my second project we demonstrate a novel excitatory effect of cannabinoids on action potential independent GABAergic transmission in the rat dentate gyrus. Specifically, we find that both WIN55, 212-2 and anadamide increase the frequency of miniature (mIPSCs) recorded from hilar mossy cells without altering event amplitude, area, rise time, or decay. The effect of WIN55, 212-2 on mIPSCs is insensitive to AM251 and preserved in CB1 knockout mice indicating that it does not depend on activation of CB1 receptors. It is also insensitive to AM630 and unaffected by capsazepine suggesting that neither CB2 nor vanilloid type 1 receptors are involved. Further, the effect of WIN55, 212-2 on mIPSCs is sensitive to suramin, suggesting that G protein coupled receptors (GPCR) play a role in mediating the facilitation. Additionally, cannabinoids do not potentiate bipolar or minimally evoked IPSCs in the presence of AM251, suggesting that the exocytotic mechanism that produces WIN55, 212-2 sensitive mIPSCs is distinct from that which produces CB1 sensitive and action potential dependent release. We also report that the effect of WIN55, 212-2 is severely reduced on limiting presynaptic calcium availability, and yet is insensitive to chelation of calcium in the postsynaptic cell. Finally, we demonstrate that depolarization of hilar mossy cells sufficient to produce activation of CB1 receptors has only a minimal effect on mIPSC frequency. Consistent, with this we also found that 2-AG had a modest effect on increasing the mIPSC frequency.Cumulatively, our results indicate that cannabinoid ligands can selectively facilitate action potential independent exocytosis of GABA in the rat dentate gyrus in a CB1 independent manner, and further emphasize that this new cannabinoid mediated signaling system is distinct from the conventional CB1 dependent systems in a number of ways.

CHAPTER 1
INTRODUCTION

The hippocampus is located in the temporal lobe of the cerebral cortex. It is one of the most heavily studied areas of the mammalian central nervous system. There are three main reasons for this. First, the hippocampus has a well defined structure at both gross and histological levels and this makes it attractive for scientific research. Second, it has long been known to play a fundamental role in many learning and age related memory disorders like Alzheimer's. Third, many studies have also implicated the role of hippocampus in epileptic disorders due to its high seizure susceptibility. One of the most interesting neurophysiological finding showing the involvement of hippocampus in memory formation was from a patient known as H.M. The patient was suffering from intractable epilepsy. For his treatment, he underwent a bilateral hippocampal removal. After the procedure H.M. suffered a permanent loss in the ability to encode new information into long-term memory. This was also observed in other patients with bilateral hippocampal damage although H.M. was the first and one of the most thoroughly studied neuroscience research subjects. These findings were published in a landmark paper by Scoville and Milner in 1957.

In order to understand the role of the hippocampus in memory formation or seizures, it is important to learn how the brain functions at a basic synaptic level. My thesis work in the broad sense concentrates on understanding these basic neurophysiological mechanisms in the hippocampus (focus on dentate gyrus). In the future, this information will allow us to determine how the synaptic mechanisms may be altered in disease models of Alzheimer's and epilepsy.

First part of this chapter includes a brief anatomical description of the hippocampus. The later part focuses in detail on the hilar mossy cells which are located in the dentate gyrus. These cells have been implicated in different theories of epileptogenesis and memory disorders

(Jefferys and Traub 1998; Santhakumar et al. 2000; Scharfman et al. 2001; Sloviter 1991). The main goal of my project was to study the synaptic circuitry associated with mossy cells.

Hippocampus.

The shape of the hippocampus resembles that of a sea horse and this is how its name was coined. In Greek "hippo" means horse and "kampos" means sea monster. It is located in the limbic system as a structure called the hippocampal formation which encompasses the dentate gyrus, the hippocampus, the subiculum, persubiculum and parasubiculum and the entorhinal cortex. The dentate gyrus consists of three layers: the granule cell layer, the molecular layer located above the granule cell layer and the polymorphic cell layer, also known as the hilus located below the granule cell layer. The hippocampus also has three pyramidal cell layers the CA1-CA3. The pyramidal layers are also divided into different strata: stratumoriens, moleculare and radiatum. The unmyelinated axons of the granule cells are known as the mossy fibers. Their name was coined "mossy" since the large filamentous extensions and varicositied of these fibers are reminiscent of moss. These fibers synapse on to the distal dendrites of the CA3 pyramidal cells and their collaterals synapse on to the proximal dendrites of the glutmatergic interneurons located in the hilus, the mossy cells.

Classic Trisynaptic Pathway

The above paragraph lists all the basic key components that are involved in the transfer of information across the hippocampus. The entorhinal cortex is the starting point of the circuit since much of the sensory information that reaches the hippocampus enters through the entorhinal cortex. Information from the entorhinal cortex is relayed to the granule cell layer through the perforant path inputs. Mossy fibers then transfer the information from the granule cells to the CA3 pyramidal cells and the hilar mossy cells. The CA3 pyramidal cells, in turn project to the CA1 through the schaffer collateral inputs. Information from the CA1 is then

passed to the subiculum and back to the deep layers of the entorhinal cortex (Fig 1-1). This pathway of information transfer that linked each region of the hippocampal formation was termed the trisynaptic pathway by Anderson and colleagues in 1971.The transfer of information that takes place through this pathway is essential for coding long term memories (ref).

The intrahippocampal connections form a *trisynaptic loop*, which is composed of the cells of dentate gyrus, CA3 and CA1 and their interconnections (Fig. 1-1) (Amaral and Witter 1995).

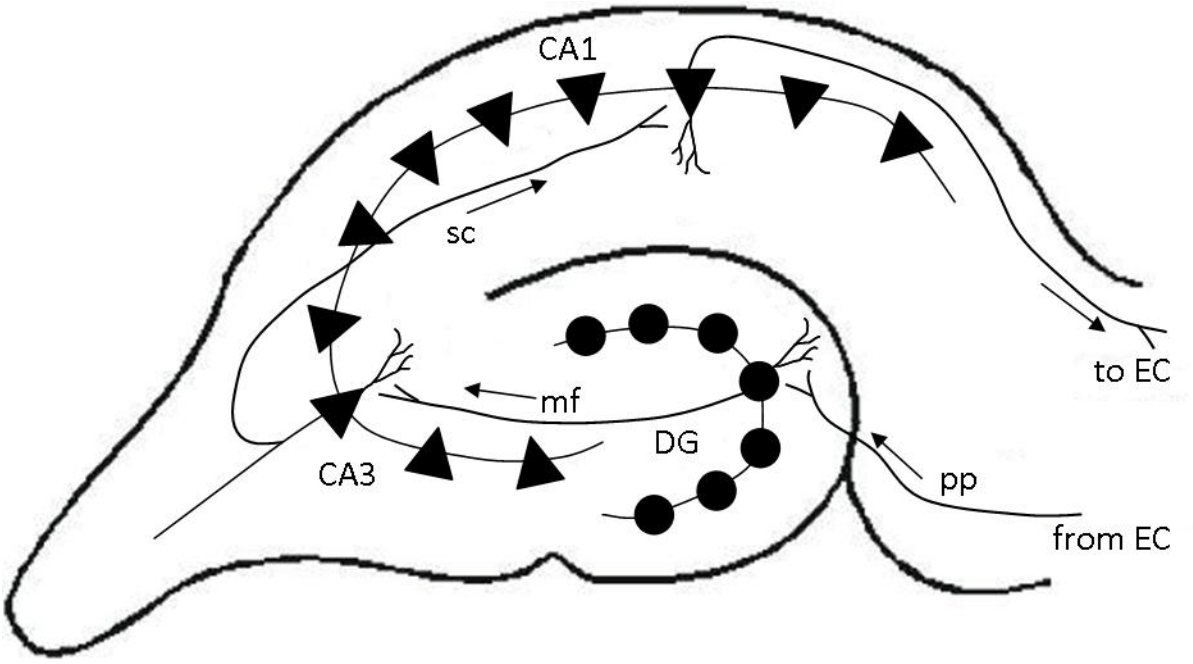

Figure 1-1. The trisynaptic loop of the hippocampus. The filled triangles represent the pyramidal cell layer (CA1 and CA3) and the filled circles represent the granular cell layer of the dentate gyrus. Abbreviations: EC = entorhinal cortex; DG = dentate gyrus; pp = perforant pathway; mf = mossy fibers; sc = Schaffer collaterals; ff = fimbria fornix.

Interneurons of the Dentate Gyrus

So far, I have only mentioned the principal cells of the hippocampus. However, the hippocampal formation also consists of several types of interneurons that serve as key players in mediating synaptic plasticity. Briefly, interneurons can be classified on the basis of their neurochemical identity, axonal arbaorizations and post synaptic targets. Immunohistochemical

studies have revealed dense labeling of GAD65-labeled axon terminals in the outer molecular layer and inner molecular layer (Halasy and Somogyi 1993; Han et al. 1993).The axon terminals in these layers have their cell bodies in the hilus. These interneurons are known as hilar perforant path-associated interneurons (HIPP) and hilar commissural/associational path associated interneuron (HICAP) respectively and contain the peptide somatostatin (Bakst et al. 1986; Katona et al. 1999). The other groups of interneurons are known as the Basket cells. These interneurons have their cell bodies in the base of the granule cell layer (GCL) or inside the GCL and their axons project to multiple granule cells (GC). The axon-axonic groups of interneurons have their cell bodies on the border of the GCL or the hilus. They project on the initial axonal segments of the granule cells. Both the basket cells and axo-axonic interneurons can exert control over the output of the granule cells and can potentially contribute to synchronization of granule cells by forming multiple contacts with them ((Miles et al. 1996). There are two other groups of interneurons that are difficult to classify. The first group is the calretinin, callbindin positive interneurons which are located in the hilus and their axons contact other interneurons. The second group consists of the somatostatin containing GABAergic interneurons whose cell bodies are in the hilus but their axons project into the medial septum where they synapse on other PV containing GABAergic inteneurons (Freund and Antal 1988). The axons of these PV neurons synapse back to GABAergic interneurons in the dentate forming a recurrent GABAergic feedback loop that regulates theta activity (Dragoi et al. 1999). GABAergic interneurons make up 50% of the population of neurons in the hilus. The other 50% of the neurons constitute the mossy cells or glutamatergic internurons ((Zappone and Sloviter 2004)reference).

Mossy Cells

Scarcity of information on the hilar mossy cells is attributed due to its low number in the hilus. Mossy cells were first recognized by Cajal (1911) and Lorente de No (1934) for their

dendrites covered with large spines. Later they were given the name “mossy cells” by Amaral (1978) due to the mossy appearance of their large spines.

Mossy Cell Anatomy

Mossy cells are usually multipolar and have tapering dendrites that remain restricted to hilus proper. Occasionally, their dendrites can be found in the molecular layer of the dentate gyrus in rats and primates where they may be innervated directly by entorhinal cortex bypassing the granule cells (Scharfman 1991). Mossy cells that do not have their dendrites in the molecular layer can receive only indirect information from the entorhinal cortex relayed by granule cells. The proximal dendrites and somata of mossy cells are covered by large spines or thorny excresences. Their spines are different from the spines on CA3 proximal apical dendrites, in the sense that, the spines on CA3 are irregular and thorny in their shape while the spines on mossy cells resemble clusters of spheres (ref).

The axons of GAD positive inhibitory interneurons innervate the somatic region of mossy cells suggesting a strong perisomatic inhibitory input to these cells. Perisomatic innervation to these cells is primarily from terminals that contain the neuropeptides PV and CCK (Acsady et al. 2000). Mossy cells also receive strong cholinergic innervations to their perisomatic and proximal dendritic inputs that arrive from the medial septum (Deller et al. 1999). Additionally, these proximal inputs are also innervated by axons of granule cells called mossy fibers whereas the distal dendrites are innervated by the non mossy fiber inputs. It’s said that there is a 100:1 ratio of granule cells to mossy cells. It is possible that at least some of this non mossy fiber component is from CA3 pyramidal cells which extend its axons back into the hilus and granule cell layer (Amaral 1978; Frotscher et al. 1991; Schwartzkroin et al. 1990). My third chapter addresses how you may differentiate responses on mossy cells that result due to activation of mossy fiber or the various types of non mossy fiber inputs.

Interest in studying mossy cells has developed more in the recent years. This is due to the several characteristics of mossy cells that make them unique among local circuit neurons in the dentate gyrus. First, they are glutamatergic rather than GABAergic (Soriano and Frotscher 1994). Second, they have septotemporal rather than laminar axonal projections (Amaral and Witter 1989; Buckmaster et al. 1992; Buckmaster et al. 1996; Jackson and Scharfman 1996; Scharfman 1995; Schwartzkroin et al. 1990). Their axons synapse on distant granule cells and aspiny dendrites of inhibitory interneruons . Third, mossy cells also receive dense innervations from mossy fiber axons of granule cells on their spiny proximal dendrites (Frotscher et al. 1991). Activation of these synapses generates large spontaneous EPSCs. Together, these features indicate a prominent role of mossy cells in regulating granule cell activity. Improper regulation of the granule cell activity may contribute to the development of seizures and certain memory disorders. Additionally, mossy cells are also extremely sensitive to excitotoxic death and this feature may make mossy cells more vulnerable to loss during seizure activity (Bekenstein and Lothman 1993; Buckmaster and Dudek 1997; Buckmaster and Jongen-Relo 1999; Buckmaster et al. 1996; Coulter 2004; Jefferys and Traub 1998; Santhakumar et al. 2000; Scharfman et al. 2001; Sloviter 1991)

Mossy Cells and Epilepsy

One of the characteristic signs of epilepsy is the loss of hilar neurons in the dentate gyrus. Specifically the death of these cells has been reported in cases of post traumatic brain injury like ischemia and seizures. The dentate gyrus constitutes both GABAergic and glutamatergic hilar interneurons (50:50) but many reports indicate that mossy cells (glutamatergic interneurons) are more vulnerable to excitotoxicity. This may be due to the fact that several of the cellular properties of mossy cells that make them unique and significant for normal physiological functioning could actually put these cells at greater risk during epilepsy. For example, mossy

cells receive massive excitatory inputs from mossy fibers and also project back to cells that provide them with strong glutamatergic transmission, thus forming a positive feedback circuit (Frotscher et al. 1991; Ribak et al. 1985; Soriano and Frotscher 1994; Wenzel et al. 1997). They have a high rate of spontaneous activity which is on an average higher than sIPSCs. They also undergo ADP (after depolarization), which is a long form of potentiation, after a period of depolarization (Strowbridge and Schwartzkroin 1996). Cumulatively, these properties could make these cells more vulnerable during various forms of injuries. For the longest time the vulnerability of mossy cells in epilepsy led many to believe that it is the loss of these cells that leads to the generation of seizures. However, there is little evidence regarding this theory. This is because until recently there was no specific marker for mossy cells. A more recent hypothesis states that all of the mossy cells don't necessarily die; rather some of them survive and play a prominent role in generating seizures (for review refer (Santhakumar et al. 2000). This hypothesis is also known as the irritable mossy cell hypothesis. For better understanding of these two hypotheses, refer to the illustrated diagrams below: (Fig 1-2)

The stimulation of the perforant path in these diagrams represents a traumatic injury or insult.

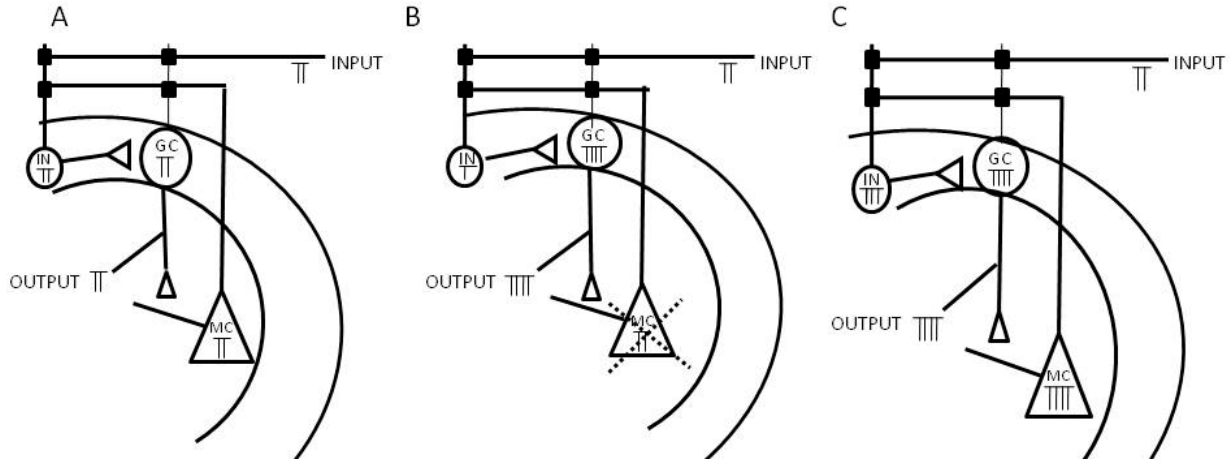

Figure 1-2. Models of epileptogenesis

Figure 1-2 (A) shows the basic connectivity's in the dentate gyrus during physiological conditions. On stimulating the perforant path inputs, both the granule cells and GABAergic interneurons are discharged. GCs in turn excite the mossy cells which project back to GCs and IN (interneurons). The GC discharge is the main signal that leaves the dentate gyrus (OUTPUT). **Panel B** illustrates the Dormant Basket Cell Hypothesis. This hypothesis states that the loss of mossy cells is the main event leading to hyperexcitability. Due to prolonged stimulation of the perforant path, there is a loss of mossy cells. This results in lack of an excitatory drive on GABAergic interneurons, making them dormant and leads to hyperexcitabillity of the GCs. **Panel C**: The irritable mossy cell hypothesis suggests that it is the survival of mossy cells which is the central event leading to hyperexcitability. Since some mossy cells are also lost, the surviving cells have a greater excitatory drive from the GCs. This leads to the formation of a hyperexcitable excitatory feedback pathway to granule cells. In addition to the above theories, there is a third theory of epileptogenesis: mossy fiber sprouting. This hypothesis states that when mossy cells are lost during hyperexcitable periods, mossy fibers tend to project on to other granule cells and form a recurrent excitatory network. This results in many granule cells discharging at the same time and causing hyperexcitability.

The above paragraph lists three theories of epileptogenesis. However, more work needs to be done on mossy cells to substantiate these hypotheses. My research project is highly relevant to the study of epilepsy or Alzheimer's as it extends our understanding of how excitability may be regulated in mossy cells. Recent discoveries regarding retrograde signaling by endogenous cannabinoids and tonic inhibition by ambient γ-aaminobutyric acid (GABA) have had a profound impact on our understanding on hippocampal and cerebellar physiology. In my study, I examined the extent to which these signaling mechanisms contribute to the regulation of synaptic

transmission in the dentate, an area intimately involved in learning and memory. Although hilar mossy cells have been strongly implicated in the etiology of temporal lobe epilepsy, very little is actually known about the role of the above singaling mechanisms in moduling their activity. My project focused on how cannabinoids, cholinergics and ambient GABA work together to regulate transmitter release from GABAergic and glutamatergic afferents to mossy cells.

Now I would also like to review some known facts about both GABAergic and endocannabinoid signaling in the dentate. This review will help us understand and appreciate the significance of my thesis work.

In the following sections, I will provide a brief overview of the endocannabinoid and the GABAergic signaling system. I will also highlight a significant body of existing evidence indicating the role of these systems in modulating neurophysiological mechanisms on different synapses in the brain, with more focus on the dentate gyrus. Additionally, I will address some novel roles of these mechanisms in the dentate. At the end of this chapter, I will provide a brief synopsis of the work done in my research project.

GABAergic Signaling System

GABA is accepted as the main inhibitory neurotransmitter in the brain. It exerts its action by activating either ligand-gated $GABA_A$ receptors, or metabotropic $GABA_B$ receptors. Activation of $GABA_A$ receptors generally produces a fast or phasic inhibition either by generating an inward chloride current or, if the membrane potential is very near the equilibrium potential for chloride, by shunting concurrent excitatory inputs. Activation of post synaptic $GABA_B$ receptors causes inhibition by increasing potassium conductance via a G-protein coupled second messenger system or via a direct action of $\beta\gamma$ subunits with the ion channels (Bowery 1993). The time course of this inhibition is slower than that mediated by $GABA_A$

receptor activation. $GABA_B$ receptors on presynaptic terminals in the CNS usually inhibit calcium influx resulting in the inhibition of exocytosis.

Presynaptic $GABA_B$ Receptors Mediated Plasticity

Some studies indicate that in certain brain regions $GABA_B$ and $GABA_A$ sites exist together, but in other areas, there are more $GABA_B$ sites than $GABA_A$ sites. For example, in the interpeduncular nucleus the density of $GABA_B$ sites is high whereas that of $GABA_A$ sites is extremely low (Chu et al. 1987) By contrast, $GABA_B$ sites appear to be absent from the cerebellar granule cell layer whereas high affinity $GABA_A$ sites are in abundance (Wilkin et al. 1981). Although our lab was the first to demonstrate an effect of presynaptic $GABA_B$ receptors in modulating transmitter release from afferents to mossy cells (Nahir et al. 2007), presynaptic effects of activating $GABA_B$ receptors have been discovered on various other synapses and in different brain areas. For example, bath application of baclofen has been found to decrease the evoked IPSPs on Schaffer collaterals in CA1 (Gerasimov and Artemenko 1990). Further, at various glutamatergic terminals, activation of $GABA_B$ receptors has shown to inhibit both calcium dependent (for review see (Chen and Regehr 2003; Dittman and Regehr 1997; Ishikawa et al. 2005; Misgeld et al. 1995; Sakaba and Neher 2003; Takahashi et al. 1998) and calcium independent release (Capogna et al. 1996; Dittman and Regehr 1996; Jarolimek and Misgeld 1992; Kolaj et al. 2004; Scanziani et al. 1992). In addition to modulating glutamate release presynaptic $GABA_B$ receptor activation can also modulate the release of other neurotransmitters like GABA, acetylcholine, 5-HT, cetecholamines and other neuropeptides like somatostatin, CGRP, substance P and many more (for review see Bowery 1993). Some studies have also reported presynaptic $GABA_B$ receptors as autoreceptors. Even though there is lack of neurochemical evidence to support this hypothesis, there is some neurophysiological evidence that points to presynaptic autoreceptor control. Mott et al. have demonstrated in the rat dentate

gyrus that the reduction in recurrent inhibition produced by tetanic stimulation of mossy fibers can be reversed by phaclofen ($GABA_B$ receptor antagonist) (Mott et al. 1990). They suggest that GABA is released during tetanic stimulation and acts on $GABA_B$ sites located on inhibitory intemeurones to reduce recurrent inhibition. In addition, some paired-pulse studies performed in hippocampal slices recorded from CA l neurones support the existence of $GABA_B$ receptors on these terminals (Davies et al. 1990). The role of $GABA_B$ receptors has also been implicated in long term potentiation (LTP), a phenomenon known to be involved in learning and memory. Mott et al. induced LTP by 5-Hz stimulation. This induction caused the $GABA_A$ inhibition to decrease, as a result of which the NMDA mediated excitation increased. A $GABA_B$ receptor antagonist, 2-OH Saclofen, prevented the reduction of inhibition, the increase of excitation, and the induction of LTP. These studies suggest that disinhibition caused by $GABA_B$ receptors is required for induction of LTP by 5-Hz stimulation. $GABA_B$ receptor modulation of synaptic plasticity occurs at frequencies in the range of the endogenous hippocampal theta rhythm, which has been shown to modulate LTP in vivo (Mott and Lewis 1991).

There is an overwhelming amount of information both on $GABA_A$ and $GABA_B$ receptor mediated plasticity (pre and post synaptic terminals). However, in the previous section I only described presynaptic $GABA_B$ receptor mediated plasticity. This is because my research work demonstrated a significant role of presynaptic $GABA_B$ receptors in inhibiting transmitter release from mossy fiber terminals to hilar mossy cells. Strikingly, to mediate the inhibition $GABA_B$ receptors were functionally activated by ambient GABA in the synaptic cleft. The following paragraph discusses the role of ambient GABA in hippocampal neurophysiology.

Ambient GABA

In recent years much more has been learnt about the GABAergic transmission, which has made the signaling mechanism much more complicated to understand. For starters, considerable

progress has been made in understanding that ambient GABA plays a role in normal synaptic function. Ambient GABA arises from action-potential dependent and/or independent signaling or even via reverse action of GABA uptake mechanisms (Brickley et al. 1996; Carta et al. 2004; Rossi et al. 2003; Wall and Usowicz 1997). When ambient GABA binds to extrasynaptic receptors, it gives rise to tonic currents. These currents have been found to be carried by $GABA_A$ receptors detected in several types of neurons. For example, studies done in the cerebellar granule cells report a form a tonic $GABA_A$ receptor mediated signaling that appears to be caused by activation of extrasynaptic $GABA_A$ receptors. In addition, they found that the large tonic conductance increased in magnitude during development as a result of GABA being trapped in the glomeruli (Brickley et al. 1996; Wall and Usowicz 1997). Further, it was found that, the cerebellum extrajunctional $GABA_A$ receptors contain the α6 and δ subunits, which contribute to the non – desensitizing high affinity receptors (Nusser et al. 1998). Interestingly, $GABA_A$ receptor mediated tonic currents in the cerebellum were found to be generated by GABA that originates from a non-vesicular source, and is modulated by cholinergics (Rossi et al. 2003). Cholinergic modulation of ambient GABA is an interesting phenomenon and is also observed in my research work in the dentate.

Tonic $GABA_A$ receptor activation has also been illustrated in the granule cells of the dentate gyrus (Nusser and Mody 2002; Overstreet and Westbrook 2001; Stell and Mody 2002). $GABA_A$ receptors in these cells also have δ and α6 subunits which have higher affinity for GABA and lower propensity to desensitization than $GABA_A$ receptors involved in phasic inhibition. Tonically active $GABA_A$ receptors are also expressed on the interneurons in the CA1 pyramidal cell layer of the hippocampus, both in the stratum oriens and stratum radiatum (Semyanov et al. 2003).Interestingly, similar to our work, tonic signialing was not observed in

the CA1 interneurons unless the extracellular GABA concentration was raised experimentally. $GABA_B$ receptors also have the basic properties necessary to make them effective detectors of ambient GABA. Tonic activation mediated by $GABA_B$ receptors has been demonstrated in the hippocampus and the cerebellum (Dittman and Regehr 1997; Isaacson et al. 1993). Presynaptic $GABA_B$ receptors on the mossy fiber terminals to the CA3 pyramidal cells are also effective detectors of ambient GABA. A study done by Voght and Reghr in 2003 demonstrated that inhibition of mossy fiber terminals to CA3 resulted due to activation of the $GABA_B$ receptors by ambient GABA that was under cholinergic control.

Notably, the functional significance of tonic $GABA_A$ receptor activation on interneurons and principal cells has been suggested in regulating the overall network excitability. It has been reported that elevation of extracellular GABA, either by perfusion or by blockade of GABA uptake, leads to an increase in the tonic current in interneurons and a decrease in their excitability (Semyanov et al. 2003). In contrast, blockade of tonic currents increased the excitability of interneurons.

In conclusion, ambient GABA appears to be of important in synaptic processing within the brain and has the ability to be detected by both high affinity $GABA_A$ receptors and exptrasynaptic $GABA_B$ receptors. In my research work, I demonstrated a significant role of ambient GABA in modulating synaptic transmission to hilar mossy cells. To summarize my results in chapter 3, we found that both mossy fiber and non-MF glutamatergic inputs to mossy cells express presynaptic $GABA_B$ receptors at their terminals that are capable of directly inhibiting glutamate release. These receptors are tonically activated by ambient GABA in in vitro preparations and can be further activated by GABA transporters. We also report that bath application of muscarinic agonists directly inhibits release of glutamate from non-MF inputs

while indirectly inhibiting mossy fiber inputs by driving action potential-dependent increases in ambient GABA. Finally, we demonstrate for the first time that hilar mossy cells express high-affinity $GABA_A$ receptors that produce tonic GABAergic currents that are sensitive to changes in concentration of ambient GABA.

Endocannabinoid Signaling System

Marrijuana and its active constituent delta-9-hydrocannabinol (Δ^9THC) are known and consumed worldwide for their therapeutic properties and at the same time they are misused by people for recreational purposes. Their dual usage has lead to huge discrepancies both socially and politically over the significance of Δ^9THC in current day medicine. Therapeutically, cannabis (marijuana) has analgesic, anticonvulsant and appetite stimulant properties. However, the use of cannabis is also associated with a number of risk factors. Many studies indicate its role in memory impairment, locomotion and nociception. Cannabis has a number of constituents in different proportions out of which cannabinoids have been known to be most pharmacologically active (Abood et al. 1993).

Endocannbinoids: Synthesis, Release, Uptake and Degradation

The active ingredient in marijuana, delta-9-hydrocannabinol (Δ^9 - THC), was first identified in 1964 (Gaoni and Mechoulam 1964). The two main endogenous ligands anadamide (AEA) and 2- arachidonyl glycerol (2-AG) were identified thereafter ((Devane et al. 1992; Sugiura et al. 1995). Some of the other endogenous ligands include virodhamine which resembles anadamide, and 2-arachidonylglyceryl ether noladin- an analogue of 2-AG. They are mostly located in the brain, plasma and peripheral tissues. Out of all the cannabinoids, noladin is present in the lowest concentrations in the brain which indicates that it may be less important to CNS function (Rodriguez de et al. 2005). Even though anandamide and 2-AG are both fatty acid derivation, they do not share the same synthetic pathway. The synthesis of anandamide involves

reactions with two enzymes. The first enzyme is N-acyltransferase. This enzyme synthesizes the lipid precursor N-arachidonyl-phosphatidylethanolamine (NAPE) in a calcium dependent manner, by carrying out a catalytic transfer of arachidonic acid from phosphatidylcholine to the head group of phosphatidylethanol amine. The second enzyme phospholipase D drives the release of anandamide from its precursor NAPE (Cadas et al. 1997; Di Marzo et al. 1999; Di Marzo et al. 1994). By contrast, the primary immediate precursor for synthesis of 2-AG appears to be diacylglycerol, which is produced from phospohotidylinositol by phospholipase C, and subsequently converted to 2-AG by diacylglycerol lipase (DAGL) (Piomelli 2003; Stella et al. 1997). So far, it is thought that the synthesis of AEA depends on a highly calcium-dependent enzyme NAPE. However, the involvement of calcium in the synthesis of 2-AG is still not clearly understood. For example, some studies indicate that in certain systems calcium dependent production of 2-AG is likely to be significantly enhanced if the calcium influx occurs co-incident with synaptic activation of certain metabotropic receptors (notably metabotropic glutamate receptors and/or muscarinic receptors), capable of activating a calcium-dependent isoform of phospholipase C (Hashimotodani et al. 2005; Maejima et al. 2005). By contrast, there are studies which indicate that activation of metabotropic receptors can drive calcium independent production of 2-AG.

On reaching the extracellular space, the uptake of anandamide can occur through facilitated diffusion (Beltramo et al. 1997; Hillard et al. 1997; Piomelli et al. 1999). This carrier mediated process is known to be fast, selective, saturable and temperature dependent. While it is suggested that the anadamide transporter also facilitates the uptake of 2-AG, the possibilities for other 2-AG transporters should not be ruled out. The degradation of both anandamide and 2-AG is mediated by fatty acid amide hydrolase (FAAH), an enzyme that has an expression pattern in

the CNS that closely corresponds to that of the CB1 receptor (Piomelli et al. 1999;(Deutsch et al. 2002)). Another route for the breakdown of 2-AG is mediated by the enzyme monoglyceride lipase (MGL) (Dinh et al. 2002).

CB1 Receptors

The first cannabinoid receptor in the CNS, CB1 was identified and cloned in 1990 (Howlett et al. 1990; Matsuda et al. 1990). It is widely expressed in the hippocampus, cortex, cerebellum and basal ganglia (Herkenham et al. 1991; Herkenham et al. 1990; Tsou et al. 1998a; Tsou et al. 1998b). Presynaptic CB1 receptors are coupled to Gi protein which activates adenylyl cyclase. It may also be directly coupled to either calcium or potassium conductances (Howlett 1998; Howlett et al. 2004). Hence, majority of the effects associated with CB1 activation are inhibitory (Hajos et al. 2000; Hofmann et al. 2006; 2008; Pitler and Alger 1992; Wilson and Nicoll 2001). The other cannabinoid receptors are CB2 and vanilloid receptors. CB2 is mostly found in the peripheral system (Munro et al. 1993). There is a lot of literature indicating the activation of vanilloid receptors by anadamide in the CNS (discussed later). Many immunohistochemical and electron microscopic techniques indicate prominent expression of CB1 receptors in the dentate. In particular, these receptors were found to co-localize with CCK-positive interneurons, many of which have cell bodies located in the subgranular zone (Frazier 2007). The distribution of CB1 receptors on CCK positive terminals in the dentate supports their role in modulating GABAergic transmission to the mossy cells and granule cells. The fact that these CB1 and CCK positive interneurons do not make synaptic contacts with other basket cells also helps in distinguishing hilar circuitry from other circuits in the hippocampus (Acsady et al. 2000).

For many years, the immunohistochemical and electrophysiological evidence supported the existence of CB1 receptors on GABAergic terminals only. However, this conventional view

did not fit with the clinical evidence that supports Δ^9THC as an antiepileptic (Wada et al. 1973). The disagreement with the physiological data on cannabinoid receptors and clinical evidence of Δ^9THC curing epilepsy is now being addressed. More recently, newer C-terminal antibodies have been developed with a better ability to detect CB1 receptors on glutamatergic terminals (Katona et al. 2006; Marsicano et al. 2003; Monory et al. 2006). One such striking study using dual-labeling in situ hybridization techniques has shown strong co-localization of mRNA for CB1 and for the vesicular glutamate transporter type 1 (VGLUT1) both in the soma of hilar mossy cells and in presynaptic terminals forming asymmetric synaptic contacts in the inner molecular layer (Monory et al. 2006). Our lab also provides electrophysiological evidence which supports selective expression of CB1 receptors on glutamtergic terminals to CA3 cells (Hofmann et al. 2008). This study showed that CB1 receptors were identified on AC inputs and not on mossy fiber terminals to CA3 cells. Interestingly, some studies also reported that deletion of CB1 receptors from glutamatergic cortical neurons, but not GABAergic neurons led to an increase in susceptibility to seizures (Monory et al. 2006). Cumulatively, the above mentioned recent findings of CB1 expression on glutamatergic afferents will help bridge the gap in literature on the physiological effects of CB1 receptor activation on glutamatergic afferents.

Original Role of Cannabinoids

The conventional role of cannabinoids has been indicated in inhibiting calcium dependent release from glutamatergic and GABAergic terminals. In the following paragraphs, I describe the evidence that supports the above statement. The first evidence that indicates the role of exogenous cannabinoids in modulating GABAergic transmission, to dentate granule cells, was reported in 2000 by Hajos et al. His studies showed that bath application of WIN55, 212 reduced the amplitude of evoked IPSCs (eIPSCs) by 36 ± 6% of baseline in wild type but not CB1 -/- mice (Hajos et al. 2000). Another study in 2003 reported that the reduction of sIPSCs by WIN55,

212-2 in human dentate granule cells is sensitive to the application of AM251 (Nakatsuka et al. 2003). A break-through in endocannabinoid research was achieved in 2001, when it was reported that endocannabionids can act as retrograde messengers, in a phenomenon known as depolarization induced suppression of inhibition (DSI) (Wilson and Nicoll 2001). DSI is a form of short term plasticity in which endocannabinoids released from the post synaptic cell on depolarization diffuse backwards across the synaptic cleft, to bind to CB1 receptors on GABAergic interneurons and transiently inhibit them. This phenomenon was also discovered on other inhibitory synapses in different areas of the brain like basal ganglia, amygdala, cerebellum, substantia nigra etc (Bodor et al. 2005; Hofmann et al. 2006; Isokawa and Alger 2005; Trettel and Levine 2003; Zhu and Lovinger 2005). Further, DSI in granule cells was completely characterized in 2005 (Isokawa and Alger 2005). This study reported that direct depolarization of a single dentate granule cell reduces the amplitude of both spontaneous and evoked IPSCs (stimulation in the molecular layer).Moreover, they also found that bath application of WIN55, 212-2 and CP55, 942 occluded DSI and in addition found that DSI was blocked in the presence of SR141716A. They also showed that there was a strong correlation between the magnitude of DSI and the extent of depolarization induced increases in calcium. Strikingly, their study illustrated that DSI is dependent on calcium release ryonodine sensitive internal calcium stores, as depletion of those stores reduced both internal calcium and the magnitude of DSI. Further, our lab also reported the first evidence of DSI in the hilar mossy cells (Hofmann et al. 2006). Specifically, the study showed that DSI of eIPSCs in hilar mossy cells is dependent on both post synaptic calcium and presynaptic CB1 receptors. Additionally, a strong correlation between the magnitude of DSI in hilar mossy cells and depolarization duration was found. DSI was also enhanced by bath application of carbachol. Interestingly, using dual patch clamp techniques our

lab also showed that there are tight spatial constraints on diffusion of endocannabinoids released from hilar mossy cells. This phenomenon targeted only a calcium dependent release process from GABAergic interneurons. Cumulatively, the above mentioned results suggest a prominent role for endocannabinoids in modulating calcium dependent release from GABAergic interneurons to different post synaptic targets in the hippocampus. However, there is very little evidence in the literature for the role of exogenous and endogenous cannabinoids in modulating excitatory transmission in the dentate gyrus despite its potential use as an antiepileptic.

As I briefly mentioned earlier, our lab has recently published a paper which reports DSE in CA3 pyramidal cells. The results indicate that CB1 dependent inhibition of A/C mediated transmission occurs subsequent to both bath application of exogenous agonists and depolarization mediated release of endogenous agonists (Hofmann et al. 2008). By contrast, MF projections to CA3 pyramidal cells appear to lack functional CB1 receptors. Nevertheless, we cannot rule out the possibility of non CB1 receptors on the mossy fiber terminals. There are some earlier studies that indicate the expression of CB1 receptors on perforant path inputs to granule cells. One particular study demonstrated that bath application of WIN55, 212-2 produced a rightward shift in the input/output curve, and a reduction in paired pulse facilitation, of perforant path synaptic potentials recorded from the outer third of the molecular layer (Kirby et al. 1995). There is very limited literature available on the role of endocannabinoids in modulating glutamate release from mossy cell axons. Recent immunohistochemical studies do support the existence of CB1 receptors on these glutamatergic axons. There are a few neurophysiological studies which also reinforce the exisitence of CB1 receptors on mossy cell axons. For example, a recent study showed that evoked IPSCs in granule cells (stimulation in the inner molecular layer) were inhibited by bath application of WIN55, 212-2. Notably, an

interesting study also illustrated that the CB1 knockouts that are selective for glutamatergic neurons reduce the threshold for kainic acid seizures (Marsicano et al. 2003; Monory et al. 2006).

Collectively, these results suggest a strong role for ECs in modulating excitatory circuits in the hippocampus, a rich future for research on EC dependent modulation of excitability, and real potential to make significant strides towards a better understanding of neurological disorders of both memory and excitability.

Novel Cannabinoid Receptors

A final area worth discussing is on the unidentified cannabinoid receptors. This topic is very relevant to my research project as a large part of my work was on the novel cannabinoid receptor that mediates facilitation of action potential independent excoytosis (discussed later). There is some interesting literature available on the role of novel cannabinoid receptors in modulating transmission at various synapses. Although 2-AG is present in a 200 fold higher concentration than AEA in the brain and it is widely accepted that 2-AG is the retrograde messenger responsible for the production of DSI and DSE, the role of anandamide should not be overlooked (Stella et al. 1997; Sugiura et al. 1995). This is because interestingly, anadamide has been known to act at non-CB1 receptor sites (for review see (Chevaleyre et al. 2006). One such site where anandamide binds is the VR1 receptor (vanilloid receptor). Recent immunohistochemical studies have indicated a high expression of vanilloid receptors in the brain, especially in the dentate gyrus (Toth et al. 2005). In other areas of the brain activation of the VR1 receptor by anadamdie has also resulted in modulation of synaptic transmission (Marinelli et al. 2003; Marinelli et al. 2002). Some other targets of anandamide include α7 containing nicotinic acetylcholine receptors, 5-HT3 receptors and muscarinic acetylcholine receptors (Oz 2006). Interestingly, a recent study done on brain membranes from CB1 -/- mice

reported the ability of cannabinoid agonists to stimulate GTPγS binding (Breivogel et al. 2001b; Di Marzo et al. 2000). Many cannbinoid agonists were tested, however only WIN55, 212-2 and anadamide were effective. Further, studies done in the hippocampus and whole brain preparations indicated that WIN55, 212-2 and anandamide stimulate GTPγS binding which was insensitive to the application of the CB1 receptor antagonist SR141716A. Interestingly, the binding also had a distribution distinct from CB1 and CB2. Importantly, the recent discovery of NAPE-PLD (synthetic enzyme responsible for AEA production) was also identified in the excitatory presynaptic terminals in several areas of the CNS (Nyilas et al. 2008). Notably, the distribution of NAPE-PLD, like FAAH (an enzyme that breaks down AEA), and the EC mediated yet CB1 receptor independent binding of [35s]GTPγS noted above, overlaps with but likely does not perfectly match the distribution of CB1 receptors. The CA1 is another area where CB1 independent effects have been identified. Several studies in this area have demonstrated that the glutamatergic Schaffer collateral pathway (from CA3 pyramdial cells to CA1 pyramidal cells) is not only sensitive to inhibition by WIN55, 212-2 but still persists in CB1-/- animals and further is insensitive to the application of the CB1 antagonist SR141716A (Hajos and Freund 2002a; b; Hajos et al. 2001). However, this result has been contradicted by many other studies and there are no conclusive results on this issue (Kawamura et al. 2006; Takahashi and Castillo 2006)

Collectively, these studies support the existence of novel cannabinoid like receptor targets. Additionally, we also believe that many of those unidentified sites can be activated by anadamide. The above findings are in favor of my data discussed in detail in chapter 4. In brief, we found a novel effect of cannabinoids in increasing the action potential independent spontaneous exocytosis of GABA without affecting action potential dependent exocytosis. This

unusual effect of cannabinoids is not mediated by CB1, CB2, or TRPV1 receptors. Further, we found that the increased mIPSC frequency is likely to depend on cannabinoid mediated increases in presynaptic calcium concentration. The retrograde messenger responsible for DSI is likely not the optimal ligand for endogenous activation of this novel cannabinoid dependent signaling system. Interestingly, the results indicate that anandamide was more efficacious in mediating the potentiation of action potential independent GABAergic events than 2-AG. Strikingly, the cannabinoid ligand mediated facilitation was sensitive to the application of suramin, strongly suggesting the role of a G protein coupled receptor. All together, our results suggest an unusal effect on action potential independent GABAergic currents that is not mediated by the conventional CB1 receptor.

CHAPTER 2
METHODS

Hippocampal Slice Preparation

Male Sprague-Dawley rats between 18 and 25 days of age were anesthetized using a single intraperitoneal injection of ketamine / (80-100mg/kg) and were decapitated using a small animal guillotine. Their brains were rapidly removed and immersed in an ice cold solution (ACSF). Horizontal sections of the hippocampus were cut at 300 µm using a Pelco Series 3000 Vibratome (Pelco, Redding, CA) and immediately submerged in an incubator containing ACSF preheated to 30-35ºC. Following a 30 minute incubation period, slices were allowed to equilibrate to room temperature for an additional 30 minutes before use. ACSF for sectioning and incubation contained in (mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 2.5 $MgSO_4$, 10 D-glucose, 1 $CaCl_2$, and 25.9 $NaHCO_3$, saturated with 95% O_2 and 5% CO_2. In some experiments hippocampal slices were prepared from male C57BL/6 J CB1 knockout mice, aged between 20 and 25 days. In most cases, mice were mildly anesthetized with halothane or isoflurane prior to ketamine injection; slice preparation was otherwise identical to that described above All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida and conformed to animal welfare guideline issued by the National Institutes of Health.

Whole Cell Recording

After incubation, slices are transferred to a recording chamber that is perfused with ACSF at a constant rate of 2ml/min and maintained at 30°C. ACSF used for recordings is saturated with 95% O_2 and 5% CO_2. It contains in (mM): 126 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.5 $MgSO_4$, 11 D-glucose, 2.4 $CaCl_2$ and 25.9 $NaHCO_3$. For experiments done in chapter 3, unless otherwise stated the ACSF also contained 50µM Picrotoxin [PTX, aminobutyric acid type A receptor ($GABA_AR$) antagonist. In chapter 4, the ACSF used for the experiment presented in Fig… was identical to

that described and used during both incubation and dissection. Slices were visualized by infrared differential interference contrast (IR DIC) microscopy using an Olympus interference contrast (IR DIC) microscopy using an Olympus BX51W1 microscope. Whole cell voltage-clamp recordings were performed using micropipettes pulled from borosilicate glass using a Flaming/Brown electrode puller (Sutter P-97, Sutter Instrument, Novato, CA). The inner tip diameter was approximately 1 µm and the electrode tip resistance was between 3-5 MΩ when filled with the following internal solutions.

The internal solution used in chapter 3 contains (in mM): 140 $CeMeSO_3$, 1$MgCl2$. 3NaCl, 0.2 Cs-EGTA, 10 HEPES, 4 diNa-ATP, 0.3 Na-GTP and 5 QX-314 Cl. The internal used in chapter 4 contains (in mM): 90 or 105 $CeMeSO_3$, 55 CsCl, 1 $MgCl_2$, 0.2 EGTA, 10 HEPES, 2 diNa-ATP, 0.3 Na-GTP and 5 QX-314 Cl. Both internals were pH adjusted to 7.3 using CsOH and volume adjusted to 290-315mOsm.

To visualize the cells by fluorescence microscopy, sulforhodamine 101 (~ 63 µM) was added to the internal solution each day before starting the experiment. The typical access resistance during whole cell recording ranged between 10 and 40 MΩ and was uncompensated.

In chapter 3 the local application experiments were performed using a Picospritzer III (General Valve, Fairfield, NJ) and the pipettes used for spritzing generally had larger diameters. For the experiment presented in Fig 3-2, (Glu spritz), a 10-ms spritz at approximately 20 psi was applied at 0.05 Hz using standard pipettes loaded with 50-200 µM glutamate. For experiments in which DCG-IV was exogenously applied, identical applications were applied using pipettes loaded with 1 µM DCG-IV.

In chapter 4, Fig 4-5, IPSCS were evoked using a bipolar stimulator or with an ACSF filled patch pipette that was connected to a constant current stimulus isolator (World Precision

Instruments, Sarasota, FL). The current intensity varied between 25 and 100 μA; stimulation duration was 0.1ms. Stimuli were generally delivered at a frequency of 0.2 Hz.

Voltage-clamp experiments were performed using an Axon Multiclamp 700A or 700B amplifier (Molecular Devices, Sunnyvale, CA). Data were sampled at 20 KHz, filtered at 2 KHz, and digitally recorded by a Digidata 1322A A/D converter using Clampex v. 9 (Molecular Devices, Sunnyvale, CA). Most chemicals were obtained from Tocris Cookson (Ellisville, MO) or Sigma-Aldrich (St. Louis, MO) with the exception of Ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetoxymethyl Ester (EGTA-AM) which was obtained from Calbiochem (San Diego, CA).

Identification of Mossy Cells

Hilar mossy cells were identified using a wide range of physiological and anatomical criteria as outlined in several previous publications (Frazier et al., 2003; Hofmann et al., 2006; Nahir et al., 2007). In brief, a typical mossy cell appeared larger than other types of hilar cells when visualized under IR DIC, had large whole cell capacitance >200pF, and displayed both high frequency and large amplitude (>100pA) spontaneous excitatory postsynaptic currents (sEPSCs) when voltage clamped at -70 mV in the absence of glutamate receptor antagonists. The cell was considered to be a mossy cell only if it met all the above mentioned criteria. At the end of each experiment, cells were examined with fluorescence microscopy to confirm that they were both multipolar and spiny (with thorny excrescences being particularly prominent on the proximal dendrites).

Identification of Mossy Fiber Inputs to Mossy Cells

Standard recording pipettes were filled with ACSF and connected to a constant-current stimulus isolator (World Precision Instruments, Sarasota, FL) for use in minimal stimulation experiments. Current intensity varied between 25 and 100 μA; stimulation duration was 0.1 ms.

Stimuli were generally delivered at a frequency of 0.2 Hz, with rare exceptions at 0.33 or 0.1 Hz. To ensure isolation of only one or a few synaptic inputs, responses were accepted for further analysis only if they displayed a sharp stimulus threshold, as indicated by a change from mostly failures to mostly success in $\lesssim$10 µA, with no significant increase in amplitude over the next 10 µA (FIG 2-1A) In experiments that involved paired pulses the interstimulus interval was 60 ms.

Mossy fiber inputs to mossy cells were identified by four separate criteria: rise time; latency; frequency facilitation; and sensitivity to DCG-IV, a potent group II mGluR agonist (Kamiya et al. 1996;(Ohishi et al. 1995; Yoshino et al. 1996) see FIG 2-1B and C). Of these, sensitivity to DCG-IV was the major criterion. Acceptable responses exhibited a minimum of 80% block by DCG-IV. The only exception to that standard was for cells that were treated with DCG-IV before full recovery from baclofen. In those cases, a 50% block by DCG-IV was considered acceptable. Although DCG-IV was typically applied by bath at a concentration of 1 µM at the end of each experiment, in some cases, 1 µM DCG-IV was delivered before further experimentation to the surface of a slice just above a patched cell by local application (10 s at $\sim$ 20 psi; e.g., Fig 3-2).

In addition to sensitivity to DCG-IV, we also considered rise time, latency, and frequency facilitation observed in response to a five-pulse train of stimuli delivered at 16.67 Hz as diagnostic criteria helpful in identifying mossy fiber inputs. In general, mossy fibers exhibited faster rise times, shorter latencies (e.g., 1.17 ± 0.09 ms, n = 19, vs. 1.86 ± 0.25 ms, n = 7, P = 0.003), and more robust frequency facilitation than non-MF inputs that failed the DCG-IV test or that were isolated in the continual presence of DCG-IV. In every cell, individual sweeps that lacked a smooth rising phase, showed multiple peaks, or were contaminated by sEPSCs were manually removed from analysis.

Independent Measurement of Phasic and Tonic Currents from Continuous Voltage-Clamp Recordings of sIPSCs

Changes in spontaneous inhibitory postsynaptic currents (sIPSCs; phasic current) are most often measured using traditional event detection. Changes in tonic current, on the other hand, are most often measured as differences in holding current (I_{hold}). However, such measurements must be carefully obtained to prevent contamination by sIPSCs ((Prenosil et al. 2006; Scimemi et al. 2005). Alternatively tonic currents have been characterized using noise analysis (Brickley et al. 1996; Mtchedlishvili and Kapur 2006) or by fitting a Gaussian curve to an all-points histogram of sampled data (Glykys and Mody 2006a; Glykys et al. 2006; Petrini et al. 2004; Wall and Usowicz 1997). Unfortunately, these techniques generally fail to simultaneously describe changes in both tonic and phasic currents. For the current study, we developed custom software in OriginC that accomplishes that goal. Our method is directly based on that described in a recent report from Glykys and Mody (2006b).

In brief, continuous time series data were recorded at 20 kHz, downsampled to 5 kHz, and analyzed in 5-s intervals. For each consecutive interval, an amplitude histogram was constructed from all 25,000 data points. A nonlinear least-squares fit (NLSF) was then performed on a portion of each amplitude histogram using a Gaussian function of the form

$$y = \frac{Ae^{-2(x-x_c)^2/w}}{w\sqrt{\pi/2}} \qquad (2\text{-}1)$$

where A is the area under the normal curve, w is the width at half-peak amplitude, and x_c is the x value at the center of the curve. Importantly, the portion of the amplitude histogram used for NLSF fitting excluded all histogram bins left of center that had fewer than half the number of counts in the largest bin. Because physiological noise in the absence of spontaneous IPSCs is expected to be normally distributed, x_c from the best-fit curve was accepted as the mean tonic

current for each bin. To calculate phasic current we relied on the fact that for a purely Gaussian distribution, x_c of the best-fit curve obtained as above will be equal to the mean value of all the data in the interval. Thus in intervals with little or no spontaneous activity, $\sum x - x_c, \forall x \approx 0$. By contrast, in intervals with appreciable spontaneous activity (arising from increased sISPC frequency and/or amplitude), spontaneous inward currents create a leftward skew to the amplitude histogram that will not affect x_c from the best-fit curve, but that will cause a significant shift in the sum of $(x - x_c)$ away from 0. Therefore this sum was considered to represent total phasic current per interval.

Glykys and Mody tested this technique against real and simulated sIPSCs and reported it to be sensitive to minor (~15%) changes in sIPSC parameters. We verified our own code in two ways. First we demonstrated that adding a standing current to all points selectively modulates tonic current, whereas doubling the amplitude of all points >2 SD from the mean selectively modulates phasic current (data not shown). Second, we plotted phasic and tonic currents analyzed in 1-s intervals on top of raw sIPSCs as in Fig2-2.These plots emphasize the ability of the tonic current to accurately identify I_{hold} regardless of sIPSC frequency and also demonstrate that phasic current clearly increases with event frequency, showing sensitivity to as little as one event.

Data Analysis

Spontaneous miniature post synaptic currents (mIPSCs and mEPSCs) were detected using appropriate parameters in MiniAnalysis v. 6.03 (Synaptosoft, Decatur, GA) and for some experiments in chapter 4 (Fig 4-6) mIPSCs were also detected using a software written in OriginC by CJF. The event detection parameters were identical to that set in MiniAnalysis. Within individual cells a Kolmogorov-Smirnov (K-S) test was used to assess the significance of any changes in miniature frequency or amplitude. To determine the average effect across all cells, the frequency and amplitude data were binned in 30s bins, normalized to a two minute

baseline period prior to bath application of the test compound, and averaged across all cells. A two tailed one sample t-test was then used to evaluate the significance of the test compounds (null hypothesis: mean = 1, for data normalized to the baseline mean). Post synaptic currents that were generated using a bipolar or minimally evoked electrode were analyzed in Clampfit version 9 (Molecular Devices, Sunnyvale, CA). The minimally evoked recordings were examined on a sweep by sweep basis to select sweeps that showed clean, single peak, short latency responses, that had a smooth rising phase and short latency and were not contaminated by spontaneous activity. Significance of the test drugs was checked using a two tailed one sample t-test (null hypothesis: mean = 1, for data normalized to baseline mean).

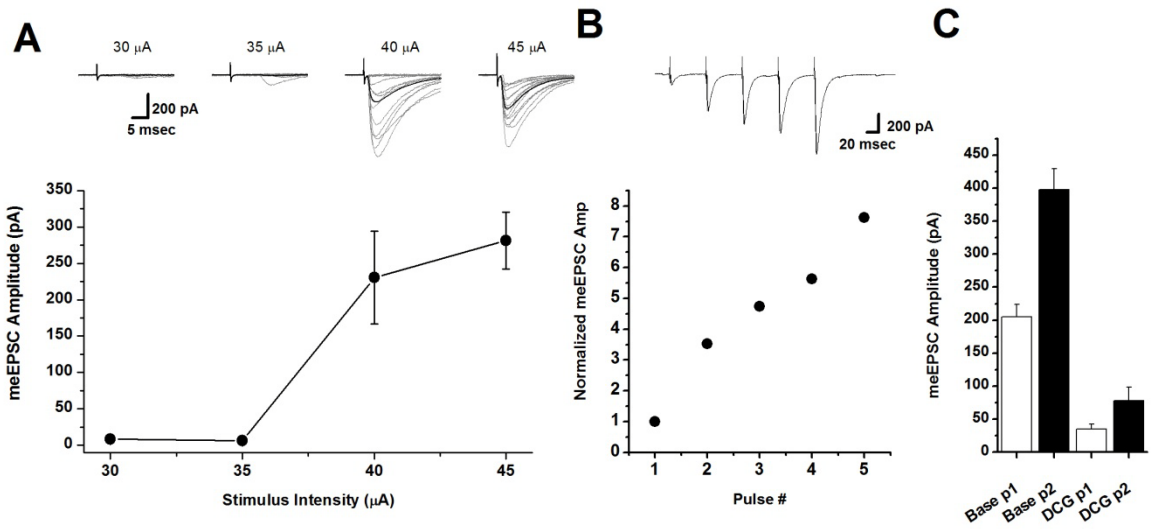

Figure 2-1. .Isolation of minimally evoked mossy fiber (MF)–mediated excitatory postsynaptic currents (EPSCs). *A*: example of a minimally evoked EPSC (meEPSC) with a sharp stimulus threshold between 35 and 40 μA. Stimulation duration was 0.1 ms. *Insets*: individual responses at each stimulus intensity (gray), with the average response overlaid in black. Scatterplot indicates average amplitude and SE of the sweeps shown. *B*: response in *A* also demonstrated strong frequency facilitation when stimulated with a 5-pulse train at 16.7 Hz (stimulation intensity = 45 μA). *Inset*: average of 5 trials, whereas the scatterplot indicates the peak amplitude of each pulse normalized to pulse 1 (p1) and measured from the average sweep. *C*: this response also demonstrated strong sensitivity to inhibition by bath application of 1 μM (2*S*, 2'*R*, 3'*R*)-2-(2', 3'- dicarboxycyclopropyl) glycine (DCG-IV). Specifically, p1 was inhibited by 83%, whereas p2 was blocked by 80%. Paired-pulse ratio increased from 1.9 to 2.26. All panels in this figure represent data from a single representative experiment. Latter 2 features (as indicated in *B* and *C*) identified this response as being of MF origin.

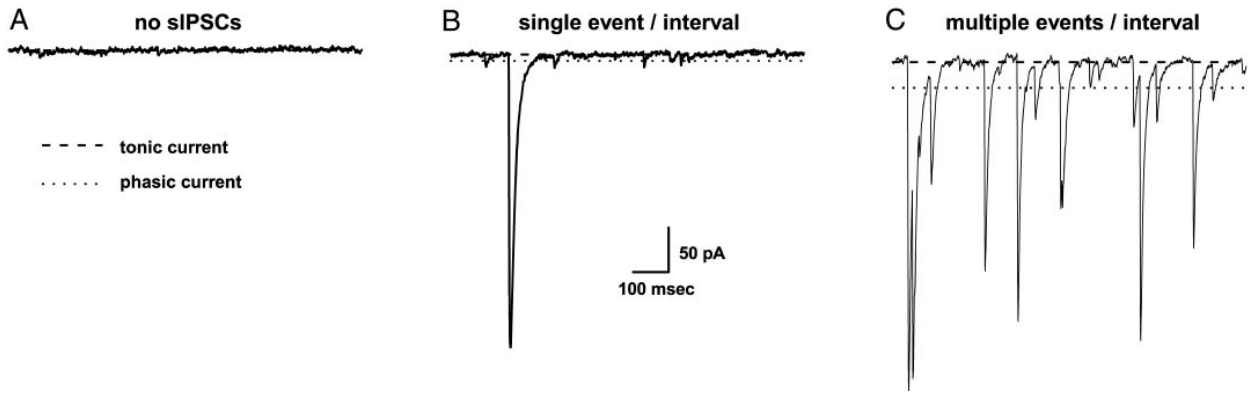

Figure 2-2. Simultaneous measurement of phasic and tonic currents. *A*: in the absence of spontaneous activity, tonic current (dashed lines) accurately reflects the holding current (*I*hold), whereas phasic current (dotted lines) calculated as described in METHODS is near 0. For simplicity, phasic current is plotted in all panels as an offset from tonic current. *B*: phasic current shows excellent sensitivity to spontaneous activity. In this panel, a single event produces a phasic current of 7.3 pA when calculated over a 1-s interval. *C*: phasic current clearly increases with the level of spontaneous activity, whereas tonic current continues to accurately report *I*hold even during periods of very high event frequency. Legend and scales apply to all panels. Data are spontaneous inhibitory postsynaptic currents (sIPSCs) taken from baseline (*A* and *B*), and early carbachol (CCh) period (*C*) of cell shown in Fig 3-6A.

CHAPTER 3
AMBIENT GABA AND CHOLINERGICS MODULATE EXCITATORY TRANSMISSION TO HILAR MOSSY CELLS

Introduction

One of our motives for starting this project was to examine how cholinergics regulate glutamatergic transmission from excitatory afferents to hilar mossy cells. Cholinergic innervations of the hippocampus arrive mainly through the fornix/ fimbria and originate in the medial septal nuclei and the vertical limb of the nucleus of the diagonal band of Broca. Their effects are mediated by activation of muscarinic and nicotinic receptors which are expressed on both the principal cells and interneurons of the hilus (Albuquerque et al. 1997; Levey et al. 1995). Early studies have implicated a role of cholinergics in learning and memory. For example, previous reports indicate that loss of cholinergic innervations may underlie some of the pathology in Alzheimer's disease (Bartus et al. 1982). Evidence also suggests that drugs which bind to acetylcholine receptors in the hippocampus can induce changes in synaptic plasticity and thereby affect learning and memory (Hasselmo et al. 1995). In the present study, we are demonstrating an effect of cholinergics on excitatory afferents to hilar mossy cells.

As I mentioned in the Introduction hilar mossy cells are the only glutamatergic non-prinicipal cells in the hippocampus or dentate gyrus that have a strong contralateral projection, are extremely sensitive to excitotoxicty, and are strongly implicated in the etiology of epilepsy (refer introduction chapter). Mossy cells are also innervated by monosynaptic excitatory inputs of both dentate granule cells and CA3 pyramidal cells (Coulter 2004; Frotscher et al. 1991; Ishizuka et al. 1990; Ribak et al. 1985). Through these feed-back excitatory connections mossy cells have been suggested to play important roles in both normal signal processing in learning and memory, as well as in seizure propagation. Thus, these cells are driven by the axon collaterals of some of the most heavily studied synaptic pathways in the CNS. For example,

extensive work has been done on the mossy fiber pathway to the CA3 pyramidal cells .This pathway is well known for its tight spatial localization, strong frequency facilitation, and clear sensitivity to metabotropic glutamate receptor (mGluR) agonists, possible role as a "detonator synapse", developmental plasticity of transmitter phenotype, and unusal N-methyl-D-aspartate-independent and presynaptic form of long-term potentiation (Henze et al. 2000; Nicoll and Schmitz 2005; Urban et al. 2001). Considerable work has also been demonstrated on the collateral projections of mossy fibers to CA3 interneurons. These synapses are anatomically and functionally different from mossy fiber synapses to CA3 pyramidal cells (Lawrence et al. 2004; Lei and McBain 2002; Pelkey et al. 2006; Toth et al. 2000). By sharp contrast, very limited work has been done on the mossy fiber collateral projections to mossy cells even though they form the intermediate synapse of the trisynaptic pathway. This was the second motive for conducting the present study. We wanted to bridge this gap in the literature by expanding our understanding on the physiology or pharmacology of the different excitatory afferents to hilar mossy cells, whether they are collaterals of the mossy fiber inputs or inputs from the CA3 pyramidal cells.

One of the initial studies completed on the mossy fiber synapse to hilar mossy cells demonstrated that mossy fibers to hilar mossy cells showed significant NMDA receptor independent LTP. Additionally, it was the first study to address short term plasticity's like frequency facilitation and paired pulse facilitation which are characteristics of mossy fibers (Lysetskiy et al. 2005). My current work is an effort to expand our knowledge in this area by examining how both cholinergic and GABAergic signaling mechanism modulate excitatory afferents to the hilar mossy cells. In this project we specifically examined how both the GABAergic and cholinergic mechanisms work together to modulate excitatory transmission

from collaterals of mossy fiber inputs and CA3 pyramidal cells. We report that there are $GABA_B$ receptors on both mossy fiber and non mossy fiber inputs to hilar mossy cells but only $GABA_B$ receptors on mossy fiber inputs play a role in mediating an indirect inhibition of mossy fiber inputs to hilar cells. This finding was consistent with work done by Voght and Regher in the CA3 area. On the contrary we found that non mossy fiber inputs are inhibited directly by $GABA_B$ and muscarinic receptor activation. We also provide the first clear demonstration that hilar mossy cells express high-affinity $GABA_A$ receptors capable of responding to changes in ambient GABA and present evidence that in this system cholinergic changes in ambient GABA are tightly linked to action-potential dependent inhibitory neurotransmission.

Results

Mossy Fiber Inputs to Hilar Mossy Cells Express $GABA_B$ Receptors.

Mossy cells were clamped at -70mv with a $CeMeSo_3$ internal. In order to isolate action potential independent events or miniature excitatory post synaptic currents (mEPSC) we bath applied 1μM TTX. Under these conditions bath application of Baclofen, a $GABA_B$ receptor agonist reduced the mEPSC frequency reduced (to 65.9 ± 4.64% of baseline, n=9, $P<0.0001$; Fig 3-1C) without affecting mEPSC amplitude (99.6 ± 4.26% of baseline, n = 9, $P>0.05$, Fig 3-1D). On pretreatment with CGP, a $GABA_B$ antagonist the reduction in frequency with baclofen was completely abolished (frequency: 99.2 ± 12.07% of baseline, $P>0.05$; Fig 3-1C; amplitude 90.1 ± 6.11%, $P>0.05$, n=8, Fig 3-1D). On repeating the experiment with an extracellular solution that contained 1mM Ca, 2.5mM Mg and 200mM Cd, a voltage gated calcium channel blocker, baclofen was unable to reduce the frequency of mEPSCs (frequency: 99.3 ± 12.37%, $P>0.05$; amplitude:100.3 ± 4.95%, $P>0.05$; n=6; data not shown). These experimental conditions were designed to further reduce the probability of observing calcium-dependent exocytosis. Overall, these findings clearly indicate that there are presynaptic $GABA_B$ receptors present on

glutamatergic afferents to hilar mossy cells but these experiments do not specify which glutamatergic afferent expresses the $GABA_B$ receptors. In order to identify the mossy fiber inputs from the non mossy fiber inputs we used a minimal stimulator coupled which certain pharmacological tools like bath application of DCG-IV, a mgluR II agonist, frequency facilitation and kinetic properties of the post synaptic responses (refer Methods). Bath application of baclofen reduced the amplitude of minimally evoked responses of mossy fiber inputs to 9.9 ± 1.37% of baseline (n=14, P<0.0001; Fig 3-2B) and consistent with a presynaptic site of action we found that bath application of baclofen increased the failure rate from 11.3 ± 3.02 to 75.9 ± 4.97% and coefficient of variation of mEPSCs (CV) from 0.79 ± 0.09 to 1.36 ± 0.11 (n=14, P<0.001 in both cases; data not shown). We have also confirmed that these responses were mediated from mossy fiber inputs because of their sensitivity to DCG-IV. Together, these results suggest that there are $GABA_B$ receptors present on mossy fiber inputs to mossy cells. To further reinforce this conclusion, we showed that an identical application of baclofen had no significant effect on mossy cell responses to local application of exogenous glutamate (50-200µM, 96.4 ± 9.7% of baseline, n=10; summarized in Fig 3-2B)

Presynaptic $GABA_B$ Receptors on mf Terminals to Hilar Mossy Cells are Sensitive to Changes in Ambient GABA and are Tonically Active.

In the presence of 50µM PTX, bath application of 1-[2-[[(diphenylmethylene) imino] oxy] ethyl]-1, 2, 5, 6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), a GABA uptake inhibitor reduced the amplitude (to 40.85 ± 11.49% of baseline levels, n=6, P<0.03; Fig 3-3B) which reversed on wash out. This effect was mediated by a presynaptic $GABA_B$ receptor since the reduction in amplitude was accompanied by an increase in failure rate (from 8.5 ± 4.4 to 22.9 ± 6.43%, n= 6, P = 0.03; Fig 3 – 3C) and coefficient of variation (from 0.74 ± 0.08 to 1.04 ± 0.11, n = 6, P< 0.01; Fig 3-3D). Additionally, we also found that these receptors undergo tonic

activation since in the presence of 50µM PTX, bath application of CGP increased the minimally evoked amplitude (to 254.7 ± 33.5% of baseline, n=7, P< 0.005; Fig 3-4B). Concurrent with a CGP mediated potentiation, we observed a decrease in the failure rate (from 33.3 ± 8.39 to 7.0 ± 3.1%, n=7, P<0.01; Fig 3-4C) and CV (from 0.99 ± 0.09 to 0.64 ± 0.06, n=7, P<0.01; Fig 3-4D) confirming its presynaptic nature. Note that in two of nine cases bath application of CGP produced much milder increases in amplitude (to 110 ± 2.90% of baseline) without consistent changes in failure rate and CV.

Bath Application of Muscarinic Agonists Inhibits Mossy Fiber Inputs to Hilar Mossy Cells by Activation of Presynaptic $GABA_B$ Receptors.

As I mentioned in the introduction of this chapter, that early literature indicated the role of cholinergic receptors in inhibiting excitatory transmission to prinicipal cells. In our next experiment we wanted to test the hypothesis if these putative muscarinic receptors on mossy fiber inputs to hilar mossy cells may provide an additional mechanism of presynaptic inhibition of mossy fiber transmission. Consistent with our hypothesis, in 11 out of 12 cells tested bath application of muscarine reduced the amplitude of evoked post synaptic currents to 52.0 ± 5.69% of baseline (n=11, P<0.01; data not shown). Concurrent with this decrease we saw an increase in CV from 0.52 ± 0.06 to 0.63 ± 0.07 (n=11, P= 0.05; data not shown). Further reinforcing the presynaptic nature, we found that 3µM carbachol failed to reduce the responses produced by exogenously applied glutamate (100.1 ± 6.74% of baseline, n=9, P>0.05; data not shown). Strikingly, the minimally evoked amplitude of 6 cells that were reduced by muscarinic agonists to 55.9 ± 6.81% showed a complete reversal with CGP 52432 (to 108 ± 11.6% of baseline, n=6, P<0.01; Fig 3-5B). This result is more consistent with an indirect mechanism of muscarinic inhibition, one that does not involve activation of muscarinic acetylcholine receptors (mAchRs) directly on the terminal. However, because previous experiments indicated that mossy

fiber inputs to hilar mossy cells are subject to tonic inhibition by ambient GABA, we also tested the ability of muscarinic agonists to inhibit mossy fiber transmission to mossy cells in slices pretreated with CGP. Under those conditions, no muscarinic inhibition of mossy fiber transmission was observed, and in fact, a slight but statistically insignificant increase in meEPSC was observed (to 119.9 ± 21.9%, n = 8, P = 0.39; data not shown).

Bath Application of Muscarinic Agonists Produces Action Potential Dependent Increases in Ambient GABA that are Strongly Correlated with Increases in Spontaneous IPSCs.

Thus far, we found that preseynaptic $GABA_B$ receptors on mossy fiber terminals to hilar mossy cells are clearly implicated in muscarinic mediated inhibition. This result raised two interesting questions. 1) Can we detect hypothetical changes in ambient GABA that do not depend on mossy fiber terminals? 2) If cholinergics do raise ambient GABA, then what is the exact mechanism? We answered both these questions by using an in house technique that allowed us to quantify both phasic and tonic currents simultaneously in a period of high spontaneous activity. In our experimental conditions tonic currents are produced by activation of high affinity extrasynaptic $GABA_B$ receptors and phasic currents are caused by activation of post synaptic $GABA_A$ receptors. For this experiment, mossy cells were voltage clamped at -70mv using an internal that contained 60mM chloride. We isolated sIPSCs by bath applying glutamate antagonists NBQX and APV. The spontaneous currents that were caused by the activation of post synaptic $GABA_A$ receptors were inward.

After recording a 10 minute baseline, 3µM carbachol was bath applied (see Fig 3-6A for sample recording). In 6 cells out of 10 we found that carbachol caused a sustained increase of frequency and in some cases amplitude as well (data not shown). These changes clearly reflected as an increase in "phasic current" of 4.29 ± 1.68 Pa/5-s intervals (n = 6, P = 0.05; Fig 3-6C). The average phasic current increased to 767.4 ± 280.9% of baseline. Notably, the increase in the

phasic current was also accompanied by a shift in the holding current to 118.0 ± 5.74% of baseline. The change in the holding current is reflected as a change in the tonic current. The average increase in tonic current was of 23.2 ± 4.61 pA (n = 6, P<0.005; Fig 3-6C)

Results from this experiment supports the hypothesis that changes in tonic current observed in this manner are directly caused by muscarinic agonist induced action potential dependent increases in ambient GABA and subsequent activation of high affinity $GABA_A$ receptors expressed by hilar mossy cells. For instance, in these experiments we saw that PTX reversed both the increase in phasic and tonic currents that was induced by carbachol. PTX restored tonic current (to 97.6 ± 3.14% of baseline levels Fig 3-6C) and often reduced phasic current to below baseline levels. (E.g. to 53.7 ± 23.5% of baseline, n = 6, P = 0.11, Fig 3-6C). Further, there was also an extremely strong correlation in the analysis of mean change in both phasic and tonic currents across all three experimental conditions (R^2 = 0.996, slope = 5.48, P = 0.002; Fig 3-6D). Importantly, cells that did not show an increase in phasic current on bath application of carbachol also failed to show a significant increase in tonic current. The average tonic current in those cells increased to 103 ± 5.31% of baseline, P = 0.53).

In order to eliminate possible contributions of post synaptic mAchRs in mediating increases in phasic and tonic currents by CCh, we designed an experiment in which we blocked both the glutamatergic and GABAergic responses by washing on 10µM NBQX, 40µM APV, 10µM CGP 52432 and 50µM PTX. We also bath applied 1µM TTX and used an ACSF that contained low calcium and high Mg (refer methods). Under those conditions we found that I_{hold} did not change on bath application of carbachol. The holding current was 100 ± 0.75% of baseline (n=3, P = 0.71; data not shown). On repeating this experiment in the absence of PTX we found that carbachol failed to increase both phasic and tonic currents, strongly suggesting that

action potential independent mechanisms likely do not contribute substantially to CCh mediated changes in ambient GABA.

Non Mossy Fiber Glutamatergic Inputs to Hilar Mossy Cells are Directly Inhibited by BOTH $GABA_B$ Receptors and Muscarinic Agonists

Mossy fiber inputs only represent a certain percent of excitatory inputs to the hilar mossy cells. We also wanted to observe how the putative non mossy fiber inputs respond to $GABA_B$ and muscarinic agonists. To answer this question, we observed all the runs that were collected for Fig 4-7 but did not pass the criteria for mossy fiber inputs. Such an analysis demonstrated several similarities and one prominent difference. Similar to the mossy fiber inputs the putative non mossy fiber inputs were strongly inhibited by baclofen (to 18.2 ± 3.4% of baseline, n=7, $p<0.01$; data not shown) and, in two out of four cases showed clear increases in amplitude after the bath application of CGP to 379 ± 72.3% of baseline; data not shown; two cells failed to show robust increases). Further, in 23 out of 26 cells, bath application of muscarinic agonists reduced the minimally evoked amplitude to 47.0 ± 4.68% of baseline. However, strikingly, unlike the mossy fiber inputs, putative non mossy fiber inputs to mossy cells failed to reverse on CGP application. Specifically, in 9 cells that failed to pass the mossy fiber criteria, bath application of muscarine reduced the meEPSC amplitude to 40.6 ± 7.55% of baseline. Subsequent application of CGP virtually unchanged this value. These cases were still sensitive to the application of DCG-IV. The evoked amplitude in the 9 cases that lacked CGP effect was reduced to 47.8 ± 5.9% of baseline in the presence of DCG-IV. This suggests that our minimally evoked responses had mixed mossy fiber and non mossy fiber origins. Therefore, we designed an experiment in which we pretreated the slices with DCG-IV and CGP. Under these conditions bath application of 3-10 μM CCh reduced the amplitude of non mossy fiber mediated meEPSCs to 55.4 ± 8.49% of baseline (n=12, $p<0.01$, Fig 3-7B) and an average increase in CV (from 0.88 ± 0.06 to 1.07 ±

0.10, n=12, p=0.12). In another set of experiments we also tested the bath application of baclofen on meEPSCs in slices pretreated with DCG-IV. We found that baclofen reduced the amplitude of meEPSCs to 30.8 ± 4.1% of baseline (Fig 3-7B). Consistent with this result, we saw an increase in both failure rate (from 19.0 ± 7.26 to 67.0 ± 5.71%) and CV (from 0.7 to 1.11, n=7, $p<0.01$ in both cases). Cumulatively, these results suggest that non mossy fiber inputs to mossy cells also express presynaptic $GABA_B$ receptors that are subject to tonic inhibition by ambient GABA, but only non mossy fiber inputs express presynaptic mAchRs capable of directly inhibiting glutamatergic transmission at the terminal.

Origin of Non-mf Inputs

The minimal stimulation technique used in experiments so far helped us identify mossy fiber inputs from non mossy fiber inputs but it did little to distinguish between the different types of non mossy fiber inputs. We know from our existing results that mossy cells receive non mossy fiber glutmatergic inputs from both CA3 pyramidal cells and perforant path inputs. In order to identify these inputs selectively we used different experimental strategies. For example, to selectively activate CA3 inputs, to mossy cells we stimulated the CA3 pyramidal cell layer using a bipolar stimulator (0.1ms duration, generally <100μA) in the presence of both DCG and CGP. Under these conditions bath application of CCh reduced the evoked amplitude to 56.7 ± 7.11% of baseline, $P<0.001$, Fig 3-7C) which reversed to 71.8 ± 9.48% of baseline, $P = 0.01$ within 15min of bath application of atropine.). These results suggest that CA3 glutamatergic inputs to hilar mossy cells undergo direct muscarinic inhibition. Importantly, to reduce bursting these experiments were done in the absence of PTX. Therefore in order to eliminate the possibility that the CCh-mediated reduction in evoked amplitude is as a result of increases in ambient GABA and subsequent activation of presynaptic $GABA_A$ receptors we also demonstrated that N0-711

had no effect on EPSCs generated under identical conditions (106 ± 16.8% of baseline, n = 6, P = 0.72).

We activated perforant path inputs to mossy cells by stimulating the middle to outer perforant path, also in the presence of DCG and CGP. Notably, on stimulating this layer we failed to find detectable EPSCs in hilar mossy cells in 75% of the attempts, even when stimulus intensity surpassed 200µA. This was not as a result of strong DCG sensitivity of these inputs, as from previous literature we know that perforant path inputs have intermediate sensitivity to DCG and also identical stimulation of these inputs produced detectable responses in granule cells. Cells in which small detectable EPSCs were observed showed an insignificant reduction in the EPSC amplitude with bath application of CCh. The evoked amplitude reduced (by 10.4 ± 0.57%, n = 3, P = 0.003; data not shown). Together, these results indicate that minimally evoked EPSCs of non mossy fiber origin described earlier are more likely to represent afferent input from CA3 pyramidal cells than from the perforant path. Nevertheless, we cannot rule out gluatamatergic inputs from other mossy cells.

Discussion

In this study we found that glutamatergic transmission to hilar mossy cells can be modulated by both cholinergic and GABAergic mechanisms. Our results determined that $GABA_B$ receptors are expressed on both mossy fiber and non mossy fiber afferents to hilar mossy cells which are capable of directly inhibiting glutamate release from those inputs. These receptors can also be tonically activated by ambient GABA and are also sensitive to changes in ambient GABA that may be induced by blocking GABA transporters. We also demonstrated that cholinergic agonist directly inhibit non mossy fiber inputs while indirectly inhibiting mossy fiber inputs by driving action potential induced increases in ambient GABA. Finally, this is the

first study to demonstrate the expression of high affinity $GABA_A$ receptors on mossy cells that can be activated by tonic GABA and are also sensitive to changes in ambient GABA.

$GABA_B$ Agonists Inhibit mEPSCs

Our very first experiment illustrates inhibition of miniature EPSC frequency by baclofen that is reversed by CGP. The amplitude remains unchanged, confirming a presynaptic site of action of baclofen. Even though this is the first study to demonstrate presynaptic $GABA_B$ mediated inhibition of glutamatergic afferents to hilar mossy cells, this plasticity is quite common in other areas of the CNS. For example, $GABA_B$ mediated inhibition has been demonstrated at various glutamatergic synapses in a both calcium dependant (for review see (Chen and Regehr 2003; Dittman and Regehr 1997; Ishikawa et al. 2005; Misgeld et al. 1995; Sakaba and Neher 2003; Takahashi et al. 1998) and calcium independent (Capogna et al. 1996; Dittman and Regehr 1996; Jarolimek and Misgeld 1992; Kolaj et al. 2004; Scanziani et al. 1992) manner. Another interesting aspect of the present study is that $GABA_B$ mediated inhibition was largely eliminated when the external solution (ACSF) contained low Ca, high Mg and 200 µM Cd^{2+} to further reduce the probability of having calcium dependent events. This result suggests that $GABA_B$ receptor activation preferentially inhibits a calcium-dependent release process at these synapses. In 2003, Lei and McBain also reported a similar $GABA_B$ mediated inhibition of glutamatergic afferents to stratum radiatum interneurons in CA3, although, in their study, additional Kcl was required to evoke calcium dependent and baclofen sensitive mEPSCs. On the other hand, Scnaziani et al. (1992) demonstrated $GABA_B$ mediated inhibition of Cd^{2+} insensitive mEPSCs in CA3 pyramidal cells. Together, these results suggest that the mechanism of $GABA_B$ mediated inhibition may change from synapse to synapse. It may also vary based on the post synaptic target, even though the axons are collaterals of each other.

For the longest time, it's been assumed that miniature events in the presence of TTX in normal ACSF are completely calcium independent. However, recent literature contradicts that idea and there has been growing precedent for the idea of calcium dependent exocytosis in the presence of TTX at various synapses. For example, in our results, it is noteworthy that mEPSCS recorded from hilar mossy cells in the presence of TTX retained significant calcium dependency. In addition, in cerebellar Purkinje cells CB1 mediated inhibition was eliminated (Yamasaki et al. 2006) and a direct reduction in mIPSC frequency was observed in a subset of hilar mossy cells (Hofmann et al. 2006) on perfusing calcium-free external solution containing BAPTA-AM.

Different Mechanism of Inhibitions on Mossy Fiber vs. Non Mossy Fiber Inputs

Our results using minimal stimulation techniques show that cholinergic agonists modulate both mossy fiber and non mossy fiber inputs by different mechanisms. We found that mossy fiber inputs lack presynaptic muscarinic receptors and express $GABA_B$ receptors that are capable of detecting ambient GABA. Cholinergic agonists bind to the somatic muscarinic receptors on GABAergic interneurons and induce action potential dependent increases in ambient GABA that activates the $GABA_B$ receptors on mossy fiber terminals and leads to glutamatergic inhibition. This finding complements a very recent report of CCh-induced and yet GABA-mediated effects on mossy cell gain (Kerr and Capogna 2007). It is also consistent with previous work done in the CA3 area, by another group of scientists. They showed that mossy fiber inputs to CA3 also express $GABA_B$ receptors and are subject to tonic inhibition by ambient GABA that is mediated by both $GABA_B$ and $GABA_A$ receptors (Hirata et al. 1992; Ruiz et al. 2003; Vogt and Nicoll 1999; Vogt and Regehr 2001). Earlier studies done by William and Johnston, 1990 on modulation of glutamate release from mossy fiber terminals to CA3 pyramdial cells by cholinergic agonists indicated a direct role of presynaptic muscarinic receptors on those

terminals. However, a more recent study specifies an indirect mechanism dependent on $GABA_B$ receptors that is consistent with our finding.

Notably, our results are the first to show that levels of ambient GABA around mossy fiber terminal zones in the hilus are tightly coupled to action potential dependent increases in ambient GABA. Consistent with this finding, work done in the developing and mature cerebellum also supports a clear link between sIPSCs mediated by ambient GABA (Brickley et al. 1996; Carta et al. 2004; Kaneda et al. 1995; Wall and Usowicz 1997). Additionally, cell culture studies of both cerebellar and hippocampal neurons show TTX mediated reductions in tonic GABAergic currents (Leao et al. 2000; Petrini et al. 2004). Work done in the hippocampus also implicates a direct link between KA-induced increases in sIPSCs with $GABA_A$ mediated changes in holding current (Frerking et al. 1999).

While examining the mechanism of inhibition of non mossy fiber inputs, we found that these inputs can undergo presynaptic inhibition by both presynaptic $GABA_B$ receptors and muscarinic acetylcholine receptors (mAchRs). We further wanted to identify the origin of these non mossy fiber inputs but our experiments were not designed to distinguish between the types of non mossy fiber inputs. In order to address this question more directly, we changed our stimulating locations to specifically isolate inputs from the CA3 pyramidal cell layer or the perforant path. Our results indicated that EPSCs recorded from hilar mossy cells on stimulating the CA3 layer with a bipolar stimulator are inhibited by bath application of CCh, even in the presence of CGP 52432 and DCG-1V. Although, these experiments were done in the absence of PTX, increases in ambient GABA mediated by NO-711 failed to produce comparable inhibition of these CA3-evoked EPSCs. By contrast, stimulation of the perforant path inputs failed to result in any detectable responses in the hilar mossy cells. The cells that did show some small

detectable responses lacked a significant reduction on bath application of CCh. Cumulatively, these results point towards the fact that the non mossy fibers axons to hilar mossy cells represent collaterals of CA3 inputs than the perforant paths. Nevertheless, we cannot disregard the role of mossy cell inputs to other mossy cells, although there is evidence suggesting that granule cells and molecular layer interneourons are the likely primary targets of mossy cell axons (Wenzel et al. 1997)

It is worth noting, that even though functionally active $GABA_B$ receptors are expressed on non mossy fiber inputs to mossy cells, these inputs are still directly inhibited by activation of presynaptic muscarinic receptors on the same terminal. Based on these results, we hypothesized that spatial factors most likely would account for the apparent discrepancy. This is because changes in ambient GABA responsible for inhibiting glutamate release from MF terminals are action potential dependent. If spatial factors was the reason, then mAchR expressing interneurons would likely terminate on more proximal dendrites of mossy cells, where they would be closer to MF inputs than non MF inputs. We also cannot eliminate the possibility that $GABA_B$ receptors on non mossy fiber inputs may have lower sensitivity to changes in ambient GABA than those expressed on MF terminals. Finally, both mAchR's and $GABA_B$ receptors on non MF terminals may share a common presynaptic signaling pathway for inhibiting transmitter release on the terminal. This would result in full or partial occlusion of one another.

One last interesting question raised in this study is what could be the functional implication of this plasticity? How would it fit into regulating excitability of mossy cells? To understand this in depth more work needs to be done in this area. But some speculations can be made which I have assembled in the last chapter.

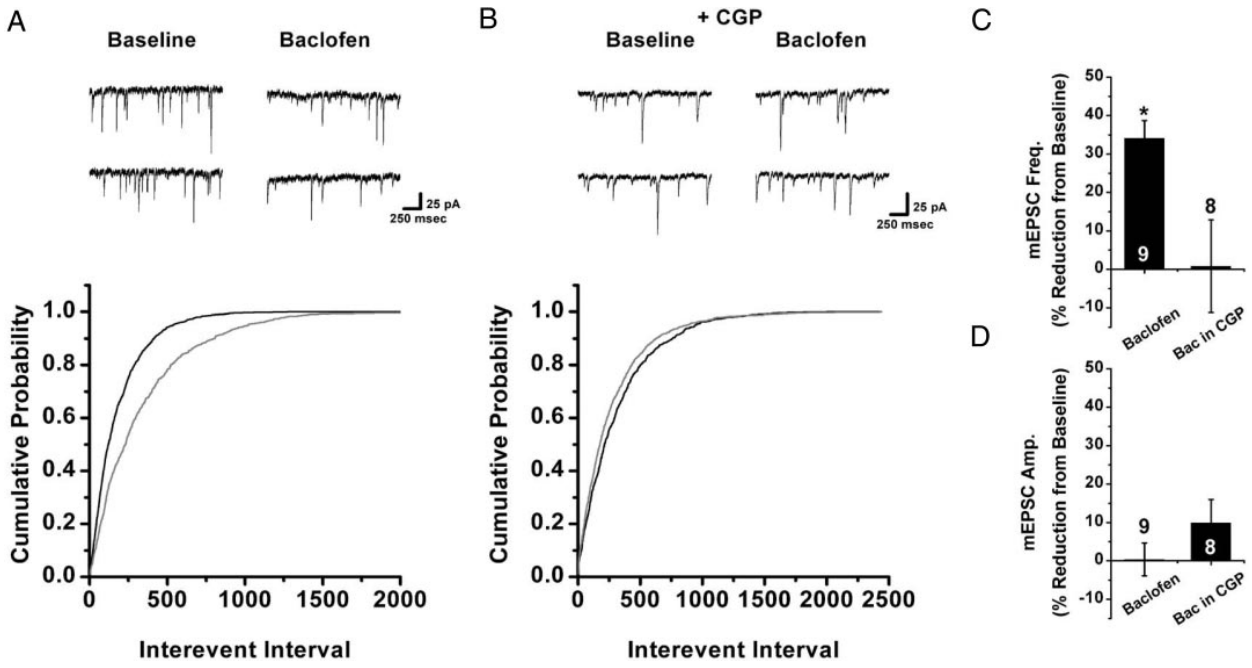

Figure 3-1. (RS)-4-Amino-3-(4-chlorophenyl)butanoic acid (baclofen) reduces miniature excitatory postsynaptic current (mEPSC) frequency without affecting amplitude. A: cumulative probability histogram (CPH) for a sample cell. Baclofen (10 µM) significantly increased the interevent interval (IEI) of mEPSCs [baseline IEI: 179.5 ± 4.31 ms; baclofen IEI: 328.2 ± 10.92 ms; $P < 0.001$, Kolmogorov–Smirnov (K-S) test] with only a negligible change in amplitude (baseline amplitude: 43.3 ± 0.89 pA; baclofen amplitude: 41.4 ± 1.22 pA; data not shown). *B*: CPH for a sample cell pretreated with 5 µM [3-[[(3,4-dichlorophenyl)methyl]amino]propyl] (diethoxymethyl) phosphinic acid (CGP 52432). Baclofen had no significant effect on mEPSC interevent interval ($P = 0.06$, K-S test) or amplitude ($P = 0.05$, K-S test; data not shown). *Insets*: consecutive 2-s intervals during either the baseline or after 6 min of baclofen wash-in. *C*: summary graph of baclofen's effect on frequency. Baclofen significantly reduced mEPSC frequency (34.1 ± 4.64%, $n = 9$, $P < 0.0001$), whereas pretreatmentwith 5µM CGP 52432 completely blocked this effect (0.8 ± 12.07%, $n = 8$, $P > 0.05$). *D*: summary graph of baclofen's effect on amplitude. Baclofen didnot significantly affect the amplitude in either control conditions (0.4 ± 4.26% reduction from baseline, $n = 9$, $P > 0.05$) nor did pretreatment with 5µM CGP52432 (9.9 ± 6.11% reduction from baseline, $n = 8$, $P > 0.05$).

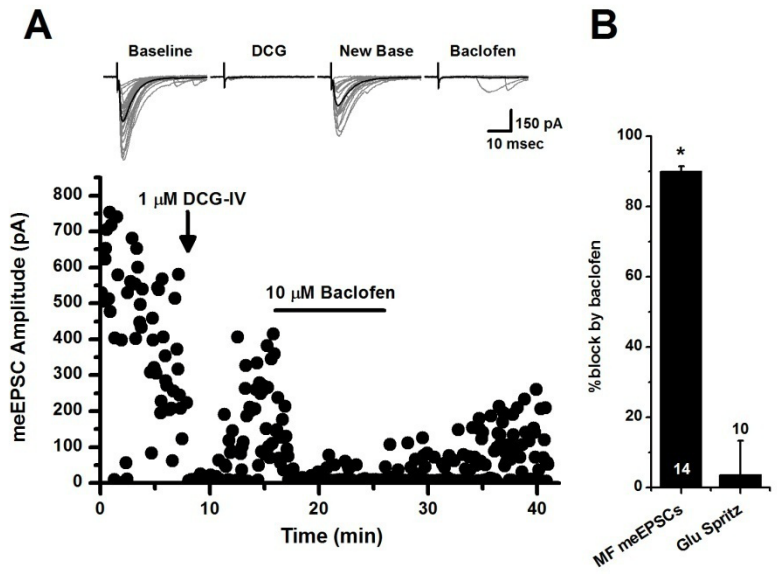

Figure 3-2. Baclofen blocks meEPSCs from isolated mossy fiber terminals by presynaptic γ-aminobutyric acid type B receptors (GABABRs). A: effect of 10 μM baclofen in a representative cell. A 10-s local application of 1 μM DCG-IV completely blocked meEPSCs (1.8 ± 0.7% of baseline). After recovery from local application of DCG-IV, bath application of 10 μM baclofen reduced meEPSC amplitude to 10.4 ± 1.0% of baseline. During thebaclofen washout, meEPSC amplitude showed considerable recovery (to 68.3 ± 4.9% of baseline). Insets: individual traces from the relevant periodsare shown in gray, with the average trace overlaid in black. *B*: summary graphcomparing baclofen's effect on MF meEPSCs and exogenous glutamate spritz. Cells treated with DCG-IV either before or after baclofen were pooled andshowed significant inhibition by the $GABA_B$ agonist (90.1 ± 1.37%, $n = 14$, $P < 0.0001$). By contrast, baclofen failed to inhibit exogenously appliedglutamate (50–200 μM; 3.6 ± 9.7% block, $n = 10$).

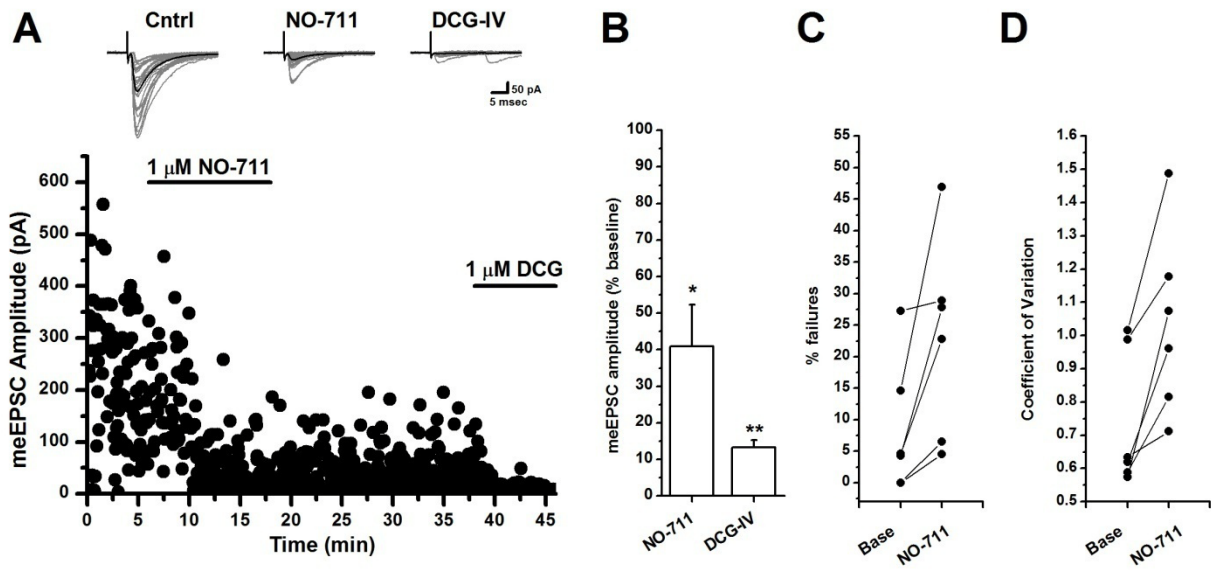

Figure 3-3. Presynaptic GABABRs on MF terminals respond to increases in ambient GABA. A: single-cell recording shows that bath application of 1μM 1-[2-[[(diphenylmethylene)imino]oxy]ethyl]-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride (NO-711), a potent GABA uptake inhibitor, reversibly depressed average meEPSC amplitude, strongly suggesting that ambient GABA directly inhibits mossy fiber inputs. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: summary graph of effects of NO-711 and DCG-IV on meEPSCs. In 6 cells, NO-711significantly inhibited meEPSC amplitude (to 40.9 ± 11.49% of baseline, $P < 0.03$), whereas subsequent DCG-IV treatment further reduced the amplitude (to 13.2 ± 2.01% of baseline, $P < 0.01$). Error bars indicate SE. C and D: summary plots for the effect of NO-711 on failure rate and coefficient of variation (CV). Connected points represent data from a single cell. In 6 cells, NO-711 significantly increased the average failure rate (from 8.5 ± 4.4 to 22.9 ± 6.43%, $n = 6$, $P = 0.03$) and CV (from 0.74 ± 0.08 to 1.04 ± 0.11, $n = 6$, $P < 0.01$).

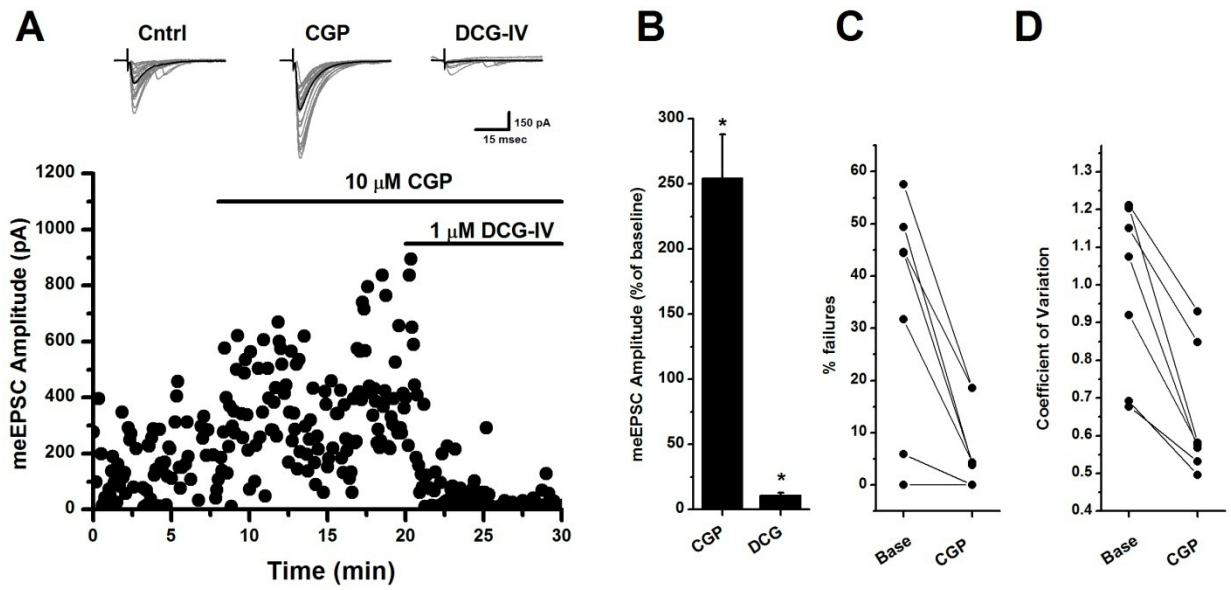

Figure 3-4. Mossy fiber inputs to mossy cells are subject to tonic inhibition by ambient GABA, mediated through GABABRs. A: effects of 10 μM CGP 52432 and 1 μM DCG-IV in a representative cell. CGP 52432 significantly increased the average meEPSC amplitude (to 211.4 ± 14.29% of baseline) and decreased the failure rate (0 failures in CGP 52432). Subsequent bath application of DCG-IV reduced meEPSC amplitude by 95.6 ± 1.9% and significantly increased the failure rate (to 75.5%), suggesting the responses resulted from mossy fiber stimulation. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: summary graph of the effect of CGP 52432 on meEPSCs. In 7 of 9 cells, CGP 52432 significantly increased meEPSC amplitude (to 254.7 ± 33.5% of baseline, $P < 0.005$), whereas DCG-IV almost completely blocked all meEPSCs (10.8 ± 2.0% of baseline, $P < 0.005$). Error bars indicate SE. C and D: summary plots for the effect of CGP 52432 on failure rate and CV. Connected points represent data from a single cell. CGP 52432 significantly reduced the average meEPSC failure rate (from 33.3 ± 8.39 to 7.0 ± 3.1%, $n = 7$, $P < 0.01$) and CV (from 0.89 ± 0.09 to 0.58 ± 0.06, $n = 7$, $P < 0.01$).

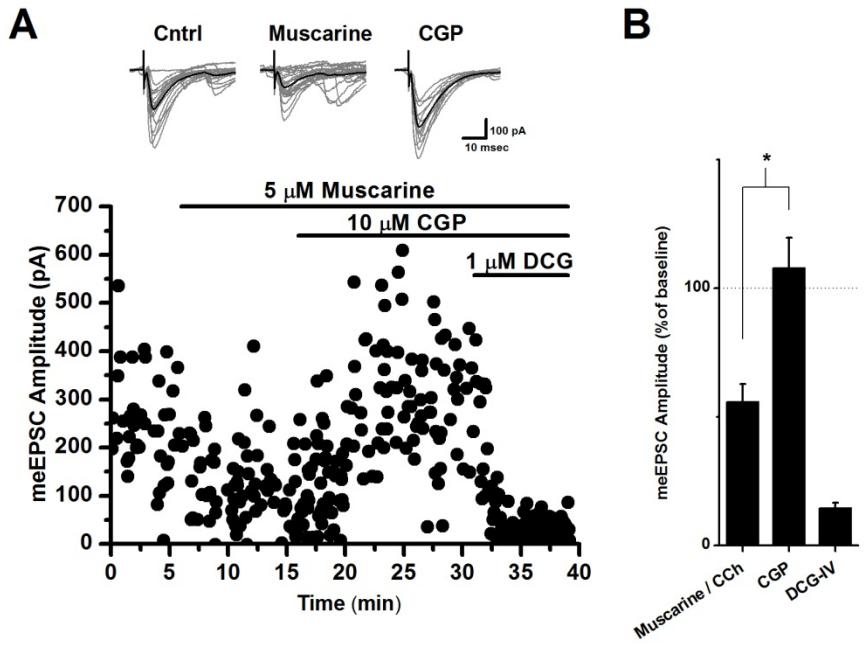

Figure 3-5. Muscarinic inhibition of mossy fibers is reversed by a selective GABABR antagonist. A: single-cell recording shows that bath application of 5 μM muscarine decreased meEPSC amplitude. This effect was reversed by 10 μM CGP 52432, implying that muscarinic receptors indirectly inhibit neurotransmitter release from mossy fibers. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: in 6 cells, muscarinic agonists (5 μM muscarine or 3 μM CCh) reduced average meEPSC amplitude (to 55.9 ± 6.81% of baseline), whereas 10 μM CGP 52432 reversed this effect (to 108 ± 11.6% of baseline, $P < 0.01$). Bath application of 1 μM DCG-IV significantly reduced meEPSC amplitude (to 14.7 ± 1.86% of baseline, $P < 0.05$).

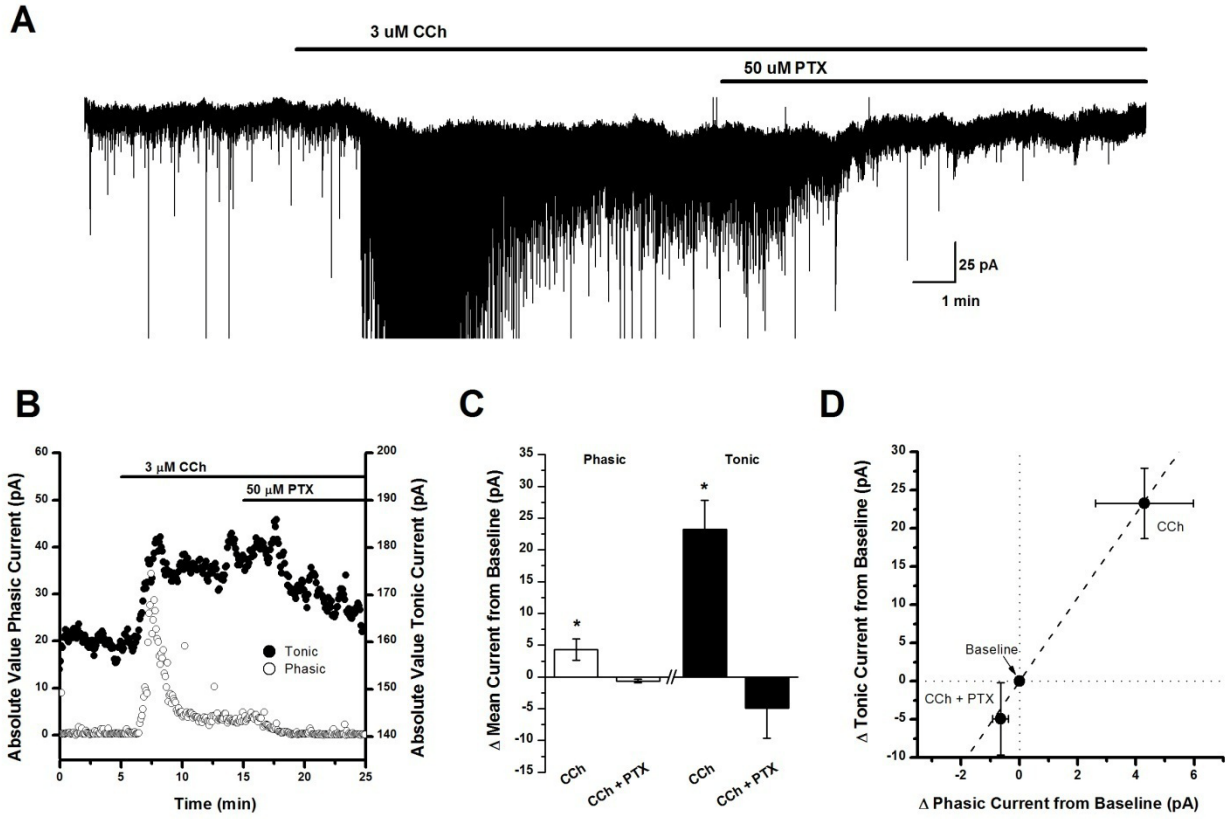

Figure 3-6. CCh induced increases in sIPSCs produce significant increases in GABAA-mediated tonic inhibition of hilar mossy cells. A: sample current trace from a mossy cell treated with CCh and picrotoxin (PTX). Shortly after the initial CCh (3 μM) application, a sudden, dramatic increase in both sIPSC frequency and amplitude was observed. Effect on sIPSCs was accompanied by a negative shift in the Ihold. Both phenomena were reversed by application of 50 μM PTX. Note: data clipped to allow for visualization of changes in both phasic and tonic currents. B: effects of CCh and PTX on tonic (black dots) and phasic (white dots) currents in the same sample cell calculated as described in METHODS. Bath application of 3 μM CCh significantly increased both tonic and phasic currents (by 15.81 and 3.3 pA, respectively; $P < 0.001$ in both cases), whereas 50 μM PTX significantly reversed these effects ($P< 0.001$ in both cases). C: summary graph of effects of CCh and PTX on phasic (white bars) and tonic (black bars) currents. In 6 of 10 cells, CCh significantly increased the phasic current (by 4.29 ± 1.68 pA from baseline, $P = 0.05$), whereas PTX reversed this effect (-0.65 ± 0.27 pA from baseline, $P > 0.05$). CCh also significantly increased the tonic current (by 23.2 ± 4.61 pA from baseline, $P < 0.005$), whereas PTX reversed this increase (- 4.91 ± 4.76 pA from baseline, $P> 0.05$). Error bars indicate SE. D: average changes in tonic current were plotted against changes in phasic current for each condition and fit by linear regression. Calculated slope is 5.48 with $R2 = 0.996$, $P= 0.002$, indicating a strong correlation between phasic and tonic currents across all 3 experimental conditions.

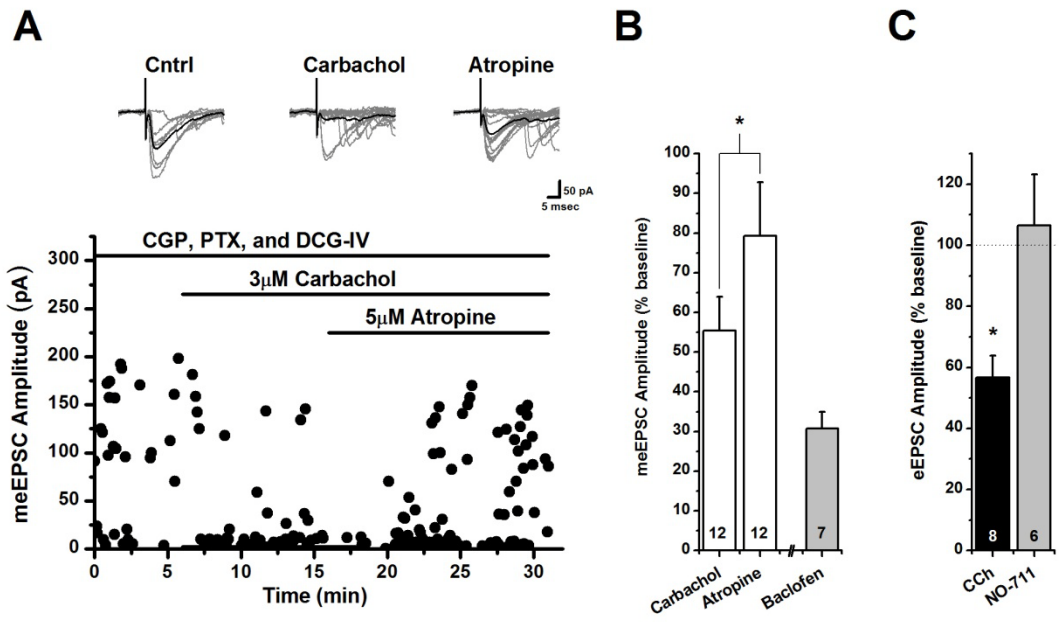

Figure 3-7. Non-MF inputs to mossy cells express both GABABRs and muscarinic acetylcholine receptors (mAChRs). A: in a representative cell pretreated with PTX (50 µM), CGP 52432 (10 µM), and DCG-IV (1 µM), bath application of 3 µM CCh reduced the average meEPSC amplitude, whereas bath application of atropine (5 µM) rescued meEPSCs to near-baseline levels. Insets: individual traces from the relevant periods are shown in gray, with the average trace overlaid in black. B: summary plot of independent effects of muscarinic and GABAB-receptor activation observed in 12 cells. White bars: bath application of CCh (3–10 µM) reduced the average meEPSC amplitude (55.4 ± 8.49% of baseline, n = 12, $P < 0.01$), whereas atropine (5 µM) partially but significantly reversed this effect (to 79.4 ± 13.4%, n = 12, $P = 0.02$). Gray bar: in a separate group of 7 cells pretreated with PTX (50 µM) and DCG-IV (1 µM), bath application of baclofen (10 µM) significantly reduced the average meEPSC amplitude (30.8 ± 4.1% of baseline, $P < 0.0001$). C: summary plot indicating that CA3-evoked EPSCs, recorded from hilar mossy cells in the presence of DCG-IV and CGP 52432, were significantly reduced by bath application of 3 µM CCh, yet no similar effect was produced by bath application of NO-711 in 6 separate experiments.

CHAPTER 4
NON CB1 EFFECTS ON GABAERGIC TRANSMISSION

Introduction

The introductory chapter of this document presents a tremendous amount of information that has been discovered over the last few years on the role of exogenous and endogenous cannabinoids in modulating synaptic transmission in the CNS (refer the introduction chapter for review). The most important finding on endocannabinoids to date has been on their ability to be retrograde messengers and transiently inhibit action potential dependent release by activation of presynaptic CB1 receptors. This phenomenon was first seen on GABAergic interneurons and was termed DSI (Depolarization induced suppression of inhibition). Since the initial discovery that cannabinoids act as retrograde messengers, CB1 dependent DSI has been discovered on many inhibitory synapses in different areas of the brain such as basal ganglia, amygdala, cerebellum, substantia nigra etc (Bodor et al. 2005; Hofmann et al. 2006; Isokawa and Alger 2005; Trettel and Levine 2003; Zhu and Lovinger 2005). Further, our lab also reported DSI of mossy cells in the dentate gyrus (Hofmann et al. 2006). More recently, a few studies have also indicated CB1-mediated modulation of excitatory afferents. This is because newer antibodies have revealed the expression of CB1 receptors on glutamatergic terminals (Katona et al. 2006; Marsicano et al. 2003; Monory et al. 2006). A similar inhibitory phenomenon on these terminals termed depolarization induced suppression of excitation or DSE has now been described in several areas of the brain e.g. cerebellum, ventral tegmental area, thalamus, amygdala and in the hippocampus (for review refer (Chevaleyre et al. 2006). A recent paper from our lab provides physiological evidence of the selective expression of functional CB1 receptors on glutamatergic terminals (Hofmann et al. 2008).

So far, from the above mentioned literature, we find that virtually all the original effects of cannabinoids mediated by the activation of CB1 receptors are inhibitory and target the action potential dependent exocytosis (for review refer to (Chevaleyre et al. 2006; Marsicano and Lutz 2006). However, in this project, I will describe an effect of cannabinoids that is very different from its conventional effects. Specifically, we find that bath application of WIN55, 212 or anandamide produces a clear increase in the frequency of miniature IPSCs recorded from hilar mossy cells in rat dentate gyrus, without altering event amplitude, area, rise time or decay. The effect differs from previously reported effects on synaptic transmission in many respects. First, it is not mediated by CB1 receptors, or vanilloid type 1 receptors, and it is still present in CB1-/- animals. Second, it selectively modulates action potential independent exocytotic events. Third, it promotes exocytosis rather than inhibits it. Fourth, it appears to depend critically on a CB mediated increase in presynaptic calcium concentration rather than an inhibition of calcium influx, and fifth, it is only weakly invoked by postsynaptic stimulation that produces robust DSI. Therefore, we believe this manuscript represents the initial description of a new form of cannabinoid mediated modulation of synaptic transmission.

Results

WIN55, 212-2 Potentiates Action Potential Independent Events through a CB1 Receptor Independent Manner

Our first experiment was designed to examine the effect of WIN55, 212-2 on action potential independent GABA currents from interneruons to mossy cells. The cells were voltage clamped at -70mV. GABAergic mIPSCs were isolated by bath applying 1micromolar TTX and the glutamatergic antagonists NBQX or DNQX and APV. In the presence of TTX and glutamate antagonists the frequency of mIPSCs was approximately 3 Hz. To enhance the frequency to a mean rate of 15.2 ± 2.65 Hz we bath applied a calcium independent secretagogue ruthenium red.

Under these conditions, bath application of 5µM WIN55, 212-2 increased the ruthenium red enhanced frequency (to 129 ± 7.31% of baseline, p=0.003, n=10, Fig 4-1B and C) with no change in amplitude or post synaptic kinetic properties (FIG 4-1C). These results suggest a presynaptic site of action of WIN55, 212-2. To reinforce a lack of effect of WIN55, 212-2 on the post synaptic cell we conducted a separate set of experiments that were designed to measure the effect of WIN55, 212-2 on the cells intrinsic properties every 5 seconds. In these experiments we found that WIN55, 212-2 had no change on access resistance, input resistance or holding current in 8 cells tested in the presence of DNQX, APV and TTX (data not shown). The conventional role of cannabinoids is to inhibit action potential dependent release by the activation of CB1 receptors. In the present study, we found that the excitatory effect of WIN55, 212-2 on mIPSCs was not blocked in the presence of the CB1 receptor antagonist AM251 and was still present in the CB1 -/- animals (Fig 4-2A and C). Further, preincubating the slices with 1 µM AM630, a CB2 antagonist or 10 µM capsazepine (CPZ), a vanilloid receptor antagonist, did not block in increase of mIPSCs with WIN55, 212-2 (Fig 4-2B). These results suggest that WIN55, 212-2 potentiates the action potential independent events by activating an as yet unidentified receptor subtype. However, in order to substantiate these results we conducted a number of control experiments.

Ruthenium Red Doesnot Enable or cause the WIN55, 212-2 Mediated Effects on mIPSCS

We first wanted to eliminate the hypothesis that the increase in frequency of mIPSCs by WIN55, 212-2 does not have an unexpected dependency on ruthenium red. To test this hypothesis, we bath applied ruthenium red for a long time period in the absence of WIN55, 212-2. Specifically, the frequency of mIPSCs 10-14 minutes post drug application was 106 ± 5.18% of baseline (n=5, p=0.30, Fig 4-3A and C) with no change in amplitude. This result verifies that ruthenium red does not cause or enable the effect of WIN55, 212-2. In another set of experiments

we bath applied WIN55, 212-2 on mIPSCs in the absence of ruthenium red. Under these conditions we found that WIN55, 212-2 increased the frequency to 225 ± 28.8% of baseline in 7 out of 17 cells (Fig 4-3B and C). These results reinforce that ruthenium red is not required to produce the WIN55, 212-2 mediated increases in mIPSCs. Since the basal frequency i.e. in the presence of TTX and absence of ruthenium red was low and the success rate also reduced, bath application of WIN55, 212-2 produced significantly larger increases in normalized frequency with much greater variability in experiments.

Notably, in the absence of ruthenium red, the success rate was reduced to 41%. It is also clear that there was no difference in the baseline event frequency (2.8 ± 0.65 Hz vs. 2.7 ± 0.23 Hz, p=0.80), baseline event amplitude (19.7 ± 9.00 pA vs. 19.45 ± 8.29 pA, p=0.86),initial input resistance (183 ± 24MΩ vs. 167 ± 16 MΩ, p=0.60), initial access resistance (11.0 ± 1.2 MΩ vs. 11.6 ± 0.85 MΩ, p=0.71) and initial holding current (-159 ± 36 pA vs. -136 ± 14.4 pA, p=0.518) among runs that show WIN55, 212-2 mediated increases in mIPSC frequency and those that do not. These results clearly and strongly suggest that some negative synapses exist among GABAergic interneurons to hilar mossy cells. We also hypothesized that in the presence of ruthenium red a higher percentage of afferents were active, there by increasing the probability of having synapses that respond to WIN55, 212-2. Therefore, to increase the success rate and reduce the variability in experiments, the use of ruthenium red, in our judgment was advantageous in some experiments.

Anandamide also Increases the mIPSC Frequency from GABAergic Terminals without altering Amplitude, Area, Rise Time, or Decay

Next, we wanted to demonstrate that the increase of action potential independent mISPCs is not unique to WIN55, 212-2. If the effect of WIN55, 212-2 is mediated by activation of a CB-like receptor then an endogenous ligand should also be able to produce a similar result. To that

end, we found that bath application of 0.5 µM anandamide increased the mIPSC frequency to 151 ± 9.7% of baseline, n=5, p=0.016, Fig 4-4) without affecting the amplitude or post synaptic kinetic properties. Further, we also found that 2µM capsaicin failed to produce a similar increase in frequency (102 ± 16.4% of baseline, n=5, 0.92, data not shown). This result is consistent with the failure of capsazepine to block the effects of WIN55, 212-2, and reinforces the hypothesis that the effects of WIN55, 212-2 are mediated by an as yet unidentified CB-like receptor.

WIN55, 212-2 has no Effect on Action Potential Dependent Exocytosis in the Presence of the CB1 Receptor Antagonist AM251

So far, the above data suggests that bath application of cannabinoid agonists selectively modulate action potential independent exocytosis by activating a non CB1, CB2 and vanilloid receptor. Next, we wanted to examine the effect of cannabinoid agonists on action potential dependent exocytosis. We reasoned that cannabinoids can potentiate action potential dependent IPSCs but the CB1 mediated inhibition may over whelm the non CB1 mediated potentiation. To prove this hypothesis, we designed an experiment in which action potential dependent events were evoked by using a bipolar stimulator and minimal stimulator in separate experiments in the presence of AM-251. Bath application of WIN55, 212-2 or anandamide failed to change the amplitude or pair pulse ratio of eIPSCs under these conditions (Fig 4-5). These results reinforce the hypothesis that cannabinoid agonists selectively modulate mIPSC frequency via a CB1 receptor independent manner and the exocytotic process of the action potential dependent events is mechanistically distinct from the conventional action potential dependent exocytosis. To reinforce this hypothesis, we also found literature suggesting distinct chemical pools for action potential dependent and independent exocytosis. Further, these pools may have distinct calcium sensitivities as well.

Calcium ions Play a Role in the WIN55, 212-2 Mediated and CB1 Receptor Independent Effects on mIPSCs

For a long time it was assumed that events in the presence of TTX are presumably calcium independent. But recent literature challenges that idea and reports that TTX-sensitive events still have a certain degree of calcium dependency. Therefore, as our next step, we conducted experiments in which the calcium levels were manipulated. We used five different approaches to change the levels of calcium: In two of these manipulations we bath applied a voltage gated channel blocker, cadmium or lowered the amount of calcium in the ACSF to reduce the availability of external calcium. In the other two approaches we globally chelated calcium by preincubating the slices with BAPTA-AM or EGTA-AM. In our fifth manipulation, we selectively wanted to chelate calcium only in the post synaptic cell without affecting the presynaptic calcium availability. To achieve this we back filled the cell with a 10mM BAPTA internal solution.

The ability of WIN55, 212-2 to facilitate mIPSC frequency is dramatically reduced with global chelation of internal calcium or reduction in the availability of external calcium (Fig 4-6). However, a detailed consideration of this data can be more complicated than that. It is important to note that all of these experiments were conducted in the absence of ruthenium red. This choice eliminated concern about unanticipated effects of ruthenium red on calcium homeostasis in general or voltage gated calcium channels in particular, but also meant that we had to contend with a control dataset (as presented in Fig 4-3B and Fig 4-6A-B) that had a low baseline frequency and greater variability in response to WIN55, 212-2. Because of these features, a careful analysis of all experiments that altered calcium availability required a consideration of changes in both population effect and success rate. Success rate was defined as the percentage of experiments (in each experimental group) in which bath application of WIN55, 212-2 caused a

sustained increase in baseline frequency of ≥ 1 Hz. The average baseline frequency across all groups was 2.3 ± 0.15 Hz, n=55. For a less stringent measure of success rate, see the inset panel in Fig 4-6D and Fig 4-6 legend. The population effect was defined as the average change in baseline frequency across all runs (including both successes and failures).

When these variables are considered independently, it is clear that global manipulations of calcium availability reduced both the population effect and the success rate (Figure 4-6 C). For example, there was no significant effect if WIN55, 212-2 on mIPSC frequency (measured across all cells) in low calcium external, in 200 μM $Cd^{2+,}$ or the following incubation in BAPTA-AM. However, significant increases in mIPSC frequency (again measured across all cells) were observed in control conditions, and in cells backfilled with 10mM BAPTA. A smaller, but statistically significant increase in mIPSC frequency was noted in cells preincubated in EGTA-AM. Similarly, success rate (measured by 1Hz rule) ranged from a high of 66% in cells backfilled with BAPTA (50% in the control group) to a low of 15% in low calcium external solution. Importantly, success rate was lower than control in every experimental group that involved global manipulation of calcium availability (Fig 4-6C)

While these results are quite striking, in our view an independent analysis of either population effect or success rate is potentially misleading and runs the risk of underestimating the overall loss of effectiveness of WIN55, 212-2. Therefore we next defined the effectiveness of WIN55, 212-2 in each experimental group as the product of the success rate and the population effect. Thus, as the success rate approaches 0, so does the effectiveness of WIN55, 212-2. By contrast, as success rate approaches 100%, the effectiveness of WIN55, 212-2 approaches the population effect. We believe this is not only a reasonable approach to the data at hand, but that it in fact provides the cleanest and most accurate view of the overall effect of our various

calcium manipulations. The result of this analysis are presented in Fig 4-6D, where it becomes apparent that the overall effectiveness of WIN55, 212-2 is reduced to <20% of control conditions by every global manipulation of calcium, and yet is comparatively unaffected by chelation of calcium exclusively in the postsynaptic cell. In our view, this data strongly suggests that there is a key role for calcium ions, likely in the presynaptic terminal, in producing WIN55, 212-2 mediated, and yet action potential independent, facilitation of mIPSCs. Some more detailed questions regarding interpretation of this dataset are considered in the discussion.

Depolarization of DSI Positive Hilar Mossy Cells Produces a Small, but Comparatively Long Lasting, Increase in mIPSC Frequency.

So far we demonstrated that cannabinoid agonists can increase mIPSC frequency. Next, we wanted to examine if a similar facilitation of mIPSCs can be produced by endogenous cannabinoids. From previous work in our lab, we know that depolarization of hilar mossy cells results in the release of cannabinoids which activate presynaptic CB1 receptors and cause depolarization induced suppression of inhibition DSI (Nahir et al. 2007). To be able to examine the effect of endogenous cannabinoids on mIPSCs, our first step was to determine if the clamped cell can produce DSI. To test for DSI, the mossy cells were voltage clamped at -70mV using a high Cl internal. Evoked IPSCS were generated using a bipolar stimulator in the presence of ionotropic glutamate receptor antagonists and carbachol. After recording a stable baseline, mossy cells were depolarized from -70 to 0mV for five seconds to test for DSI. Cells that produced robust DSI (to 45.2 ± 4.7% of baseline, n=6, $p<0.001$, Fig 4-7A) were considered to be releasing cannabinoids. These DSI positive cells were then treated with TTX and ruthenium red as in Fig 1. After recording a baseline under these conditions, the cells were given an identical series of depolarizations while we monitored mIPSC frequency and amplitude.

Our results from these experiments indicate that depolarization of hilar mossy cells which is sufficient to produce robust DSI causes a minimal but statistically significant increase in the frequency of miniature events. This mIPSC frequency increased (to 106 ± 2.3% of baseline when measured 0-12 seconds after depolarization, n=6, p=0.04), with no change in amplitude (101 ± 1.45% of baseline, n=6, p=0.48, Fig 4-7B-C) These results suggest that the retrograde messenger responsible for DSI can activate the unidentified CB1-like receptor but it may not be the optimal endogenous ligand to produce the novel plasticity described here. We further tested this hypothesis more directly by examining the effects of 2-AG on mIPSC frequency as this ligand is considered to be the retrograde messenger responsible for inducing DSI (reference). Bath application of 2-AG at 30μM increased the frequency to (115 ± 4.31% of baseline, n=4, p=0.04, Fig 4-8). Although this increase was statistically significant the overall effect was considerably smaller than that produced by AEA (data presented in Fig 4-4). It is also note worthy that the concentration of 2-AG used in this experiment was 60 fold higher than that of AEA.

Smaller Doses of WIN55, 212-2 and its Analog WIN55, 212-3 also Produce Facilitation of mIPSCs

Thus far, our data clearly supports the finding that cannabinoid ligands increase mIPSC frequency through a non CB1, CB2 or vanilloid receptor. But could this effect of CB ligands be mediated in a non specific manner, by altering the membrane fluidity as opposed to a specific ligand protein interaction? Work done in this manuscript till now already supports the specific ligand protein interaction theory . However, the following experiments were done to further reinforce this hypothesis. Effects of various ranges of WIN55, 212-2 and its analog WIN55, 212-3 (50-5nM) were examined on mIPSCs. We found that both WIN55, 212-2 and WIN55, 212-3 were efficacious at these different nanomolar concentrations that cannot reasonably be expected

to produce broad changes in membrane fluidity or other widespread non-specific action. (Fig 4-9). Notably, these results also demonstrate a lack of stereoselectivity for WIN55, 212-2 mediated potentiation of mIPSCs.

The effect of Cannabinoid Ligands on mIPSCs is Mediated through a GPCR.

In a final series of experiments we wanted to determine if the CB-like receptor discussed in this manuscript is in fact a GPCR. Specifically, we tested the ability of suramin (G protein uncoupler) to block the ability of WIN55, 212-2 in facilitating mIPSCs. We found that on preincubating the slices with 20μM suramin for 20minutes, WIN55, 212-2 was unable to increase the frequency of mIPSCs. The average frequency across all cells dropped from 154 ± 19.1 % of baseline (n=17, Fig 4-3B) in control conditions to 100 ± 20.3% (n=10, Fig 4-10A and C) in the presence of suramin. Concurrently, the success rate reduced from 41% in control conditions to 0% in suramin. This result strongly suggests that the effects of WIN55, 212-2 are sensitive to suramin. However, suramin is also known to be an effective P2X receptor antagonist. Therefore, as a positive control we tested the effect of another P2X receptor antagonist PPADS on the WIN55, 212-2 mediated facilitation of mIPSCs. We pretreated the slices with PPADS for 20 min, in a manner identical to suramin experiments. Under these conditions WIN55, 212-2 clearly increased the average frequency of mIPSCs to 169 ± 34% of baseline across all 12 cells tested (Fig 4-10B and C). Further, the frequency increased by 1Hz in 5 of 12 cells. This led to success rate of 42% and in these 5 cells the average frequency increased to 264 ± 60% of baseline ($p=0.05$). Nevertheless, the increase in frequency caused by WIN55, 212-2 in the presence of PPADS is not significantly different from the effect of WIN55, 212-2 in control conditions ($P=0.54$).

These results indicate that P2X receptors do not play a role in the WIN55, 212-2 mediated facilitation of mIPSCs and the ability of suramin to block WIN55, 212-2 mediated facilitation is most likely due to its effect on GPCRs and not P2Rs.

Interestingly, we also found that the basal frequency in the presence of suramin was reduced (from 2.72 ± 0.286 Hz, n=17 to 0.67 ± 0.19 Hz, n=10, $p<0.01$) but not in the presence of the P2X receptor antagonist (2.2 ± 0.31 Hz, $p=0.18$ vs. control conditions). To eliminate the hypothesis that the low basal frequency affected the success rate in suramin, we determined whether there was a significant effect of WIN55, 212-2 in the best 40% of the responders in suramin, but there was no significant effect.

Discussion

This study demonstrates a novel effect of cannabinoid ligands in the CNS. In particular the cannabinoid agonist WIN55, 212-2 potentiates mIPSCs by presynaptic activation of a non CB1, CB2 or vanilloid receptor TRPV1. The endogenous ligand anandamide also facilitates mIPSCs through a presynaptic site of action. The other endogenous ligand 2-AG, was much less effective than anandamide in increasing the frequency of mIPSCs and endogeous release of 2-AG by depolarization resulted in a very modest but significant increase of mIPSCs. Further, we found that cannabinoid ligands selectively modulate the action potential independent exocytosis and yet still retain a high degree of calcium dependence. Finally, this novel effect was produced even at nanomolar concentrations by both WIN55, 212-2 and its analog WIN55, 212-3, and importantly is mediated by activation of an as yet unidentified G protein coupled receptor.

Effect of WIN55, 212-2 on mIPSCs does not Involve a Non-specific Interaction but is Possibly Mediated by an Unidentified GPCR

One question raised from these findings is if there is a possibility of a non specific effect of WIN55, 212-2 in facilitating mIPSCs? From our data we can confidently state that the effect of

cannabinoids on mIPSCs involves a specific ligand-protein interaction. The following points support that conclusion 1) some synapses to hilar mossy cells do not respond to CB agonists with increases in mIPSC frequency. 2) 2-AG is a much less efficacious agonist that AEA (refer Fig 4-8), despite comparable lipophilicity. 3) Both WIN55, 212-2 and its analog WIN33, 212-3 appears to work quite well at nanamolar concentrations. These small concentrations cannot be expected to produce alterations in membrane fluidity. Further, suramin also blocks the WIN55, 212-2 mediated increases. This finding strongly suggests that WIN55, 212-2 increases mIPSC frequency through activation of a GPCR. Interestingly, a recent study also reported that some cannabinoid agonists also affect TASK1 channels (Maingret et al. 2001). The study indicated that anandamide, its analog methanandamide and WIN55, 212-2 were most efficacious in inhibiting TASK1, where as the other powerful cannabinoid compounds like 2-AG and HU210 were not. This profile is very similar to that of our unidentified receptor. So, to eliminate the hypothesis that WIN55, 212-2 and anadamide mediated facilitation could be as a result of activation on TASK1, we designed an experiment in which we blocked the TASK1 channel with bupivicane and then applied the cannabinoid agonists. Under these conditions, we found that WIN55, 212-2 was still able to produce facilitation of mIPSCs comparable to control experiments shown in Fig 4-1(data not shown). These results suggest that TASK1 channels possibly do not mediate the novel effect of cannabinoids.

The unidentified receptor in this study is likely to have higher affinity for WIN55, 212-2 and anandamide than 2-AG. The known cannabinoid receptors do not share this profile, however some literature does point towards the existence of such a receptor. For e.g. a study done on brain membranes from CB1 -/- mice reported the ability of cannabinoid agonists to stimulate GTPγS binding. Many cannbinoid agonists were tested, however only WIN55, 212-2 and

anadamide were effective (Breivogel et al. 2001b; Di Marzo et al. 2000). Further, WIN55, 212-2 and anandamide stimulate GTPγS binding which was insensitive to the application of the CB1 receptor antagonist SR141716A. Interestingly, the binding also had a distribution distinct from CB1 and CB2. This result further support the hypothesis that there are some unidentified cannabinoid like receptors that could possibly be G protein coupled. In the future, it would be very interesting to investigate the effect of WIN55, 212-2 and anadamide stimulated GTPγS binding on action potential independent transmission.

Role of Calcium Ions in WIN55, 212-2 Mediated Facilitation

As discussed in the last chapter, for many decades it's been assumed that TTX sensitive events are presumably calcium independent. This hypothesis has been challenged at many levels. For example, in my previous project (chapter 3), the results indicated that in the presence of low external calcium and 200μM cadmium, baclofen failed to inhibit mEPSCs (Nahir et al. 2007). This phenomenon has also been observed in other preparations. It is worth noting that recent work at the calyx of held suggests that different calcium sensors play a role in modulating calcium dependent synchronous and asynchronous release (Sun et al. 2007). Importantly, other studies have indicated that different pools of vesicles with distinct calcium sensitivities regulate action potential dependent and independent exocytosis (Groemer and Klingauf 2007; Sara et al. 2005; Wasser and Kavalali 2008). Consistent with this idea, our results also illustrate a certain dependency of action potential independent exocytosis on levels of calcium ions. Specifically, we found that different manipulations of calcium levels severely reduced the ability of WIN55, 212-2 to facilitate mIPSCs. However, chelating calcium exclusively in the post synaptic terminal did not. These results suggest that WIN55, 212-2 mediated facilitation of mIPSCs requires an increase in presynaptic calcium concentration. This is an important finding of the current manuscript, however there are other points in these set of experiments that are worthy of some

discussion. For example, we found that not only did the calcium chelator BAPTA-AM block the WIN55, 212-2 mediated facilitation, but it was also severely reduced by EGTA-AM. It is known that both these compounds have different rates of chelating. Typically, sensitivity of a calcium dependent event to EGTA-AM is expected to increase with some distance between the calcium source and the relevant binding protein. Indeed, numerous studies have now indicated that EGTA-AM is more effective at blocking synchronous release that depends on the buildup of residual calcium between action potentials, than synchronous release, which is directly coupled to action potential evoked calcium influx (Atluri and Regehr 1998; Hagler and Goda 2001; Otsu et al. 2004). Thus in our view, the sensitivity of WIN55 212-2 mediated facilitation of mIPSCs to EGTA-AM suggests that this form of novel plasticity is dependent on small changes in presynaptic calcium concentration which is derived from a source at some distance from releasable vesicles. Therefore, in the future, it will be interesting to investigate the role of calcium stores in regulating the cannabinoid ligand mediated facilitation of action potential independent exocytosis. There is other evidence in the literature, in addition to those discussed above that support the idea of calcium stores playing a role in mediating action potential independent transmission (Collin et al. 2005).

However, our result with cadmium contradicts the above hypothesis. We did not expect cadmium to block the ability of WIN55, 212-2 in facilitating mIPSC frequency. This is because cadmium is a voltage gated calcium channel blocker. But these channels were already blocked by ruthenium red (Gomis et al. 1994; Trudeau et al. 1996b) which clearly does not prevent the facilitation of mIPSCs by WIN55, 212-2. At present, we cannot explain this inconsistency, but we do hypothesize that cadmium ultimately affects the calcium homeostasis in a way that ruthenium red does not.

Source and Mechanism of Release of the Cannabinoid Ligand Mediating Potentiation of Action Potential Independent Transmission

Another interesting question is on the identity of the endogenous cannabinoid ligand mediating the facilitation of mIPSCs. On depolarizing the DSI positive cells we find only a minimal increase in the mIPSC frequency. While not shown on this synapse but some previous literature on short term plasticity's (DSI, DSE) indicate that 2-AG is the retrograde messenger released on depolarization. In the present study, we also find that bath application of 2-AG produced a significantly smaller effect than anandamide despite being applied at a dose 60 fold higher than AEA. Previous literature suggests that anandamide is a partial agonist on CB1 receptors and is not very abundant in the CNS. Importantly, more recent evidence has also suggested that anandamide exerts non CB1, CB2 mediated effects and efforts are being made to identify those receptors (for review see (Di Marzo et al. 2002)). Cumulatively, these results indicate that anandamide may be a more optimal ligand than 2-AG in mediating the novel plasticity described here. In addition, we also hypothesize that the endogenous activation of this system will depend on mechanisms that regulate production of anandamide (activity dependent or not) than those that are responsible for production of 2-AG.Interestingly, a synthetic enzyme responsible for the production of anandamide was reported in the terminals of mossy fibers with high expression in the hilar region of the dentate gyrus. This idea is being investigated in our lab and more work needs to be done to substantiate these conclusions.

Functional Significance of the Novel Cannabninoid Plasticity

Finally, does this unusal plasticity mediated by cannabonoids have any functional significance? Previous literature indicates the role of action potential independent synaptic transmission in clustering of postsynaptic glutamate receptors (Saitoe et al. 2001), formation of dendritic spines (McKinney et al. 1999), and related maintenance of synaptic connections in the

absence of action potentials (McKinney et al. 1999).Additionally, a number of studies also report the ability of miniature events to affect firing rates (Carter and Regehr 2002) and inhibitory tone (Lu and Trussell 2000). There may be many functions of action potential independent events but what they have in common is that all the above physiological mechanisms are driven by low levels of released neurotransmitter. For future studies, we hypothesize that the novel plasticity discussed in this study may have the ability to increase ambient GABA which could functionally activate a number of extrasynaptic $GABA_B$ receptors and high affinity $GABA_A$ receptors identified in my last project (Nahir et al. 2007). Further work need to be done to prove this hypothesis.

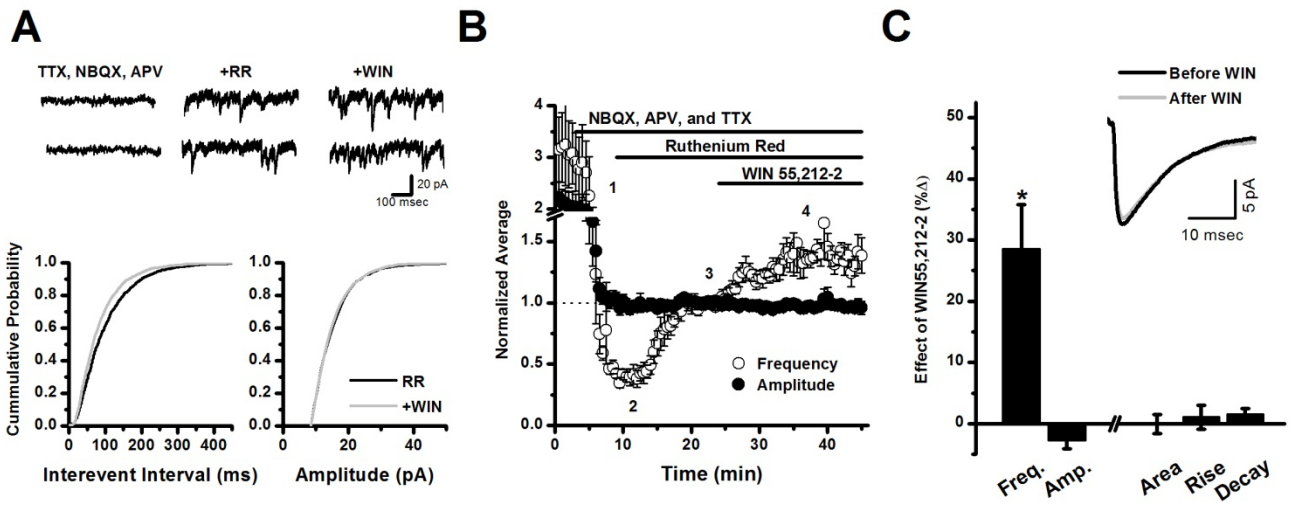

Figure 4-1. Bath application of cannabinoid agonist WIN55,212-2 causes an increase in mIPSC frequency without altering amplitude. A: Top panels are raw data traces representative of mIPSCs recorded in various experimental conditions (see labels). Note that mIPSC frequency increases with bath application of ruthenium red (50 µM), and then further increases with bath application of WIN55,212-2 (5 µM). Lower panels are cumulative probability histograms constructed from the same cell. In this cell WIN55,212-2 caused a decrease in interevent interval (IEI) from 100 ± 2.18 ms to 83.2 ± 1.17 ms ($p<0.001$, Kolmogorov-Smirnov (K-S) test) with no change in amplitude (baseline 15.9 ± 0.2 pA; WIN55,212-2 15.6 ± 0.1 pA ; $p=0.35$, K-S test). B) Event frequency (open circles) and average amplitude (closed circles) were calculated in 30 second bins, normalized to the level observed immediately prior to application of WIN55,212, and averaged across five cells. Numbers indicate different conditions observed over the course of the experiment as follows: (1) Mossy cells display large amplitude and high frequency miniature EPSCs immediately after patching in control conditions. (2) Application of NBQX, APV, and TTX blocks both ionotropic glutamate receptors and action potentials, leaving much lower frequency, and much smaller, miniature IPSCs. (3) Subsequent application of ruthenium red produced an increase in mIPSC frequency that stabilized within ~15 min, and (4) Bath application of WIN55,212-2 in the presence of ruthenium red produces a further increase in event frequency, without altering event amplitude. Note again that data were normalized to the stable level observed in NBQX, APV, TTX, and ruthenium red. In some panels in later figures similar experiments are illustrated from this point forward. C) Summary plot for all cells tested by WIN55,212-2 in the presence of ruthenium red (n=10) indicating that other event parameters (area, rise time, and decay) were also unaffected by WIN55,212-2. Inset shows the average of all mIPSCs from a representative cell during the baseline and measurement periods.

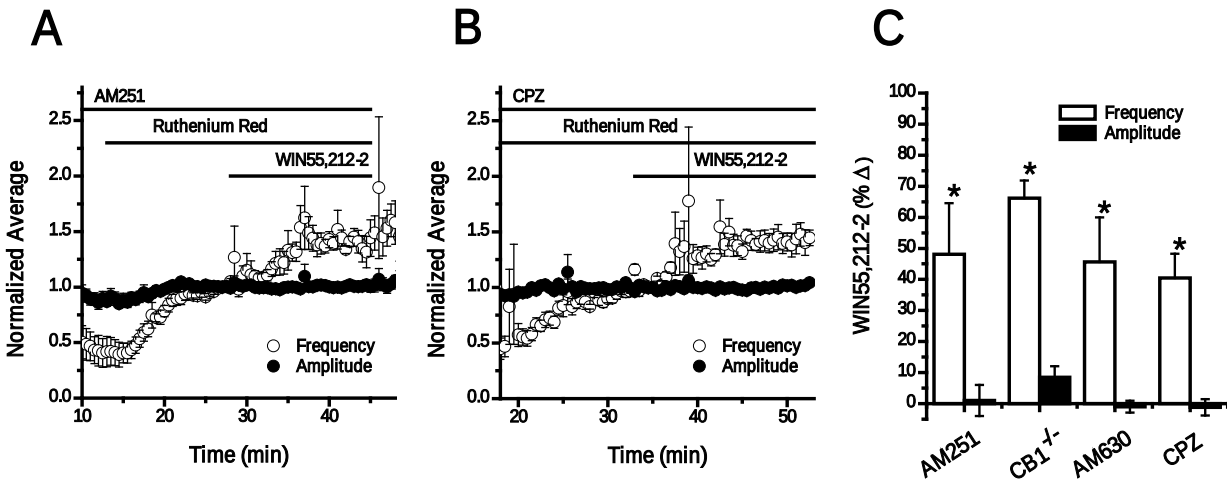

Figure 4-2. WIN55, 212-2 mediated facilitation of mIPSCs is not dependent on CB1, CB2, or TRPV1 receptors. A) In the presence of 5 µM AM251, bath application 5 µM WIN55, 212-2 increased the frequency of mIPSCs to 148 ± 16.5% of the level observed in ruthenium red ($p=0.03$ $n=7$), without altering amplitude. B) In the presence of 10 µM capsazepine (CPZ), a TRPV1 antagonist, WIN55,212-2 potentiated the frequency to 140.4 ± 7.85% of the level observed in ruthenium red ($p=0.01$, $n=4$). C) Summary plot indicating the WIN55,212-2 mediated increases in mIPSC frequency were observed not only in the presence of AM251 and CPZ, but also in CB1-/- animals (166 ± 5.66% of baseline, $n=3$, $p=0.007$) and in the presence of 1 µM AM630, a CB2 receptor antagonist (146 ± 14.3% of baseline, $n=5$, $p=0.03$). Data were analyzed and presented as described in Fig. 1B.

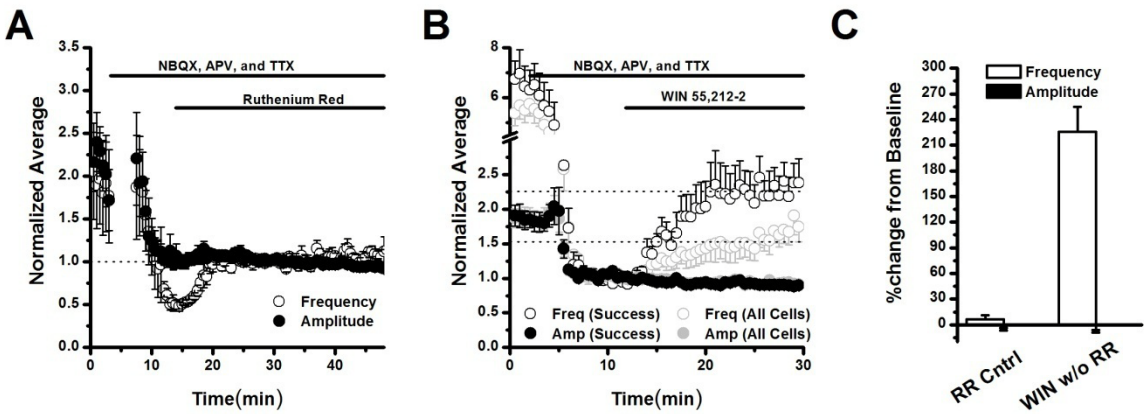

Figure 4-3. Ruthenium red does not independently produce, and is not required to observe, WIN55, 212-2 mediated increases in mIPSC frequency. A) 50 µM ruthenium red produces an increase in miniature IPSC frequency that remains stable in the absence of WIN55,212-2 (106 ± 5.18%, measured 10-14 minutes after normal time for WIN55,212-2 application, p=0.3 ,n=5). Data in this plot are aligned by the time of ruthenium red application. Data is not shown from 3 to 7 minutes because the amount of time between application of ionotropic glutamate receptor antagonists and ruthenium red was not constant in all cases (minimum: 6 min, maximum: 10 min). B) WIN55, 212-2 mediated increases in mIPSC frequency can still be observed in the absence of ruthenium red (mIPSC freq: 237.6 ± 16.81% of control, p=0.01, n=3 of 6, successes only, open circles, see main text), with no corresponding change in amplitude (filled circles). When averaged across all cells (successes and failures), mIPSC frequency was increased by WIN55, 212-2 to 173 ± 30.0% baseline (open gray circles, n=6, p=0.059). C) Summary: RR in the absence of WIN55, 212-2 had no late effect on event frequency, while WIN55, 212-2 still increased event frequency in the absence of RR in 50% of trials. For the analysis in panels B-C, successes are runs in which bath application of WIN55, 212-2 increased event frequency by ≥ 1 Hz (measured 10-14 minutes after application).

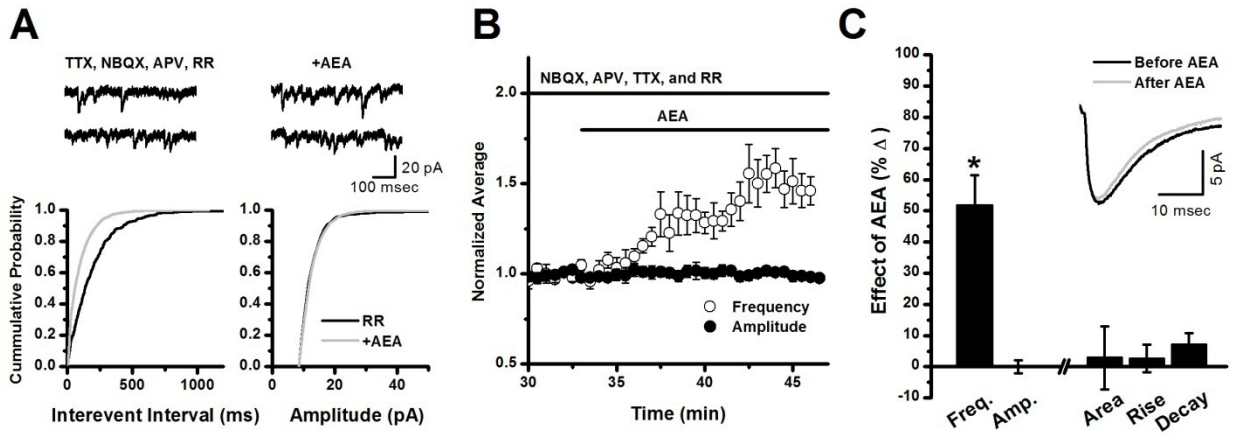

Figure 4-4. Bath application of AEA also selectively increases mIPSC frequency without altering amplitude, area, rise time, or decay. A) Top panels are raw data traces representative of mIPSCs recorded in NBQX, APV, TTX, and ruthenium red (left traces) and in the same cell following addition of 0.5 µM AEA (right traces). Lower panels are cumulative probability histograms constructed from the same cell. In this cell AEA caused a clear decrease in interevent interval (IEI) without altering event amplitude. B) Across five cells tested, AEA significantly increased mIPSC frequency (to 151.6 ± 9.7% of baseline, $p<0.01$, $n=5$), without altering event amplitude. Data in this panel is analyzed and presented as described in the legend for Fig. 1B. C) Summary plot indicating that other event parameters (area, rise time, and decay) were also unaffected by AEA. Inset shows the average of all mIPSCs from a representative cell during the baseline and measurement periods.

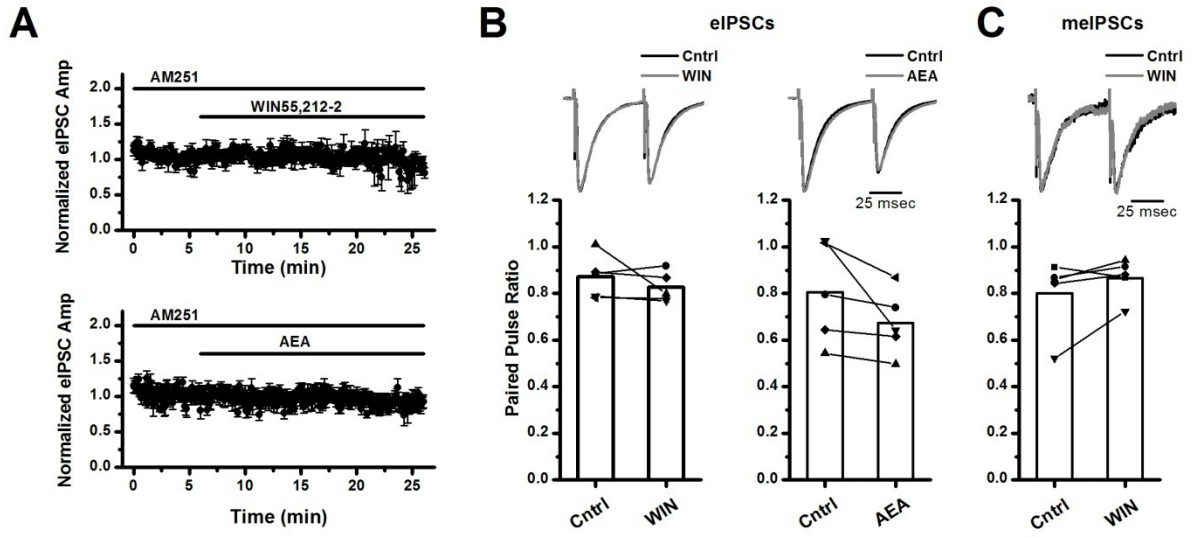

Figure 4-5. Cannabinoid agonists have no effect on action potential dependent exocytosis in the presence of the CB1 receptor antagonist AM251. A) Evoked IPSCs were generated at a rate of 0.33 Hz using a bipolar (panel A-B) or minimal (glass monopolar) stimulator (panel C) placed in the hilus. NBQX and APV were present for all experiments. A) Bath application of 5 µM WIN55,212-2 or 0.5 µM AEA had no significant effect on evoked IPSC amplitude in slices pretreated with 5 µM AM251. B) Similarly, under these conditions, CB agonists had no effect on the paired pulse ratio of responses generated with a bipolar stimulator. C) WIN55,212-2 also had no effect on PPR of minimally evoked responses (meIPSCs). Raw data traces in panels B and C are normalized to the P1 peak.

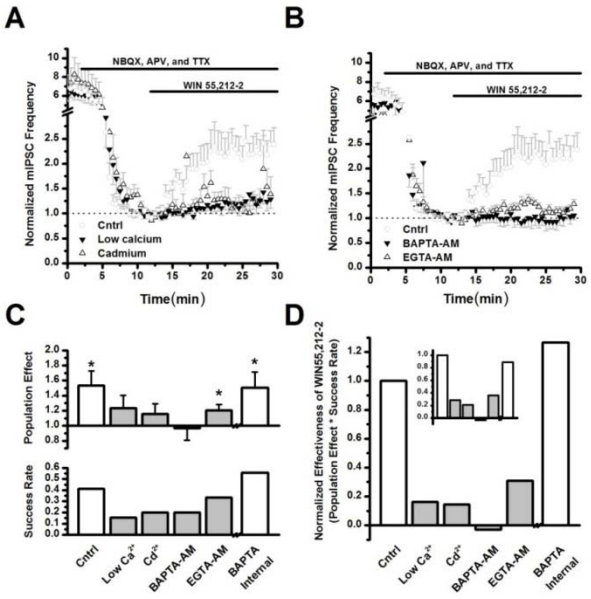

Figure 4-6. WIN55, 212-2 mediated increases in mIPSC frequency are likely to depend on increases in presynaptic calcium concentration. NBQX, APV and TTX were present for all experiments. A) Under control conditions (open circles) WIN55,212-2 significantly increased mIPSC frequency (data from Fig 3B, successes only). By contrast, WIN55,212-2 mediated enhancement of mIPSC frequency was severely reduced by bath application of low Ca^{2+} external solution (see methods) or 200 µM Cd^{2+} (n=13 and 10 respectively, data shown is average of all experiments). B) Similarly, preincubation with either 100 µM EGTA-AM or 100 µM BAPTA-AM (n=12 and 5 respectively) severely reduced the effect of WIN55,212-2 on mIPSC frequency. C) Top panel: Effect of WIN55,212-2 on mIPSC frequency, averaged across all cells tested (both successes and failures), is shown for every experimental condition. Bottom panel: similar plot showing success rate for bath application of WIN55,212-2 in every experimental condition. Success rate was defined as the percentage of trials where WIN55,212-2 caused an increase in mIPSC frequency of at least 1 Hz, measured from 10-14 minutes after onset of application. Both the population effect and the success rate are reduced by global manipulations of calcium (gray bars) but largely spared by selective chelation of calcium in the postsynaptic cell (10 mM BAPTA internal, open bar on the right). D) Effectiveness of WIN55,212-2 in every experimental condition is expressed as the product of the success rate and the population effect. Values are normalized to control conditions. Effectiveness of WIN55,212-2 was severely reduced by all global manipulations of calcium, but comparatively unaffected by chelation of calcium in the postsynaptic cell. Inset shows result of an identical analysis where success rate was defined as the percentage of cells that produced a statistically significant increase in baseline frequency ($p<0.05$) over the same time period. This was a less restrictive test of success, but the overall results of the analysis were quite similar. Bars are presented in an identical order.

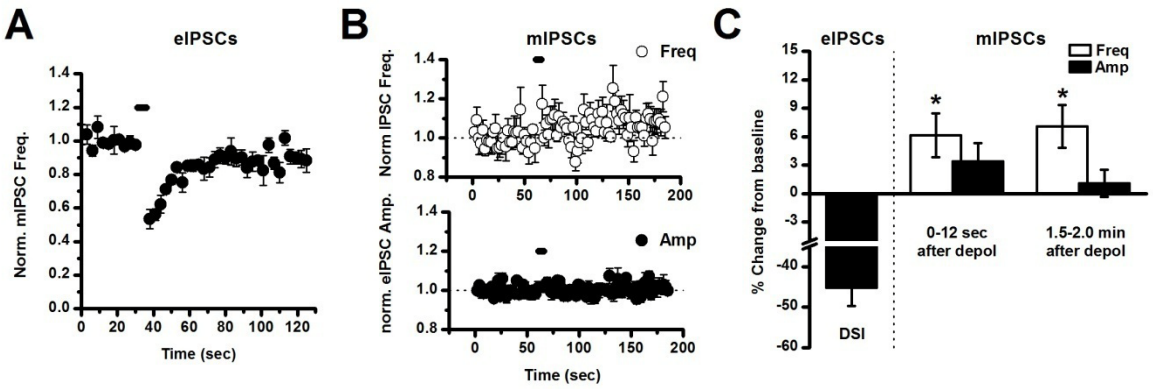

Figure 4-7. Depolarization of DSI positive hilar mossy cells produces a small, but comparatively long lasting, increase in mIPSC frequency. DNQX and APV are present for all experiments. Evoked IPSCs were generated at a rate of 0.33 Hz using a bipolar stimulator. A) In the presence of bath applied CCh (3 µM), depolarization of mossy cells from -70 to 0 mV for 5 s (bar) caused a significant reduction of the mean eIPSC amplitude (DSI, by 45.21 ± 4.49%, n=6, $p<0.001$, measured over first 6 seconds after depolarization). B) After application of 1 µM TTX and 50 µM ruthenium red, identical depolarization of these same cells produced a small, but statistically significant increase in mIPSC frequency (106 ± 2.3% of baseline when measured 0-12 seconds after depolarization, n=6, $p=0.04$), without altering amplitude. Interestingly, this change was also clearly longer lasting than DSI (107 ± 2.26% of baseline, n=6, $p=0.04$ when measured 1.5-2.0 minutes after depolarization). C) Summary plot indicating the effect of depolarization on evoked IPSCs vs. mIPSCs in the same population of cells (n=6).

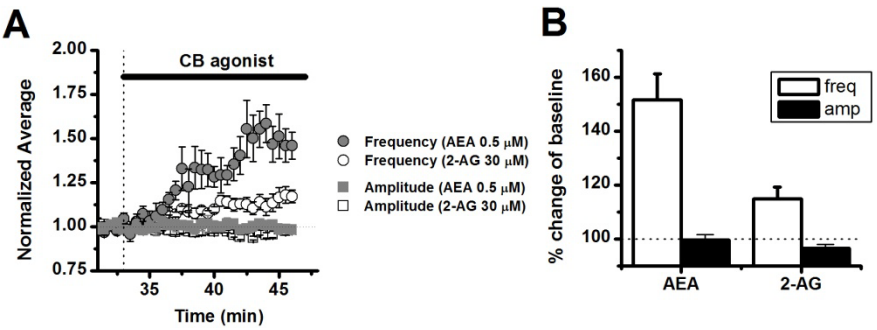

Figure 4-8. Anandamide is more efficacious than 2-AG in causing the WIN55, 212-2 mediated facilitation. A) Average scatter plot compares the effects of 30micromolar 2-AG and 0.5 micromoplar AEA on frequency and amplitude (open circles). AEA significantly increased the frequency (to 151.6 ± 9.71%, n=5, p=0.016) with no change in amplitude (grey circles). 2-AG at a 60 fold higher dose increased the frequency (to 115 ± 4.31%, n=4, p=0.04), strongly suggesting that the effect was considerably smaller than that produced by AEA. B) Summary plot indicates the effect of both endocannabinoids on frequency and amplitude.

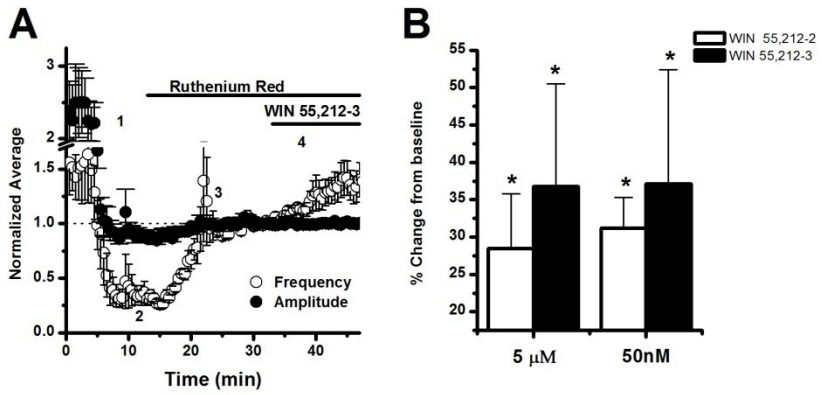

Figure 4-9. WIN55, 212-2 mediated potentiation of mIPSCs lacks stereoselectivity A) The analog WIN55, 212-3 significantly increased the mIPSC frequency (to 136 ± 13.7%, n=8, p=0.03) with no change in amplitude, strongly suggesting that the effect is essentially identical to that produced by WIN55, 212-2. B) Summary plot indicates the effect of WIN55, 212-2 and its analog on mIPSC frequency at two different doses.

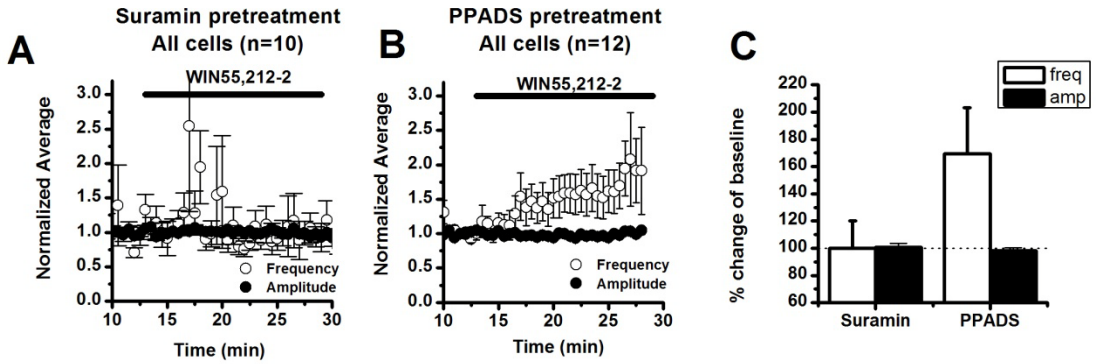

Figure 4-10. Effect of WIN55, 212-2 is mediated through activation of a G protein coupled receptor. A) Preincubation of the brain slices with suramin dramatically blocked the ability of WIN55, 212-2 to increase mIPSC frequency. B) In the presence of the P2X antagonist PPADS, the mIPSC frequency significantly increased (to 169 ± 34%, n=12, $p<0.05$), strongly suggesting the effects of suramin on WIN55, 212-2 mediated facilitation is by acting on the GPCRs and not P2X receptors. C) Summary plot compares the average effects of suramin and PPADS on frequency and amplitude

CHAPTER 5
DISCUSSION

The primary goal of my thesis was to extend our understanding of the communication between cells that are thought to be intimately involved in the initiation of epileptic seizures or memory disorders like Alzheimer's disease. We were also interested in examining how the communication between those cells is modulated by compounds with great therapeutic potential like cholinergics and GABAergics. Currently, most cholinergic agonists used for the treatment of Alzheimer's disease exert their actions by acting at acetylcholine receptors to inhibit of acetylcholine esterase and to increase the amount of acetylcholine The therapeutic potential of $GABA_B$ agonists has also been demonstrated as antidepressants where as the $GABA_B$ antagonists are implicated as antiepileptics and used for memory consolidation (Bowery 1993). In my project we were very interested in examining how these therapeutic agents could regulate transmitter release from key synapses involved in the etiology of epilepsy and memory disorders.

In the present study, we found that muscarinics indirectly inhibit mossy fiber inputs to mossy cells by activating $GABA_B$ receptors. By contrast, muscarinics directly inhibit non mossy fiber inputs (AC synapses) by direct activation of muscarinic receptors. These findings extend the differences in the modulatory profile of mossy fiber and AC synapses. For example, it has long been known that unlike AC synapses, mossy fiber synapses are sensitive to the application of mGluRII agonists DCGIV. Further, they also differ in short term plasticities like frequency facilitation (Kamiya et al. 1996; Manzoni et al. 1995). In our study, we also used these two characteristics to distinguish between these inputs (Nahir et al. 2007). An interesting aspect of our study was that action potential induced increases in ambient GABA play a significant role in regulating transmitter release from mossy fiber terminals to mossy cells. Action potentials act as one source of raising the levels of ambient GABA in the synaptic cleft and have also been

observed in other areas of the brain. However, action potential independent mechanisms for regulating tonic GABA have also been suggested in some studies (Brickley et al. 1996; Carta et al. 2004; Rossi et al. 2003; Wall and Usowicz 1997). A third source of changing levels of tonic GABA is by using certain GABA uptake inhibitors like NO-711(Fig 3-3), which was also used in our study. We found that presynaptic $GABA_B$ receptors on mossy fiber terminals and high affinity $GABA_A$ receptors on mossy cells were able to detect or respnd to the low levels of ambient GABA. Notably, presynaptic $GABA_B$ receptors on non mossy fiber inputs did not respond to ambient GABA. The exact reason for this difference is still not known but in our view the presynaptic $GABA_B$ receptors on AC inputs to mossy cells could have lower sensitivity to changes in ambient GABA or spatial factors could account for this apparent discrepancy. Further, it can be speculated that the manner in which alterations in the firing pattern of a presynaptic cell affects transmission depends on the use-dependent plasticity of the synapse. For future work, it would be interesting to investigate why these two excitatory afferents that synapse on to the same post synaptic target respond so differently to the same neurotransmitter. Intriguingly, these two glutamatergic inputs also differ in the expression of the cannabinoid receptor CB1. Our lab provides electrophysiological results indicating the existence of CB1 on AC inputs to mossy cells and not mossy fibers (Hofmann et al. 2008).

My first manuscript, Chapter 3, has contributed a substantial amount of information on how the two most heavily studied pathways in the hippocampus (mossy fiber and AC) can be regulated by different signaling mechanisms. Extensive work has been done on the mossy fiber pathway. This is because it heavily innervates the principal cells involved in the trisynaptic pathway and the collaterals of mossy fiber innervate the mossy cells which plays a key role in the initiation of seizures. As I mentioned in my introduction, the mossy fibers to CA3 are well

known for its tight spatial localization, strong frequency facilitation, clear sensitivity to metabotropic glutamate receptor agonists, possible role as a "detonator synapse" and unusual N-methyl-D-aspartate-independent and presynaptic form of long-term-potentiation (for review see (Henze et al. 2000) . Interestingly, a number of recent reports have highlighted the fact that mossy fibers release GABA in addition to glutamate. These reports indicate that in young rats (till 23 days) mossy fibers release GABA. As adults (>23 days), mossy fibers switch to releasing glutamate as their main neurotransmitter (Gutierrez 2002; 2003; Gutierrez and Heinemann 2001; Gutierrez et al. 2003). In addition to this, when seizures are induced in older animals mossy fibers release GABA as their principal neurotransmitter (Gutierrez and Heinemann 2001). This may be the body's protective mechanism against induction of seizures. If the results of these studies are in fact true, then it would be interesting to see how cholinergics can modulate exocytosis from mossy fiber terminals that release GABA in younger animals. If mossy fiber terminals in older animals do release GABA during seizures, then would cholinergics modulate this exocytosis and affect the output of mossy cells during hyperexcitability?

Ultimately, what is the functional significance of the plasticity documented in Chapter 3? We found that cholinergics inhibit the release of glutamate to mossy cells by activating the presynaptic $GABA_B$ receptors. $GABA_B$ antagonists are implicated in memory consolidation. The possibility of cognitive improvement stems from the observations of that $GABA_B$ antagonists can facilitate long-term potentiation in hippocampal slices when provoked by high frequency stimulus trains (Olpe and Karlsson 1990). Studies published by Schwartzwelder et al, 1987) also suggest that baclofen can decrease memory acquisition and retention in rats (Swartzwelder et al. 1987). By contrast, an antagonist may play an opposite role, although no information is available at present. Based on these findings, we can also hypothesize that cholinergics may modulate LTP

from mossy fiber terminals to mossy cells. Cholinergics increase ambient GABA in an action potential dependent manner which activates the $GABA_B$ receptors and inhibits them. This could affect the excitatory drive on mossy cells and possibly affect LTP. Conversely, if the level of available ambient GABA in the synaptic cleft is low or the $GABA_B$ receptors on mossy fiber terminals are inactive, then that will lead to increased glutamatergic transmission from mossy cells and make mossy cells fire more and possibly increase LTP. However, these experiments need to be tested for conclusive results. Further, we were the first to show that ambient GABA raised by cholinergics generates tonic GABAergic currents in mossy cells by activating the high affinity $GABA_A$ receptors on these cells, possibly affecting their firing rate. Recent work also indicates the presence of high affinity $GABA_A$ receptors on mossy fibers to CA3. Although, till date, there is no evidence indicating the presence of high affinity $GABA_A$ receptors on mossy fibers to mossy cells, but the possibility of $GABA_A$ receptors on mossy fibers being modulated by ambient GABA should not be eliminated. Interestingly, the indirect mechanism of synaptic modulation has also been found on other synapses. Previous studies have indicated that muscarine can also facilitate mossy fiber inputs to CA3. Muscarine does this by increasing the firing rate of dentate granule cells, which in turn facilitate mossy fiber synapses. The sensitivity of mossy fibers to muscarine results due to the low threshold for frequency facilitation in this synapse (Regehr et al. 1994; Salin et al. 1996). Therefore, we can speculate that if the $GABA_B$ receptors on mossy fibers are blocked and the granule cells connections are preserved in the hippocampal slice, we would expect that glutamatergic transmission to mossy cells will increase and increase the excitability of postsynaptic hilar cells.

Based on the above hypotheses, we believe that further studies into specific mechanisms that regulate mossy cell excitability by cholinergic or GABAergic signaling systems will be

necessary to develop a more complete understanding of hippocampal function or dysfunction ranging all the way from memory consolidation to epileptogenesis.

In my second project (chapter 4), we demonstrated a novel effect of cannabinoids that is mediated by a non CB1, CB2 or vanilloid receptor. Additionally, it is also potentiated by anadamide and is sensitive to the application of suramin, strongly suggesting that it may be mediated by activation of an unidentified G protein coupled receptor (GPCR). Our results indicate that WIN 55, 212-2 mediates facilitation of mIPSCs by activating a GPCR. Interestingly, the success rate of finding WIN55, 212-2 mediated facilitation was reduced from 41% in control conditions to 0% in the presence of suramin (a G protein uncoupler). Previous studies have identified WIN55, 212-2 stimulated and yet AM251 insensitive increases in GTPγ S binding in several areas of the CNS, including hippocampus (Breivogel et al. 2001a; Di Marzo et al. 2000). This result reinforces the notion that the unidentified CB-like receptors remain to be found and also confirms that at least some of them are in fact GPCRs. In the future, efforts are going to be made to identify the specific G protein involved. However, recent data from our lab indicates that a certain protein kinase plays a role in causing the WIN55, 212-2 mediated facilitation, downstream of the activation of the unidentified GPCR (unpublished data, Mack Hofmann). This hypothesis stems from the fact that some studies suggested an involvement of protein kinase A in facilitating action potential independent events. (Trudeau et al. 1996a; Trudeau et al. 1996c; Trudeau et al. 1998). We hypothesize that activation of the GPCR by a cannabinoid agonist is going to lead to the activation of protein kinase A in an adenylate cyclase/cAMP dependent manner. PKA will then phosphorylate proteins necessary for exocytosis, hypothetically snap 25. So far, the work done in our lab demonstrates that in the presence of a protein kinase blocker H-89, WIN55, 212-2 was unable to produce an increase in

mIPSCs. Further, bath application of a cAMP agonist facilitated the mIPSC frequency, comparable to the control. Additionally, we also found that bath application of forskolin occluded the effect of WIN55, 212-2. This is because forskolin is an activator of PKA. These studies are still incomplete and need higher n values and more controls to substantiate this hypothesis. Cumulatively, these results do suggest that the signaling cascade for the increase in action potential independent exocytosis by WIN55, 212-2 and anandamide involves the activation of PKA. Hence, we speculate that the G protein involved may be Gs since it is known to activate PKA. It is noteworthy that the WIN55, 212-3 analog used in these studies also potentiated mIPSC frequency. Although, the lack of stereoselectivity was not expected by us, we believe that this lack of steroselectivity does not go against our GPCR hypothesis. This is because there are numerous prior examples of specific GPCR ligand interactions that fail to show strong stereoselectivity in functional assays. In fact, the lack of stereoselectivity reinforces the conclusion that the WIN55, 212-2 mediated potentiation is not CB1 dependent.

We found that preincubation in either BAPTA-AM or EGTA-AM blocks WIN55, 212-2 mediated increase in mIPSC frequency, yet this same effect is completely insensitive to blockade of voltage gated calcium channels by ruthenium red, and still present (although reduced) following bath application of 200µM cadmium. Overall, we believe that these results strongly suggest a role of calcium release from internal calcium stores. Additionally, we can also hypothesize that the calcium involved in the WIN55, 212-2 mediated facilitation is directly dependent on release from IP3 sensitive stores. We could test for both these hypotheses by using thapsigargin for depleting the calcium stores or use an IP3 receptor antagonist 2-aminoethoxydiphenylborane (2-APB) (Li et al. 2008). If these hypotheses are correct then the

WIN55, 212-2 mediated facilitation would be sensitive to the application of either thapsigargin or 2-APB.

Intriguingly, both BAPTA-AM and EGTA-AM severely reduced the facilitation by WIN55, 212-2 even though they chelate calcium at different rates. Further, we also found that bath application of WIN55, 212-2 had no effect on action potential dependent exocytosis. Cumulatively, these results raise two interesting questions. First, is the WIN55, 212-2 mediated effect on mIPSCs dependent on a calcium sensor separate from that involved in action potential dependent exocytosis? Second, is the separate calcium sensor located in the same or distinct vesicle pool (action potential independent vs. dependent (readily releasable pool))? The first question stems from the fact that the WIN55, 212-2 mediated increase in mIPSC frequency was not only sensitive to BAPTA-AM but also EGTA-AM, which implies that calcium is acting at some distance from the release site. In addition, WIN55, 212-2 did not potentiate eIPSCs in the presence of AM251, implying that the WIN55, 212-2 mediated increases in cytosolic calcium were not robust enough to modulate action potential dependent exocytosis. The second question, on the location of the calcium sensor was raised based on our results which show a lack of an effect of WIN55, 212-2 on action potential dependent exocytosis. The result lead us to speculate that cannabinoids may target a vesicular pool different from the one normally subjected to action potential dependent release and CB1 mediated inhibition. It is of interest to compare our hypotheses to several elegant studies that have suggested that miniature synaptic events in other preparations do in fact largely come from a distinct pool with a unique calcium sensor (Groemer and Klingauf 2007; Sara et al. 2005; Wasser and Kavalali 2008). However, there is no common agreement on this issue. In the future, it would be interesting to investigate the possibility of

different calcium sensors for separate release mechanisms (action potential dependent or independent).

Overall, our data with suramin does indicate that the WIN55, 212-2 mediated potentiation is through the activation of an unidentified GPCR. Other studies that show stimulation of GTPγS binding with WIN55, 212-2 and anadamide in CB1-/- mice also support our GPCR results. However, we cannot rule out other non-GPCR targets. Specifically, different studies have suggested that additional molecular targets for endocannabinoids exist and further cannabinoids can alter the excitability of neurons by binding to these novel sites. Some of these novel targets include voltage-gated ion channels including Ca^{2+}, Na^{+} and various types of K^{+} and ligand-gated ion channels such as serotonin type 3, nicotinic acetylcholine, and glycine receptors. In addition, modulatory effects of endocannabinoids on other ion-transporting membrane proteins such as transient potential receptor-class channels, gap junctions and transporters for neurotransmitters have also been demonstrated (for review refer (Oz 2006)). It is worth mentioning that most of the effects of pharmacologically relevant concentrations of endocannabinoids on the above mentioned target proteins were inhibitory, with the exception of few like calcium activated K channels, Glycine receptors and NMDA receptors. Although the mechanisms of these effects are currently not clear, it is likely that these direct actions of endocannabinoids are due to their lipophilic structures. In another set of experiments we also ruled out one such non-GPCR target of cannabinoids, the TASK1 channel (Maingret et al. 2001). This channel had a profile similar to our unidentified receptor. However, the effect of WIN55, 212-2 on mIPSCs was not blocked in the presence of bupivicane, the TASK1 channel blocker. Nevertheless, we still cannot eliminate the possibility of these novel sites for endocannabinoids mediated effects. Further, as discussed

in chapter 4 in detail, we have ruled out the possibility of any non specific action of cannabinoid ligands in mediating the increase of action potential independent exocytosis.

In the present study, we found that WIN55, 212-2 and the endoegenous ligand anandamide potentiate action potential independent exocytosis through a CB1 receptor independent manner. Our results indicate that ananadamide was more efficacious in producing the facilitation than 2-AG even though 2-AG was applied at a much higher dose. In addition to this we also found that depolarization of the mossy cells to release endogenous cannabinoids (presumably 2-AG) resulted in robust DSI, but produced a very modest increase in mIPSC frequency. Cumulatively, these results suggest that unlike the CB1 receptor, the novel CB-like receptor may not be sensitive to 2-AG or the amount of 2-AG that is released on depolarization of mossy cells may be sufficient to activate the CB1 receptor, but may not be sufficient to activate the novel CB-like receptor. However, proper dose response curves need to be constructed for anandamide, 2-AG and some other specific NAEs including N-palmitoylethanloamine (PEA) and N-oleoylethhanolamine (OEA), in order to substantiate this conclusion. Consistent with the idea that anandamide is a more optimal ligand for physiologically activating the novel receptor; we are also hypothesizing that NAPE-PLD, recently found in the presynaptic terminals of glutamatergic axons (Nyilas et al. 2008) may assist in the release of AEA that can cause increase in mIPSCS by binding to the novel receptor. However, at this point we cannot eliminate the hypothesis that anandamide released from mossy fiber terminals in our experimental design may not be sufficient to activate the novel CB-like receptor. Further, we can also not eliminate certain spatial constraints between mossy fiber terminals and GABAergic interneurons. If our experimental design for releasing anandamide is successful, then we do expect to observe increases in mIPSC frequency to hilar mossy cells. While the optimal ligand may be

anandamide, it is worth pointing out, that the synthetic pathway for anandamide described above and in the introduction is also likely to be involved in the production of a number of other N-acylethanolamines (NAEs) such as PEA and OEA (Okamoto et al. 2004). While there is no evidence in indicating CB1 receptor activation by these compounds (Felder et al. 1993), their potential involvement in CB1 receptor independent signaling remains to be determined.

Finally, what are the functional consequences of increased miniature synaptic transmission by cannabinoids? Decades ago, in the neuromuscular junction, miniature events were instrumental in the development of modern quantal theory of synaptic transmission. However, in the past, this release mechanism has received very little attention compared to other forms of synaptic transmission. But in the recent years, interest on this topic has been growing and numerous functional roles of action potential independent transmission have been discovered (refer discussion in chapter 4). In the present study, we hypothesize that the increased action potential independent exocytosis produced by cannabinoid agonists can affect the excitability of mossy cells in several different ways. First, the cannabinoid dependent increased ambient GABA can target tonic $GABA_B$ receptors on mossy fiber terminals to mossy cells and inhibit it (Nahir et al. 2007). This would decrease the excitatory drive on mossy cells. Second, previous literature indicates the expression of high affinity $GABA_A$ receptors on mossy fiber terminals to CA3. If this is infact true for mossy fibers to mossy cells, then activation of tonic $GABA_A$ receptors would serve as an additional way of inhibiting these terminals and increasing the excitability of mossy cells. Third, in addition to modulating presynaptic receptors on mossy fiber terminals, we also expect the raised ambient GABA to target post synaptic high affinity $GABA_A$ receptors on mossy cells and decrease excitability of these cells. The original work that demonstrated activation of high affinity $GABA_A$ receptors by ambient GABA involved an examination of the

effects of postsynaptic receptor activation on holding current (Glykys and Mody 2007; Mtchedlishvili and Kapur 2006; Yeung et al. 2003). Earlier studies also support the existence of extrajunctional $GABA_A$ receptors in the cerebellar granule cells, dentate granule cells and CA1 stratum radiatum interneurons (refer Introduction). Finally, in addition to activating presynaptic and extrasynaptic $GABA_B$ receptors we also expect the raised ambient GABA to target the high affinity presynaptic $GABA_A$ receptors on GABAergic interneruons and undergo autoreceptor inhibition. These receptors were recently were recently identified in our lab (data collected by Casie Lindsly). Overall, we expect that cannabinoids can affect the firing properties of mossy cells by regulating the action potential independent release. The future goal of our lab is to conduct more experiments to prove the above described hypotheses.

One obvious question from observing this unusual plasticity would be if these novel CB-like receptors are located on the excitatory terminals to mossy cells. Our lab does have some data which does suggests that bath application of WIN55, 212-2 increases mEPSCs to mossy cells. However, based on those results we cannot predict if there is any selective expression of the novel CB-like receptors on the excitatory inputs. We also need to increase the n value of these experiments and run other controls to substantiate this conclusion. Based on the data we have, it would be interesting to speculate that if the tonic $GABA_B$ receptors on mossy fiber terminals are blocked by a $GABA_B$ receptor antagonist then an activity dependent release of cannabinoids or bath application of cannabinoid agonists in the presence of a CB1 receptor blocker AM251 would lead to an increase in glutamate transmission to hilar mossy cells and thereby increase their excitability.

It will be very interesting to determine how the endocannabinoid mediated yet CB1 receptor independent increases in miniature transmission may be altered during chronic and

acute models of drug abuse. It would then be intriguing to compare the effects of marijuana on the known original CB1 mediated plasticity vs. the novel CB1 independent plasticity. Till date, the only study done on the effect of chronic doses of cannabinoids is on LTP. In these studies it was found that blockade of LTP following chronic exposure of THC was recovered 3 days following THC withdrawal, but was only partially reversed at this time, and was still significantly impaired at 2 weeks of withdrawal. Acute exposure of THC also impairs endocannabinoid mediated LTD and I-LTD both in the NAc and hippocampus (Hoffman et al. 2007; Mato et al. 2004). Hence, we know that acute and chronic doses of THC affect synaptic plasticity's in the key areas of the brain involved in learning and memory and therefore it would be interesting to determine the role of novel CB-like receptors in these drug abuse models. Finally, it would be also be very exciting to investigate the role of these novel CB-like receptors in neurogenesis. This area has received a lot of attention in the last decade. Recent literature supports a role for CB1-dependent increases in neurogenesis (Di Marzo et al. 2002; Jiang et al. 2005; Kim et al. 2006). However, paradoxical results also indicate the involvement of non CB1 receptors in neurogenesis. For example, one study reported an anandamide mediated reduction in neurogenesis in the dentate gyrus (Rueda et al. 2002). Further, in 2004 Jin et al. found that SR141716A-mediated increases in neurogenesis were preserved in CB1 knockouts, but absent in VR1 knockouts (Jin et al. 2004). Together, these results point towards a VR1 mediated neurogenesis. This idea is also consistent with the previous finding that anandamide and SR141716A interact with VR1 receptors (Zygmunt et al. 1999). However, we also found from my current data that anadamide is very efficacious in activating the novel GPCR receptor. Although at this point we do not know the sensitivity of the novel CB-like receptor to

SR141716A, we should not eliminate the hypothesis that this novel GPCR can also affect neurogenesis.

In the end, we found that both the cholinergic and endocannabinoid signaling systems can regulate excitability of mossy cells by modulating transmitter release from its afferents. My research work does not provide any exact role of these signaling mechanisms in the treatment of epilepsy but it does provide us with fundamental information on how synaptic transmission to mossy cells can be regulated under normal physiological conditions. We hope that these findings will be useful for identifying therapeutic targets in chronic models of drug abuse and other disease models like epilepsy and Alzheimer's.

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BIOGRAPHICAL SKETCH

Chinki Bhatia was born to Mr.C.G.Bhatia and Mrs. Indu Bhatia . She completed her high school studies from The Indian School, Bahrain, 1996. She received bachelor's in pharmacy from the Birla Institute of Technology, Mesra, Ranchi in May 2002. During the following years she joined the University of Sydney to do a master's in In vitro Fertilization. In July 2004, she entered the Graduate School at The University of Florida. She received her Doctorate in August, 2009.