Modulation of Hippocampal Endocannabinoid Plasticity by Homer Proteins and Delta(9)-Tetrahydrocannabinol

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Abstract

The endocannabinoid (eCB) system comprised of lipophylic signaling molecules, postsynaptic production enzymes, and presynaptic G-protein coupled receptors (GPCR) has recently emerged as a key mediator in a broad range of neuronal plasticity. Initial interest in the system stemmed from, Δ^9-tetrahydrocannabinol (THC) the main psychoactive ingredient in the illicit drug marijuana which elicits various effects on the central nervous system (CNS) through interactions with the eCB system. Further study described a nearly ubiquitous yet tunable system that may underlie many CNS functions. Studying the eCB system offers insight into the basic science of synaptic transmission but more importantly the mechanism by which postsynaptic determinants may influence their synaptic inputs to adapt to changing CNS conditions. The objective of these studies was to determine the properties of THC that dictate its relationship to the intact eCB system and to characterize how specific members of the homer family of postsynaptic scaffolding proteins impact differing forms of eCB-mediated plasticity.

Activation of the cannabinoid receptor-1 (CB1R) by THC or eCBs leads to several presynaptic consequences, but most importantly to inhibition of voltage-gated calcium channels (VGCC) and thus a reduction of action potential induced neurotransmitter release. In the first study presented here, we described a voltage-mediated switch by which THC acts as an agonist of CB1R or as an antagonist to effects at CB1R mediated by eCBs. Using patch clamp electrophysiology to study excitatory synaptic transmission we discovered that the rate at which the presynaptic

neuron is depolarized has drastic effects on THC mediated inhibition. Excitatory postsynaptic currents (EPSCs) evoked at 0.1 Hz were suppressed by THC, however THC did not effect EPSCs evoke at 0.5 Hz. Experiments using other CB1R agonists Win55212,2 (Win-2) and 2-Arachydonylglycerol (2-AG) did not demonstrate sensitivity to stimulus rate. THC application at 0.5 Hz stimulation antagonized the inhibition produced by Win-2. THC but not Win-2 inhibited influx of calcium through VGCCs in a rate dependent manner. Win-2 but not THC shifted the voltage-dependent activation of tail currents. THC but not Win-2 mediated inhibition of VGCCs displayed mild prepulse facilitation. THC antagonized depolarization-induced suppression of excitation (DSE). These findings illustrate the complex interplay between THC and the eCB system and suggest that behavioral responses to THC may stem from a combination of both CB1R activation and inhibition of eCB action at CB1R.

Postsynaptic induction of the eCB system and subsequent production of eCBs may be achieved via several interrelated molecular mechanisms that focus around glutamatergic ionotropic and metabotropic signaling and postsynaptic intercellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Homer, a family of postsynaptic scaffolding proteins, is present in the hippocampus and has been found to organize many of the structural and mechanistic proteins necessary for several forms of synaptic plasticity. In the second study we describe a homer isoform-specific switch in eCB plasticity that primes neurons for ionotropic mediated production of eCBs but shifts them away from metabotropic eCB production. Transfection of hippocampal cultures with homer 1a

(H1a) enhances DSE. Transfection of cultures with H1a inhibits metabotropic suppression of excitation (MSE). In combination experiments on the same neuron with DSE followed by MSE, DSE is enhanced and MSE is inhibited in H1a expressing neurons. Brain derived neurotrophic factor (BDNF) induces formation of H1a mRNA and leads to functional expression of H1a which dissociates homer 1c-GFP puncta. BDNF mediates an enhancement of DSE and inhibition of MSE similar to that found in H1a transfected neurons. These findings identify H1a as a crucial mediator of eCB signaling that may be induced by BDNF and can lead to an enhancement of depolarization mediated eCB production. The second study delineated how postsynaptic homer proteins can influence differential paths to activating eCB mediated synaptic plasticity.

The physiological manifestations of eCB signaling have tentacles that extend from neurotransmitter release to higher brain function through the regulation of Ca^{2+} channels and induction of synaptic plasticity. These studies are directed toward understanding the way in which THC interacts with the eCB system, and the postsynaptic scaffolding protein complement which induces changes to eCB-mediated plasticity. Delineating these concepts may lead to better understanding of higher brain function and will offer new targets for therapeutic development.

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Abbreviations

$[Ca^{2+}]_i$	Intercellular calcium concentration
2-AG	2-arachydonylglycerol
AEA	Anandamide
AM251	N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamid
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
BDNF	Brain-derived neurotrophic factor
CB1R	Cannabinoid receptor-1
CC	Coiled coil
CNS	Central nervous system
DAG	Diacylglycerol
DAGL	Diacylglycerol lipase
DSE	Depolarization-mediated suppression of excitation
DSI	Depolarization-mediated suppression of inhibition
eCB	Endocannabinoid
eCB-LTD	Endocannabinoid-mediated long-term depression
EPSC	Excitatory postsynaptic current
FDA	Food and Drug Administration
GPCR	G-protein coupled receptor
GRK	G-protein receptor kinase

H1a	Homer 1a
H1c	Homer 1c
H1c-GFP	Homer 1c-GFP
H2a	Homer 2a
H3a	Homer 3a
I_{Ca}	Calcium current
IP_3	Inositol 1,4,5-trisphosphate
IPSC	Inhibitory postsynaptic current
ISI	Inter spike interval
LTD	Long-term depression
LTP	Long-term potentiation
mAchR	Muscarinic acetylcholine receptor
MAPK	Mitogen-activated protein kinase
MGL	Monoacylglycerol lipase
mGluR	Type 1 metabotropic glutamate receptor
ms	Milliseconds
MSc	Multiple Sclerosis
MSE	Metabotropic suppression of excitation
MSI	Metabotropic suppression of inhibition
mV	Millivolts
nA	Nano amperes
NAPE-PLD	N-acyl phosphatidylethanolamine phospholipase D

NMDAR	N-methyl-D-aspartic acid receptor
pA	Pico amperes
PIP2	Phosphatidylinositol 4,5-bisphosphate
PLCβ	Phospholipase Cβ receptor
RT-PCR	Reverse transcriptase – polymerase chain reaction
RyR	Ryanodine receptor
SR141716A	Rimonabant
THC	Δ^9-tetrahydrocannabinol
VGCC	Voltage-gated calcium channel
Win-2	Win55212,2

Summary of Manuscripts

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Chapter One: Introduction

The use of Marijuana (*Cannabis Sativa*) as a modifier of human consciousness has been demonstrated throughout recorded human history. The Marijuana plant has functioned as an herbal remedy and an adjunct to spiritual ceremonies as far back as 5000 years (Mechoulam, 1986). Currently, recreational use of the drug can be found worldwide with users pursuing the psychological high achieved by inhalation or ingestion of Marijuana and its derivatives. Consumption leads to a euphoric state, followed by sedation, changes to the perception of self and environment, and loss of motor coordination (Abood and Martin, 1992). Marijuana mediates psychoactive effects through a handful of phytocannabinoid compounds, the most potent of which is Δ^9-Tetrahydrocannabinol (THC).

Despite historical use, THC was not identified by medical science until the mid 20th century (Mechoulam and Gaoni, 1967) and not until the early part of the last decade was the cannabinoid receptor (CB1R) identified and cloned (Matsuda et al., 1990). Purification of endogenous ligands for CB1R commenced and two lipophilic molecules, Anandamide (AEA) and 2-Arachydonyl Glycerol (2-AG) were soon identified as common receptor agonists that were released postsynaptically and affected presynaptic CB1R (Devane et al., 1992; Sugiura et al., 1995; Stella et al., 1997). Thus, the endocannabinoid (eCB) system is comprised of presynaptic CB1R, endogenous ligands, postsynaptic synthesis machinery, and eCB degrading enzymes. The aim of this work is to describe the contribution of the homer family of postsynaptic scaffolding proteins to the mechanism of eCB-mediated plasticity and to characterize how THC may interact with the eCB system to cause its psychotropic effects.

I. Cannabinoid Pharmacology

THC is one of approximately 60 cannabinoid compounds synthesized by the Cannabis plant and is responsible for most of the psychotropic effects seen following human or animal consumption (Felder and Glass, 1998; Hall and Solowij, 1998). Despite the influence of THC on the central nervous system (CNS), it is not the only mediator of change in animals or human beings who consume cannabis. Thus, THC is not considered the sole active ingredient in marijuana smoke. Cannabis consumption mediates multiple physiological effects and are manifested both in the short and long term.

The short-term effects of cannabis consumption are many, and vary from person to person. They include many effects extended from CNS control of physiology and thus are most likely caused by THC, but also influence other processes. After inhalation of cannabis smoke, cannabinoids, especially THC enter the circulation and begin to alter CNS function. Users report a host of experiences that include euphoria, loss of short-term memory, cognitive dysfunction, motor incoordination, and possible psychosis. Physiologically users experience tachycardia, orthostatic hypotension, and increased appetite (Abood and Martin, 1992). As will be discussed later, the ubiquitous distribution of CB1R readily lends itself to widespread cognitive dysfunction upon activation.

Persistent use of cannabis has a host of consequences which are hard to separate from the apathy and general malaise of the long-term drug user (Abood and Martin, 1992). Users are more prone to cancers of the lungs and mouth (Sarafian et al., 1999), immune suppression (Dittel, 2008), lowered sperm counts (Nahas et al.,

2002), and developmental abnormalities if taken as an adolescent or while pregnant (Trezza et al., 2008). Users develop tolerance to THC, and often maintain use of the drug from habit, rather than from intoxicative pleasure (Fan et al., 1994; Bass and Martin, 2000). Despite disregard and disinformation in popular culture regarding its addictive potential, THC is addictive (Kelly et al., 1994). Experiments in both animal and human subjects report reinforcing effects of THC administration (Martellotta et al., 1998; Haney et al., 2008) and withdrawal symptoms upon abstinence (Jones et al., 1981; Haney et al., 2005; Lichtman and Martin, 2005). Although cannabis consumption affects multiple physiological processes through several cannabinoid compounds, THC remains the central player in CNS cannabinoid pharmacology.

Cannabinoids have potential as therapeutic agents for a broad range of maladies (Pertwee, 2007, 2009). Despite the ongoing discussion on the use of the cannabis plant as a stand alone therapy, and both the legal and ethical issues surrounding it (Seamon, 2006), researchers have pushed forward in identifying pathologies that are assuaged by use of synthesized or purified cannabinoid compounds.

THC is a potent appetite enhancer and antiemetic that may be used in AIDS wasting and cancer chemotherapy patients. Cannabinoid derivatives are packaged and marketed as two separate preparations that have undergone clinical trial and Food and Drug Administration (FDA) approval in these capacities (Pertwee, 2009). Nabilone (trade name Cesemet), a synthetic analogue of THC, was licensed in 1981 as an antiemetic. Later THC was approved to treat chemotherapy-induced emesis (1985) and as an appetite stimulant (1992), marketed as dronabinol (trade name

Marinol). THC-induced stimulation of appetite is well established (Foltin et al., 1986; Kirkham and Williams, 2004), and its antiemetic properties were comparable to those of other pharmacological agents (Penta et al., 1981). Prescriptions are commonly filled for the latent phase of chemotherapy-induced emesis and offer a dual palliative effect of decreasing emesis while stimulating appetite. Dronabiol is currently FDA approved as a palliative agent to prevent the weight loss and wasting seen in patients with AIDS.

THC effectiveness as an appetite stimulant was the theoretical basis for rimonabant (SR141716A), the only cannabinoid antagonist to be clinically available. SR141716A was used as a CB1R antagonist for *in vitro* studies; however its impact on metabolism and appetite has catapulted it into the pharmaceutical realm (Di Marzo, 2008). The European Medicines Agency (EMEA) had accepted SR141716A to treat metabolic syndrome in the EU in June 2006. By antagonizing CB1R, SR141716A is hypothesized to decrease appetite in a way opposite to the stimulation of appetite seen when THC activates CB1R. Clinical trials found a 4-6 kg loss in total body weight and significant improvement in measures of metabolic syndrome including increased HDL, increased glucose tolerance, decreased triglycerides as well as reductions in waist circumference (de Kloet and Woods, 2009). Adverse psychiatric effects were found in patients without previous histories, and thus FDA approval for SR141716A was denied in the US. Recently (November 2008), the EMEA withdrew marketing authorization for rimonabant following persistent reports of adverse effects, and has essentially eliminated its use.

THC is a compound almost ideally suited as a neuroprotective agent especially in the case of excessive excitation. Excitotoxicity of neurons follows disproportionate release of glutamate that often stems from stroke, neurodegenerative disease, or other traumatic brain injury. Glutamate over-activates postsynaptic receptors (AMPA, NMDA, and Kainate) and causes unmitigated damage due to excess postsynaptic Ca^{2+} (Arundine and Tymianski, 2003). THC has been shown to reduce Ouabain (a Na^{+}/K^{+} - ATPase inhibitor) induced excitotoxicity in rat neonates (van der Stelt et al., 2001) and NMDA-mediated retinal toxicity (El-Remessy et al., 2003) in CB1R dependant manner. In addition, THC is neuroprotective through mild inhibition of presynaptic glutamate release mediated by CB1R (Shen and Thayer, 1998b; Lundberg et al., 2005; Gilbert et al., 2007). This attenuation of synaptic transmission lowers excitatory output, but does not grossly inhibit glutamatergic neurotransmission that may result in unwanted effects seen in more efficacious synaptic blockade (Aarts and Tymianski, 2003; Heresco-Levy, 2003).

Patients diagnosed with multiple sclerosis (MSc) have also found some respite in cannabinoid-based pharmaceuticals. Early experiments in a rodent model of MSc found THC treated mice displayed few signs of MSc, and had a greater survival rate (Lyman et al., 1989). Human clinical trials however were less promising, and shied away from a THC therapy. Later studies did show a statistical enhancement of MSc relief after combinations of THC and cannabadiol (a non-CB1R activating phytocannabinoid) were administered (Wade et al., 2004). In 2005, the FDA approved a 1:1 combination of THC and cannabadiol to treat spasticity and neuropathic pain (Pertwee, 2009). Marketed under the name Sativex, it is a

sublingual spray that has shown some objective improvement of MSc symptoms (Costa, 2007).

THC has undergone trials as an antinocioceptive agent (Costa, 2007). It has been found to have comparable potency and act synergistically with morphine (Welch and Dunlow, 1993; Pugh et al., 1996). THC has succeeded in paradigms for both acute pain and chronic pain/hyperalgesia and may be administered orally, intravenously, or through direct CNS application (Hosking and Zajicek, 2008). THC alleviation of nocioceptive responses are CB1R dependent as CB1R antagonists and $CB1R^{-/-}$ mice demonstrated increased response to nocioceptive stimuli (Ledent et al., 1999; Zimmer et al., 1999).

As with other G-protein coupled receptor (GPCR) systems, both full and partial endogenous agonists for CB1R have been identified. They are synthesized postsynaptically from lipid precursors, and mediate biological responses in the presynaptic neuron through activation of presynaptic CB1R. The two most prominent endogenous agonists for CB1R are AEA and 2-AG. Both are readily synthesized and purified from brain extracts, and are inducible messengers of eCB signaling. AEA is a partial agonist of CB1R and was identified first from pig brain extracts (Devane et al., 1992). It binds CB1R with high affinity (K_i = 52 nM) and is enzymatically inactivated by fatty acid amide hydroxylase (FAAH) localized in postsynaptic dendrites and soma (Egertova et al., 2003; McKinney and Cravatt, 2005). Long thought to be a product of transacylation of phosophatidylinositol and subsequent degradation by N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), it was recently discovered that NAPE-PLD knockout mice retained normal levels of

AEA (Leung et al., 2006). Subsequent research identified that instead α/β hydroxylase 4 and phospholipase Cβ (PLCβ) may be the catalytic enzymes for AEA (Liu et al., 2006; Simon and Cravatt, 2006). Despite initial interest, AEA has been downplayed as an eCB with the potential to modulate central synaptic plasticity (Sugiura et al., 2006). Rather, AEA plays an active role in pain neurotransmission and inflammation (Schlosburg et al., 2009). However due to its low concentration in brain tissue, weak agonist properties, and poor specificity (Di Marzo et al., 2002), AEA may not effectively modulate CNS cannabinoid signaling.

2-AG is the likely mediator of central eCB synaptic plasticity (Sugiura et al., 2006). It was discovered soon after AEA (Mechoulam et al., 1995; Sugiura et al., 1995), was found at concentrations up to 800 times higher than AEA in brain extracts (Kondo et al., 1998; Jung et al., 2005; Sugiura et al., 2006), and fits the mold as the endogenous CB1R activator. 2-AG is a full agonist and is synthesized "on demand" after production of diacylglycerol (DAG) from aracidonate containing membrane phospholipids which are heavily represented in synaptosomes (Marsicano et al., 2003; Piomelli et al., 2007). Subsequent enzymatic catalysis to 2-AG by DAG lipase completes the synthesis reaction. 2-AG binds CB1R with a modest K_i of 15 μM, and elicits significant cellular responses in neurons (see following section on CB1R). Although other endogenous agonists exist for CB1R (PEA, Virhodamine) none have the potential of 2-AG as an inducible activator of CB1R.

THC is partial agonist of CB1R and only weakly stimulates G-protein release upon receptor activation (Chaperon and Thiebot, 1999; Shen and Thayer, 1999; Breivogel and Childers, 2000a). Despite its weak properties at CB1R, THC is

allowed for scientific testing only under strict DEA oversight in the United States. THC binds quite readily to CB1R, with a K_i value of 37-199 nM (Agurell et al., 1986). However due to its combination of weak receptor activation and strong affinity, it has the well demonstrated effect of occluding or antagonizing full CB1R agonists (Blackstone and Sheng, 1999; Kelley and Thayer, 2004; Straiker and Mackie, 2005). Likewise its lipophilicity makes experiments on its short-term effects difficult and precludes removal of the drug from cellular systems (Lundberg et al., 2005). The challenging physical properties of THC in combination with the regulatory culture surrounding it, has precipitated the synthesis and use of other cannabinoid compounds to better dissect THC actions within the CNS. Even under ideal conditions these synthetic agonists can only represent THC effects *in vivo* and cannot completely mimic them.

The discovery of CB1R led to a veritable explosion of agonists by the pharmaceutical industry. Despite a long list of candidate molecules with varying chemical characteristics, Win55212,2 (Win-2) has remained the workhorse of the cannabinoid investigator. Win-2 has broached many of the scientific and legal hurdles encountered by researchers of cannabinoid pharmacology offering an effective activator of CB1R without all the mess (lipophilicity issues) and legal ramifications inherent to THC research. Win-2 is a potent full CB1R agonist that has a strong binding affinity for CB1R (K_i = 62 nM) and elicits potent release of G-proteins from the receptor (Childers, 2006) leading to inhibition of presynaptic Ca^{2+} channels (Twitchell et al., 1997; Shen and Thayer, 1998a). It is soluble in water and thus agonist effects are reversible over short periods (Shen and Thayer, 1999;

Kouznetsova et al., 2002). It is readily bioavailable and mimics many physiological characteristics of THC (Compton et al., 1992). It is used in biochemical, neurophysiological, and behavioral paradigms, and has proven a versatile pharmacological tool (Davies et al., 2002). Although Win-2 is often substituted for THC in biological experiments, it has been noted that Win-2 does not fully mimic THC effects in a number of systems.

Selective antagonists of CB1R have been developed. SR141716A was the first widely available for basic research, and soon moved into clinical trials (Rinaldi-Carmona et al., 1994b; Barth and Rinaldi-Carmona, 1999). In research circles SR141716A remains a powerful tool to dissect CB1R pharmacology. SR141716A is a selective antagonist of CB1R with a potent affinity (Kd = 1.2 nM) and in general antagonizes CB1R agonist effects both *in vitro* and *in vivo* (Pertwee, 2005). Questions of its specificity and potential agonist effects have clouded use as an antagonist of CB1R in recent years (Jarbe et al., 2002; De Vry and Jentzsch, 2004). More commonly N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251) is used experimentally as a CB1R antagonist and is a structural analogue of SR141716A. It is a CB1R specific antagonist and exhibits potent inhibition of CB1R (K_i=7.5nM) and readily reverses cannabinoid mediated effects, yet does not seem to posses any agonist properties. Many classic CB1R antagonists (including AM251 and SR141716A) have been recently reclassified as inverse agonists that induce effects opposite to CB1R activation most likely through inhibition of tonic eCB receptor activation. So-called

"neutral" CB1R antagonists that do not influence CB1R have begun to be developed (Pertwee, 2005).

In Chapter Two we describe a presynaptically driven activity dependent switch for THC at CB1R. Weak THC activation of CB1R is mitigated and results in effective antagonism of the receptor from both exogenous and endogenous cannabinoid agonists. This clarifies the ongoing dilemma of how THC seems to exhibit both agonist and antagonist properties, and suggests THC agonist properties are so weak that it primarily antagonizes the eCB system.

II. Cannabinoid Receptor Type I (CB1R)

The discovery of THC as the active ingredient in Cannabis led to intensified research into its method of action within the mammalian CNS. Early theories on THC method of action included the inhibition of Na^+ channels (Turkanis et al., 1991), non-specifically changing membrane fluidity and conductance (Pertwee, 1988), as well as the possibility of a classic receptor system directly activated by THC. After much debate, binding of a radiolabeled cannabinoid agonist, CP 55,940, led to the cloning of an orphan GPCR designated as the cannabinoid receptor (Matsuda et al., 1990). Subsequent research has identified other receptors activated by cannabinoids including a second cannabinoid receptor present in glial and immune populations (CB2R), vanilloid receptors activated by AEA, and others (Smart et al., 2000; Mackie and Stella, 2006). These other cannabinoid receptors modulate pain, immune response, and other less well characterized systems, but their description is outside the scope of this review.

CB1R is a member of the seven transmembrane G-protein coupled receptor (GPCR) super-family (Matsuda et al., 1990). It is coupled to inhibitory G-proteins ($G_{i/o}$) (Howlett et al., 1986; Bidaut-Russell et al., 1990; Childers et al., 1992; Compton et al., 1993), but may associate with G_s when $G_{i/o}$ interaction is blocked by pertussis toxin (Glass and Felder, 1997; Felder and Glass, 1998). Activation of CB1R with exogenous or endogenous cannabinoids causes dissociation of $G_{i/o}$ from CB1R and mediation of downstream targets that can effect both short-term and long-term changes to neuronal physiology (Figure 1.1). CB1R activation augments the mitogen-activated protein kinase (MAPK) signaling cascade and negatively regulates

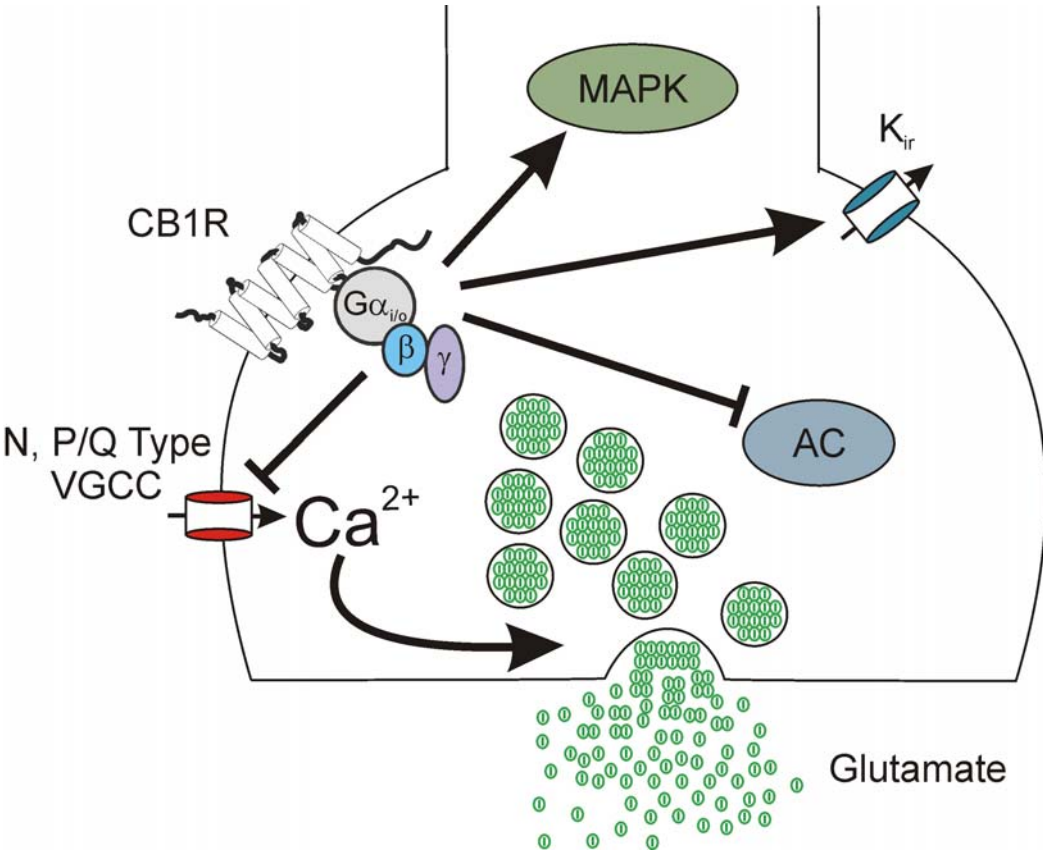

Figure 1.1. Downstream targets of cannabinoid receptor-1 (CB1R) activation. CB1R agonists precipitate multiple downstream effects to modulate short term and long term effects on synaptic transmission. The mitogen-activated protein kinase (MAPK) pathway is stimulated which influences long term transcription in the presynaptic neuron. Inward rectifying potassium channels (K_{ir}) are activated and enhance repolarization. Adenylate cyclase (AC) is inhibited. Most importantly Ca^{2+} conductance through N and P/Q type voltage gated calcium channels (VGCC) are inhibited which directly depresses neurotransmitter release.

adenylate cyclase (AC). In the short term however, CB1R immediately affects the biophysical mechanics of neurotransmission. Inward rectifying K^+ channels (Henry and Chavkin, 1995; Guo and Ikeda, 2004) and A-type K^+ currents (Deadwyler et al., 1993) enhance neuronal repolarization while N and P/Q-type voltage gated calcium channels (VGCC) are inhibited, lowering presynaptic Ca^{2+} influx and subsequent release of neurotransmitters (Twitchell et al., 1997; Shen and Thayer, 1998a). Coupling of CB1R activation to G-proteins is inefficient (Sim et al., 1996b; Breivogel et al., 1997) and thus $G_{i/o}$ interaction with downstream effectors may be more important to presynaptic effects than the quantity of receptors present. CB1R is highly expressed in many regions of the mammalian brain including basal ganglia, substantia nigra, globus pallidus, cerebellum, and hippocampus (Herkenham et al., 1991; Jansen et al., 1992; Mailleux and Vanderhaeghen, 1992; Thomas et al., 1992; Matsuda et al., 1993) as well as in peripheral tissue. Expression levels are approximately equal to those of the ionic glutamate receptor and GABA receptor, and CB1R has been identified as the most abundant GPCR in the brain (Devane et al., 1988; Herkenham et al., 1990; Howlett et al., 2002; Piomelli, 2003). Thus, THC activation of CB1R and attenuation of synaptic transmission at these synapses can lead to widespread malfunction of normal CNS processing. Despite some debate, CB1R is presynaptically localized throughout the CNS in heterogeneous populations of both excitatory and inhibitory boutons (Kawamura et al., 2006). Early articles pointed to this expression profile (Herkenham et al., 1990; Matsuda et al., 1990) and further anatomical, electron microscope (EM), and electrophysiological experiments confirmed its presynaptic localization (Freund et al., 2003). Although widely

expressed, CB1R is far from ubiquitous within neuronal populations of CNS structures. In the hippocampus alone, the three formations (CA3, CA1, and dentate gyrus) display varied CB1R expression (Katona et al., 2006). Heterogeneous populations of both inhibitory and excitatory neurons also express different levels of CB1R throughout the CNS, making electrophysiological experiments crucial to determining a cell-by-cell relationship between presynaptic and postsynaptic eCB physiology. In the hippocampus, excitatory neurons display strong CB1R expression throughout but; interestingly only a small subset of GABAergic neurons express CB1R (Katona et al., 2006). For several years, a third cannabinoid receptor was proposed in the hippocampus, but later found to be an aberration of mouse transgenics (Hajos et al., 2001; Hajos and Freund, 2002). In addition, a widely disseminated CB1R specific antibody pointed to hippocampal expression only on the small percentage of GABAergic neurons, but the antibody omitted strong glutamatergic expression (Kawamura et al., 2006).

Tolerance to the behavioral effects of cannabinoid agonists is well-established (Pertwee et al., 1993). CB1R desensitization *in vitro* is evidenced by reductions in GTPβS binding after agonist treatment (Sim et al., 1996a) and desensitization of cannabinoid inhibition of AC (Dill and Howlett, 1988). Studies confirmed this with the discovery of G-protein receptor kinase (GRK) phosphorylation leading to β-arrestin mediated internalization via clathrin coated pits (Hsieh et al., 1999; Jin et al., 1999; Kouznetsova et al., 2002). CB1R desensitization in the hippocampus can occur at sub-chronic or chronic dosing (Kouznetsova et al., 2002; Luk et al., 2004; Lundberg et al., 2005) and leads to desensitized responses to normal cannabinoid

effects on neurotransmitter release and excitatory networks (Kouznetsova et al., 2002; Lundberg et al., 2005). Several reports demonstrated THC mediated desensitization of eCB signaling despite no short-term effect of THC on excitatory postsynaptic currents (EPSC) (Straiker and Mackie, 2005, 2006). The authors argued that low receptor density might have masked the direct effects of THC while prolonged exposure led to adequate CB1R desensitization.

In Chapter Two I describe how the weak agonist properties of THC at CB1R and the voltage dependence of presynaptic N-type VGCCs combine to limit THC inhibition of EPSCs to slow firing synapses, but not fast firing synapses. Although THC interaction with CB1R was established in the early 1990's, it was not until a decade later that the physiological consequences of eCB signaling were realized.

III. Endocannabinoid Plasticity

Around the same time eCBs became known to biological science as endogenous activators of CB1R, an interesting neurological phenomenon in the hippocampus was discovered (Pitler and Alger, 1992; Pitler and Alger, 1994). Dubbed depolarization induced suppression of inhibition (DSI), hippocampal neurons that were stimulated for extended periods transiently inhibited neurotransmitter release from presynaptic inputs. Concurrently a similar effect was noted in excitatory (DSE) and inhibitory purkinje cells of the cerebellum (Llano et al., 1991; Vincent et al., 1992; Vincent and Marty, 1996). This transient effect could be induced through stimulating trains of action potentials or extended postsynaptic depolarizations for multiple seconds. This phenomena enjoyed few proponents until it was demonstrated to be mediated by retrograde eCB signaling in two landmark papers (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Shortly thereafter, eCB actions were noted in inhibitory and excitatory synapses of the cerebellum (Kreitzer and Regehr, 2001; Maejima et al., 2001a), hippocampus (Ohno-Shosaku et al., 2001; Straiker and Mackie, 2005), cortex (Bacci et al., 2004), and striatum (French et al., 1997). Further study has revealed two roads to eCB production through depolarization or activation of postsynaptic metabotropic receptors. These stimulatory pathways lead to five forms of eCB plasticity that have underscored a major mechanism of adaptation in neuronal networks.

DSE/I results from depolarization of postsynaptic neurons, eCB production in a Ca^{2+} sensitive manner, diffusion of eCBs to presynaptic CB1R, and inhibition of presynaptic neurotransmitter release (Figure 1.2). The enzymes involved in

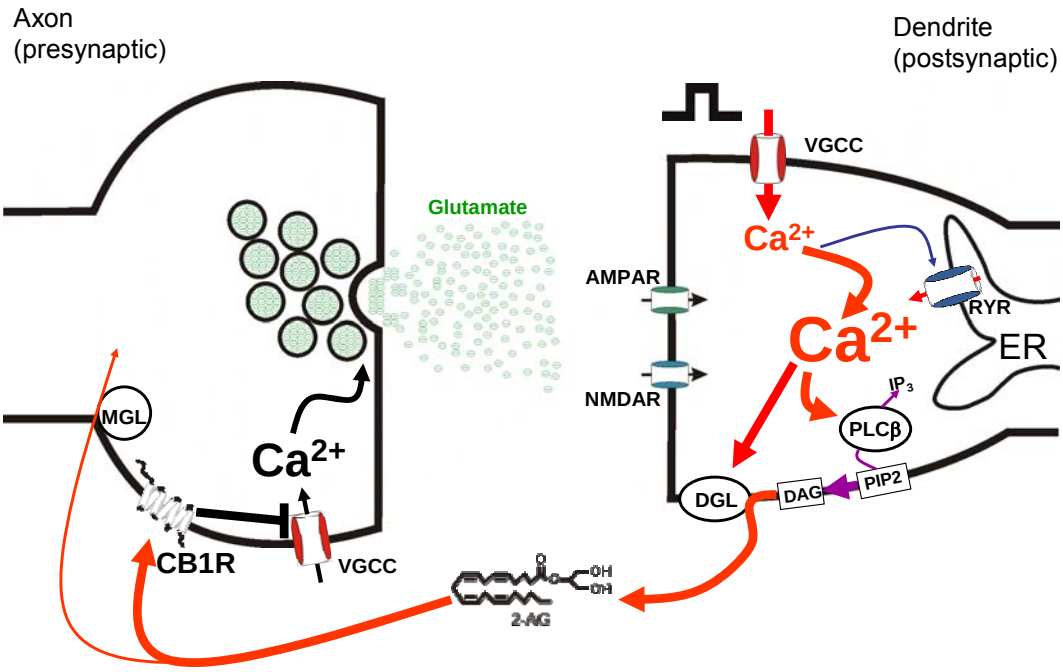

Figure 1.2. Depolarization induced suppression of excitation (DSE). Prolonged depolarization of postsynaptic neuron leads to Ca^{2+} influx through voltage gated calcium channels (VGCC). Perhaps assisted by Ca^{2+} release from the endoplasmic reticulum (ER) through ryanodine receptors (RYR), postsynaptic Ca^{2+} then enhances activity of phospholipase Cβ (PLCβ) and diacylglycerol lipase (DGL). PLCβ cleaves membrane phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG is then catalyzed by DGL into 2-arachydonylglycerol (2-AG), an endocannabinoid that diffuses to presynaptic cannabinoid receptors (CB1R) and inhibits influx of presynaptic Ca^{2+} through VGCC. Consequently glutamate containing vesicle fusion is inhibited and less glutamate is released to activate postsynaptic AMPA and NMDAR channels. Excess 2-AG is catalytically inactivated by monoacylglycerol lipase (MGL).

cannabinoid mobilization for DSE/I have been identified and convincingly localized to postsynaptic membranes (Katona et al., 2006). PLCβ cleaves the membrane bound phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), into DAG and inositol 3,4,5-triphosphate (IP_3). Synthesis of 2-AG is undertaken by DGL which enzymatically cleaves DAG into 2-AG. 2-AG then diffuses to the presynaptic membranes and activates CB1R. Following synthesis and diffusion of eCBs, 2-AG degradation occurs mostly at the behest of presynaptic MGL, but two other enzymes (α/β hydroxylase 6 and 12) have activity for 2-AG and may serve as redundant degradation enzymes or at special eCB synapses (Blankman et al., 2007). Several papers found cyclooxygenase-2 (COX-2), a lipid metabolizing enzyme, may contribute to 2-AG degradation and DSE/I, but no localization studies have been undertaken (Straiker and Mackie, 2005; Sang et al., 2006). CB1R, MGL and DGL are correspondingly found at synaptic terminals where DSE/I are present (Freund et al., 2003; Gulyas et al., 2004; Straiker and Mackie, 2005), although the presence of CB1R does not always predict DSE/I. Current theories on the mechanisms of DSE/I involve Ca^{2+} mediated activity increases in PLCβ and DGL (Hashimotodani et al., 2005; Maejima et al., 2005; Hashimotodani et al., 2008), although there are reports of DSE/I even under conditions of enzymatic blockade (Hashimotodani et al., 2005; Maejima et al., 2005; Edwards et al., 2006).

The source of calcium for DSE/I has been strongly debated. Depolarization of postsynaptic neurons on the order of seconds will mediate influx of Ca^{2+} through VGCCs. Presumably much of the Ca^{2+} flux will be mediated by L-type VGCC, as they inactivate slowly and thus allow sustained current, despite prolonged

depolarization. L-type VGCC are localized to somatic and dendritic structures, and more recently to the perisynaptic area of the postsynaptic density (PSD) (Tippens et al., 2008). NMDA mediated production of eCBs at inhibitory synapses of the hippocampus has been demonstrated, but the authors were unable to separate NMDA activation from other VGCC responses (Ohno-Shosaku et al., 2007). While entry via L-type VGCC may constitute the bulk of the Ca^{2+} for DSE/I , additional release of ER Ca^{2+} through ryanodine receptors (RyR) contributes to the magnitude of DSE/I in the hippocampus (Straiker and Mackie, 2005; Isokawa and Alger, 2006) and eCB mediated neuroprotection (Zhuang et al., 2005b). An elegant study by Isokawa's group found localization of RyR and L-type VGCC in the hippocampus and that depolarization induced Ca^{2+} release from RyR was impeded by both RyR antagonism and L-type VGCC blockade (Berrout and Isokawa, 2009). Thus, Ca^{2+} sensitization of RyR stores may augment the Ca^{2+} present for initiation of DSE/I.

The second major stimulus of eCB production in CNS neurons is activation of postsynaptic metabotropic receptors and results in eCB plasticity known as metabotropic suppression of excitation (MSE) or inhibition (MSI) (Figure 1.3). First thought to be a simple corollary of DSE/I, MSE/I has retained subtle yet substantial differences in the method of activation in postsynaptic membranes. MSE/I flows from metabotropic receptors through PLCβ and produces DAG as a substrate for DGL to produce 2-AG (Maejima et al., 2001a; Varma et al., 2001; Ohno-Shosaku et al., 2002a; Robbe et al., 2002). 2-AG is then free to diffuse to presynaptic CB1R and inhibit neurotransmitter release in a manner similar to DSE/I. Interestingly only G_q coupled muscarinic acetylcholine receptors (mAchR, M_1-M_3) and G_q coupled type 1

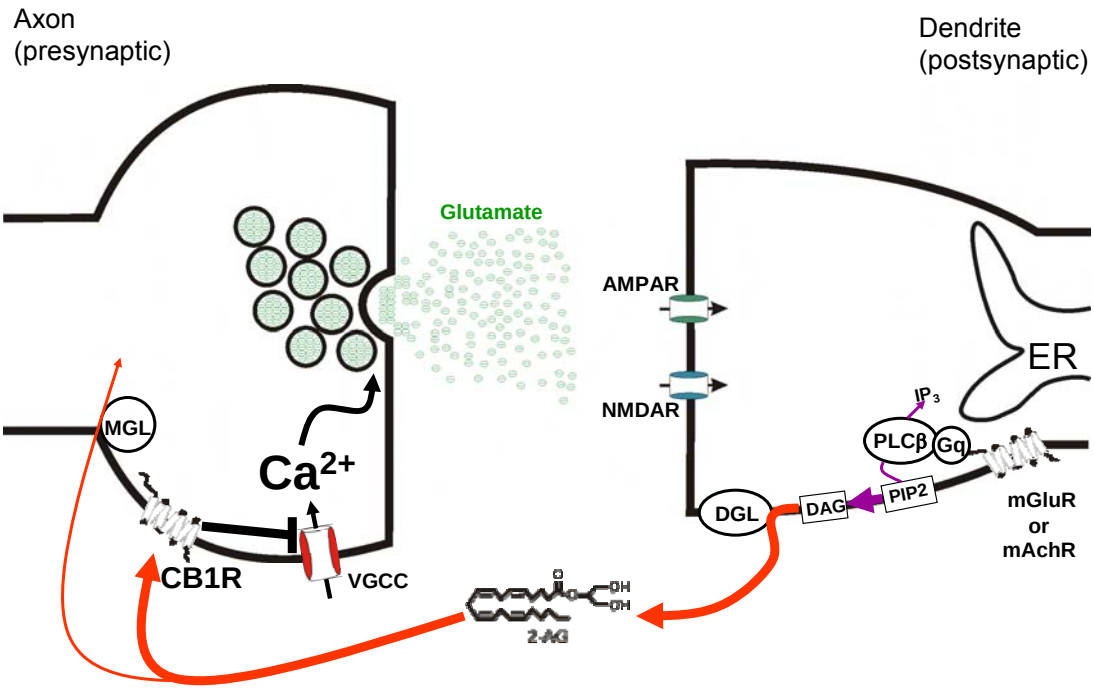

Figure 1.3. Metabotropic suppression of excitation (MSE). Activation of postsynaptic metabotropic receptors leads to production of eCBs. Muscarinic acetylcholine receptor (mAchR) or metabotropic glutamate receptor (mGluR) activation leads to G_q mediated activation of phospholipase Cβ (PLCβ). PLCβ cleaves membrane bound phosophoinositicde 1,6 bisphosphate (PIP2) and produces inositol-3-phosphate (IP_3) and diacylglycerol (DAG). Diacyglycerol lipase (DGL) then uses DAG to produce 2-AG which is free to diffuse to presynaptic CB1R.

metabotropic glutamate receptors (mGluR, mGluR1a,5) have been found to initiate eCB responses in the CNS (Kreitzer and Regehr, 2001; Straiker and Mackie, 2006). PLCβ knockouts prevent mAchR and mGluR mediated MSI (Hashimotodani et al., 2005).

Unlike DSE/I, the necessity of Ca^{2+} in MSE/I has been questioned. Reports in the hippocampus and cerebellum point to Ca^{2+} as an assisting factor which greatly enhances release of eCBs (Kim et al., 2002; Ohno-Shosaku et al., 2003; Ohno-Shosaku et al., 2005). Activation of metabotropic receptors leads to production of DAG, but also results in release of IP_3, an agonist of IP_3 receptors (IP_3R), and a mediator of Ca^{2+} release from the ER. Currently there is no known role for IP_3 or IP_3R in eCB signaling despite coupling to mGluRs (Brakeman et al., 1997a).

While DSE/I and MSE/I are relatively brief, lasting seconds to minutes, they may underlie or initiate eCB mediated plasticity that influence synaptic strength for minutes or even hours. Such changes to synaptic strength use various signaling mechanisms, and lead to long-term potentiation (LTP) or depression (LTD) of synaptic transmission (Malenka and Bear, 2004). Activation of NMDAR can change synaptic strength by modifying the postsynaptic response to neurotransmitters and result in LTD. However, a subset of LTD responses that emanate from the postsynaptic site and inhibit presynaptic neurotransmitter release are in fact eCB mediated (Gerdeman et al., 2002; Robbe et al., 2002; Sjostrom et al., 2003). Electrophysiological experiments demonstrated the presynaptic nature of this form of LTD. Induction shifted the paired pulse ratio (PPR), decreased mini EPSC (mEPSC) frequency but not amplitude, increased synaptic failures, and was absent in $CB1R^{-/-}$

mice (Gerdeman et al., 2002; Robbe et al., 2002; Chevaleyre and Castillo, 2003; Sjostrom et al., 2003). Experiments in postsynaptic neurons further confirmed that LTD flowing from post- to presynaptic receptors is a form of eCB plasticity. It was inhibited by postsynaptic Ca^{2+} chelators (Bender et al., 2006), inhibitors of DGL (Chevaleyre and Castillo, 2003), GDPβS-mediated inhibition of G-protein dissociation (Chevaleyre and Castillo, 2003; Bender et al., 2006), and L-type VGCC blockers (Calabresi et al., 1992; Tang et al., 2001; Wang et al., 2006). Thus, eCBs mediate many forms of LTD (eCB-LTD) resulting in prolonged reduction of presynaptic neurotransmitter release.

eCB-LTD is found at many central synapses (Chevaleyre et al., 2006) and each brain region seems to differ in its induction specificity. Both repetitive synaptic activity that leads to mGluR activation or direct pharmacologic agonists of mGluR can lead to eCB-LTD (Bolshakov and Siegelbaum, 1994; Robbe et al., 2002; Azad et al., 2004). Application of CB1R agonists alone (without presynaptic stimulation) does not initiate LTD, rather a second mechanism must be activated concurrently to initiate a CB1R mediated LTD response. Several candidates have been put forward including slight postsynaptic depolarization (Kreitzer and Malenka, 2005), presynaptic NMDA receptors (Sjostrom et al., 2003), and presynaptic activation (Heifets et al., 2008), but no solid conclusion has yet been reached.

eCB plasticity in all of its forms represents a non-classical view of synaptic networks and the bidirectional effects that may be initiated to change synaptic strength. While great strides have been made, a crucial question of THC effect on eCB plasticity has not been fully addressed. It is well accepted that THC is a partial

agonist of CB1R (Chaperon and Thiebot, 1999; Shen and Thayer, 1999; Breivogel and Childers, 2000b), and due to its partial agonist properties it antagonizes or occludes the effects of more efficacious agonists (Shen and Thayer, 1999; Kelley and Thayer, 2004). To date THC has been shown to inhibit both MSE and DSE in autaptic hippocampal cultures (Straiker and Mackie, 2005, 2006), but these same reports found no direct effect of THC on neurotransmission, despite demonstrable THC mediated CB1R desensitization. Inhibition of LTP was noted in acute brain slices from THC treated rats, but the authors saw continued antagonizing effects despite removal of the drug and substantial recovery time (Mato et al., 2004). Effects of THC *in vivo*, while mostly represented by substitution with Win-2 occasionally yield results that are consistent with both agonist and antagonist properties of THC (McMahon and France, 2003; De Vry and Jentzsch, 2004; Pamplona et al., 2006). To date the rules that govern THC dual agonist/antagonist properties have not been sufficiently explored.

In Chapter Two I describe eCB mediated synaptic plasticity induced by depolarization (DSE). THC modulates eCB plasticity in hippocampal cultures. The *in vivo* consequences of these modifications remain unclear.

IV. Homer Proteins and the Postsynaptic Density

Recent investigations have credited postsynaptic scaffolding proteins in the PSD with regulating excitatory synaptic transmission and plasticity (Okabe, 2007). Postsynaptic signaling cascades (including that of the eCB system) rely on preassembled pathways, and require optimal spatial and functional arrangement. The homer family of postsynaptic scaffolding proteins has recently emerged as central to the organization of functional synaptic transmission and plasticity at excitatory synapses. Surprisingly postsynaptic homer and eCB-related proteins overlap in both binding partners and functional pathways.

The PSD is a physical and theoretical division associated with functional glutamatergic synapses (Okabe, 2007). It is a three dimensional (3D) structure comprised of ion channels, metabotropic receptors, ER proteins, and intercellular signaling proteins. A number of structural proteins that form a lattice upon which signaling cascades may be organized maintain the 3D shape of the PSD (Kreienkamp, 2002). PSD-95 associates with NMDAR and through adapter proteins to AMPAR (Kornau et al., 1995; El-Husseini et al., 2000; Ehrlich and Malinow, 2004), and is considered a significant marker of functional excitatory synapses. Shank and actin form linkages to PSD-95 and homer proteins which incorporate signaling cascades including mGluR-IP3R (Brakeman et al., 1997b). Homer proteins structurally assemble signaling cascades in the PSD through tetramerization with shank (Hayashi et al., 2009). Thus, the role homer proteins play in signal organization and plasticity may underlie neurological function (Brakeman et al., 1997a; Kato et al., 1998; Tu et al., 1998).

Homers are structural proteins that form heteromers and link many pathways involved in postsynaptic excitatory neurotransmission. They are well conserved across animal species including one gene in fruit fly, three in mouse, and three in human (Shiraishi-Yamaguchi and Furuichi, 2007). In humans, homer genes are alternately spliced to form at least 22 individual homer isoforms, that are either long or short (Figure 1.3). Long isoforms contain both an ENA/VASP homology (EVH1) domain which associates with signaling proteins, as well as a C-terminal coiled coil (CC) domain which mediates homer to homer interactions and functional apposition of related signaling proteins including shank (Xiao et al., 1998; Tadokoro et al., 1999; Hayashi et al., 2006). Short isoforms possess an EVH1 domain, but no CC domain and thus uncouple CC-homer assemblies in a dominant negative manner (Xiao et al., 1998; Roche et al., 1999). Homer genes, while present in many organ systems, are predominantly found in the CNS (Shiraishi et al., 2004; Shiraishi-Yamaguchi and Furuichi, 2007). Most central structures express disparate combinations of CC-homer isoforms (Xiao et al., 1998; Shiraishi et al., 2003; Shiraishi et al., 2004), including the hippocampus whose formations display a heterogeneous mix of Homer 1b/c, Homer 2a/b and Homer 3a/b (Shiraishi et al., 2004). CC-homers are enriched in dendrites, and often co-localize with mGluRs (Ango et al., 2000; Shiraishi et al., 2003). CC-homers are essentially constitutively expressed, although changes in expression under pathological or drug induced states have been noted (Swanson et al., 2001; Szumlinski et al., 2004; Szumlinski et al., 2008). Unlike CC-homers, short homers (H1a and Anai-3) are not highly expressed in CNS structures. Instead, their expression is induced by various stimuli, particularly during times of intense synaptic

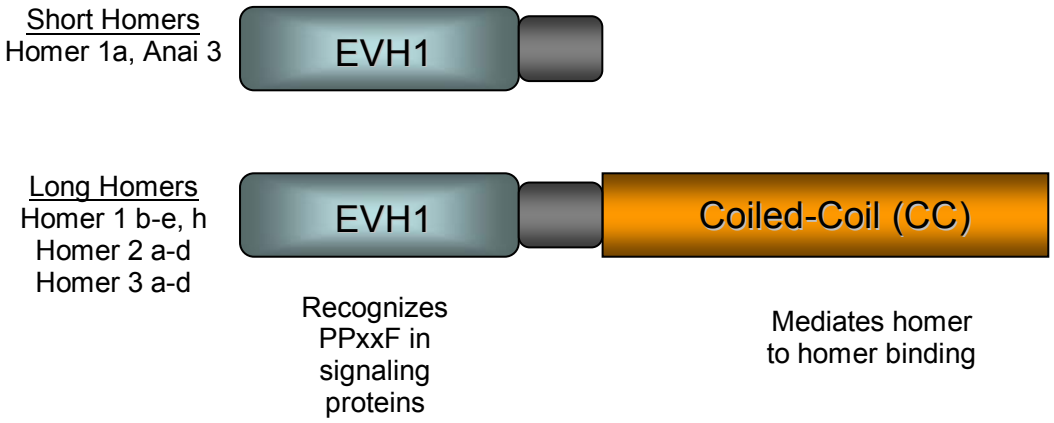

Figure 1.4. Structural overview of Homer Proteins. Homer proteins are most commonly categorized as "long" or "short". Both long and short homers contain an Ena/Vasp homology domain type 1 (EVH1) that recognizes the consensus sequence PPxxF in postsynaptic density signaling and structural proteins. These have been found to include type 1 mGluR, IP_3R, RyR, and shank. Long forms additionally have a coiled-coil domain (CC) which forms homer to homer linkages and organizes signaling cascades on which EVH1 binding proteins may interact. Because short homers have an EVH1 domain, but cannot crosslink to other homers, they function as dominant-negative uncouplers of homer structural and functional assemblies.

activity (Brakeman et al., 1997a; Kato et al., 1997) or growth factor activation (Kato et al., 2003; Kammermeier, 2008).

The EVH1 domain links homers to signaling proteins important for synaptic functionality and plasticity (Worley et al., 2007). EVH1 recognizes proline-rich motifs, especially PPxxF (Beneken et al., 2000; Irie et al., 2002). Binding targets of the EVH1 domain include mGluR (Brakeman et al., 1997a; Kato et al., 1998; Tu et al., 1998), PLCβ (Nakamura et al., 2004; Hwang et al., 2005), L-Type VGCC (Olson et al., 2005; Yamamoto et al., 2005), RyR (Soloviev et al., 2000; Hwang et al., 2003), IP3R (Tu et al., 1998; Yuan et al., 2003), DGL (Jung et al., 2007), Shank (Tu et al., 1999), Actin (Shiraishi et al., 1999) and place them in configurations optimal for signal throughput.

Homer proteins have been linked to synaptic plasticity throughout the CNS and are often associated with responses to drugs of abuse and addiction (Szumlinski et al., 2004; Szumlinski et al., 2008). The most common link to synaptic plasticity is thought to be mGluR mediated Ca^{2+} release through IP_3R in the ER (Worley et al., 2007). In general, CC-homers stabilize mGluR in the PSD and H1a functionally uncouples mGluRs from downstream effectors (Tu et al., 1998; Kammermeier and Worley, 2007; Kammermeier, 2008). Growing evidence suggests homers may play roles in a host of processes related to excitatory synaptic plasticity. Homer 1b and shank regulate the maturation of dendritic spines while H1a inhibits localization of NMDAR, AMPAR and shank (Sala et al., 2001; Sala et al., 2003). The effect of H1a on synaptic transmission has offered conflicting results. Several reports found an H1a-mediated reduction in AMPAR/NMDAR current amplitudes, while others saw

an increase in AMPAR signaling dependant on the ratio of H1a to Homer 1b (Van Keuren-Jensen and Cline, 2006). Homer 2a (H2a) has been linked to proper membrane localization of DGL, the enzyme responsible for 2-AG production (Jung et al., 2007). Despite interactions with many of the players involved in eCB signaling (mGluR, DGL, RyR, VGCC) it remains to be seen how different complements of homer proteins effect eCB-mediated synaptic plasticity.

Drugs of abuse induce changes to homer protein expression within the CNS (Worley et al., 2007; Szumlinski et al., 2008). Homers interact and functionally link proteins that are also involved in eCB signaling including mGluR (Katona and Freund, 2008). Interestingly some of the same stimuli that initiate homer protein expression also have been linked to synaptic plasticity that could potentially be eCB mediated. In Chapter Three we describe the contribution of the homer family of postsynaptic scaffolding proteins to eCB-mediated plasticity in the hippocampus and characterize a H1a gate that favors DSE over MSE.

V. Rationale for Studies

The eCB system plays a prominent role in synaptic plasticity and survival in normal, pathological, and drug induced states. It represents one of the major mechanisms by which postsynaptic neurons can influence presynaptic inputs.

THC is a common drug of abuse that has potential as a therapeutic compound for several neuronal pathologies. Previous studies have characterized THC effects on excitatory synaptic transmission (Shen and Thayer, 1999; Straiker and Mackie, 2005). In Chapter Three I tested the hypothesis that THC may act as an agonist or antagonist of eCB signaling depending on the firing rate of the presynaptic neuron. Using electrophysiology we determined that the inherent voltage sensitivity of N and P/Q-type VGCCs combined with weak THC agonist properties govern THC effects at CB1R. Furthermore, in the presence of exogenous or endogenous CB1R agonists, THC mainly functions as an antagonist. Use of marijuana is widespread and growers of street preparations strive for higher THC content (Compton et al., 2004). Full characterization of THC agonist/antagonist properties in light of eCB signaling will help to not only understand THC in regards to abuse, but also shed light on potential therapies using THC and other cannabinoid agonists.

Homer proteins provide a scaffold for functional pathways in excitatory synapses of the CNS. Previous reports have linked homer isoforms to synaptic plasticity, but few have studied these effects in light of eCB signaling. In Chapter Three I test the hypothesis that H1a gates the mechanism for eCB induction by uncoupling mGluR-mediated eCB production while enhancing depolarization-mediated eCB production. Using electrophysiology, molecular biology, and confocal

imaging we determined that H1a does indeed induce hippocampal neurons to display this mechanistic divide in eCB signaling. Additionally we found that brain derived neurotrophic factor (BDNF) represents a physiological link by which neurons can stimulate H1a production and enhance DSE while blocking MSE. DSE mediates eCB production at active synapses through global Ca^{2+} entry, while MSE is a synapse specific effect driven by persistent firing of one synapse (Brenowitz and Regehr, 2007). CNS pathology involves both widespread neuronal dysregulation and more subtle changes to synaptic strength and survival**.** Understanding the homer protein mediation of specific eCB plasticity may give rise to therapies that direct eCB plasticity to synapses requiring widespread or specific eCB modulation.

In neurons, eCBs modulate synaptic plasticity, reduce excitability, and regulate Ca^{2+} homeostasis. These studies delineate THC interaction with both CB1R and the eCB system and establish H1a as an inducible modulator of postsynaptic eCB production.

Chapter Two: Modulation of excitatory synaptic transmission by Δ^9-tetrahydrocannabinol switches from agonist to antagonist depending on firing rate. Mol Pharmacol. 2009 Apr;75(4):892-900

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Δ^9-Tetrahydrocannabinol (THC), the principle psychoactive ingredient in marijuana, acts as a partial agonist on presynaptic CB1 cannabinoid receptors to inhibit neurotransmitter release. Here we report that THC inhibits excitatory neurotransmission between cultured rat hippocampal neurons in a manner highly sensitive to stimulus rate. THC (1 μM) inhibited excitatory postsynaptic currents (EPSCs) and whole-cell I_{Ca} evoked at 0.1 Hz but, at 0.5 Hz THC had little effect. The cannabinoid receptor full agonists Win55212-2 (100 nM) and 2-arachidonylglycerol (1 μM) inhibited EPSCs independent of stimulation at 0.1 or 0.5 Hz. THC occupied CB1 receptors at 0.5 Hz, but the receptors failed to couple to presynaptic Ca^{2+} channels. Consequently, 1 μM THC blocked the inhibition of EPSC amplitude by Win55212-2 when EPSCs were evoked at 0.5 Hz. A depolarizing prepulse to 0 mV reversed THC inhibition of I_{Ca} but, reversal of the inhibition produced by Win55212-2 required a pulse to +80 mV, suggesting that the voltage-dependent reversal of Gβγ inhibition of voltage-gated Ca^{2+} channels accounts for the frequency dependence of cannabinoid action. THC blocked depolarization-induced suppression of EPSCs evoked at 0.5 Hz, indicating that it inhibited retrograde endocannabinoid signaling in a frequency-dependent manner. Thus, THC displayed a state-dependent switching from agonist to antagonist that may account for its complex actions in vivo.

I. Introduction

Δ^9-Tetrahydrocannabinol (THC), the active agent in the medication dronabinol and the principal psychoactive ingredient in marijuana, exerts its effects on the central nervous system (CNS) via cannabinoid type 1 receptors (CB1R) (Chaperon and Thiebot, 1999). CB1R are G protein-coupled receptors (GPCRs) that activate K^+ channels and mitogen activated protein kinases and inhibit adenylyl cyclase and voltage-gated Ca^{2+} channels (VGCCs) (Howlett, 2005). The short-term inhibition of synaptic transmission by cannabinoids is primarily mediated by inhibition of presynaptic VGCCs (Brown et al., 2004). Endocannabinoids (eCBs) produced by postsynaptic neurons diffuse in the retrograde direction where they too, act on presynaptic CB1R to inhibit excitatory and inhibitory synaptic transmission (Lovinger, 2008). The effects of THC on synaptic transmission and eCB signaling are not entirely explained by it simply mimicking eCBs.

THC is a partial agonist of CB1R as indicated by weak stimulation of GTP-γS binding in rodent brain (Breivogel and Childers, 2000b) and modest inhibition of excitatory postsynaptic currents (EPSCs) (Shen and Thayer, 1999). The CB1R antagonist rimonabant completely blocked THC-mediated inhibition of glutamatergic synaptic transmission. THC produces a sub-maximal response even at concentrations that saturate CB1Rs; thus, THC will attenuate the actions of full agonists, including eCBs (Kelley and Thayer, 2004). In addition to its low intrinsic activity, THC is highly lipophilic, which imbues it with properties incompatible with many experimental techniques including poor washout and insufficient penetration of brain slices (Lundberg et al., 2005; Lovinger, 2008). Thus, more water soluble full CB1

agonists such as [(*R*)-(+)-[2,3-dihydro-5-methyl-3[(4-morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazinyl]-(1-naphthalenyl)methanone mesylate salt] (Win55212-2) are widely used for in vitro studies (Eissenstat et al., 1990). However, the recreational and clinical use of THC provides a compelling reason to study this compound specifically. In the few studies to examine the acute effects of THC on synaptic transmission, the magnitude of the responses varied widely (Shen and Thayer, 1999; Straiker and Mackie, 2005). The low intrinsic activity of THC confers a high sensitivity to CB1R density (Selley et al., 2001). However, there is another important aspect to cannabinoid action that might explain the varied effects reported for THC.

Increases in firing rate will overcome the inhibition of neurotransmitter release produced by activation of presynaptic GPCRs (Brenowitz et al., 1998; Frerking and Ohliger-Frerking, 2006). Indeed, the presynaptic inhibition produced by Win55212-2 is attenuated at high firing rates (>20 Hz) (Foldy et al., 2006). Because THC is widely used in humans, has weak and variable affects on synaptic transmission and has potentially complex interactions with the eCB signaling system, we examined the effects of firing rate on the presynaptic inhibition produced by THC.

Here, we tested the hypothesis that increases in presynaptic firing rate would attenuate THC-mediated inhibition of excitatory synaptic transmission and that at high firing rates THC would antagonize eCB signaling. Our data indicate that modest increases in stimulus frequency had a profound effect on THC-mediated effects. At low firing rates, THC exhibited classical agonist properties. In contrast, at elevated firing rates THC occupied CB1R, but did not effectively couple to VGCCs and thus,

THC acted as an antagonist. This state-dependent switching from agonist to antagonist may account for the complex actions of THC in vivo.

II. Materials and Methods

Materials. Dulbecco's modified Eagle medium (DMEM) and sera were purchased from InVitrogen (Carlsbad, CA). THC was obtained from the National Institute on Drug Abuse (Research Triangle Institute, Research Triangle Park, NC). Bicuculline methochloride and 2- arachidonylglycerol were purchased from Tocris Cookson (Ellisville, MO). All other reagents were purchased from Sigma (St. Louis, MO).

*Cell Culture***.** Rat hippocampal neurons were grown in primary culture as described previously (Pottorf et al., 2006) with minor modifications. Fetuses were removed on embryonic day 17 from maternal rats, anesthetized with CO_2, and killed by decapitation under a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Hippocampi were dissected and placed in Ca^{2+} and Mg^{2+}-free HEPES buffered Hanks' salt solution, pH 7.45. HEPES buffered Hanks' salt solution was composed of the following (in mM): HEPES 20, NaCl 137, $CaCl_2$ 1.3, $MgSO_4$ 0.4, $MgCl_2$ 0.5, KCl 5.0, KH_2PO_4 0.4, Na_2HPO_4 0.6, $NaHCO_3$ 3.0, and glucose 5.6. Cells were dissociated by trituration through a series of flame-narrowed Pasteur pipettes, pelleted and re-suspended in DMEM without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (10 μg/mL and 100 μg/mL, respectively). Dissociated cells then were plated at a density of 10,000–15,000 cells/dish onto a 25-mm-round cover glass precoated with matrigel (250 μL, 0.1mg/mL). Neurons were grown in a humidified atmosphere of 10% CO_2 and 90% air at 37°C, and fed on days 1 and 6 by exchange of 75% of the media with DMEM supplemented with 10% horse serum and

penicillin/streptomycin. Cells used in these experiments were cultured without mitotic inhibitors for a minimum of 12 days.

EPSC recordings. 6-Cyano-2,3-dihydroxy-7-nitroquinoxaline-sensitive EPSCs were recorded using the whole-cell configuration of the patch-clamp technique (Kouznetsova et al., 2002). Pipettes (Narishige) with open resistances of 3–5 MΩ were filled with solution that contained (in mM): K-gluconate 120, KCl 15, $MgCl_2$ 6, EGTA 0.2, HEPES 10, Na_2ATP 5, pH 7.3 with KOH, 290 mOsm/kg. Recordings were performed at room temperature (22 ^oC) in an extracellular solution that contained (in mM); NaCl 140, KCl 5, $CaCl_2$ 9, $MgCl_2$ 6, glucose 5, HEPES 10, bicuculline methochloride 0.01, pH 7.4 with NaOH, 325 mOsm/kg. Solutions were applied by a gravity-fed superfusion system. Membrane potential was held at −70 mV and monosynaptic EPSCs evoked with a bipolar platinum electrode (FHC Inc, Bowdoinham, ME) placed near a presynaptic neuron. Voltage pulses (0.1 ms) were applied at a fixed rate of either 0.1 Hz or 0.5 Hz using a Grass S44 stimulator with a SIU-5 stimulus isolation unit (Astro-Med Inc., West Warwick, RI). For DSE recordings, EPSCs were evoked at 0.5 Hz. DSE was elicited by depolarizing the postsynaptic cell to 0 mV for 15 s followed by continued recording at 0.5 Hz. A second DSE was elicited 5 min after the first response.

VGCC recordings. Whole-cell I_{Ca} recordings were performed using pipettes filled with (in mM); $CsMeSO_4$ 145, HEPES 10, BAPTA 10, MgATP 5, Na_2GTP 1, pH 7.35 with CsOH, 315mOsm/kg. Seals were formed in; NaCl 140, KCl 5, $CaCl_2$ 9, $MgCl_2$ 6, glucose 5, HEPES 10 pH 7.4 with NaOH, 325 mOsm/kg and then switched to buffer to isolate I_{Ca} that contained (in mM); TEACl 143, $CaCl_2$ 5, $MgCl_2$ 1, HEPES

10, Glucose 10, 0.1% bovine serum albumin, pH 7.4 with TEAOH, 325 mOsm/kg. Series resistance was compensated by a minimum of 75%. Membrane potential was held at -80 mV and currents evoked by stepping to 0 mV for 40 ms every 2 or 10 s (0.5 or 0.1 Hz) as noted. Recordings were not corrected for leak as there was essentially no current at the 0 mV test potential in the presence of 200 μM Cd^{2+}. For tail current recordings, membrane potential was stepped in 10 mV intervals from -40 mV to +70 mV for 20 ms followed by repolarization to -40 mV for 20 ms with the resultant tail current peak amplitude analyzed after subtracting leak current measured in 200 μM Cd^{2+}. Voltage-dependent reversal of cannabinoid inhibition of I_{Ca} was tested by comparing a control current evoked by stepping from -80 mV to 0 mV for 40 ms to a current evoked 500 ms later following a 40 ms pre-pulse to either 0 mV or +80 mV. Voltage sensitivity of cannabinoid inhibition was also assayed using paired 20 ms steps from -80 mV to 0 separated by a 10 ms return to -80 mV.

Data Acquisition and Analysis. Currents were amplified using an Axopatch 200A; for EPSC and I_{Ca} recordings respectively, data were filtered at 2 and 1 kHz and digitized at 11 and 5 kHz with a Digidata interface controlled by pClamp software (MDS Analytical Technologies, Toronto, Canada). Tail current and voltage sensitivity recordings were digitized at 50 kHz and filtered at 10 kHz. Leak currents were digitally subtracted from corresponding tail currents using Clampfit 9.0 and curves fit using Microcal Origin 6.0 (Northampton, MA). Access resistance and leak currents were monitored continuously, and the recording excluded if either changed significantly. In EPSC experiments sweeps preceded or followed by spontaneous synaptic currents were excluded from analysis. To calculate percent inhibition of

EPSC and I_{Ca} amplitudes, the mean peak current from the sweeps collected during the 1 min preceding drug application was compared to the average peak current during the final min of drug treatment, the time at which inhibition was maximal. I_{Ca} elicited at 0.5 Hz underwent significant rundown that was well described by a monoexponential decay function (r^2=0.99). Thus, to calculate changes in current amplitude, rundown was determined by fitting an exponential equation to the 3 min preceding drug application; control current amplitude (0 % inhibition) was defined as the value extrapolated to the time at which drug inhibition was calculated.

All data are presented as mean ± SE. Significance was determined using Student's t test or ANOVA with Bonferroni post test for multiple comparisons.

III. Results

THC inhibition of EPSCs is modulated by stimulus frequency

The effects of CB1R agonists were studied on excitatory synaptic transmission between rat hippocampal neurons in culture. Synaptic currents were recorded from a postsynaptic cell held at -70 mV in the whole-cell configuration of the patch-clamp. Stimulation of the presynaptic neuron with an extracellular electrode at a rate of 0.1 Hz evoked reproducible EPSCs. Application of 1 μM THC inhibited EPSC amplitude by 43 ± 10 % (n=7) (Fig. 2.1A). This level of inhibition by a maximally effective concentration of THC is consistent with the partial agonist properties of THC acting on CB1Rs (Shen and Thayer, 1999). Five minute pretreatment with 300 nM N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251), a CB1 antagonist, reduced THC-induced inhibition of EPSC amplitude to 5 ± 4 % (n=3, $p<0.01$) consistent with THC acting on CB1 receptors. Interestingly, increasing the stimulation rate to 0.5 Hz reduced the inhibition produced by THC to only 15 ± 2 % (n=6), which was significantly different from that seen at the 0.1 Hz stimulus rate (Fig. 2.1B, $p<0.01$). Due to the inherent lipophilicity of THC, effective washout from the preparation was not possible and thus THC was irreversible over the time course studied here. We next examined the frequency dependence of EPSC inhibition by the full agonists Win55212-2 (100 nM) and 2-arachidonylglycerol (2-AG) (1μM) (Fig. 2.2). In contrast to THC, Win55212-2 inhibition of EPSC peak amplitude was not different at 0.1 Hz versus 0.5 Hz (Fig. 2.2A,B) as indicated by a 69 ± 4 % and 58 ± 7 % inhibition of peak current, respectively (n=6). The inhibition produced by the eCB

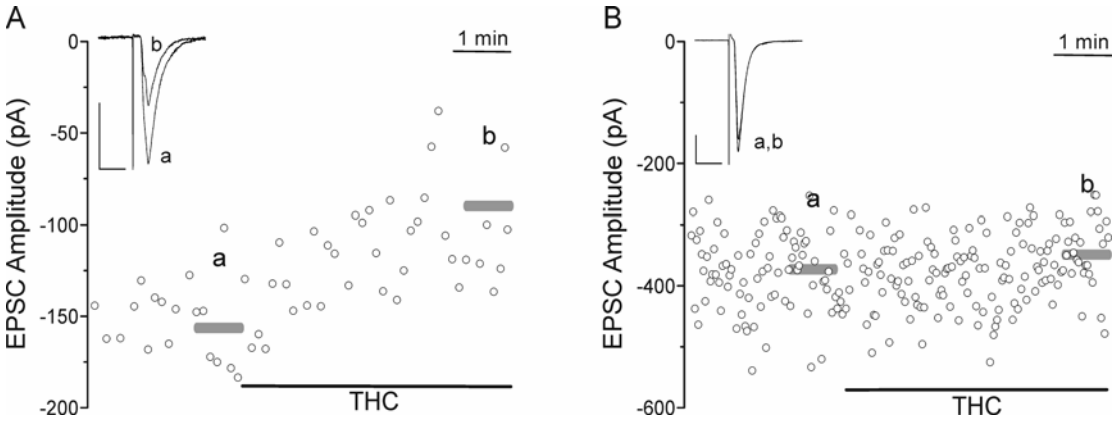

Figure 2.1. Increased stimulus rate blocks THC inhibition of EPSC amplitude.

A bipolar concentric electrode placed near a presynaptic neuron was used to evoke EPSCs in a postsynaptic cell held at -70 mV in whole-cell voltage-clamp. Representative plots show peak EPSC amplitude versus time. EPSCs were evoked every 10 s (0.1 Hz) (A) or every 2 s (0.5 Hz) (B). 1 μM THC was applied during the time indicated by the black horizontal bars. Gray bars indicate the time and amplitude of the corresponding averaged EPSCs displayed in the insets, denoted as (a) or (b). Insets, vertical bar represents 100 pA and horizontal bar represents 20 ms.

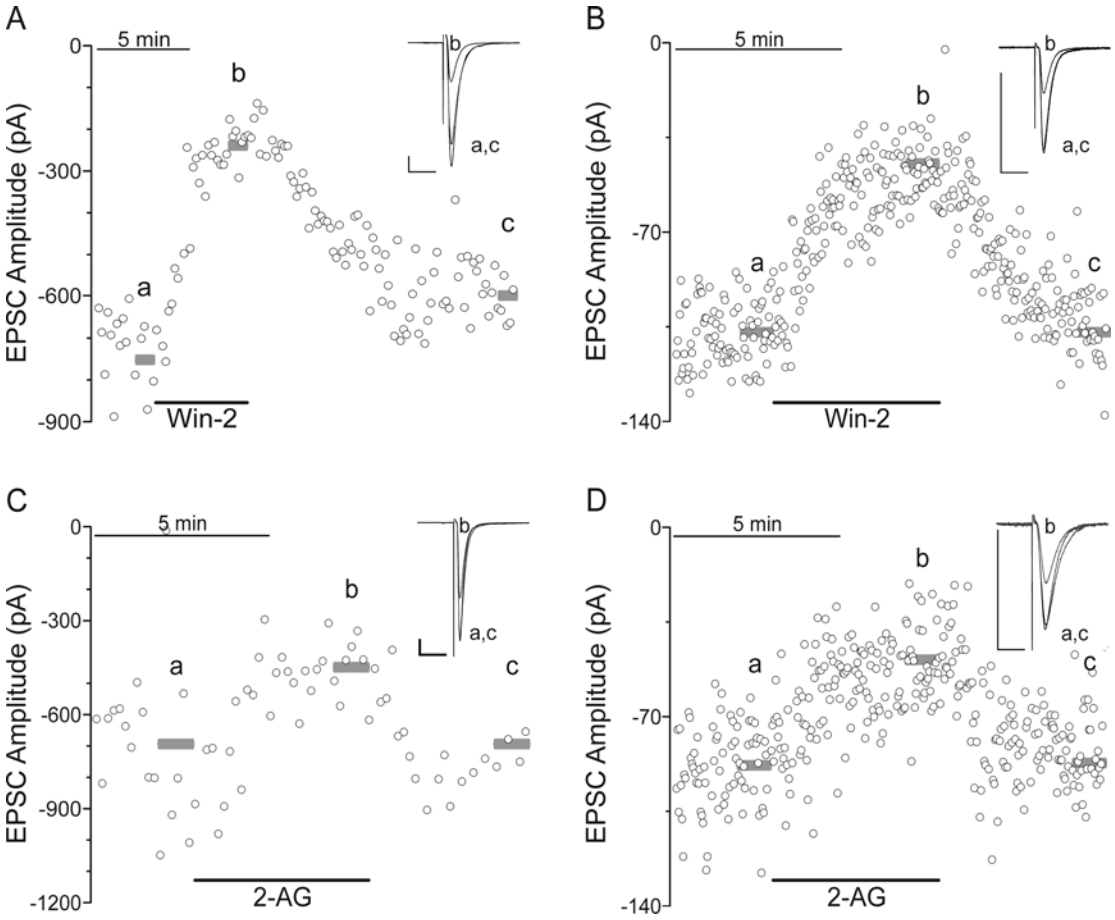

Figure 2.2. Win55212-2 and 2-arachidonylglycerol inhibit EPSC amplitude similarly at 0.1 and 0.5 Hz stimulus rates. Representative plots show peak EPSC amplitude versus time. EPSCs were evoked every 10 s (0.1 Hz) (A, C) or every 2 s (0.5 Hz) (B, D). Win55212-2 (Win-2) and 2-arachidonylglycerol (2-AG) were applied during the times indicated by the black horizontal bars. Gray bars indicate the time and amplitude of the corresponding averaged EPSCs displayed in the insets, denoted as (a), (b), or (c). Insets, vertical bar represents 100 pA and horizontal bar represents 20 ms.

agonist 2-AG was also similar at 0.1 and 0.5 Hz, producing 36 ± 9 % and 40 ± 15 % inhibition, respectively (n=6). If THC were occupying all of the CB1Rs as would be expected for a maximally effective concentration of a partial agonist, then THC might be expected to antagonize the effects of a full agonist (Shen and Thayer, 1999; Kelley and Thayer, 2004; Straiker and Mackie, 2005). Accordingly, when applied in the continued presence of THC, Win55212-2 failed to affect EPSC amplitude (8 ± 6 %, n=6, $p<0.001$; Fig. 2.3A,B). Thus, THC blocked the inhibition normally produced by 100 nM Win55212-2 even under conditions in which THC had little effect by itself. These data are consistent with the hypothesis that agonists with low intrinsic activity are particularly sensitive to attenuated presynaptic inhibition by increases in stimulation rate.

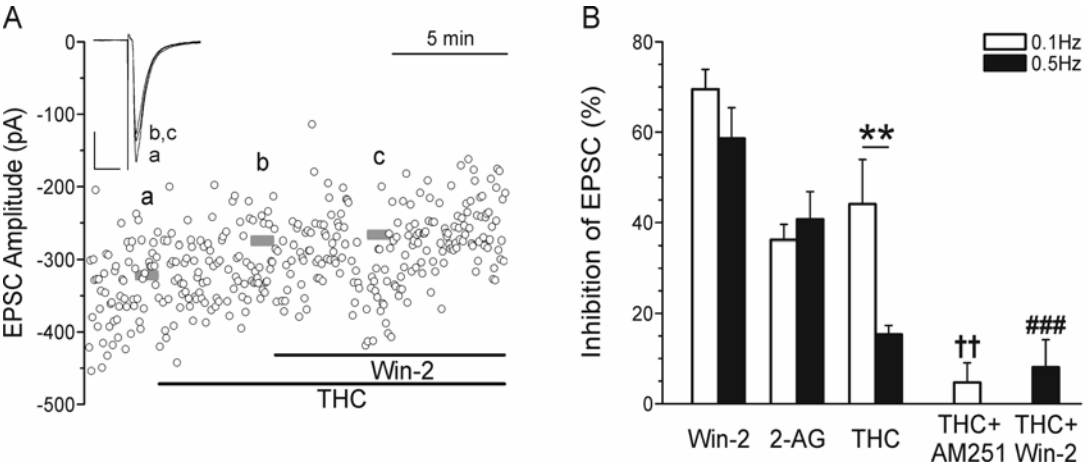

Figure 2.3. THC antagonizes Win55212-2 inhibition of EPSCs evoked at 0.5 Hz. (A) Representative plot shows peak EPSC amplitude versus time. 1 μM THC and 100 nM Win55212-2 (Win-2) were applied during the times indicated by the black horizontal bars. Gray bars indicate the time and amplitude of the corresponding averaged EPSCs displayed in the inset, denoted as (a), (b), or (c). Inset, vertical bar represents 100 pA and horizontal bar represents 20 ms. (B) Bar graph shows percent inhibition of EPSCs evoked at 0.1 (open bars) or 0.5 Hz (solid bars) treated with 100 nM Win55212-2 (Win-2), 1 μM 2-arachidonylglycerol (2-AG), 1 μM THC or 300 nM AM251 as indicated. **, $p<0.01$, 0.1 Hz versus 0.5 Hz; ††, $p<0.01$, compared to THC at 0.1 Hz; ###, $p<0.001$, compared to Win-2 at 0.5 Hz.

THC inhibition of I_{Ca} depends on depolarization rate

Activation of presynaptic CB1Rs inhibits N and P/Q type VGCCs (Lovinger, 2008) resulting in the attenuation of the evoked release of neurotransmitter. Because THC prevented the inhibition of evoked EPSCs by full agonists, we postulated that the reduced inhibition of EPSC amplitude by THC at 0.5 Hz was due to impaired inhibitory coupling to VGCCs. To test this hypothesis we elicited whole-cell I_{Ca} from hippocampal neurons at frequencies of 0.1 and 0.5 Hz. I_{Ca} was evoked from a holding potential of -80 mV by a 40 ms depolarizing step to 0 mV (Fig. 2.4). 100 nM Win55212-2 inhibited whole-cell I_{Ca} evoked at 0.1 Hz by 51 ± 6 % (n=5). Stimulation at 0.5 Hz produced significant Ca^{2+} current rundown that in control recordings was well described by an exponential function (r^2=0.99, n=4). Thus, to correct for Ca^{2+} current rundown in drug studies, 3 min of baseline preceding addition of drug was fit to a single exponential equation and extrapolated to the time at which drug inhibition was calculated (Fig. 2.4B,D). 100 nM Win55212-2 inhibited I_{Ca} evoked at 0.5 Hz by 46 ± 4 % (n=5)(Fig 3.4B,E). THC (1 μM) inhibited whole-cell I_{Ca} elicited at 0.1 Hz by 26 ± 3 % (n=5). (Fig. 4C,E). Similar results have been demonstrated in CB1R expressing neurons using common eCBs including 2-AG (Guo and Ikeda, 2004). However, when the currents were evoked at 0.5 Hz THC had no effect (n=4) (Fig. 2.4D,E). Thus, THC inhibition of I_{Ca} was highly sensitive to stimulus frequency ($p<0.01$).

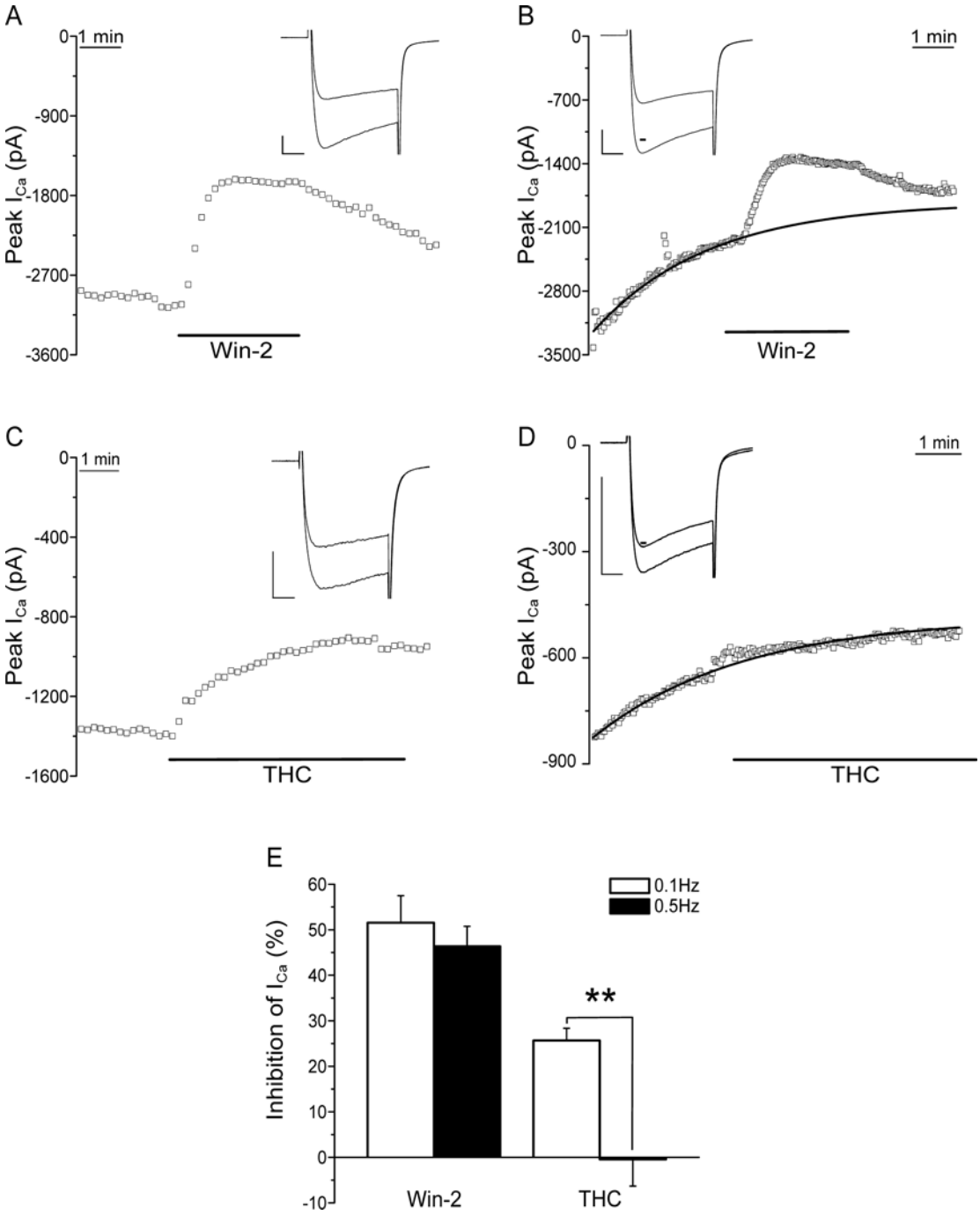

Figure 2.4. THC inhibition of VGCC is dependent on stimulus rate.

A-D, representative plots show peak I_{Ca} versus time. I_{Ca} were evoked by a depolarizing step from -80 mV to 0 mV for 40 ms at either 0.1 Hz (A,C) or 0.5 Hz

(B,D). 100 nM Win55212-2 (Win-2) or 1 μM THC were applied during the times indicated by the black horizontal bars. For recordings in which the depolarizing stimulus was applied at 0.5 Hz (B, D) current rundown was determined by fitting an exponential equation to the 3 min preceding drug application; control current amplitude (0 % inhibition) was defined as the value extrapolated to the time at which drug inhibition was calculated. Insets show the mean current trace from the sweeps collected during the 1 min preceding drug application superimposed on the averaged current traces during the final min of drug treatment. Inset, vertical bars represent 500 pA and horizontal bars represent 20 ms. The small horizontal lines in the insets to B and D represent the extrapolated curve fit during drug treatment. (E) Bar graph shows inhibition of I_{Ca} evoked at 0.1 Hz (open bars) or 0.5 Hz (solid bars) by Win55212-2 (Win-2) and THC. **, $p<0.01$ THC effects at 0.1 versus 0.5 Hz.

THC inhibition of VGCCs is more sensitive to voltage than inhibition produced by Win55212-2

Previous studies have demonstrated that inhibition of VGCCs by Gβγ is voltage-dependent (Bean, 1989; Ikeda, 1996; Agler et al., 2003). We hypothesized that inhibition of I_{Ca} by THC would be more easily reversed by depolarization than inhibition mediated by Win55212-2. The voltage-dependent activation of I_{Ca} was determined in the absence (control) or presence of Win55212,2 (100 nM) or THC (1 μM; Fig. 2.5A-B). Tail currents were recorded at a holding potential of -40 mV after depolarizing test pulses from -40 mV to +70 mV. Activation curves were well described by a single Boltzmann function: $I/I_{max}=1/[1 + \exp\{(V_{0.5}-V_m)/k\}]$. V_m is defined as the activating potential, *k* is the slope factor and $V_{0.5}$ is the half-maximum activation potential. Under control conditions $V_{0.5}$ was -3 ± 1 mV (n=6). Win55212,2 (100 nM) caused a positive shift in the voltage dependence of activation ($V_{0.5}$ = 8 ± 1 mV; n=3) while activation kinetics in the presence of THC (1μM) were comparable to control ($V_{0.5}$ = -3 ± 1 mV; n=5). To determine whether this difference resulted from an increased sensitivity of THC inhibition of I_{Ca} to depolarization relative to that produced by Win55212,2, we studied the effects of depolarizing pre-pulses on drug-induced inhibition of I_{Ca} (Fig. 2.5C-E). A conditioning prepulse to 0 mV facilitated I_{Ca} in the presence of THC, while the facilitation ratio in the presence of Win55212-2 was comparable to that of control (Fig. 2.5C-D). The ratio of the peak amplitude of the first I_{Ca} to the second I_{Ca} (following prepulse) equaled 0.73 ± 0.01 (n=27) under control conditions, 0.75 ± 0.03 in the presence of Win55212,2 (100 nM; n=12) and 0.81 ± 0.02 in the presence of THC (1μM; n=6).

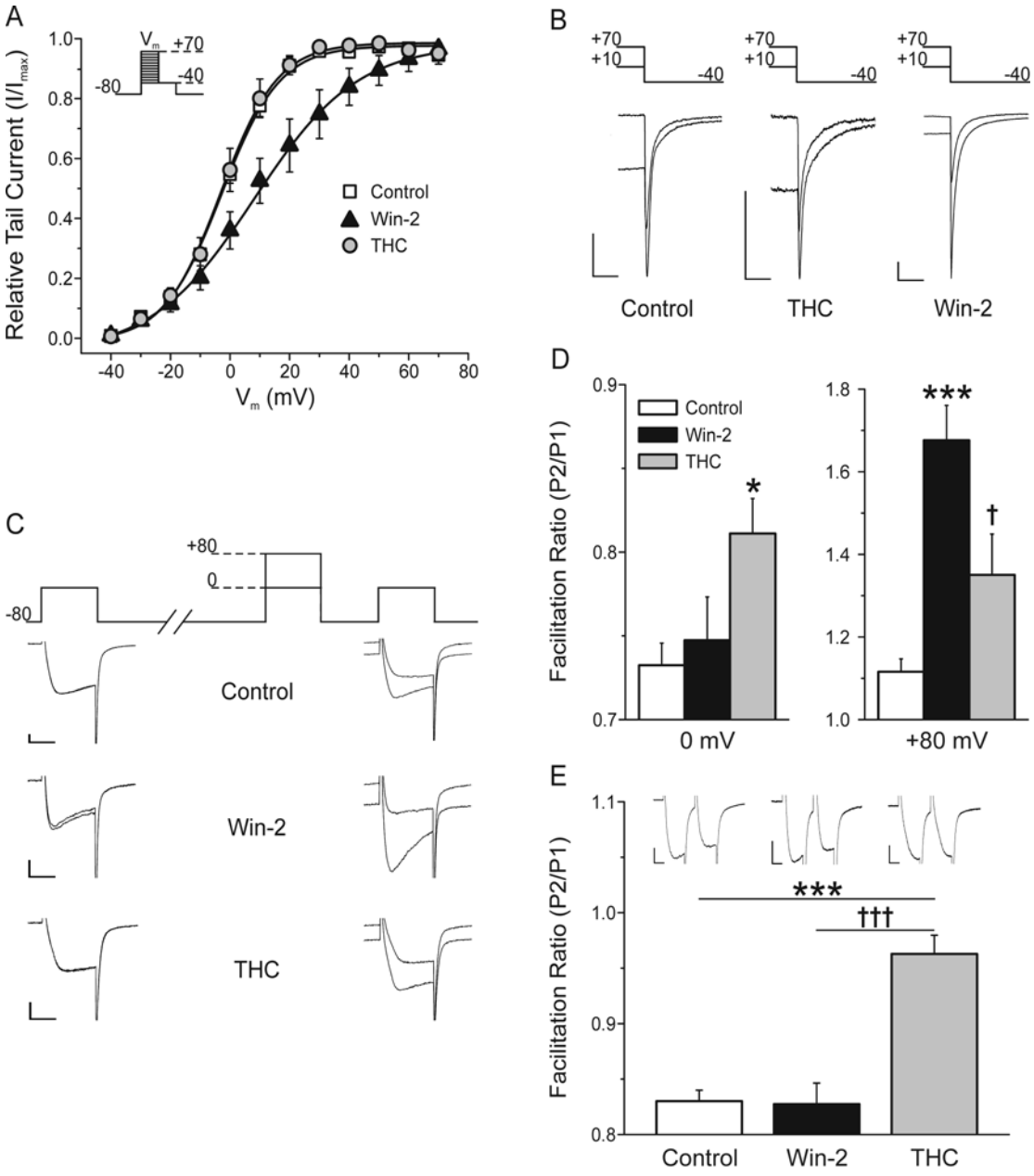

Figure 2.5. THC inhibition of ICa is more sensitive to voltage than inhibition by Win55212-2. (A) Plots display I_{Ca} activation curves in the absence (control; ■) or presence of 1 μM THC (●) or 100 nM Win55212-2 (Win-2;▲). Voltage protocol is displayed in inset. Normalized tail current amplitudes are plotted versus the voltage of a test prepulse and the solid lines describe curves fit to the data with a single

Boltzmann function: $I/I_{max}=1/[1 + \exp\{(V_{0.5}-V_m)/k\}]$. Calculated values for $V_{0.5}$ and *k* were respectively, -3 mV and 10 mV for control, -3 mV and 10 mV in THC, and 8 mV and17 mV in Win55212-2. (B) Representative tail currents evoked from +10 mV and +70 mV prepulses are overlaid. Vertical bars represent 1 nA and horizontal bars represent 1 ms. (C) Representative I_{Ca} resulting from prepulse voltage protocol (top). A prepulse to 0 mV facilitated I_{Ca} in the presence of THC. A prepulse to +80 mV facilitated I_{Ca} in the presence of both THC and Win-2. Vertical bars represent 200 pA and horizontal bars represent 20 ms. (D) Bar graphs summarizes facilitation of I_{Ca} by a prepulse to either 0 mV or +80 mV as described in C. *, $p<0.05$ THC versus control at 0 mV; ***, $p<0.001$ Win-2 versus control at +80 mV; †, $p<0.05$ THC versus control at +80 mV. (E) Bar graph displays the ratio of I_{Ca} amplitudes evoked by paired depolarizations to 0 mV. Representative traces show currents for each respective condition. Vertical bars represent 200 pA and horizontal bars represent 10 ms. ***, $p<0.001$ THC versus control; †††, $p<0.001$. THC versus Win-2.

A conditioning prepulse to +80 mV relieved both THC and Win55212-2-mediated suppression of I_{Ca} (Fig. 2.5C-D). The facilitation ratio for control experiments equaled 1.11 ± 0.03 (n=35) versus 1.67 ± 0.08 in the presence of Win55212,2 (100 nM; n=12) and 1.35 ± 0.10 in the presence of THC (1 μM; n=6). A second protocol using paired depolarizations to 0 mV verified that THC-induced inhibition of I_{Ca} is more sensitive to mild depolarization than Win55212-2 (Fig. 2.5E). The facilitation ratio for control (n=41) and Win55212,2 (100 nM, n=12) treated currents were 0.83 ± 0.01 and 0.83 ± 0.02, respectively and the ratio for THC (1 μM; n=10) was 0.96 ± 0.02. Thus, THC mediated inhibition of I_{Ca} is more sensitive to positive shifts in membrane potential than inhibition produced by Win55212-2.

THC inhibits DSE

The frequency dependence of THC-induced inhibition of I_{Ca} and EPSCs suggests that THC may act as an agonist or antagonist depending on the firing rate of the synapse. This frequency dependent shift in efficacy could have profound effects on eCB signaling. To examine the effects of THC on CB1R activation by the retrograde actions of eCBs we employed the depolarization-induced suppression of excitation (DSE) protocol previously described for hippocampal neurons in culture (Ohno-Shosaku et al., 2002b; Straiker and Mackie, 2005). In these experiments, EPSCs were evoked at 0.5 Hz and endocannabinoid production was induced by depolarizing the postsynaptic neuron to 0 mV for 15 s. Immediately following depolarization, a transient DSE (DSE 1) lasting 60 to 90 s was observed with inhibition of EPSCs reaching 46 ± 8 % (Fig. 2.6A,D; n=8). Following 5 min of continued stimulation (0.5

Hz) a second depolarization to 0 mV was applied (DSE 2) (Fig. 2.6B,D). Under control conditions DSE2 produced a 47 ± 10 % inhibition of EPSC amplitude (n=4), similar to DSE1. DSE was completely blocked by pretreatment (5 min) with 300 nM AM251 (data not shown). Similar to the actions of the antagonist, in synaptic pairs treated for 5 min with 1μM THC, DSE2 was completely blocked (6 ± 6 %; n=4) (Fig. 2.6C,D). THC significantly reduced DSE relative to control ($p<0.001$).

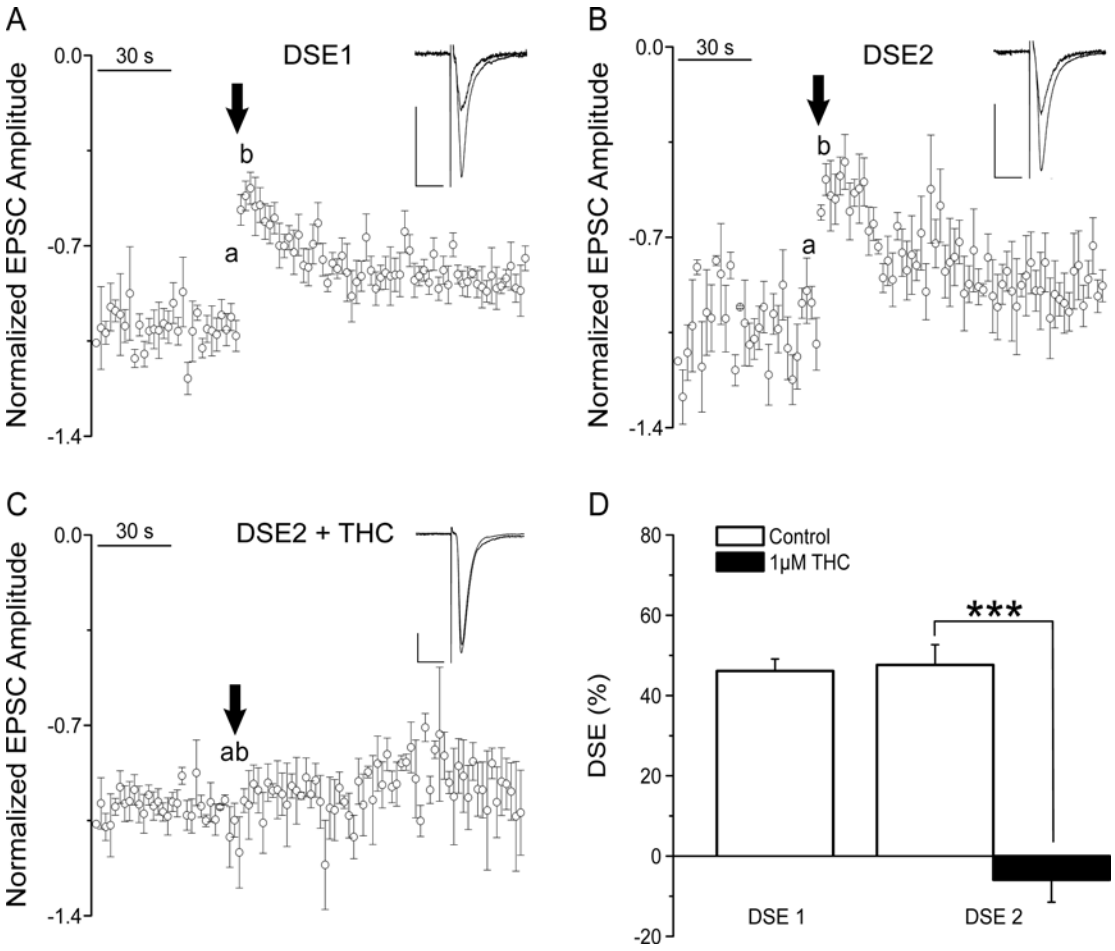

Figure 2.6. Inhibition of DSE by THC. A-C, EPSCs were evoked at 0.5 Hz. Plots show mean EPSC amplitudes, normalized to the 15 responses immediately before depolarization, plotted versus time. The postsynaptic cell was depolarized to 0 mV for 15 s at the times indicated by arrows. A, an initial control DSE (DSE1) was elicited in each recording. A second postsynaptic depolarization (DSE2) was evoked after 5 min treatment with vehicle (B) or 1 μM THC (C). D, bar graph shows the mean magnitude of DSE1 and DSE2 in the absence (open bars) and presence of 1 μM THC (solid bar). Percent DSE was calculated according to the following equation, % DSE=100($EPSC_{control}$-$EPSC_{DSE}$)/$EPSC_{control}$ where $EPSC_{control}$ is the average amplitude of the 15 EPSCs immediately prior to depolarization and $EPSC_{DSE}$ is the

average amplitude of the two EPSCs immediately after depolarization. ***,$p<0.001$ DSE2 in absence relative to in the presence of THC.

IV. Discussion

The effects of THC on synaptic networks are complex and not fully explained by agonist actions. In the current study, we show that the effects of THC on glutamatergic synapses between hippocampal neurons switch between agonist and antagonist actions on CB1Rs, depending on stimulus rate. When EPSCs were evoked at 0.1 Hz, THC acted as a weak agonist of CB1Rs to inhibit synaptic transmission. In contrast, when stimulated at 0.5 Hz, THC had little direct effect and actually acted as an antagonist capable of blocking the actions of exogenously applied and endogenously produced cannabinoids. Voltage-sensitive coupling of CB1Rs to VGCCs caused these state-dependent actions of THC. The extreme sensitivity of the actions of THC to firing frequency was not previously appreciated and the concept that agonists of low intrinsic activity might act as antagonists during certain patterns of activity has broad implications for the many drugs that act on presynaptic GPCRs to inhibit neurotransmitter release.

Frequency-dependent coupling of CB1Rs to presynaptic VGCCs

Activation of CB1Rs inhibits EPSCs and sensitive IPSCs between hippocampal neurons (Shen et al., 1996; Hajos et al., 2000). Generally, presynaptic inhibition mediated by GPCRs can be overcome by increases in firing rate (Brenowitz et al., 1998; Frerking and Ohliger-Frerking, 2006). Indeed, increasing the presynaptic firing rate to >20 Hz reversed Win55212-2 inhibition of inhibitory postsynaptic currents (IPSCs) (Foldy et al., 2006). In the current study, inhibition of EPSCs by THC was completely blocked by a modest increase in presynaptic stimulus rate to 0.5

Hz. Thus, the inhibition mediated by THC is some 40 times more sensitive to firing rate than that mediated by full CB1R agonists. We speculate that the high sensitivity of THC-mediated inhibition of synaptic transmission to firing frequency contributes to the variable effects reported for this drug in in vitro models. The only experiments to describe THC-mediated inhibition of synaptic transmission were performed at a stimulus rate of 0.1 Hz or less (Pertwee et al., 1996; Shen and Thayer, 1999; Azad et al., 2008) or employed intermittent burst-type stimulus protocols with prolonged inter stimulus intervals (Pertwee et al., 1992). Interestingly, a partial agonist at 5HT3 receptors was previously shown to display a frequency-dependent reduction in the efficacy for inhibition of neurotransmitter release (Van der Vliet et al., 1988), suggesting that a general property of presynaptic inhibition produced by agonists of low intrinsic activity may be an especially high sensitivity to firing rate.

The short-term effects of cannabinoid agonists on synaptic transmission are primarily mediated by inhibition of presynaptic VGCCs (Lovinger, 2008). Because the relationship of presynaptic $[Ca^{2+}]_i$ to vesicular release is a power function, small changes in Ca^{2+} influx have large effects on neurotransmission. CB1Rs couple to VGCCs via the βγ subunits of inhibitory G-proteins (Agler et al., 2003). We found that increased firing rate reduced THC inhibition of whole-cell I_{Ca} through VGCCs. Frequency dependent relief of VGCC channel inhibition has been attributed to the accumulation of residual Ca^{2+} in the presynaptic terminal at high frequencies (Kreitzer and Regehr, 2000) and might contribute to the frequency dependence of THC inhibition of EPSCs but, would not account for the frequency-dependent modulation of VGCCs. The most likely explanation for the reduction in CB1R

coupling to VGCCs is the relief of G-protein-mediated inhibition that occurs during repetitive physiological stimuli (Brody et al., 1997). This attenuation of GPCR-mediated inhibition of VGCCs results from voltage-dependent reversal of Gβγ coupling to N and P/Q type Ca^{2+} channels (Bean, 1989; Ikeda, 1996; Agler et al., 2003). We observed a positive shift in the activation curve for I_{Ca} in the presence of Win55212-2, consistent with a voltage-sensitive coupling between CB1R and VGCC. In the presence of THC I_{Ca} was facilitated by a small depolarizing prepulse, indicating that THC inhibition of VGCC was even more sensitive to voltage than that produced by Win55212-2. Thus, the inefficient release of βγ subunits evoked by THC (Breivogel and Childers, 2000b) relative to full agonists, appears to make THC-mediated synaptic inhibition extremely sensitive to voltage.

THC and the endocannabinoid system

If the attenuation of THC-mediated synaptic inhibition results from a frequency dependent reduction in βγ coupling to VGCCs, then the binding of THC to CB1Rs would not necessarily be affected by stimulus rate. Indeed, at the high (0.5 Hz) stimulus rate THC clearly occupied the receptor, even though it failed to affect synaptic transmission, as indicated by block of Win55212-2-mediated inhibition of EPSC amplitude. This observation has significant implications for how THC interacts with the eCB system. We found that THC acted as an antagonist in DSE experiments, presumably because the DSE protocol requires fast stimulus rates in order to resolve the inhibition of EPSCs produced by the transient production of eCBs. Our results agree with those of Straiker and Mackie (2005) in that THC

antagonized the eCB system. However, they did not observe an effect of THC at low stimulus frequency, presumably the combination of low receptor density and low agonist efficacy prevents effective CB1R coupling to VGCC. Chronic exposure to THC desensitized CB1Rs, consistent with studies that found prolonged treatment with THC in vivo produced a functional desensitization that impaired synaptic plasticity and prevented Win55212-2 mediated inhibition of IPSCs (Mato et al., 2004; Hoffman et al., 2007). The desensitization of CB1Rs, mediated by βγ activation of G-protein receptor kinases (Jin et al., 1999; Kouznetsova et al., 2002), would not be expected to display the frequency (voltage) dependence described for coupling to VGCCs.

In contrast to presynaptic inhibition mediated by autoreceptors that respond to released neurotransmitter, THC modulates a receptor in which the endogenous ligand is distinct from that which mediates synaptic transmission. Thus, we envision at least four states under which THC will exert different effects on synaptic transmission. At low firing frequencies THC will mimic the actions of eCBs to inhibit synaptic transmission. If eCBs are present their actions would be occluded. In contrast, at high firing rates THC will have little direct effect. When eCB production is stimulated and the synapse is firing at a high frequency, THC will block the actions of eCBs.

THC affects firing patterns and behavior

The inhibition of low frequency EPSCs by THC (as shown in Fig. 2.2.1A) is predicted to remove the slow component of a complex firing pattern. The idea that the frequency-dependence of drug-induced presynaptic inhibition applies a high-pass

filter to affected synapses has been previously suggested (Frerking and Ohliger-Frerking, 2006). For example, baclofen (a $GABA_B$ receptor agonist) acted to enhance the contrast between low and high frequency field excitatory postsynaptic potentials during application of physiological spike trains taken from animals in a delayed non-matched to sample behavioral paradigm. The drug preferentially inhibited field excitatory postsynaptic potentials that followed long inter spike intervals (ISI) at CA3 to CA1 synapses. The average firing rate of CA3 neurons was near 1 Hz and the change in ISI near 0.1 Hz, close to the frequencies over which THC displayed dramatic changes in efficacy in the current study. Interestingly, cells exhibiting longer ISIs (near 0.1 Hz) tended to fire in response to specific cues and were inhibited by baclofen, while neurons that integrated multiple inputs had shorter ISIs (near 1 Hz) and were less sensitive to baclofen. Our data suggest that THC might also enhance the importance of high frequency integrative inputs relative to more specific low frequency inputs.

THC has broad actions in the CNS, most of which are reversed by CB1R antagonists (Rinaldi-Carmona et al., 1994a; Chaperon and Thiebot, 1999). However, some behavioral responses to THC suggest actions more complex than simple agonist effects. THC suppresses certain operant behaviors in a manner not effectively reversed by rimonabant, a CB1R antagonist (De Vry and Jentzsch, 2004; McMahon et al., 2005). The eCB system also participates in complex hippocampal functions including memory extinction (Zhuang et al., 2005a). THC retarded extinction of an adverse associative memory (Ashton et al., 2008) in contrast to acceleration induced by Win55212-2 (Pamplona et al., 2006) and similar to the slowed extinction found in

CB1R knockout animals (Marsicano et al., 2002). A human study found THC actually enhanced a spatial working memory task for females and increased intrusion errors in spatial span tasks in both males and females (Makela et al., 2006). THC antagonism of eCB mediated plasticity might underlie these effects, although linking synaptic transmission experiments to behavior is highly speculative.

V. Conclusions

We have shown that the effects of THC on neurotransmission depend on the firing rate of the synapse and the presence of eCBs. Because the actions of THC are more sensitive to firing rate than highly efficacious cannabinoid agonists such as Win55212-2, we caution that results obtained in vitro with full agonists may not accurately reflect the more complex actions of THC in vivo. Going forward, it will be important to resolve the electrophysiological and behavioral consequences for presynaptic inhibition produced by agonists of differing intrinsic activities.

Chapter Three: Homer 1a gates the induction mechanism for endocannabinoid-mediated synaptic plasticity

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At hippocampal excitatory synapses, endocannabinoids (eCBs) mediate two forms of retrograde synaptic inhibition that are induced by postsynaptic depolarization or activation of metabotropic glutamate receptors (mGluRs). The homer family of molecular scaffolds provides spatial organization to regulate postsynaptic signaling cascades, including those activated by mGluRs. Expression of the homer 1a (H1a) immediate early gene produces a short homer protein that lacks the domain required for homer oligomerization, enabling it to uncouple homer assemblies. Here, we report that H1a differentially modulates two forms of eCB-mediated synaptic plasticity, depolarization-induced suppression of excitation (DSE) and metabotropic suppression of excitation (MSE). Excitatory postsynaptic currents were recorded from cultured hippocampal neurons and DSE evoked by a 15 s depolarization to 0 mV and MSE evoked by a type I mGluR agonist. Expression of H1a enhanced DSE and inhibited MSE at the same synapse. Many physiologically important stimuli initiate H1a expression including brain derived neurotrophic factor (BDNF). Treating hippocampal cultures with BDNF increased transcription of H1a and uncoupled homer1c-GFP clusters. BDNF treatment blocked MSE and enhanced DSE. Thus, physiological changes in H1a expression gate the induction pathway for eCB-mediated synaptic plasticity by uncoupling mGluR from eCB production.

I. Introduction

The endocannabinoid (eCB) system is an important mediator of synaptic plasticity in the CNS. eCBs produced postsynaptically diffuse in a retrograde direction to act on presynaptic cannabinoid receptor-1 (CB1R) and inhibit neurotransmitter release (Lovinger, 2008). Production of eCBs is initiated by two distinct yet related postsynaptic mechanisms: depolarization and metabotropic receptor activation. These processes induce short-term plasticity at excitatory synapses, termed depolarization-induced (DSE) or metabotropic (MSE) suppression of excitation. DSE requires Ca^{2+} influx via voltage-gated Ca^{2+} channels (VGCC), which may be amplified by ryanodine sensitive Ca^{2+} stores, and subsequent Ca^{2+}-stimulated production of 2-arachydonylglycerol (2-AG) (Isokawa and Alger, 2006). Type I metabotropic glutamate receptors (mGluRs) produce MSE by activating phospholipase Cβ (PLCβ) to release diacylglycerol (DAG) that is hydrolyzed by DAG lipase (DGL) to 2-AG (Maejima et al., 2001b; Straiker and Mackie, 2006).

The Homer family of postsynaptic scaffolding proteins is central to synaptic development, organization, and plasticity (Worley et al., 2007) and has been associated with neuronal disease and addiction (Szumlinski et al., 2006; Szumlinski et al., 2008). Homer proteins contain a N-terminal Ena/VASP homology domain 1 (EVH1 domain) that recognizes target proteins involved in eCB signaling including: mGluR, PLCβ, ryanodine receptors (RyR), and L-Type VGCCs (Worley et al., 2007). Homer proteins couple signaling molecules in the postsynaptic density (PSD) by oligomerizing through their C-terminal coiled coil (CC) domain and cross-linking with other structural proteins in the PSD such as Shank and PSD-95 (Tu et al., 1999).

Thus, the combination of EVH1 interaction with signaling proteins and CC-domain-mediated oligomerization leads to the structural and functional apposition of synaptic proteins. Three homer genes are subject to alternative splicing yielding an array of protein products (Shiraishi-Yamaguchi and Furuichi, 2007). Expression of the short homer isoform 1a (H1a) uncouples CC-homer structures (Xiao et al., 1998). H1a contains an EVH1 domain but lacks the CC-domain, enabling it to act in a dominant-negative capacity. H1a is an immediate early gene induced by glutamate (Sato et al., 2001), brain derived neurotrophic factor (BDNF) (Kato et al., 2003), activation of adenylyl cyclase (Kammermeier, 2008), stimuli that induce long-term potentiation (Kato et al., 1998), and exploratory behavior (Brakeman et al., 1997a). Indeed, the rapid induction and dominant-negative properties of H1a make it a powerful modulator of postsynaptic function.

Several studies suggest a link between Homer proteins and eCB signaling. Homer 2a (H2a) properly localized the 2-AG synthetic enzyme DGL in Neuro-2a cells (Jung et al., 2007) and mGluRs are attached to homer scaffolds. The inhibition of eCB-mediated long-term depression (LTD) by cocaine correlated with the expression of CC-homers (Fourgeaud et al., 2004). Thus, there are intriguing hints linking homer proteins to eCB signaling but this putative relationship has not been fully examined.

Here we tested the hypothesis that H1a regulates the induction of eCB mediated synaptic plasticity. Our data indicate that H1a expression suppressed metabotropic-mediated while it enhanced depolarization-mediated eCB production.

Furthermore, treatment with BDNF elevated H1a levels demonstrating a physiological mechanism to regulate eCB-mediated synaptic plasticity.

II. Materials and Methods

Materials. Dulbecco's modified Eagle medium (DMEM) and sera were purchased from InVitrogen (Carlsbad, CA). BDNF was obtained from R&D Systems (Minneapolis, MN). Bicuculline methochloride and dihydroxyphenylglycine (DHPG) were purchased from Ascent Scientific (Princeton, NJ). All other reagents were purchased from Sigma (St. Louis, MO).

*Cell Culture***.** Rat hippocampal neurons were grown in primary culture as described previously (Pottorf et al., 2006) with minor modifications. Fetuses were removed on embryonic day 17 from maternal rats euthanized with CO_2 under a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guide for the care and use of laboratory animals. Hippocampi were dissected and placed in Ca^{2+} and Mg^{2+}-free HEPES buffered Hanks' salt solution, pH 7.45. HEPES buffered Hanks' salt solution was composed of the following (in mM): HEPES 20, NaCl 137, $CaCl_2$ 1.3, $MgSO_4$ 0.4, $MgCl_2$ 0.5, KCl 5.0, KH_2PO_4 0.4, Na_2HPO_4 0.6, $NaHCO_3$ 3.0, and glucose 5.6. Cells were dissociated by trituration through a series of flame-narrowed Pasteur pipettes, pelleted and re-suspended in Dulbecco's modified Eagle medium (DMEM) without glutamine, supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 μ/mL and 100 μg/mL, respectively). Dissociated cells then were plated at a density of 10,000–15,000 cells/dish onto a 25-mm-round cover glass precoated with matrigel (250 μL, 0.1mg/mL). Neurons were grown in a humidified atmosphere of 10% CO_2 and 90% air at 37°C, and fed on days 1 and 6 by exchange of 75% of the media with DMEM supplemented with 10% horse serum and

penicillin/streptomycin. Cells used in these experiments were cultured without mitotic inhibitors for a minimum of 11 days.

Excitatory postsynaptic current (EPSC) recordings. 6-Cyano-2,3-dihydroxy-7-nitroquinoxaline (CNQX)-sensitive EPSCs were recorded using the whole-cell configuration of the patch-clamp technique (Kouznetsova et al., 2002). Pipettes (Narishige) with open resistances of 3–5 MΩ were filled with solution that contained (in mM): K-gluconate 120, KCl 15, $MgCl_2$ 6, EGTA 0.2, HEPES 10, Na_2ATP 5, pH 7.3 with KOH, 290 mOsm/kg for DSE recordings and 113mM K-gluconate, 15mM KCl, 6mM $MgCl_2$, 10mM BAPTA, 10mM HEPES, 5mM Na_2ATP, 6.85mM $CaCl_2$, pH 7.3 (KOH), 290 mOsm for MSE experiments. Recordings were performed at room temperature (22 ^oC) in an extracellular solution that contained (in mM); NaCl 140, KCl 5, $CaCl_2$ 9, $MgCl_2$ 6, glucose 5, HEPES 10, bicuculline methochloride 0.01, 0.5% BSA, pH 7.4 with NaOH, 325 mOsm/kg. Solutions were applied by a gravity-fed superfusion system. Membrane potential was held at −70 mV and monosynaptic EPSCs evoked with a bipolar platinum electrode (FHC Inc, Bowdoinham, ME) placed near a presynaptic neuron. Voltage pulses (0.1 ms) were applied at a fixed rate of 0.5 Hz using a Grass S44 stimulator with a SIU-5 stimulus isolation unit (Astro-Med Inc., West Warwick, RI). DSE was elicited by depolarizing the postsynaptic cell to 0 mV for 15 s followed by continued recording at 0.5 Hz. MSE was induced by superfusion of 1 μM DHPG.

I_{Ca} recordings. Whole-cell recordings of currents through voltage-gated Ca^{2+} channels (VGCCs) were performed using pipettes filled with (in mM); $CsMeSO_4$ 145, HEPES 10, BAPTA 10, MgATP 5, Na_2GTP 1, pH 7.35 with CsOH, 315mOsm/kg.

Seals were formed in; NaCl 140, KCl 5, $CaCl_2$ 9, $MgCl_2$ 6, glucose 5, HEPES 10 pH 7.4 with NaOH, 325 mOsm/kg and then switched to buffer to isolate I_{Ca} that contained (in mM); TEACl 143, $CaCl_2$ 5, $MgCl_2$ 1, HEPES 10, Glucose 10, 0.1% bovine serum albumin, pH 7.4 with TEAOH, 325 mOsm/kg. Series resistance was compensated by a minimum of 75%. Membrane potential was held at -80 mV and currents evoked by stepping to 0 mV for 200 ms every 10 s (0.1 Hz). Recordings were not corrected for leak as there was essentially no current at the 0 mV test potential in the presence of 200 μM Cd^{2+}.

Data Acquisition and Analysis. Whole-cell currents were amplified using an Axopatch 200A. For EPSC and I_{Ca} recordings respectively, data were filtered at 2 and 1 kHz and digitized at 11 and 5 kHz with a Digidata interface controlled by pClamp software (MDS Analytical Technologies, Toronto, Canada). Access resistance and leak currents were monitored continuously, and the recording excluded if either changed significantly. In EPSC experiments sweeps preceded or followed by spontaneous synaptic currents were excluded from analysis. To calculate percent inhibition of EPSC and I_{Ca} amplitudes, the mean peak current from the sweeps collected during the 1 min preceding drug application was compared to the average peak current during the final min of drug treatment, the time at which inhibition was maximal.

DNA constructs and transfection. The coding regions for Homer1a and 1c were amplified by PCR from the Marathon-ready mouse brain cDNA library (Invitrogen) using the 5' primer ATGGGGGAGCAACCTATCTTCAGC and 3' primer TTACTTAATCATGATTGCTGAATTG combination for Homer1a, and the

5' primer ATGGGGGAGCAACCTATCTTCAGC and 3' primer TTAGCTGCATTCCAGTAGCTTGGC combination for Homer1c. PCR products were subsequently cloned into the pcDNA3.1/V5-His-TOPO vector or the pcDNA3.1/NT-GFP-TOPO vector (Invitrogen) according to manufacture specifications. Homer2a was amplified from human cDNA with an additional N-terminal c-myc tag using the 5' primer ACAAGCTTATGGAACAAAAACTTATTTCTGAAGAAGATCTGGGAGAACAG CCCATCTT and 3' primer CGGCCGCCTAGTTATCGGTGCCCAGCT. PCR products were subsequently cloned into pcDNA3.1 vector via the 5' HindIII and 3' NotI restriction digest sites. Similarly, Homer3a was PCR amplified from mouse cDNA and cloned into pcDNA3.1 using the 5' primer ACAAGCTTATGGAACAAAAACTTATTTCTGAAGAAGATCTGTCCACAGCC AGGAACA and 3' primer GCGGCCGCCTAGGGTGCTGCCTCTGCCA. All constructs were propagated in *Escherichia coli* Top-10 strain (Invitrogen), isolated using Maxiprep kits (Qiagen) and sequenced.

Hippocampal neurons were transfected with plasmid DNA using the calcium phosphate procedure described by Xia et al (Xia et al., 1996), with some modifications. Briefly, 9-11 days after plating, hippocampal neurons were placed in serum-free DMEM (1 ml per dish) supplemented with 1 mM sodium kynurenate, 10 mM $MgCl_2$ and 5 mM HEPES, pH 7.5 for 30 min. The conditioned media was saved. A DNA/calcium phosphate precipitate was prepared by mixing 3.8 µl of 2 M CaCl2, 1 µg DNA per plasmid and dH_2O in a volume of 30 µl with an equal volume of 2x HEPES buffered saline (274 mM NaCl, 10 mM KCl, 1.4 mM Na_2HPO_4, 15 mM D-

glucose, 42 mM HEPES, pH 7.05). The precipitate was allowed to form for 30 min at room temperature (22^{o}C) before addition to the cultures. Sixty microliters of the DNA/calcium phosphate precipitate were added drop-wise to each dish. Dishes were returned to the 10 % CO_2 incubator. After 1.5 hrs the incubation was stopped by washing cells three times with 3 ml per well of DMEM (10 mM $MgCl_2$, 5 mM HEPES). The saved conditioned medium was added back to each well, and the cells were returned to a 10 % CO_2 incubator at 37^{o}C. The transfection efficiency ranged from 2 to 11%.

Quantitative real-time reverse transcription-PCR (Q-RT-PCR) experiments. RNA was extracted from hippocampal cultures using an RNA isolation kit (Stratagene, La Jolla, CA). Q-RT-PCR was performed on 100 ng of isolated RNA using a SYBR Green QRT-PCR kit (Stratagene). Homer 1a primers (5'-CAAACACTGTTTATGGACTG-3' and 5'-TGCTGAATTGAATGTGTACC-3') were designed against the unique 3' mRNA end of homer 1a, not present in the long-splice isoforms of homer (i.e. homer 1c). In addition to using equal amounts of total RNA as defined by UV spectroscopy we also included an internal reference control (GAPDH) to account for the integrity of mRNA. QuantiTect primers (Qiagen, Valencia, CA) were used for amplification of GAPDH mRNA. PCR cycling and detection was performed on a Mx3005P PCR System and quantitiative analysis performed with MxPro-Mx3005P Software v4.01 (Stratagene).

*Confocal Microscopy***.** 48-72 hours following transfection with expression vectors for DsRed2 and Homer 1c fused to green fluorescent protein (H1c-GFP) (Inoue et al., 2007) the Petri dish with cells was sealed with Parafilm and imaged with

an Olympus Fluoview 300 laser scanning confocal microscope (Melville, NY, USA) attached to an inverted Olympus IX70 microscope equipped with a PlanApo 60 x oil emersion objective (NA 1.40) (Tokyo, Japan). For all experiments, an 8 step (1 μm/step) z-series was performed. GFP was excited at 488 nm with an Argon laser and imaged at 530 nm (10 nm bandpass). DsRed2 was excited at 546 nm with a He-Ne laser and imaged at 605 nm (75 nm bandpass). Cells were returned to the incubator immediately after imaging.

Image Processing. To count and label homer 1c-GFP puncta an automated algorithm was created using MetaMorph 6.2 image processing software (Waataja et al., 2008). First, maximum z-projection images were created from the DsRed2 and GFP image stacks. Next, a threshold set 1 s.d. above the image mean was applied to the DsRed2 image. This created a 1-bit image that was used as a mask via a logical AND function with the GFP maximum z-projection. A top-hat filter (80 pixels) was applied to the masked H1c-GFP image. A threshold set 1.5 s.d. above the mean intensity inside the mask was then applied to the contrast enhanced image. Structures between 8 and 80 pixels (approximately 0.37 to 3.12 μm in diameter) were counted as puncta. The structures were then dilated and superimposed on the DsRed2 maximum z-projection for visualization. Changes in the number of H1c-GFP puncta were presented as a percent of the initial puncta count; n was the number of cells, each from separate cover glass.

All data are presented as mean ± SE. Significance was determined using Student's t test or ANOVA with Bonferroni post test for multiple comparisons.

III. Results

eCBs modulate synaptic strength following postsynaptic synthesis and retrograde diffusion to act on presynaptic CB1Rs. In hippocampus, 2-AG synthesis can be induced by activation of type I mGluRs that couple to the PLCβ cascade or by activation of VGCCs (Maejima et al., 2001b; Straiker and Mackie, 2006). Elements of this pathway interact with members of the homer family of molecular scaffolds (Nakamura et al., 2004; Hwang et al., 2005). However, the level of homer interaction with the eCB pathway induced by either mGluRs or VGCCs is largely unknown. Here we examined the role of homer proteins in regulating 2-AG production at excitatory synapses between hippocampal neurons in culture. Synaptic currents were recorded from a postsynaptic neuron held at -70 mV using the whole-cell configuration of the patch-clamp technique. EPSCs were evoked by stimulation of the presynaptic neuron with an extracellular electrode (0.5 Hz). MSE was induced by superfusion of the type I mGluR agonist DHPG and DSE was evoked by a 15 s test pulse to 0 mV.

H1a inhibits MSE

Activation of postsynaptic mGluRs stimulates the PLCβ cascade producing 2-AG that inhibits EPSCs by activating presynaptic CB1Rs (Fig 3.1A). Application of 1 μM DHPG for 2 min inhibited EPSCs by 51 ± 4 % (n=12) (Fig. 1B, F). DHPG-mediated inhibition was blocked completely by preincubation with the CB1R antagonist N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; 300nM; n=3). Transfection of neurons with an

expression construct for H1a resulted in a significant loss of DHPG mediated inhibition to 12 ± 10% (Fig. 1C, F; n=4; $p<0.001$). H1a, which lacks the C-terminal coiled-coil (CC) domain present in full length members of the homer family (Fig 3.1A; inset), is expected to uncouple type 1 mGluR from other homer binding proteins (Xiao et al., 1998). In contrast, expression of H2a, which contains the CC domain, did not change DHPG mediated inhibition (50 ± 5 %) (Fig. 1D, F; n=3). Interestingly, expression of Homer 3a (H3a) which, like H2a has both binding domains also attenuated DHPG mediated inhibition of EPSCs to 10 ± 8% (Fig 3.1E, F; $p<0.001$). Thus H1a and H3a expression interfere with mGluR mediated eCB production machinery while H2a does not.

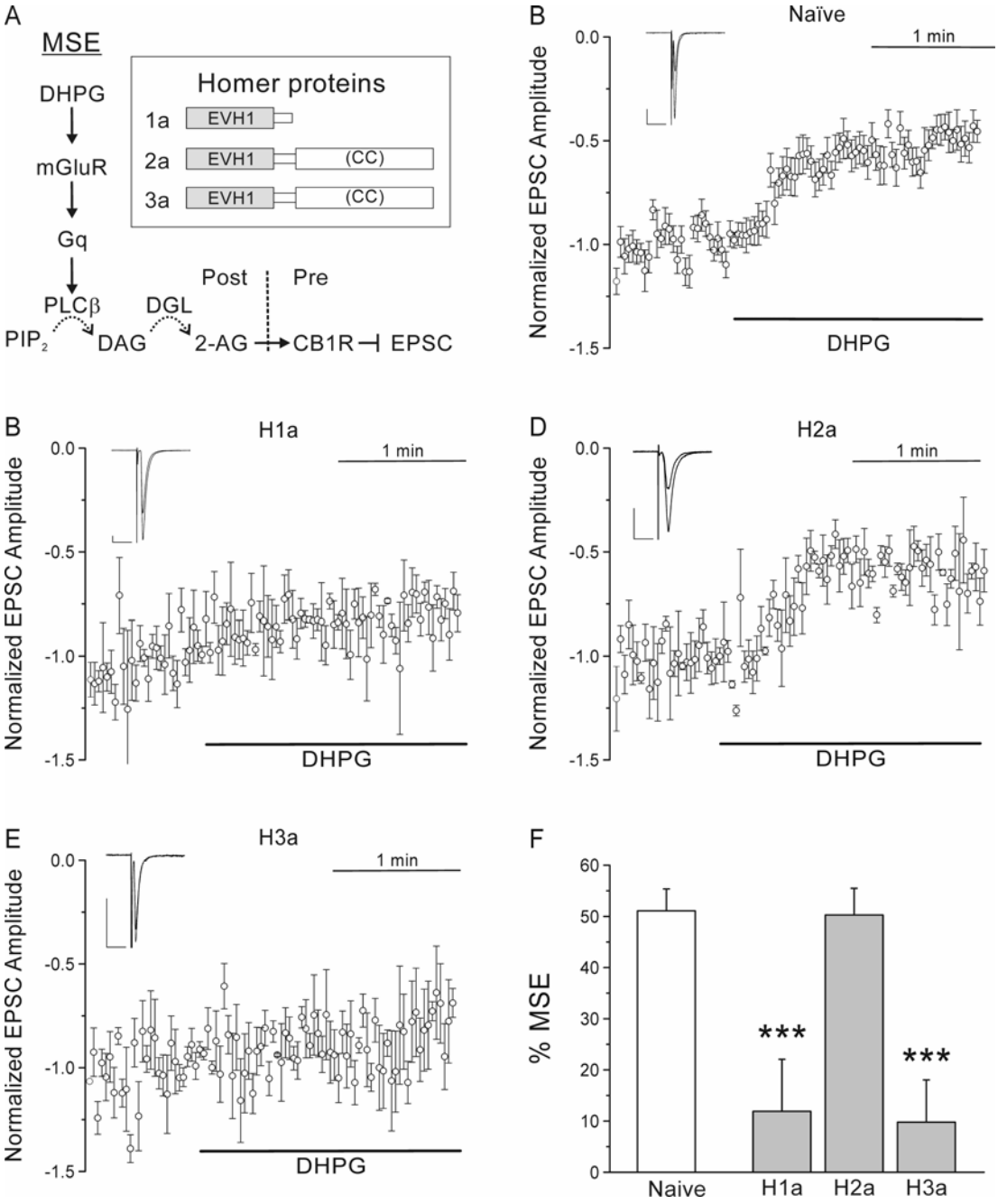

Figure 3.1. H1a expression inhibits MSE. (A) Schematic representation of MSE. Activation of type I mGluRs with DHPG leads to Gq-mediated activation of PLCβ. PLCβ enzymatically cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) producing DAG. DGL hydrolyzes DAG to 2-AG. 2-AG diffuses in a retrograde direction

across the synapse and inhibits EPSCs by activation of presynaptic CB1R. Inset, homer proteins used in this study. Homer proteins exist in short and long forms. The N-terminal EVH1 domain allows binding to target proteins (*e.g.* mGluRs, PLCβ, RyRs) and the C-terminal coiled coil (CC) domain allows oligomerization through homer-homer interactions. (B-E) A bipolar concentric electrode placed near a presynaptic neuron was used to evoke EPSCs in a postsynaptic cell held at -70 mV in whole-cell voltage-clamp. Plots show mean EPSC amplitudes versus time for naive neurons (B) or those expressing H1a (C), H2a (D), or H3a (E), normalized to 30 responses immediately before DHPG application. 1 μM DHPG was applied during the time indicated by the black horizontal bars. Insets display averaged EPSCs before and 2 min after DHPG application. Vertical bar represents 50 pA and horizontal bar represents 20 ms. (F) Bar graph shows percent MSE for naive neurons (open bar) and those expressing the indicated homer proteins (gray bars). Percent MSE was calculated according to the following equation, $\%MSE=100(EPSC_{control}-EPSC_{MSE})/EPSC_{control}$ where $EPSC_{control}$ is the average amplitude of the 30 EPSCs immediately prior to DHPG superfusion and $EPSC_{MSE}$ is the average amplitude of the last 30 EPSCs during 2 min superfusion with DHPG. ***, $p<0.001$, H1a and H3a compared to naive responses.

H1a enhances DSE

Depolarization of hippocampal neurons evokes postsynaptic Ca^{2+} influx via VGCCs that induces eCB synthesis (Fig 3.2A) (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Because expression of H1a and H3a inhibited MSE, we postulated that DSE would be similarly inhibited by these homer isoforms. To test this hypothesis we induced DSE by depolarization of the patch pipette (0 mV, 15 s) and monitored changes in EPSC amplitude. Immediately following depolarization, a transient DSE lasting 60 to 90 s was observed with inhibition of EPSCs reaching 45 ± 3 % (Fig. 2B,F; n=16). DSE was completely blocked by pretreatment with 300 nM AM251 (n=3). Unexpectedly, expression of H1a increased the maximal depolarization induced inhibition of EPSCs to 64 ± 7 % (Fig 3.2C,F; n=8; $p<0.01$). Thus, H1a elicited an effect on DSE opposite to that observed for MSE, consistent with the idea that MSE requires a specific spatial configuration of the PLCβ signaling cascade (Fig 3.1A) and that DSE is actually inhibited by long forms of homer (Fig 3.2A). DSE in H2a and H3a transfected neurons behaved similar to MSE with respective inhibition of 43 ± 8 % (Fig 3.2D,F; n=5) and 23 ± 4 % (Fig 3.2E,F; n=4; $p<0.01$). We considered the possibility that H1a expression may be enhancing DSE through increased Ca^{2+} influx via L-type VGCCs in the postsynaptic neuron (Yamamoto et al., 2005). However, the fraction of Ca^{2+} current inhibited by 10 μM nimodipine was not significantly different in naive relative to H1a expressing cells (42 ± 3% and 38 ± 6 %, respectively; n=5), suggesting that H1a mediated enhancement of DSE occurs downstream from the VGCC. Overall, H1a expression enhanced DSE in contrast to its marked inhibition of MSE.

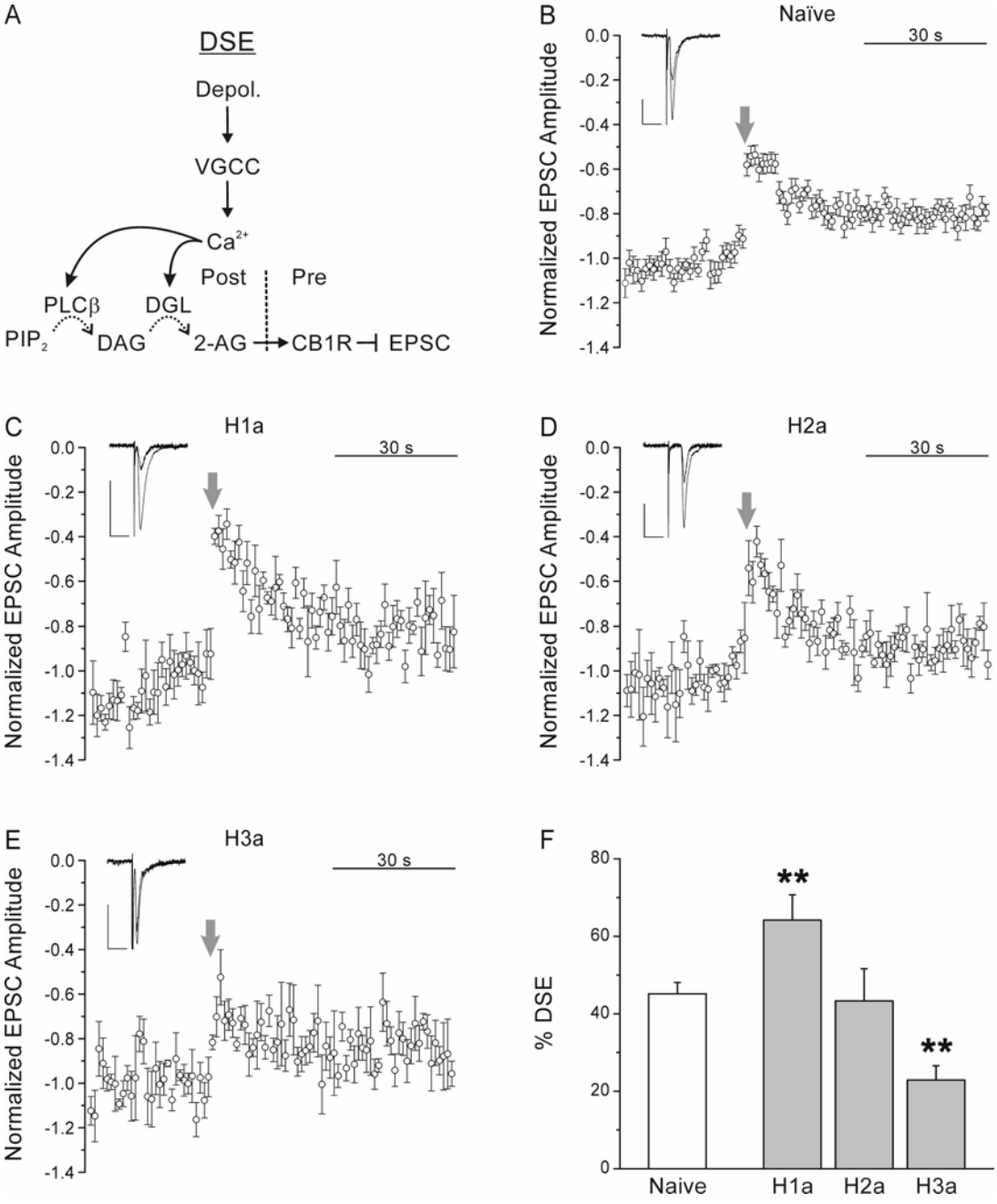

Figure 3.2. H1a expression enhances DSE. (A) Schematic representation of DSE. Depolarization of the postsynaptic neuron evokes Ca^{2+} influx via VGCCs. Ca^{2+} stimulates both PLCβ and DGL producing 2-AG that activates presynaptic CB1R to inhibit EPSCs. (B-E) EPSCs were evoked by stimulation of the presynaptic neuron. The postsynaptic cell was depolarized to 0 mV for 15 s at the times indicated by the

arrows to induce eCB production. Plots show mean EPSC amplitudes versus time for naive neurons (B) or those expressing H1a (C), H2a (D), or H3a (E), normalized to the 15 responses immediately before depolarization. Insets display averaged EPSC from the 15 sweeps before and 2 sweeps immediately after a 15 s depolarization. Vertical bar represents 50 pA and horizontal bar represents 20 ms. (F) Bar graph shows the mean magnitude of DSE for naive neurons (open bar) and those expressing the indicated homer proteins (gray bars). Percent DSE was calculated according to the following equation, $\%\ DSE=100(EPSC_{control}-EPSC_{DSE})/EPSC_{control}$ where $EPSC_{control}$ is the average amplitude of the 15 EPSCs immediately prior to depolarization and $EPSC_{DSE}$ is the average amplitude of the 2 EPSCs immediately after depolarization. **, $p<0.01$, H1a and H3a relative to naive.

H1a overexpression enhances DSE while suppressing MSE in the same neuron

The hypothesis that H1a enhances depolarization-induced, while suppressing mGluR-induced production of eCBs, suggests a critical role for H1a in eCB-mediated plasticity. Thus, DSE and MSE were compared in series to verify that the H1a-induced shift in eCB signaling occurs in the same neuron. Combination experiments on naive cells yielded DSE of 42 ± 4 % with a corresponding MSE of 38 ± 8 % (Fig 3.3A,C; n=8). Consistent with our hypothesis, similar experiments in H1a expressing cells displayed enhancement of DSE and almost complete inhibition of MSE (Fig 3.3B,C). EPSCs evoked from H1a expressing cells were inhibited by 64 ± 13 % following postsynaptic depolarization but, subsequent treatment with 1 μM DHPG produced only 8 ± 6 % inhibition (n=4; $p<0.05$). These experiments suggest that H1a uncouples mGluRs from the 2-AG synthetic machinery, possibly DGL, without impairing the activity of the individual enzymes because DSE remained fully functional. Thus, H1a appears capable of switching the induction mechanism for eCB signaling.

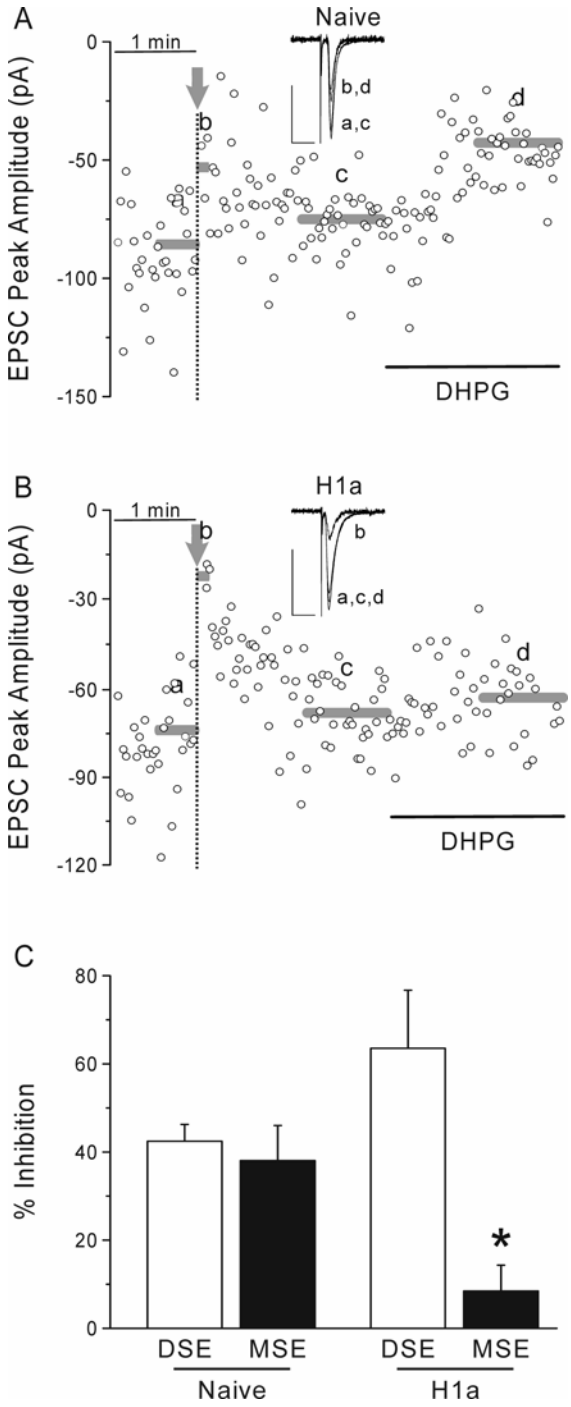

Figure 3.3. H1a shifts neurons toward depolarization mediated production of eCBs. (A-B) Representative plots show peak EPSC amplitude versus time for naive (A) and H1a (B) expressing cells. Control EPSCs were evoked every 2 seconds (0.5 Hz) for 1

minute. The postsynaptic cell was then depolarized to 0 mV for 15 s at the times indicated by arrows. 1 μM DHPG was applied during the time indicated by the black horizontal bars. Gray bars indicate the time and amplitude of the corresponding averaged EPSCs displayed in the insets, denoted (a), (b), (c), or (d). Insets, vertical bar represents 50 pA and horizontal bar represents 20 ms. (C) Bar graph shows the mean magnitude of DSE (open bar) and MSE (solid bar) for naive and H1a transfected cells. *, $p<0.05$, homer 1a MSE versus naive MSE.

BDNF treatment increases expression of H1a, enhances DSE and inhibits MSE

We next addressed the question of whether H1a-mediated changes in eCB signaling could be observed under more physiologically relevant conditions than those employed in the preceding studies in which homer was over-expressed. A number of stimuli increase H1a expression including BDNF (Worley et al., 2007). Indeed, BDNF increases the expression of H1a in hippocampal neurons and disperses puncta formed from homer 1c (H1c) assemblies (Sato et al., 2001; Inoue et al., 2004; Inoue et al., 2007). In our experiments treating hippocampal cultures with 100 ng/mL BDNF for 4 hrs enhanced transcription of H1a mRNA. RT-PCR using H1a and GAPDH specific primers revealed a 32 ± 8.8 fold induction of H1a mRNA (Fig. 4A,B) compared to no effect on the level of GAPDH mRNA (1.0 ± 0.1 fold, n=3; $p<0.001$). To determine whether BDNF stimulation of H1a mRNA leads to functional expression of the protein, we examined the dispersement of H1c puncta using confocal imaging (Fig. 4C,D). Hippocampal cultures were co-transfected with expression vectors for DsRed2 and H1c-GFP. Cells were imaged 48-72 hrs after transfection using confocal imaging to identify H1c-GFP puncta (Fig. 4C,D). The number of fluorescent puncta was objectively counted using image processing as described in Methods. Cultures were treated with BDNF or vehicle (control) and the same cell imaged again after 4 hrs. Control cells showed a 6 ± 10% increase in the number of H1c-GFP puncta (Fig. 4C,D; n=6). In contrast, cells treated with BDNF for 4 hrs showed a -30 ± 9 % loss in puncta (Fig. 4C,D; n=7; $p<0.05$). These results confirm that BDNF treatment increases H1a transcription/translation leading to the functional uncoupling of H1c-GFP assemblies.

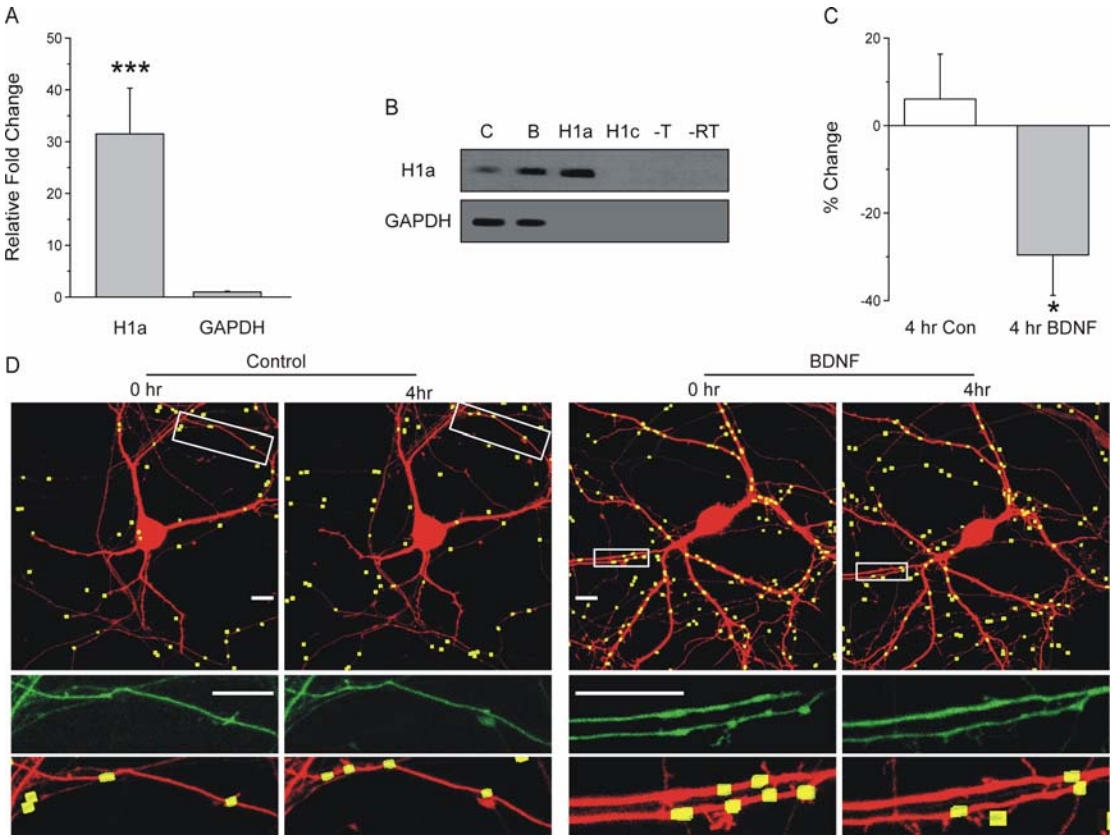

Figure 3.4. BDNF induces expression of H1a mRNA and disperses H1c-GFP puncta. (A) Bar graph displays fold induction of mRNA for homer 1a (H1a) or GAPDH using Q-RT-PCR. Hippocampal cultures were treated for 4 hours with 100 ng/mL BDNF or vehicle control prior to RNA isolation. Relative quantification algorithm was used in which changes in amplification threshold were compared between control and BDNF treated cultures. Data shown are from three separate hippocampal cultures, and Q-RT-PCR performed in triplicate. ***, $p < 0.001$ H1a versus GAPDH. (B) Gel electrophoresis of PCR products amplified from control (C) and BDNF (B) treated cultures. Positive controls of 1 ng purified H1a-pcDNA3.1 plasmid (H1a), and negative controls of 1ng purified H1c-pcDNA3.1 plasmid (H1c), no template (-T), and no reverse transcriptase added to BDNF RNA (-RT). (C) Bar

graph displays percent change in H1c-GFP puncta after 4 hr treatment with vehicle (Control) or 100 ng/mL BDNF. *, $p<0.05$. (D) Representative confocal images of hippocampal neurons expressing H1c-GFP and DsRed2 before (0 hr) and after (4 hr) treatment with vehicle (Control) or BDNF (BDNF). Processed images (top) display H1c-GFP puncta (yellow) counted using the algorithm described in Methods superimposed on the DsRed2 fluorescence image (red). Insets display the magnified image of the boxed area for unprocessed H1c-GFP image (middle) and the processed H1c-GFP/DsRed2 image (bottom). Scale bars represent 10 μm.

To test whether treatment with BDNF shifts the balance between DSE and MSE, cells were treated for 4 hours with 100 ng/mL BDNF and then examined in synaptic transmission experiments. Following treatment with BDNF, depolarization led to a 59 ± 7 % inhibition which is significantly greater than naive controls (Fig 3.5A,C; n=6; $p<0.05$) and similar to that seen in H1a transfected neurons ($p=0.62$). BDNF attenuated MSE produced by 1 μM DHPG to 16 ± 13 % that is significantly reduced relative to naive controls (Fig 3.4B,C; n=5; $p<0.01$), yet similar to the effects observed in H1a expressing cells ($p=0.82$). These data suggest that BDNF induced H1a expression that led to changes in eCB-mediated synaptic plasticity.

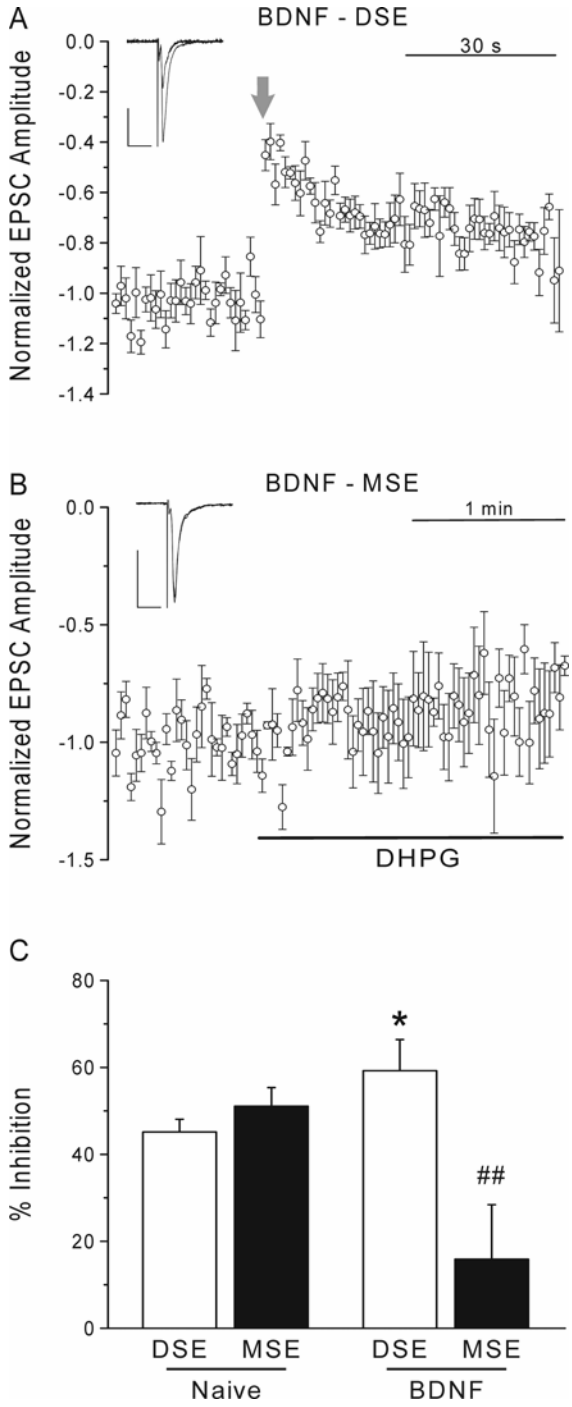

Figure 3.5. DSE was enhanced and MSE was inhibited in BDNF-treated neurons. (A) 4 hour treatment with BDNF increased the magnitude of DSE. Plot shows mean EPSC amplitudes, normalized to the 15 responses immediately before depolarization plotted versus time. The postsynaptic cell was depolarized to 0 mV for 15 s at the

time indicated by the arrow. Inset displays 15 averaged EPSCs from before and 2 EPSC from immediately after depolarization. Insets, vertical bar represents 50 pA and horizontal bar represents 20 ms. (B) BDNF treatment led to a loss of DHPG-mediated MSE. Plot shows mean EPSC amplitudes normalized to the 30 responses before superfusion of 1 μM DHPG, plotted versus time. DHPG was applied during the time indicated by the black horizontal bar. Insets display averaged EPSC from before and 2 min after DHPG application. Insets, vertical bar represents 50 pA and horizontal bar represents 20 ms. (C) Bar graph shows the mean magnitude of DSE (open bar) and MSE (solid bar) for naive and BDNF treated cultures. *, $p<0.05$ BDNF-DSE versus Naive-DSE; ##, $p<0.01$ BDNF-MSE versus Naive-MSE.

IV. Discussion

Homer proteins act as molecular scaffolds in the PSD to organize signaling cascades for effective receptor activation of downstream signaling. In the current study, we show that expression of H1a, a short isoform that disrupts homer scaffolds, modulates eCB-mediated plasticity at glutamatergic synapses in hippocampal cultures. Expression of H1a enhanced the magnitude of DSE while inhibiting MSE. Treatment with BDNF enhanced the transcription of H1a mRNA and dispersed H1c-GFP puncta indicating expression of H1a protein and uncoupling of CC-homer assemblies. Furthermore, BDNF produced a shift towards depolarization mediated eCB production similar to that seen when H1a was expressed. These results demonstrate that regulated expression of homer proteins provides a mechanism by which the balance between metabotropic and depolarization-induce eCB signaling may be spatially and temporally controlled.

Opposite effects of H1a on DSE versus MSE

H1a acts as a dominant-negative inhibitor of homer scaffolds by binding to target proteins via its EVH1 domain but, because it lacks a CC domain, fails to oligomerize with other homer proteins. Components of the eCB signaling pathway including mGluR1, mGluR5, PLCβ and DGL all bind homer EVH1 domains (Shiraishi-Yamaguchi and Furuichi, 2007); (Jung et al., 2007). H1a binding to type 1 mGluRs will induce constitutive, ligand-independent activation of the receptor (Ango et al., 2001). This may occur in the cells studied here but, it is not clear how receptor activation could inhibit MSE. If tonic mGluR activation occluded MSE, we would

have expected DSE to be inhibited as well, which was not the case. Thus, the most likely explanation for the H1a-mediated inhibition of MSE described here is an uncoupling of the type 1 mGluRs from downstream enzymes responsible for 2-AG synthesis. A previous report outlined a critical interaction between DGL and H2a (Jung et al., 2007). Linkage through a PPxxF domain on DGL was required for effective mGluR-mediated 2-AG production but, had no effect on enzymatic activity. These data are consistent with the lack of effect of H2a expression on both DSE and MSE observed in our studies. Presumably, endogenous long isoforms of homer maintain the molecular assembly required for MSE but are not limiting, consistent with the expression of H1b/c, H2a/b, and H3a/b in hippocampal neurons (Shiraishi et al., 2004). This reasoning would suggest that increased expression of long homer proteins should not affect MSE but, surprisingly H3a expression effectively blocked MSE. Expression of H3a also attenuated DSE suggesting a less selective interaction than that produced by H1a. Although the overall domain composition is shared between H3a and H2a, these proteins exhibit notable differences in their amino acid sequences. While their EVH1 domains are strongly conserved with 80% sequence identity, the CC-domains vary substantially and exhibit less than 30% identity. These differences are likely to produce unique preferences for interacting with various binding partners and/or regulation by posttranslational modification, ultimately resulting in the distinct effects of the two proteins. For instance, H3a, but not H2a, was found to contain a CaMKII phosphorylation site which selectively uncouples mGluR-Homer-IP3R complexes (Mizutani et al., 2008). This may explain the

apparent loss of MSE after H3a expression, however other CC-homers have this same site, and thus its role in eCB signaling is unclear.

H1a expression enhanced DSE. Thus, H1a expression does not grossly impair the 2-AG synthetic machinery. We suggest that DSE is less dependent on close apposition of VGCCs to PCLβ and DGL relative to the more constrained organization required for MSE. Because infusion of H1a into neocortical pyramidal cells up-regulated L-type VGCCs (Yamamoto et al., 2005) we examined the effects of H1a expression on Ca^{2+} currents. We did not observe an increase in L-type Ca^{2+} currents in cells expressing H1a although, we note that the currents were recorded from the soma with Ca^{2+} chelator in the intracellular solution. Thus, we would not have observed an increased L-type Ca^{2+} current if the H1a effect were limited to dendritic regions or was secondary to reduced Ca^{2+} dependent feedback. RyRs participate in DSE by amplifying Ca^{2+} influx mediated by VGCC (Isokawa and Alger, 2006). CC-homer proteins bind to and inhibit RyR function and their inhibition is reversed dose-dependently by H1a (Hwang et al., 2003; Westhoff et al., 2003). Thus, it is possible that H1a reduces this inhibition resulting in enhanced Ca^{2+} release from intracellular stores and more efficient DSE. Alternatively, DSE is independent of PLCβ and Ca^{2+} stores in cerebellum (Brenowitz et al., 2006) and thus, stores may play little role in the enhancement of DSE. Overall, our results demonstrate that MSE is more sensitive to H1a-induced uncoupling of homer scaffolds relative to DSE; thus, H1a can gate the induction mechanism for eCB production.

Physiological consequences of H1a-regulated induction of eCB production

The respective roles of DSE and MSE in synaptic transmission provide insight into how H1a modulation of eCB production might influence neuronal function. DSE will tend to evoke 2-AG release from the entire dendritic arbor whereas mGluR-mediated MSE is expected to be input specific (Brenowitz et al., 2006). Thus, H1a expression might potentiate the homeostatic plasticity that accompanies intense stimulation. Indeed, H1a is up-regulated during epileptic activity (Kato et al., 1998) and the eCB system is thought to provide an on-demand neuroprotection from excitotoxicity (Shen and Thayer, 1998b; Marsicano et al., 2003). Clearly, this safety mechanism provides a critical defense against aberrant synaptic activity and should not be switched off. Conversely, modulating the strength of individual synapses is thought to underlie learning and memory. Indeed, H1a expression is required for consolidation of long term fear memories (Inoue et al., 2009) and H1a enters synaptic spines in an activity-dependent manner (Okada et al., 2009). Perhaps H1a-mediated uncoupling of mGluR-mediated MSE contributes to synaptic strengthening. Consistent with the idea that H1a-mediated increases in DSE might enhance on-demand neuroprotection at the expense of single synapse plasticity were the varied effects on cognitive performance and status epilepticus that resulted from viral-mediated over-expression of H1a, H1c and H1g in the hippocampus (Klugmann et al., 2005). H1a over-expression led to learning deficits, but increased resistance to status epilepticus, while H1c and H1g enhanced cognitive performance, but had no effect on status epilepticus. Thus, our results demonstrating differential effects of

homer long and short forms on eCB plasticity parallel complex effects observed following expression of the various isoforms in vivo.

mGluR activation has been strongly linked to changes in plasticity and behavior in part, through modulation of eCB signaling (Bortolotto et al., 1995; Diagana et al., 2002; Bellone et al., 2008). Homer proteins have also been studied in behavioral experiments. Deletion of homer genes impairs behavioral plasticity in drosophila (Diagana et al., 2002) and mimics behaviors associated with cocaine induction and withdrawal in mice (Swanson et al., 2001; Szumlinski et al., 2004). Inhibition of eCB-mediated LTD in the nucleus accumbens following administration of cocaine was accompanied by expression of CC-homers (Fourgeaud et al., 2004). Thus, homer proteins are intimately linked to behavioral plasticity, particularly that induced by drugs of abuse, and the effects of homer proteins on the induction of the eCB system may play an integral role in behavioral modification.

Both homer proteins and eCBs participate in the remodeling of synaptic networks (Ehrengruber et al., 2004; Foa and Gasperini, 2009). H1a affects postsynaptic structures including those associated with H1c (Inoue et al., 2004) and inhibits spine morphogenesis (Sala et al., 2003). H1a is an immediate early gene induced by several pharmacologic and activity-related stimuli (Worley et al., 2007) including BDNF, which robustly activates H1a transcription (Sato et al., 2001). We found that BDNF increased H1a mRNA and uncoupled H1c puncta found in dendritic structures. H1a induction led to enhanced DSE at the expense of MSE. BDNF plays a critical role in synaptic development, changes in synaptic strength, and the induction and adaptation to seizures (Kuczewski et al., 2009) . eCBs also participate

in these processes (Monory et al., 2006; Lovinger, 2008). Thus, it is reasonable to speculate that BDNF-induced, homer-mediated changes in eCB signaling contribute to dynamic changes in synaptic networks.

V. Conclusion

We have shown that the postsynaptic compliment of homer proteins has profound effects on both depolarization-mediated and mGluR-mediated eCB signaling. Homer regulated changes in PSD structure provide a point of crosstalk at which the diverse signals that regulate homer immediate early gene expression can influence eCB mediated changes synaptic plasticity.

Chapter Four: Concluding Remarks

In the previous reports I discuss two systems that are intricately connected with eCB signaling. Chapter Two provides insight into the properties of THC at CB1R containing excitatory synapses of the hippocampus and provides conclusive evidence of THC interacting with both CB1R and the eCB system. Chapter Three describes the postsynaptic coalescence of homer scaffolding proteins with eCB synaptic plasticity induced by depolarization or mGluR activation. In the following pages I will discuss the study findings in detail and suggest both conclusions that may be drawn as well as future investigations that these studies suggest.

THC acts as a partial agonist of CB1R and impacts behavior through interaction with eCB signaling at central synapses. Previously THC was found to act as both an agonist of CB1R and as an antagonist of the effects of exogenous cannabinoid application in hippocampal cultures (Shen and Thayer, 1999; Straiker and Mackie, 2005). We used electrophysiology to characterize the mechanistic switch in THC inhibition of EPSC stemming from presynaptic CB1R activation. An increase in stimulation rate thwarted THC inhibition of EPSC, but THC readily occupied CB1R and antagonized activation by Win-2, a full CB1R agonist. This finding helps to clarify the varied responses to THC seen in neurotransmission experiments and supports the notion that THC will inhibit slow firing synapses while having no effect at fast firing synapses. The result of this rate specific switch will be inhibition of the early component of burst firing, a synaptic activity pattern which is prevalent in the hippocampus.

THC activates presynaptic CB1R and inhibits neurotransmitter release presumably through inhibition of presynaptic VGCC akin to the inhibition produced

by Win-2 (Shen and Thayer, 1998a). We described the sensitivity of presynaptic VGCCs to physiologically relevant THC concentrations and the voltage dependant THC modulation of Ca^{2+} current. THC inhibition of I_{Ca} was sensitive to an increased rate of depolarization and displayed a different profile in both voltage dependence and prepulse facilitation experiments compared to Win-2. This mechanism characterizes THC interaction with presynaptic VGCC and clearly differentiates its properties from those of Win-2 a common substitute for THC in scientific studies.

THC is an agonist to CB1R and inhibits activation of CB1R by applied endogenous agonists (Kelley and Thayer, 2004). DSE is a functional consequence of eCB mobilization in the hippocampus, but a study describing its relationship to THC found that THC had no agonist properties (Straiker and Mackie, 2005). In Chapter Two we describe a protocol for the induction of eCB plasticity through depolarization of postsynaptic hippocampal neurons. THC, which had shown antagonizing effects at fast stimulus rates for exogenously applied Win-2, also antagonized eCB effects following depolarization. These experiments support Straiker and Mackie's finding that THC acted as an antagonist, but proposes an activity dependant switch as the method by which agonist properties are removed at CB1R.

While Chapter Two resolves the broader issues of THC antagonism of eCB signaling it poses several interesting questions in cannabinoid pharmacology that require further study. Firstly, THC effects on neurotransmission are usually conducted in the context of fixed rate presynaptic stimulation. However, years of research have revealed that instead of a constant firing rate, complex patterns are more common, where bursts of synaptic activity may be followed by extended

intervals where essentially no presynaptic input is received. To further complicate the matter, the importance of mean firing rate has been questioned, and rather spike timing (using ISI) may be of more significance to learning and memory (Frerking and Ohliger-Frerking, 2006). *In vitro* studies of firing neurons, whether from acute brain slice or culture model would offer a reasonable approximation of THC effects *in* vivo. However, to gauge the effect of THC on real-time synaptic plasticity, behavioral studies should be considered. Since modification of synaptic firing patterns is the goal, free roaming rodents connected to electrophysiological aparati tested using THC may offer new insights into THC modification of firing patterns and shed light on how the readily reversible agonist properties of THC lead to psychotropic effects. Secondly, THC is often a political football that is surrounded by a stringent regulatory culture. The difficulty procuring THC combined with its inherent lipophilicity has made experimentation an arduous task and has driven many researchers to rely on more ideal agonists, most notably Win-2. While Win-2 does stimulate similar physiological, behavioral, and biochemical results, Chapter Two argues that THC and Win-2 are very different in their activity at CB1R synapses. THC and Win-2 stimulate $G_{i/o}$ from the receptor and inhibit I_{Ca} but the antagonist properties of THC cannot be cast aside.

Homer proteins organize the functional structures of excitatory signaling and can regulate synaptic transmission and plasticity. Previous reports have described a link between mGluR and CC-homers and revealed that H1a may uncouple this interaction (Kammermeier, 2008). In Chapter Three we used electrophysiology, molecular biology, and confocal imaging to ascertain the effect of homer proteins

commonly expressed in the hippocampus on eCB plasticity. The short homer isoform, H1a inhibited mGluR mediated eCB plasticity, and at the same time enhanced depolarization-mediated eCB plasticity. This finding offers insight into the postsynaptic makeup of homer proteins conducive to eCB signaling, and offers a precise target for the regulation of different eCB plasticity.

Homer proteins are expressed throughout the CNS and are associated with excitatory synaptic signaling. Previous reports have demonstrated eCB modulation of excitatory neurotransmission through postsynaptic depolarization (DSE) or activation of mGluR (MSE) (Maejima et al., 2001a; Ohno-Shosaku et al., 2001). In Chapter Three, we characterized the contribution of postsynaptic homer expression to DSE and MSE at excitatory hippocampal synapses. H1a and H3a inhibited MSE responses, while H2a had no effect. H1a conversely enhanced DSE. This finding offers insight into homer involvement in eCB signaling and suggests that H1a functionally uncouples mGluR from eCB production while enhancing eCBs produced through depolarization.

H1a functionally uncouples CC-homer assemblies including mGluR-IP3R and leads to changes in postsynaptic physiology. Induction of H1a has been achieved through various stimuli including treatment with BDNF (Kato et al., 2003). In Chapter Three we experimentally tested the hypothesis that BDNF increases H1a expression in hippocampal cultures. RT-PCR experiments found that H1a mRNA was induced, and the functional consequences of H1a expression (H1c-GFP dispersement) were seen. Furthermore, BDNF treatment led to an identical eCB plasticity profile as H1a expression, where MSE was inhibited and DSE was

enhanced. These results demonstrate a physiologically relevant means by which eCB plasticity may be influenced in postsynaptic neurons.

In Chapter Three we used electrophysiology, molecular biology, and confocal imaging to dissect specific eCB plasticity in the context of postsynaptic homer expression and to put forward BNDF as a physiologically relevant path to H1a induction. This chapter provides the first significant link between homer expression and changes to eCB plasticity. The potential for an interaction between these two pathways has been suggested previously (Fourgeaud et al., 2004; Jung et al., 2007; Katona and Freund, 2008), but to date none has pursued this line of research. This study opens the door to future studies on excitatory synaptic signaling by linking two major players in glutamatergic synaptic plasticity. Future studies of this interaction will likely provide a better understanding of the postsynaptic modulators of eCB signaling. Firstly, the PPxxF recognition sequence found in the EVH1 domain has many targets, including DGL (Jung et al., 2007). Interestingly both DSE and MSE experiments using H2a expressing neurons were statistically equal to controls, and thus H2a expression did not affect coupling of either Ca^{2+} mediated or mGluR mediated eCB signaling. However, H3a showed a significant decrease in both DSE and MSE responses. Localization studies of multiple homer proteins (including additional ones not tested in Chapter Three) using immunocytochemistry, backed by electrophysiology would help to identify specific homer protein interactions with eCB signaling molecules (PLCβ, DGL, mGluR, RyR, L-type VGCC). These experiments would tease apart which homers were critical to localization of eCB proteins and may further delineate a mechanism by which DSE and/or MSE may be modulated.

Secondly, H1a expression and induction by BDNF offer a critical method by which synapse specific eCB plasticity may be eliminated in favor of a neuron-wide eCB response. The behavioral consequences of this theory would be particularly interesting. Homer knockouts for specific homer proteins, especially H1a would be interesting. H1a overexpression and homer 1 and 2 genes knockout studies have been conducted (Szumlinski et al., 2005; Tappe and Kuner, 2006; Jaubert et al., 2007) resulting in increased learning defects and anxiety, however H1a knockouts remain a challenging endeavor.

In conclusion, we described two systems that modify glutamatergic cannabinoid signaling, one at presynaptic CB1R and the other in the postsynaptic density where eCBs are produced. We outlined the interaction between THC and CB1R in light of its agonist and antagonist properties. These results suggest that THC interactions with CB1R are dependant on presynaptic firing patterns, and that THC plays a dual role as an agonist of CB1R and an antagonist of eCB signaling. Furthermore, we described a mechanism by which postsynaptic neurons may both enhance eCB plasticity mediated by depolarization and limit metabotropic eCB plasticity, through the induction of H1a expression. This work links structural proteins in the PSD to changes in eCB signaling and offers a model to influence specific responses to synaptic input.

Endogenous and exogenous cannabinoids influence neuronal physiology and plasticity, and modulate behavior. These studies demonstrate novel properties of pre- and postsynaptic eCB signaling. Identifying how the eCB system is modulated may

lead to a better understanding of physiological and pathological states within the CNS.

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Appendix A

September 21, 2009

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Dear Mr. Roloff:

This is to grant you permission to include the following article in your thesis for the University of Minnesota entitled "Modulation of Hippocampmal Endocannabinoid Plasticity by Homer Proteins and Delta 9-tetrahydrocannabinol":

> Alan M. Roloff and Stanley A. Thayer, Modulation of Excitatory Synaptic Transmission by Δ^9-Tetrahydrocannabinol Switches from Agonist to Antagonist Depending on Firing Rate, *Mol Pharmacol* 2009 75: 892-900

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