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NOVEL ROLES OF THE ENDOCANNABINOID SYSTEM IN MODULATING
SYNAPTIC PLASTICITY

By

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Novel roles of the endocannabinoid system in modulating synaptic plasticity

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Learning and memory formation are invaluable processes in human life; however, the cellular mechanisms that control these phenomena are largely unknown. Synaptic plasticity, which is the ability of the synapse between two neurons to change in strength based on activity, is believed to be a key process in the formation of memories and learning. Endocannabinoids (eCB) have recently emerged as important modulators of synaptic plasticity but their precise roles and mechanisms are not well understood and many contradictions exist in the current literature. We have investigated the roles of eCBs and their primary receptor, the CB1 receptor, in the central nervous system using electrophysiological recordings in rodent hippocampus. We find that a moderate frequency 10 Hz stimulation protocol produces long-term potentiation (LTP) that is modulated by eCBs in both mice and rats; but surprisingly, the roles played by eCBs differ greatly between species. In rats, 10 Hz LTP requires CB1 receptor activation, as it is completely abolished by the CB1 antagonists AM251 and SR141716. Unlike theta burst stimulation (TBS) induced LTP, 10 Hz LTP does not require NMDA receptor activation. However, it is prevented when both NMDA and group1 mGluR receptors are blocked. The 10 Hz LTP is also independent of GABAergic synaptic inhibition, suggesting it is a novel form of excitatory synaptic plasticity mediated by the eCB system in hippocampus. In mice, we find that CB1 has an inhibitory effect on 10 Hz induced LTP. When the receptor is genetically removed in CB1 (-/-) mice or pharmacologically blocked wild type mice, 10 Hz LTP is greatly facilitated. Similar to TBS LTP, 10 Hz LTP in mice is NMDA receptor mediated. Also, the ability to achieve successful long-term depression (LTD) is decreased in CB1 (-/-) mice; yet, the magnitude of successful LTD is not changed. Together, this data supports a role for the CB1 receptor in inhibiting the induction of LTP with moderate stimulation protocols in mice, while in rats CB1 activation is required for 10 Hz LTP. Overall, our data supports that eCBs are crucial modulators of synaptic plasticity, although the roles they play may differ among species.

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CHAPTER 1: BACKGROUND AND SIGNIFICANCE

Humans have been captivated by the properties of *Cannabis sativa*, both for recreational and medicinal purposes, for thousands of years. The world's oldest known pharmacopoeia, the Pen-ts'ao Ching from China, describes its use as far back as 2727 B.C. Since then, countless cultures and societies have reported on its many uses and effects, which have included some interesting consequences on memory and learning (Murray et al., 2007). Despite this tremendous interest, it is only within the last 50 years that the mechanisms by which *Cannabis* acts on the mind and body have begun to be identified.

The endocannabinoid system is discovered

The first major breakthrough in the cannabinoid field came in 1964 when Raphael Mechoulam's lab identified Δ^9 -tetrahydrocannabinol (THC) as the major psychoactive component of *cannabis* (Gaoni and Mechoulam, 1964; Gaoni and Mechoulam, 1971). This discovery fueled an intense search for a receptor, which continued for over 20 years until the cannabinoid 1 (CB1) receptor was identified in 1988 (Devane et al., 1988) and then cloned in 1990 (Matsuda et al., 1990). A second cannabinoid receptor, CB2, was subsequently discovered and cloned in 1993 (Munro et al., 1993); however, it is predominantly located in cells of the immune system and not the central nervous system (Axelrod and Felder, 1998; Pertwee, 1997). Currently, there is great interest in the cloning of a third cannabinoid receptor, and many pieces of evidence point to its

existence in the brain (Begg et al., 2005; Lauckner et al., 2008; Mackie and Stella, 2006; Ryberg et al., 2007).

The second major breakthrough for cannabinoid research occurred when endocannabinoids (eCBs), which are endogenous ligands for the cannabinoid receptors, were discovered. Currently there are two well-characterized eCBs although many other possible ligands have been identified. The first eCB to be discovered was N-arachidonylethanolamide, named anandamide (AEA) for the Sanskrit word ananda, which means bliss (Devane et al., 1992). Several years later a second eCB, 2-arachidonoylglycerol (2-AG), was also isolated (Mechoulam et al., 1995; Sugiura et al., 1995). These eCBs share some overlap in distribution the CNS and for the most part 2-AG is found in much higher concentrations (Stella et al., 1997; Sugiura et al., 1995).

The endocannabinoids have several properties that make them unique as neurotransmitters. Most notably, they are not stored in vesicles and thus do not require vesicular fusion for release. Instead, they are membrane bound lipid precursors that are enzymatically activated and released directly into the synaptic cleft by cleavage from the membrane. Endocannabinoid release is a calcium dependent process and occurs in response to cell depolarization (Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001) or activation of group1 metabotropic glutamate receptors (mGluRs) (Ohno-Shosaku et al., 2002a; Varma et al., 2001). Although these processes occur independently, both cause an increase in intracellular calcium concentration, which is key for endocannabinoid synthesis (Cadas et al., 1996; Di Marzo et al., 1994; Stella and Piomelli, 2001; Stella et al., 1997). There is also evidence that dopaminergic and muscarinic acetylcholine

receptor activation can lead to endocannabinoid production, but these pathways are not as well characterized (Giuffrida et al., 1999; Kim et al., 2002).

Endocannabinoid synthesis & degradation

Anandamide and 2-AG are independently synthesized through multi-step processes. Anandamide synthesis begins when N-acyltransferase joins the precursor lipid phosphatidylethanolamine (PE) with an arachidonate group to create N-arachidonyl-PE. Phospholipase D (PLD) then catalyzes the hydrolysis of N-arachidonyl-PE to create anandamide. Increases in intracellular calcium initiate both the formation of N-arachidonyl-PE and its conversion to anandamide (Freund et al., 2003). 2-AG synthesis follows two possible routes both starting with the lipid precursor phosphatidylinositol (PI). In one route, PI is acted upon by phospholipase C (PLC) to create 1,2-diacylglycerol (1,2-DAG), which is then converted to 2-AG via diacylglycerol lipase. In the other route, PI is acted upon by phospholipase 1A (PL1A) to create 2-arachidonyl-lysophospholipid, which is converted to 2-AG through Lyso-PLC activity. Increases in intracellular calcium concentration have also been found to stimulate 2-AG release, likely through the above pathways (Stella and Piomelli, 2001; Stella et al., 1997).

Endocannabinoid synthesis is also unique in that it occurs postsynaptically. Such neurotransmitters are known as retrograde messengers, meaning they are released postsynaptically and then traverse the synapse “backwards” to bind presynaptic targets. This interesting mode of action was first described when a unique form of short term plasticity, termed depolarization-induced suppression of inhibition (DSI), was discovered in cerebellum (Llano et al., 1991) and hippocampus (Pitler and Alger, 1992). DSI

required depolarization of a postsynaptic neuron to inhibit neurotransmitter released by presynaptic inputs. At the time of these discoveries, it was not known which retrograde neurotransmitters were responsible for DSI. This work showed that the synapse could transmit messages in both directions and these actions may have important roles in modulating synaptic strength. Later, once the CB1 receptor had been better characterized and endocannabinoids were discovered, it was revealed that the endocannabinoid neurotransmitter system mediated DSI (Diana et al., 2002; Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Varma et al., 2001; Wilson and Nicoll, 2001).

Aside from elucidating a retrograde neurotransmission system, DSI also describes an important form of plasticity mediated by eCBs; however, before the roles of endocannabinoids in synaptic plasticity can be discussed, the termination of eCB signaling must be considered. Once released, AEA and 2-AG are taken back into neuron terminals through a carrier mediated process. Although an eCB transporter is yet to be cloned, there are several pieces of evidence supporting its existence. AEA and 2-AG uptake is saturable and these two ligands can inhibit one another's uptake, suggesting a shared transporter. Uptake is also temperature dependent, has a faster clearance rate than diffusion, and exhibits substrate selectivity (Beltramo et al., 1997; Di Marzo et al., 1994; Hillard et al., 1997). In addition, a transporter inhibitor, AM404, has been discovered. AM404 is an anandamide analog that does not activate receptors but effectively blocks both AEA and 2-AG clearance (Beltramo and Piomelli, 2000; Bisogno et al., 2001). The endocannabinoid transporter seems to be present on both neurons and glia. Its operation does not require energy expenditure from the cell, which likely makes it a process of facilitated diffusion (Beltramo et al., 1997; Hillard et al., 1997).

Once eCBs are successfully transported into neurons they are rapidly hydrolyzed. Anandamide is broken down into arachadonic acid and ethanolamine by the enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 2001; Cravatt et al., 1996). FAAH is found throughout the brain and its distribution accurately overlays known regions of endocannabinoid signaling (Desarnaud et al., 1995; Hillard et al., 1995; Thomas et al., 1997). 2-AG is mainly hydrolyzed by the enzyme monoglyceride lipase (MGL) and converted into glycerol and a fatty acid (Karlsson et al., 1997). MGL expression has also been shown throughout the brain, with the highest levels corresponding to areas of CB1 receptor expression and 2-AG release (Dinh et al., 2002).

The CB1 receptor

Just as it is important to understand how endocannabinoids are synthesized and degraded, it is equally important to understand the actions of their receptors. I will focus on CB1 and its effects on synaptic plasticity as it is the predominant receptor in the brain. The cannabinoid receptors are G-protein coupled and act through $G_{i/o}$ protein signaling. Upon activation, CB1 inhibits adenylyl cyclase activity, which decreases cytosolic cyclic AMP concentrations and activates mitogen activated protein kinases (Adams and Sweatt, 2002; Bouaboula et al., 1995). Initiation of these second messenger systems can affect gene regulation, which is important for the maintenance of long-term synaptic changes. Also, CB1 activated $G_{i/o}$ proteins inhibit L, N, and P/Q type voltage-gated calcium channels and activate inwardly rectifying potassium channels. These channel effects likely occur through direct interactions with the $\beta\gamma$ subunit of the G-

protein (Howlett et al., 2002; Pertwee, 1997). Together these actions inhibit presynaptic neurotransmitter release in the targeted cell.

CB1 is one of the most abundantly expressed GPCR receptors in the brain and has been found in many regions; with the highest levels of expression in hippocampus, cerebellum, cortex, amygdala, and basal ganglia (Freund et al., 2003; Herkenham et al., 1990; Howlett et al., 2002; Mackie, 2005). It is imperative to recognize that regions of high expression are also areas of key importance to learning and memory function. Immunohistochemical analysis shows that CB1 receptors are strongly expressed on synaptic terminals of cholecystinin-containing (CCK) inhibitory interneurons in hippocampal region CA1 (Hajos et al., 2000; Katona et al., 2001; Katona et al., 2000; Katona et al., 1999; Marsicano et al., 2002; Tsou et al., 1999). When activated they inhibit GABA release, and most of CB1's actions in hippocampus are attributed to this mechanism (Hoffman, 2000; Kreitzer and Regehr, 2001b; Levenes et al., 1998; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001).

Yet, despite the view that CB1 only acts on inhibitory interneurons in hippocampus to regulate GABA release, there is accumulating evidence supporting a role for CB1 in the modulation of excitatory signaling. Several groups have demonstrated that application of the CB1 receptor agonist WIN55,212-2 depresses presynaptic excitatory signaling, as seen by a decrease in EPSP amplitude and increased paired pulse ratio (Al-Hayani and Davies, 2000; Hajos and Freund, 2002; Hajos et al., 2001; Hoffman et al., 2005; Misner and Sullivan, 1999; Takahashi and Castillo, 2006). These effects do not occur in CB1(-/-) genetic knockout animals and they are reversed by application of CB1 antagonists (Hoffman et al., 2005; Misner and Sullivan, 1999; Takahashi and Castillo,

2006). It should also be noted that WIN55,212-2 depresses IPSC amplitudes, which supports CB1's presence on GABAergic neurons (Hajos and Freund, 2002; Hoffman and Lupica, 2000).

Aside from the WIN55,212-2 evidence, several other observations suggest that CB1 can affect glutamatergic signaling. CB1 mRNA has been found in CA3 pyramidal cells, whose axons make up the Schaffer collaterals that synapse on CA1 pyramidal cell dendrites in the stratum radiatum (Marsicano et al., 2003). The enzymatic machinery responsible for the synthesis of eCBs has also been found in glutamatergic inputs (Nyilas et al., 2008), and depolarization induced suppression of excitation (DSE), which is similar to DSI except that it inhibits presynaptic release from glutamatergic terminals, has been found in regions including hippocampus (Hajos et al., 2001; Kreitzer and Regehr, 2001b; Ohno-Shosaku et al., 2002b; Straiker and Mackie, 2005). Although there is not clear immunohistochemical data supporting CB1's existence on glutamatergic terminals in hippocampus physiological data does support this possibility.

Endocannabinoids and synaptic plasticity

Cannabinoid receptors presence on both inhibitory and excitatory neurons has important implications for the control of synaptic strength and plasticity. Synaptic plasticity is the ability of a synapse to undergo changes in strength based on prior activity, and it can be either short-term, lasting on the order of seconds to minutes, or long-term, lasting for hours or even days in some *in vivo* models. The ability of a synapse to change strength is believed to underlie the crucial processes of learning and memory formation.

There are many types of synaptic plasticity, which vary depending on the types of neurons involved, the duration of the change, the direction of change, and the brain region involved. The most widely studied form of synaptic plasticity is long-term potentiation (LTP), which was discovered in 1973 (Bliss and Lomo, 1973). In this initial report, which used recordings from the hippocampus of anesthetized rabbits, it was found that repetitive stimulation paradigms caused an increase in synaptic response that could last from 30 minutes up to 10 hours. This new phenomena exhibited pathway specificity, saturation, and was associative. These have since all been further described as traits of LTP. Overall, LTP can be defined as a sustained increase in synaptic response following the administration of specific stimulation paradigms (Malenka and Bear, 2004).

Just as it is important to strengthen synaptic connections it is equally necessary to reverse potentiation and depress a synaptic connection. Long-term depression (LTD) is described as the ability of a synapse to show a sustained decrease in response following specific stimulation paradigms. The discovery of LTD came in two parts. First, it was found that LTP could be reversed, or depotentiated, bringing synaptic transmission back to baseline levels following a potentiation (Barrionuevo et al., 1980; Fujii et al., 1991; Staubli and Lynch, 1990). Secondly, it was discovered that basal synaptic responses could be depressed with low-frequency stimulus paradigms (Dudek and Bear, 1992). LTP and LTD both can occur in many regions of the brain between many different types of synapses. Some of the major regions used to reliably study plasticity, such as hippocampus, cortex, and cerebellum, are also known to be very important for learning and memory formation.

Aside from the long-term synaptic plasticities, there are also various forms of short-term plasticity, which completely reverse and last on the order of seconds to minutes. There are two forms of short-term plasticity that are of great importance to the endocannabinoid field. These are depolarization induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE). In both DSI and DSE the release of neurotransmitter, either GABA (DSI) or glutamate (DSE), is temporarily inhibited following a brief postsynaptic depolarization. DSI was independently discovered in cerebellum (Llano et al., 1991) and hippocampus (Pitler and Alger, 1992), and as discussed previously, suggested the involvement of a retrograde messenger system, which was eventually shown to be the eCB system (Diana et al., 2002; Kreitzer and Regehr, 2001a; Ohno-Shosaku et al., 2001; Varma et al., 2001; Wilson and Nicoll, 2001). When DSE was discovered in 2001, it was also reported to follow the same induction mechanisms as DSI, except that CB1 receptors on glutamatergic inputs were targeted instead of on GABAergic neurons (Kreitzer and Regehr, 2001a).

In contrast to short term plasticity, eCBs' also mediate a form of long-term depression known as inhibitory LTD (iLTD), which suppresses GABAergic signaling over extended time periods (Chevalyere and Castillo, 2004). In both DSI and iLTD, presynaptic GABA release is inhibited through CB1 receptor activation, causing a localized block of inhibitory signaling. Several papers have supported a role for CB1 in the facilitation of LTP, likely through this mechanism. Chevalyere and Castillo (2004) showed that induction of iLTD facilitated greater LTP at the same recording site when compared to sites that did not undergo iLTD. Carlson et al (2002) also found that CB1 activation during DSI allowed LTP induction with stimulus protocols that were normally

ineffective. These data fit with CB1's currently accepted mechanism of action in hippocampus.

In contrast, there are also several papers that describe a role of CB1 in the inhibition of excitatory LTP. The CB1 agonist WIN55,212-2 has been shown to block LTP (Misner and Sullivan, 1999) and 2-AG inhibits high-frequency stimulation (HFS) induced LTP (Stella et al., 1997). In the cortex, it has been observed that CB1 activation shifts the system in favor of LTD induction while CB1 block shifts the system towards LTP induction (Auclair et al., 2000). Also, Slanina et al (2005) found that CB1 inhibited LTP induced with moderate trains of stimulations, but did not affect LTP induced with robust HFS protocols, and Hoffman et al (2007) found that chronic THC exposure inhibits both HFS and theta burst (TBS) induced LTP, which was reversed by co-administration of AM251. Interestingly this group also found that chronic CB1 block with the receptor antagonist AM251 enhanced TBS LTP but not HFS LTP.

Several behavioral studies also support a role for CB1 in LTP inhibition. The CB1 agonist HU-210 initially impaired water maze performance and LTP induction in-vivo (Hill et al., 2004), and similarly, spatial learning deficits and suppression of hippocampal pyramidal cell firing were observed in rats treated with the CB1 agonist HU-210 (Robinson et al., 2007).

Evidence for a novel cannabinoid receptor

Although the CB1 receptor is thought to be responsible for most endocannabinoid actions in the brain, there is strong evidence for a third cannabinoid receptor that may also play important roles in neuronal signaling and synaptic plasticity (Begg et al., 2005;

Lauckner et al., 2008; Mackie and Stella, 2006; Ryberg et al., 2007). Hajos et al (2001) found that the CB1 agonist WIN55,212-2 inhibited EPSCs in both wild type and CB1 (-/-) mice, suggesting a novel eCB receptor was present. It is important to note that they used adult mice of the Ledent strain (Ledent et al., 1999), which are backcrossed onto a CD1 background, as discrepancies have arisen between the different strains of mice. In direct contrast, another group (Ohno-Shosaku et al., 2002b) found that WIN55,212-2 inhibited both EPSCs and IPSCs in wild type mice but not CB1(-/-) mice; however, they used juvenile CB1(-/-) mice of the Zimmer strain (Zimmer et al., 1999), which are backcrossed onto a C57Blk6 background. This group also found that both DSI and DSE could be induced in hippocampus, although stronger depolarizations were required to induce DSE. Also, both DSI and DSE were absent in CB1(-/-) mice, which argued against the involvement of a novel CB receptor in this strain and under these conditions.

The confusion surrounding differences between species and strains of animals continued when yet another group found that wild type mice of the CD1 strain, the background for the Ledent knockouts, but not the C57Black6 strain, the background for the Zimmer knockouts, were sensitive to WIN55,212-2 (Hoffman et al., 2005). However, the most recent evidence suggests that WIN55,212-2 inhibition of fEPSPs is absent in CB1(-/-) mice of both strains, while inhibition of wild type fEPSPs remains intact (Takahashi and Castillo, 2006).

Despite these inconsistencies, several other groups have collected data to support a novel receptor's existence in CNS. Breivogel and colleagues (Breivogel et al., 2001) found that anandamide and WIN55,212-2 caused enhanced GTP γ S binding in plasma membranes of brain tissue from CB1(-/-) mice, supporting the idea that another GPCR is

activated by endocannabinoids. Also, in both wild type and CB1(-/-) mice activation of group 1 mGluR receptors has been reported to cause eCB release and mediate a short-term depression of excitatory transmission, without affecting mGluR mediated long-term depression (Rouach and Nicoll, 2003). Most recently an orphan GPCR known as GPR55 has been classified as a novel cannabinoid receptor. Ryberg and colleagues showed, using cells expressing human GPR55, that anandamide and the cannabinoid agonist CP55940 activated the receptor (Ryberg et al., 2007). More recently, an independent group has confirmed these results and found GPR55 to be activated by numerous cannabinoids (Lauckner et al., 2008). They have isolated GPR55 in the dorsal root ganglion of the spinal cord, and found that activation increases intracellular calcium while inhibiting M-type potassium channels. Further investigation will show if GPR55 is also expressed throughout the brain and if this receptor could be the elusive CB3 others have described.

Summary

The goal of this thesis is to better understand how the endocannabinoid system modulates synaptic plasticity, in particular when a moderate frequency stimulation protocol is used to induce long-term potentiation (LTP). Two sets of experiments are presented in the following manuscripts.

First, we show that brief trains of moderate frequency (10 Hz) stimulation results in an eCB-dependent LTP of excitatory synaptic transmission at rat Schaffer collateral-CA1 pyramidal synapses. This LTP is distinct from traditional NMDA receptor mediated potentiation and requires activation of cannabinoid receptors. It represents a novel form

of excitatory synaptic plasticity mediated by the endocannabinoid system in hippocampus.

Secondly, we aimed to further characterize the 10 Hz LTP using CB1 receptor knockout mice. However, this resulted in a second manuscript as we found the 10 Hz LTP to be quite different in mice. Here we found that CB1 receptor activation inhibited the 10 Hz potentiation, which was also NMDA receptor dependent, similar to traditional LTP. Together, this data supports a role for the CB1 receptor in inhibiting the induction of LTP with moderate frequency stimulation protocols. We also present data supporting the existence of a novel cannabinoid receptor, which may have a role in modulating synaptic plasticity.

CHAPTER 2

Endocannabinoid-Dependent Potentiation of Excitatory Synaptic Transmission in Hippocampus

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Abstract

Modulation of synaptic transmission by endocannabinoids (eCBs) typically involves retrograde signaling resulting in presynaptic inhibition. Here we show that brief trains of moderate frequency (10 Hz) stimulation result in an eCB-dependent long-term potentiation of excitatory synaptic transmission at rat Schaffer collateral-CA1 pyramidal synapses. This potentiation is distinct from typical theta burst stimulus induced long-term potentiation (TBS-LTP) in that it is completely abolished by the CB1 receptor antagonists AM251 and SR141716, but not by the NMDA receptor antagonist AP5. This eCB-dependent LTP is further distinguished from TBS/NMDAR-dependent LTP by the involvement of group I mGlu receptors and by marked age-dependence. The eCB-dependent LTP was saturable and reached a maximum of $34 \pm 3.8\%$ after 1-4 bouts of stimulation without occluding TBS/NMDAR-dependent LTP. This potentiation is independent of GABAergic synaptic inhibition, suggesting that it represents a novel form of excitatory synaptic plasticity mediated by the endocannabinoid system in hippocampus.

Introduction

Endocannabinoids (eCBs) have emerged as important regulators of synaptic plasticity in the brain (reviewed in Chevaleyre et al. 2006). Endocannabinoids are thought to predominantly mediate presynaptic inhibition of transmitter release via retrograde signaling. Depolarization-induced suppression of inhibition (DSI), described originally in cerebellum (Llano et al., 1991) and hippocampus (Pitler and Alger, 1992) is mediated by eCBs via transient presynaptic inhibition of GABA signaling (Kreitzer and Regehr,

2001b; Ohno-Shosaku et al., 2001; Wilson et al., 2001; Wilson and Nicoll, 2001). A similar form of short-term plasticity, depolarization-induced suppression of excitation (DSE), also involves eCB-dependent presynaptic inhibition of glutamatergic signaling via cannabinoid receptors present on glutamatergic terminals (Hajos et al., 2001; Kreitzer and Regehr, 2001b; Ohno-Shosaku et al., 2002b; Straiker and Mackie, 2005). eCB signaling is also implicated in long-term synaptic depression of inhibitory signaling in various brain regions including hippocampus (Chevalleyre and Castillo, 2003). This suppression of inhibitory signaling has been reported to facilitate induction of long-term potentiation (Carlson et al., 2002; Chevalleyre and Castillo, 2004).

The CB1 receptor, which is believed to be responsible for the majority of CNS eCB effects, is abundantly expressed throughout the brain, with high levels of expression in hippocampus (Herkenham et al., 1990; Mackie, 2005). Immunohistochemical analysis shows that CB1 receptors are strongly expressed on synaptic terminals of cholecystinin-containing (CCK) inhibitory interneurons in CA1 (Hajos et al., 2000; Katona et al., 2001; Katona et al., 2000; Katona et al., 1999; Marsicano et al., 2002; Tsou et al., 1999) and act to inhibit transmitter release (Hoffman, 2000; Kreitzer and Regehr, 2001b; Levenes et al., 1998; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). Recent data also suggests that CB1 receptors are present at low levels on excitatory presynaptic terminals (Hajos and Freund, 2002; Katona et al., 2006; Kawamura et al., 2006; Takahashi and Castillo, 2006), and there is increasing evidence for a novel CB receptor in hippocampus that may affect glutamatergic synaptic transmission (Begg et al., 2005; Lauckner et al., 2008; Mackie and Stella, 2006; Ryberg et al., 2007).

In the present study, we describe an eCB-dependent long-term potentiation (eCB-LTP) of excitatory signaling at the hippocampal Shaffer collateral-CA1 synapse that is pharmacologically and mechanistically distinct from the NMDA receptor-dependent LTP that has been widely studied at this synapse. This potentiation of excitatory signaling is not mediated by inhibition of GABA signaling, suggesting that it represents a novel form of eCB-dependent synaptic plasticity.

Experimental Methods

Animals: Transverse hippocampal slices (400 μM) were prepared from Sprague Dawley rats of indicated ages in accordance with IACUC regulations. The animals were briefly anesthetized with isoflourane, decapitated, and the brains were rapidly removed and mounted for slicing. Slices were prepared using a vibrating microtome (VT1000S Leica, Germany) in ice-cold sucrose solution saturated with 95% O_2 and 5% CO_2 containing (in mM): 79.9 NaCl, 2.5 KCl, 70 sucrose, 1.25 NaH_2PO_4 , 0.5 CaCl_2 , 7 MgCl_2 , 24.9 glucose, 25.2 NaHCO_3 , 1.0 kynurenic acid. Slices were stored in artificial cerebral spinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl_2 , 2.4 CaCl_2 , 1.2 NaH_2PO_4 , 11.4 glucose, and 21.4 NaHCO_3 at 30°.

Electrophysiology: Slices were allowed to recover at least 1 hour before being placed in a submersion-type recording chamber perfused with 95% O_2 and 5% CO_2 -saturated ACSF. Recordings were performed at 30° C. Field excitatory postsynaptic potentials (fEPSPs) were evoked by 100 μs duration stimuli in stratum radiatum with a monopolar ACSF-containing glass pipette (2-3 $\text{M}\Omega$). Stimulus strength (typically 50-100 μA) was adjusted to elicit responses 30-40% of maximum, and a stable baseline was maintained

for at least 20 minutes prior to initiation of an experiment. Responses were monitored at 0.05 Hz. The 10 Hz stimulation protocol consisted of 2 trains of 100 pulses given at 10 Hz, 20 seconds apart (Chevalayre and Castillo, 2004). Theta burst stimulation consisted of 10 trains of 5 pulses at 100 Hz with a 200 mS inter-burst interval, repeated once after a 5 second delay. fEPSPs were recorded with Axoclamp or Geneclamp amplifiers (Molecular Devices, Sunnyvale CA) interfaced to Macintosh G4 or Pentium computers via Digidata 1320s using Axograph X (Axograph) or pClamp9 (Axon Instruments) acquisition and analysis software, respectively. Data were digitized at 5 kHz and filtered at 2 kHz.

Drugs: All drugs, except SR141716 which came from NIDA (Bethesda, MD), were obtained from Tocris-Cookson (Ellisville, Missouri) and prepared daily from concentrated (>1000x) stock solutions made as specified by the manufacturer.

Statistical analysis: Data are normalized as a percentage of the baseline fEPSP amplitude (fEPSP slope analysis provided similar results) and presented as means \pm standard errors unless otherwise stated. Student's t-tests for unpaired data, accompanied by Levene's test for equal variance, were used to compare the last 10 minutes of stable responses following 10 Hz and/or theta burst stimulation among the various conditions.

Results

10 Hz stimulation induces a long-lasting fEPSP potentiation

Using a moderate frequency stimulation protocol (2 bouts of 100 pulses delivered at 10 Hz, 20 sec interburst interval), previously shown to elicit eCB-dependent iLTD without causing LTP (Chevalayre and Castillo, 2004), we observed a transient depression

Figure 1

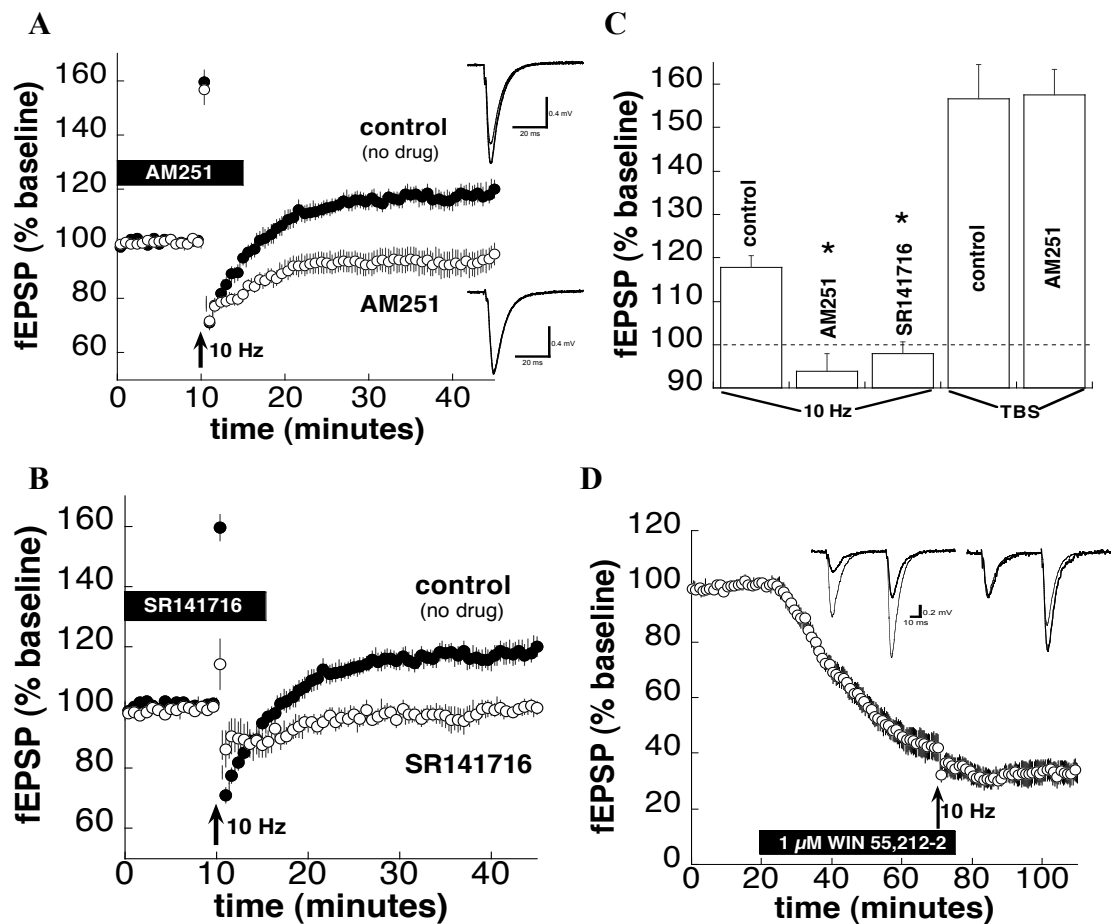


Figure 1: 10 Hz stimulation induces a long-term potentiation of hippocampal CA1 fEPSPs, which is prevented by CB1 receptor ligands.

A) Closed circles represent average normalized fEPSPs in control slices \pm standard error (n=28), open circles are data from slices recorded in the presence of 2 μ M AM251 (n=22), applied for 20 minutes prior to 10Hz stimulation. *Inset:* traces from representative experiments showing change in fEPSP for each treatment group after 10 Hz stimulation (scale bars 400 μ V by 20 mS).

B) Closed circles represent average normalized fEPSPs in control slices \pm standard error (n=28), open circles are data from slices recorded in the presence of 2 μ M SR141716 (n=8).

C) Summary of mean potentiation of fEPSP induced by 10 Hz and TBS stimulation in control slices (n=28, n=16 respectively), 2 μ M AM251 (n=22, n=8), and 2 μ M SR141716 (n=8). Both AM251 and SR141716 significantly inhibit 10 Hz potentiation ($93.9 \pm 4.0\%$ in AM251 and $98.0 \pm 2.5\%$ in SR141716 versus $118 \pm 2.7\%$ in control, $p \leq 0.01$). TBS induced LTP is not affected by 2 μ M AM251 ($158 \pm 5.9\%$, n=8, versus $157 \pm 7.9\%$, n=16, in control conditions, $p = 0.94$)

D) Application of the cannabinoid receptor agonist WIN55,212-2 (1 μ M) depresses the fEPSP amplitude by $57 \pm 4.6\%$ and 10 Hz stimulation in the presence of WIN55,212-2 depresses the response an additional $20 \pm 4.0\%$ (n=6) from the new baseline amplitude. *Inset:* PPR recordings from before (thin traces) and 45 minutes after (bold traces) WIN application. Traces on the left are normalized to the first fEPSP to reflect PPR changes (scale bars 10 mS by 200 μ V).

followed by a persistent increase in fEPSP amplitude in 21-35 day old Sprague Dawley rats (Figure 1A). This potentiation ($117.9\% \pm 2.7\%$ of baseline, $n=28$) was seen in 21 of 28 slices from 26 animals. We compared paired pulse ratio (PPR) measurements before and after 10 Hz stimulation, as changes in PPR can allude to changes in presynaptic neurotransmitter release probability. We found the baseline PPR to be 1.62 ± 0.03 ($n=28$) and following 10 Hz it was slightly reduced to 1.58 ± 0.03 ($n=28$, $p=0.03$). This reduction does correlate with an increased release probability; however, the small magnitude of change may not translate to physiological significance. With longer recording episodes the 10 Hz potentiation persisted for more than 3 hours (appendix A).

Surprisingly, the 10 Hz LTP was completely blocked by the CB1 receptor antagonist AM251 at $2 \mu\text{M}$ (Figure 1 A, C; $93.9 \pm 4.0\%$ of baseline fEPSP amplitude, $n=22$, $p<0.001$). In contrast to its potent blocking effect on fEPSP potentiation induced by 10 Hz stimulation, AM251 had no effect on theta burst-induced LTP ($158 \pm 5.9\%$ LTP in AM251 ($n=8$) versus $157 \pm 7.9\%$ LTP in control slices, $n=16$, $p=0.94$, Figure 1C). Cannabinoid receptor dependence was further substantiated by using a separate CB1 antagonist, SR141716 ($2\mu\text{M}$), which also completely blocked the 10 Hz LTP (Figure 1B,C: $98.0 \pm 2.5\%$ of baseline fEPSP amplitude, $n=8$, $p<0.001$).

To further assess cannabinoid receptor involvement in this process, we tested the effect of 10 Hz stimulation in the presence of the CB1 agonist WIN55,212-2 ($1 \mu\text{M}$). Application of the agonist decreased the fEPSP amplitude by $57 \pm 4.6\%$ in accordance with previously reported data (Al-Hayani and Davies, 2000; Hajos and Freund, 2002; Hajos et al., 2001; Hoffman et al., 2005; Misner and Sullivan, 1999; Takahashi and Castillo, 2006). 10 Hz stimulation in the presence of WIN55,212-2 did not induce

potentiation above the reduced baseline, but instead depressed the response an additional $20 \pm 4.0\%$ (Figure 1D, n=6). Theta burst-induced LTP could still be induced in WIN55,212-2 although it was reduced compared to control conditions (appendix B). Taken together these results suggest that an endocannabinoid-dependent form of LTP, distinct from theta burst induced LTP, (eCB-LTP) is elicited by 10 Hz stimulation.

10Hz-induced LTP is functionally distinct from theta burst-induced LTP

LTP at the Shaffer-CA1 synapse can be readily elicited by theta burst stimulation and it requires activation of NMDARs (Malenka and Bear, 2004). Unlike typical TBS-induced LTP, which is blocked by application of the NMDAR antagonist DL-AP5, the potentiation induced by 10 Hz stimulation was not prevented by 50 μM DL-AP5 (Figure 2 A,C; $113.3 \pm 4.4\%$ baseline fEPSP amplitude, n=13, p=0.37). At the same concentration, DL-AP5 completely blocked theta burst-elicited LTP ($157 \pm 7.9\%$ in control, n=16, and $103 \pm 2.9\%$ in AP5 n=11, p \leq 0.001).

Because group I mGluRs have been shown to stimulate postsynaptic endocannabinoid release (Maejima et al., 2001; Ohno-Shosaku et al., 2002a; Varma et al., 2001), we tested the effects of the mGluR1 antagonist LY367385 (30 μM) and the mGluR5 antagonist MPEP (10 μM). Similar to the effect of AP5, block of group I mGluRs did not significantly inhibit 10 Hz LTP (MPEP + LY367385: $114.2 \pm 3.8\%$, n=9, p=0.5) (Figure 2B,C). Since group I mGluR and NMDARs both mediate intracellular Ca^{2+} increase and could potentially synergistically activate postsynaptic eCB synthesis, we tested the effect of blocking both receptors simultaneously. In the presence

Figure 2

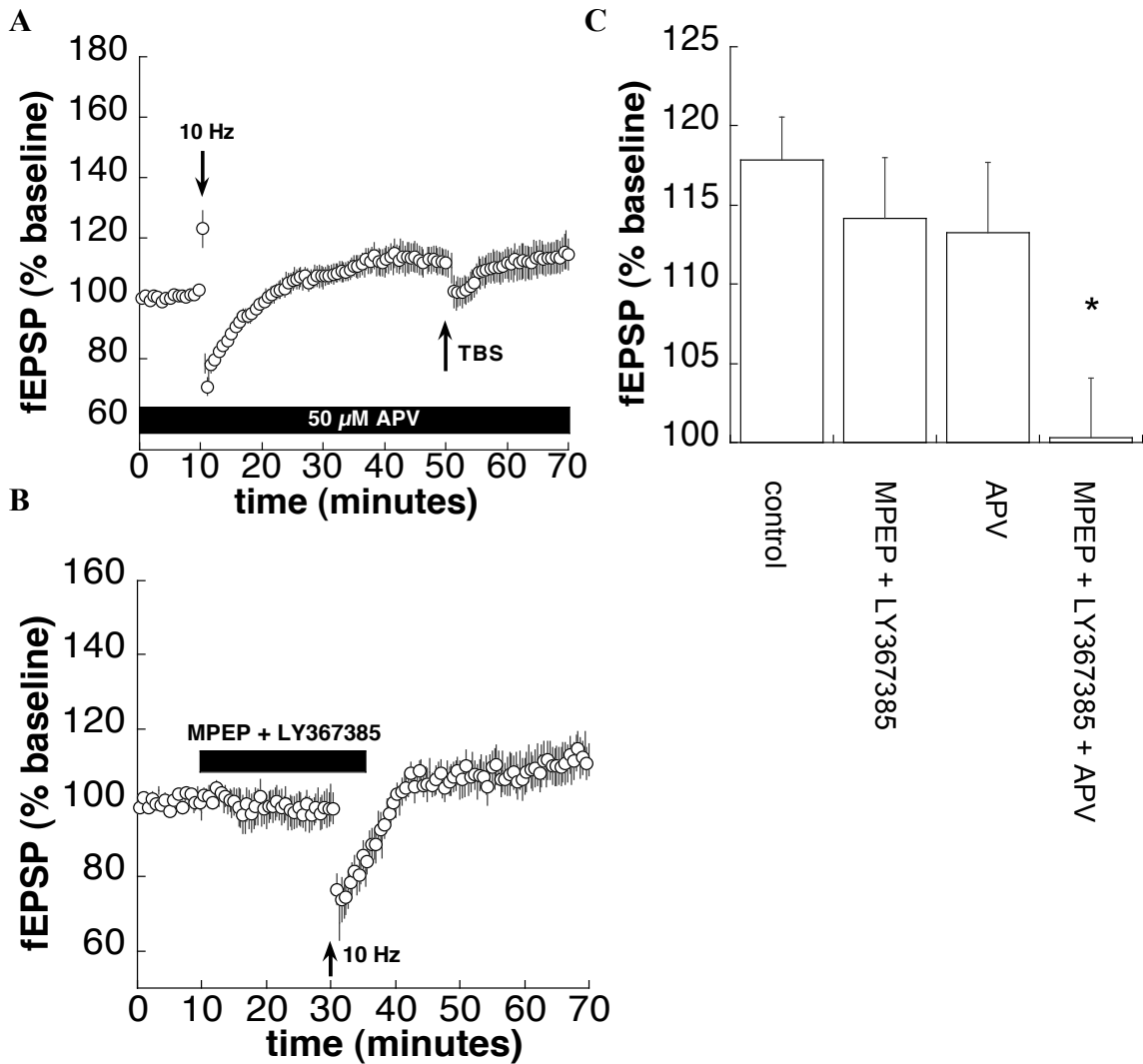


Figure 2: 10 Hz LTP is blocked by co application of NMDA and group1 mGluR antagonists

A) Averaged experiments showing 10 Hz stimulation induces eCB-LTP in the presence of 50 μ M APV while TBS LTP is completely prevented (n=13).

B) Averaged experiments showing group 1 mGluR receptor antagonists MPEP (10 μ M) and LY367385 (30 μ M) do not inhibit 10 Hz eCB-LTP (n=9).

C) Summary of mean potentiation of fEPSP (% baseline) induced by 10 Hz stimulation in control conditions and the presence of different group 1 mGluR and NMDA receptor antagonists. eCB-LTP is inhibited by a combination of 10 μ M MPEP, 30 μ M LY367385, and 50 μ M APV (100.3% \pm 3.7%, n= 10, versus 118 \pm 2.7%, n=28, in control slices, p=0.002) Individual application of these inhibitors did not change the 10 Hz potentiation (10 μ m MPEP + 30 μ m LY367385: 114.2 \pm 3.8%, n=9, p=0.50; 50 μ m APV: 113.3 \pm 4.4%, n=13, p=0.37)

50 μ M AP5, 30 μ M LY367385 and 10 μ M MPEP, 10 Hz stimulation failed to elicit eCB-LTP ($100 \pm 3.7\%$ baseline, $n=10, p=0.002$) (Figure 2C).

GABA effects on eCB-LTP

Endocannabinoid signaling in the hippocampus has been shown to result in presynaptic inhibition of GABA release (Hoffman, 2000; Kreitzer and Regehr, 2001b; Levenes et al., 1998; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). To explore the possible role of GABA signaling in eCB-LTP, we compared the effect of 10 Hz stimulation in the presence and absence of the GABA_A and GABA_B receptor antagonists picrotoxin (100 μ M) and CGP55845 (1 μ M). Blockade of both GABA_A and GABA_B receptors did not reduce 10Hz-induced potentiation ($126 \pm 7.9\%$, $n=9$). Furthermore, in the presence of GABA antagonists, 10 Hz- LTP was still blocked by AM251 ($103 \pm 3.5\%$, $n=8$, Figure 3 A,B). Together this data demonstrates that eCB-LTP is not a result of the inhibition of GABAergic signaling by CB1.

Age dependence and saturation

To better understand the characteristics of eCB-LTP we examined age dependence and its ability to saturate. We found eCB-LTP to be largely age dependent in the Sprague Dawley rats, with little to no potentiation occurring in older animals (42-98 days post natal). In this age group, 10 Hz stimulation-induced LTP was only observed in 3 out of 14 slices ($102 \pm 3.3\%$ of baseline, $n=14$; Figure 4A). Theta burst stimulation (TBS) reliably induced LTP in both age groups of animals (21-35 day old animals $157 \pm 7.9\%$ of baseline, $n=16$; 42-98 day old animals $140 \pm 6.3\%$ of baseline, $n=11, p=0.14$).

Figure 3

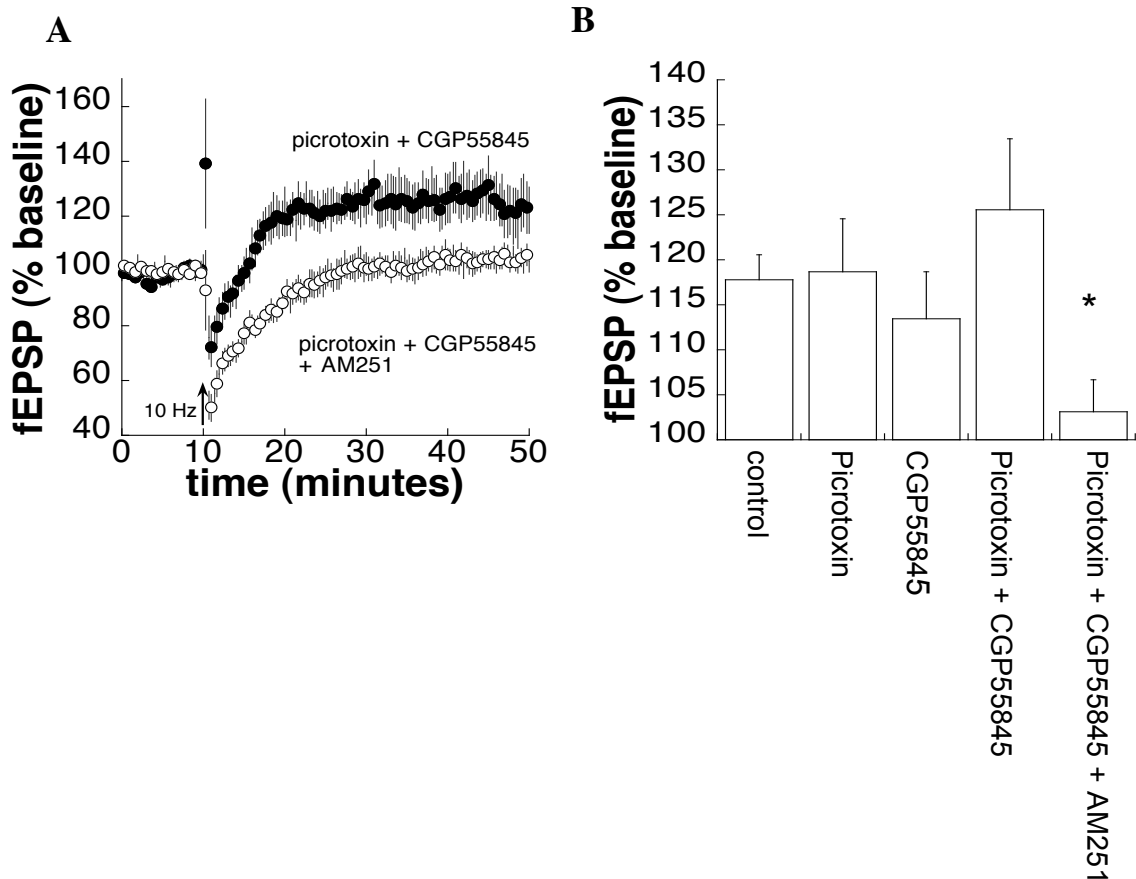


Figure 3: Inhibition of GABA signaling is not involved in eCB LTP.

A) Averaged experiments showing 10 Hz stimulation induces eCB-LTP in the presence of the GABA_A antagonist picrotoxin (100 μ M) and the GABA_B antagonist CGP55845 (1 μ M) (filled circles), which is blocked by the addition of 2 μ M AM251 (open circles).

B) Bar graph showing mean fEPSP (% of baseline amplitude) \pm standard errors following 10 Hz stimulation in control conditions (118 \pm 2.7%, n=28), 100 μ M picrotoxin (119 \pm 6.0%, n=10, p=0.88), 1 μ M CGP55845 (114 \pm 5.2%, n=9, p=0.45), 100 μ M picrotoxin + 1 μ M CGP55845 (126 \pm 7.9 %, n=9, p=0.25), 2 μ M AM251, 100 μ M picrotoxin, + 1 μ m CGP55845 (103 \pm 3.5%, n=8, p=0.01).

Figure 4

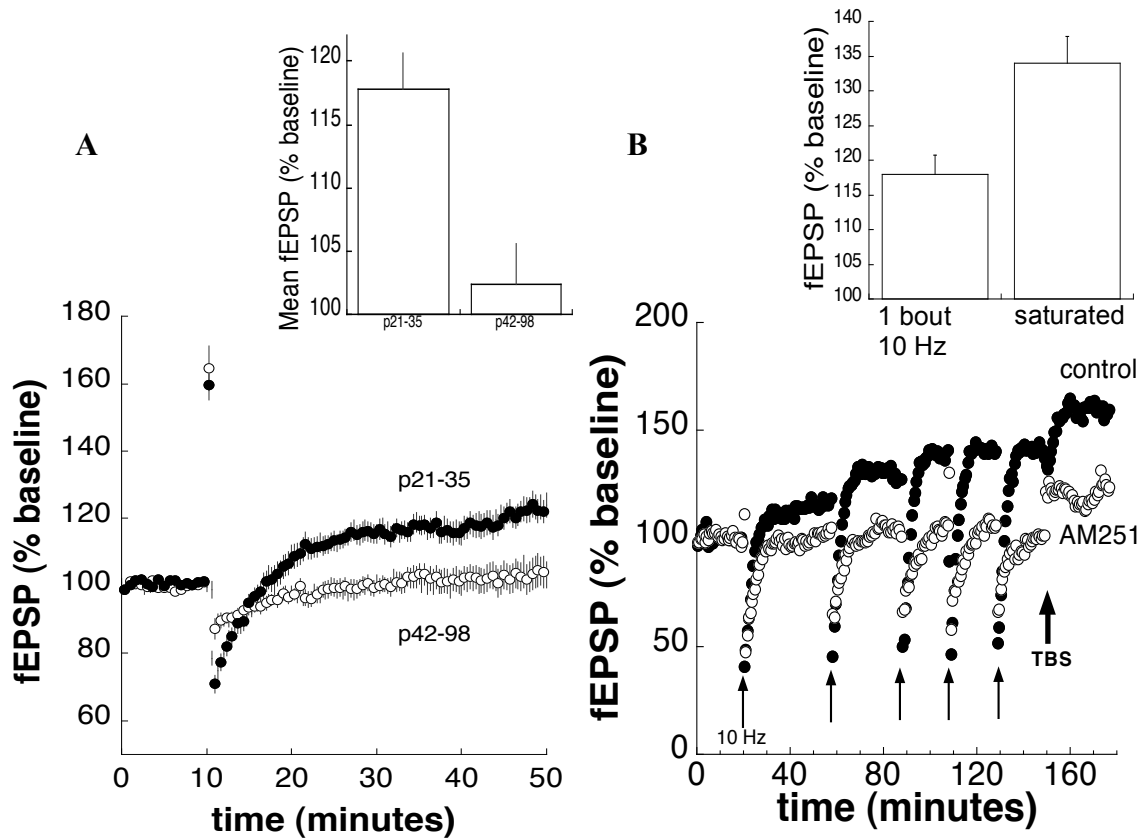


Figure 4: eCB-LTP is age dependent and saturable.

A) Filled circles represent average normalized fEPSPs in 21-35 day SD rats \pm standard error (n=28), open circles are data from 42-98 day old animals (n=14). Average fEPSP (% baseline) after 10 Hz stimulation in 21-35 day old animals is $118 \pm 2.7\%$ and is $102 \pm 3.3\%$ in 42-98 day old animals. *Inset* -Summary of mean potentiation of fEPSP induced by 10 Hz stimulation in p21-35 (n=28) and p42-98 (n=14) SD rats.

B) Representative experiment showing repeated application of the 10 Hz stimulation protocol causes saturable fEPSP potentiation, that does not occlude TBS-induced LTP (filled circles), and is blocked by $2\mu\text{M}$ AM251 (open circles) *Inset* - Averaged data showing mean saturated eCB-LTP reached after 1-4 repetitions of 10 Hz stimulation protocol ($134 \pm 3.2\%$, n=7) compared to 1 bout of 10 Hz ($118 \pm 2.7\%$, n=28).

To further explore the mechanism of eCB-LTP we sought to verify that this form of long-term potentiation could be saturated and to test whether synapses that had reached a maximal level of eCB-dependent LTP could still undergo theta burst-induced LTP. We found that repeated pairings of 10 Hz stimulation increased fEPSP amplitudes to an average saturated level of $134 \pm 3.8\%$ of baseline following 1-4 repetitions of the stimulus train (n=7; Figure 4B). Following maximal 10 Hz potentiation, theta burst stimulation further increased the fEPSP to a final level similar to what is achieved without prior 10 Hz stimulation ($157 \pm 9.5\%$; n=7, p=0.85; Figure 4B representative experiment, filled circles). The increases in fEPSP amplitude induced by repeated 10 Hz stimulation were also blocked by $2 \mu\text{m}$ AM251 while theta burst-induced LTP was not (Figure 4B, open circles). These data lend further support to the conclusion that eCB-LTP elicited by moderate frequency stimulation at the Shaffer-CA1 synapse is distinct from high frequency stimulus-induced NMDAR-dependent LTP.

Discussion

The major conclusion of this work is that a previously undescribed form of long-term potentiation of excitatory synaptic transmission exists at Shaffer collateral-CA1 pyramidal neuron synapses. This potentiation is mechanistically and pharmacologically distinct from the traditional NMDAR-dependent LTP extensively studied at this synapse (Malenka and Bear, 2004). It is induced by relatively short trains of moderate frequency stimulation (100 stimuli at 10 Hz, repeated once 20 s apart), and it appears to be endocannabinoid-mediated because it is blocked by the endocannabinoid receptor-selective antagonists AM251 and SR141716. Also, the cannabinoid receptor agonist

WIN55,212-2 occludes potentiation. The 10 Hz LTP saturably increases with repeated bouts of stimulation, resulting in potentiation of fEPSP amplitudes exceeding 30%.

The stimulation paradigm used here to elicit eCB-LTP is quite different from stimulus paradigms traditionally employed to study synaptic transmission and plasticity in the hippocampus (Bliss and Collingridge, 1993; Malenka and Nicoll, 1999). It is based on a stimulus pattern recently reported to elicit endocannabinoid-mediated LTD of GABA signaling in area CA1, which then facilitated subsequent TBS induction of LTP without directly potentiating excitatory synaptic transmission (Chevaleyre and Castillo, 2004). Differences in the recording conditions may account for the different effects on excitatory transmission observed in that study and the present study; in particular it is possible that our recording conditions reflect recruitment of a somewhat larger ensemble of release sites.

Somewhat paradoxically, depression of Shaffer-CA1 excitatory transmission is seen with the exogenously applied cannabinoid agonist WIN 55,212-2 (Misner and Sullivan, 1999), and DSE has also been recorded at this synapse (Ohno-Shosaku et al., 2002b). However, with the moderate stimulation protocols we used, endocannabinoid actions may be spatiotemporally restricted to a subset of receptors that results in excitatory potentiation rather than suppression.

Notably, hippocampal 10 Hz LTP is GABA independent, unlike previously reported eCB facilitation of HFS-induced LTP that involves suppression of GABAergic neurotransmission (Carlson et al., 2002; Chevaleyre and Castillo, 2004). Because we find that 10 Hz-induced AM251-sensitive potentiation still occurs in the presence of GABA receptor blockade, CB1 receptors on inhibitory interneurons are unlikely to be

responsible for eCB-LTP. Possible alternative mechanisms include direct modulation of glutamatergic transmission by CB1 or a related receptor, or modulation of other classes of inputs such as dopaminergic or cholinergic inputs.

Interestingly, we found that eCB-LTP was age dependent in rats, being greatly decreased beyond 6 weeks of age. However, large developmental changes in CB1 distribution have not been reported between adolescent and adult animals (Belue et al., 1995; Morozov and F. Freund, 2003; Rodriguez de Fonseca et al., 1993). There is evidence for additional cannabinoid receptors in hippocampus (Begg et al., 2005; Hajos et al., 2001; Lauckner et al., 2008; Ryberg et al., 2007) but very little is known about their possible developmental regulation or expression levels.

eCB-LTP appears to reflect a novel form of long-term potentiation of excitatory synaptic transmission in hippocampal area CA1. Further work will be required to more fully characterize the synaptic mechanisms, subtype and localization of receptors involved, and possible interactions with other forms of synaptic plasticity in this region. The properties of this form of long-term potentiation, which is elicited by moderate stimulation frequencies similar to those found *in vivo*, suggest that it may reflect an important mechanism by which endocannabinoids can influence excitatory synaptic plasticity.

CHAPTER 3

The CB1 receptor inhibits moderate frequency induced long-term potentiation in mouse hippocampus

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Abstract:

Endocannabinoids (eCB) have emerged as important modulators of synaptic plasticity in recent years; however, their precise roles and mechanisms are not well understood and many contradictions exist in the current literature. We find that a 10 Hz stimulation protocol, which has been previously demonstrated to cause eCB release and inhibitory long-term depression (iLTD) (Chevalleyre and Castillo, 2004), produces lasting potentiation in the hippocampus that is greatly facilitated in CB1 receptor knockout mice and wild type mice treated with the CB1 antagonist SR141716. This research provides evidence supporting a role for the CB1 receptor as an inhibitor of long-term potentiation (LTP) induced by a moderate-frequency stimulation. Similar to traditional LTP, 10 Hz LTP is NMDA receptor mediated as it is completely blocked by the NMDA receptor antagonist APV in wild type and CB1 (-/-) mice. LTP induced by a higher frequency theta burst stimulation (TBS) protocol is not affected by CB1. Together, this data supports a role for the CB1 receptor in inhibiting the induction of LTP at low levels of activity. This could be very important in physiological conditions since low frequency neuron firing likely occurs in the brain *in vivo*.

Introduction:

The endocannabinoid (eCB) system has recently emerged as an important modulator of synaptic plasticity (Chevalleyre et al., 2006). Endocannabinoids mainly act as retrograde messengers, traveling from their postsynaptic origin and targeting presynaptic receptors, which exert their effects largely by inhibiting neurotransmitter release in many brain structures. Currently, the most thoroughly characterized

endocannabinoids are 2-arachidonyl glycerol (2-AG) and anandamide (AEA), both of which are postsynaptically synthesized from membrane bound lipid precursors (Devane et al., 1992; Mechoulam et al., 1995; Stella et al., 1997; Sugiura et al., 1995). The endocannabinoids exert their effects through at least two cloned and widely accepted endocannabinoid receptors, CB1 (Matsuda et al., 1990) and CB2 (Munro et al., 1993). CB2 is almost exclusively located in the immune system (Axelrod and Felder, 1998; Pertwee, 1997), while CB1 is the primary receptor in the CNS. Both receptors are G-protein coupled and CB1 is one of the most highly expressed GPCRs in the brain (Herkenham et al., 1990). The CB1 receptor is thought to be responsible for most endocannabinoid actions in the brain although there is strong evidence for a third cannabinoid receptor that may also play important roles in neuronal signaling and plasticity (Begg et al., 2005; Lauckner et al., 2008; Mackie and Stella, 2006; Ryberg et al., 2007).

CB1 receptors are highly expressed in structures known to be important for learning and memory, including the cerebellum, cortex, amygdala, and hippocampus (Freund et al., 2003; Herkenham et al., 1990). The hippocampus, which is a major center for spatial learning and memory formation (Eichenbaum, 2004; Squire et al., 2004), has a very high concentration of CB1 receptors (Herkenham et al., 1990; Mackie, 2005). The majority of research demonstrates that CB1 is localized to cholecystokinin (CCK) GABAergic interneurons, where it acts to inhibit GABA release (Hajos et al., 2000; Katona et al., 2001; Katona et al., 2000; Katona et al., 1999; Marsicano and Lutz, 1999; Marsicano et al., 2002; Tsou et al., 1999), likely by direct interference with calcium channel operation (Shen and Thayer, 1998; Sullivan, 1999; Twitchell et al., 1997). The

effect of CB1 receptors on GABAergic neurons is important for at least two forms of synaptic plasticity, depolarization-induced suppression of inhibition (DSI) (Kreitzer and Regehr, 2001a; Llano et al., 1991; Ohno-Shosaku et al., 2001; Pitler and Alger, 1992; Wilson et al., 2001; Wilson and Nicoll, 2001) and inhibitory long-term depression (iLTD) (Chevalyere and Castillo, 2004). In both DSI and iLTD presynaptic GABA release is inhibited through CB1 receptor activation causing a localized disinhibition. Several papers have supported a role for CB1 in the facilitation of LTP, likely through this mechanism. Chevalyere and Castillo (2004) showed that induction of iLTD facilitated greater LTP at the same recording site when compared to sites that did not undergo iLTD. Similarly, it has also been reported that CB1 activation during DSI allowed LTP induction with stimulus protocols that were normally ineffective (Carlson et al., 2002). These data fit with the currently accepted mechanism of action of CB1 in hippocampus.

Despite the view that CB1 only acts on inhibitory interneurons and regulates GABA release, there is accumulating evidence supporting a role for CB1 in the modulation of excitatory signaling in hippocampus. The effect of the cannabinoid receptor agonist WIN55,212-2 on excitatory signaling supports this. Several papers show that WIN55,212-2 application depresses excitatory signaling, as seen by a decrease in EPSP amplitude and increased paired pulse ratio. This phenomena does not occur in CB1(-/-) animals and can be reversed by application of CB1 antagonists (Hoffman et al., 2005; Misner and Sullivan, 1999; Takahashi and Castillo, 2006). WIN55,212-2 also depresses IPSP amplitude, supporting CB1's known actions on GABAergic neurons (Hajos and Freund, 2002; Hoffman and Lupica, 2000). Aside from the WIN55,212-2

data, several other pieces of evidence suggest that CB1 can affect glutamatergic signaling. CB1 mRNA has been found in CA3 pyramidal cells, whose axons make up the Schaffer collaterals that synapse on CA1 pyramidal cell dendrites in the stratum radiatum (Marsicano et al., 2003). Also, the enzymatic machinery responsible for the synthesis of eCB has also been found in glutamatergic inputs (Nyilas et al., 2008).

Similarly there are multiple papers that show a role of CB1 in inhibition of excitatory long-term potentiation. The CB1 agonist WIN55,212-2 and the endogenous ligand 2-AG have been shown to block LTP (Misner and Sullivan, 1999; Stella et al., 1997). In the cortex, it was reported that CB1 activation shifts synapses in favor of LTD induction while CB1 block shifts them towards LTP induction (Auclair et al., 2000). Slanina et al (2005) showed that CB1 inhibited LTP induced with moderate trains of stimulations, short 100 Hz bursts, and theta burst stimulation, but did not affect LTP induced with robust high frequency stimulation protocols. Chronic THC exposure has been shown to inhibit both HFS and TBS induced LTP, which can be reversed by co-administration of AM251. Interestingly this study also found that chronic CB1 block with AM251 enhanced TBS LTP but not HFS LTP (Hoffman et al., 2007). Several behavioral studies also support a role for CB1 in LTP inhibition. Hill et al (2004) found that the CB agonist HU-210 initially impaired water maze performance and LTP induction in-vivo. Similarly, another group found spatial learning deficits and suppression of hippocampal pyramidal cell firing in rats treated with the CB1 agonist HU-210 (Robinson et al., 2007).

In this study we add to the accumulating data supporting a role for CB1 LTP inhibition. We use a moderate frequency 10 Hz LTP induction protocol, described by Chevaleyre & Castillo (2004) to induce eCB release and cause iLTD, to induce a NMDA

receptor dependent LTP, which is inhibited by CB1 receptor activation. In addition, we find that CB1 affects the ability to induce LTD, and we present data supporting the existence of a novel cannabinoid receptor.

Experimental Methods

Animals: Transverse hippocampal slices (350 μ M) were prepared from C57BL/6 wild type or CB1(-/-) mice of indicated ages in accordance with IACUC regulations. CB1(-/-) mice (a generous gift from Drs. Marsicano and Lutz, Germany) were backcrossed onto the C57Black6 background (Zimmer et al., 1999). At the time of the experiments, all mouse pups were 21-35 days old with the exception of the LTD experiments: these mice were 14-21 days old. The animals were briefly anesthetized with isoflourane, decapitated, and the brains were rapidly removed and mounted for slicing. Coronal slices were prepared using a vibrating microtome (VT1000S and VT1200S Leica, Germany) in ice-cold sucrose solution saturated with 95% O₂ and 5% CO₂ containing (in mM): 79.9 NaCl, 2.5 KCl, 70 sucrose, 1.25 NaH₂PO₄, 0.5 CaCl₂, 7 MgCl₂, 24.9 glucose, 25.2 NaHCO₃, 1.0 kynurenic acid. Slices were stored in artificial cerebral spinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 1.2 MgCl₂, 2.4 CaCl₂, 1.2 NaH₂PO₄, 11.4 glucose, and 21.4 NaHCO₃ at 30° C.

Electrophysiology: Slices were allowed to recover at least 1 hour before being placed in a submersion-type recording chamber perfused with 95% O₂ and 5% CO₂ saturated ACSF. Recordings were performed at 30° C. Field excitatory postsynaptic potentials (fEPSPs) were evoked by 100 μ s duration stimuli in stratum radiatum with a monopolar ACSF-containing glass pipette (2-3 M Ω). Stimulus strength (typically 50-100 μ A) was

adjusted to elicit responses 30-40% of maximum for LTP experiments and 60-70% of maximum for LTD experiments. A stable baseline was maintained for at least 20 minutes prior to initiation of an experiment. Responses were monitored at 0.05 Hz. The 10 Hz stimulation protocol consisted of 2 trains of 100 pulses given at 10 Hz, 20 seconds apart (Chevalleyre and Castillo, 2004). Theta burst stimulation consisted of 10 trains of 5 pulses at 100 Hz with a 200 mS inter-burst interval, repeated once after a 5 second delay. 5 Hz stimulation consisted of 2 trains of 100 pulses given at 5 Hz, 20 seconds apart. The LFS-PPR LTD stimulation protocol consisted of 1200 paired pulse stimulations, 50 mS interpulse interval, delivered at 1 Hz. fEPSPs were recorded with Axoclamp or Geneclamp amplifiers (Molecular Devices, Sunnyvale CA) interfaced to Macintosh G4 or Pentium computers via Digidata 1322s using Axograph X (AxographX, Dr. John Clements) or pClamp9 (Axon Instruments) acquisition and analysis software, respectively. Data were digitized at 5 kHz and filtered at 2 kHz.

Drugs: All drugs were obtained from either Tocris-Cooksin (Ellisville, Missouri) or Ascent Scientific (Weston-Super-Mare, UK) and prepared daily from concentrated (>1000x) stock solutions made as specified by the manufacturer.

Statistical analysis: Data are normalized as a percentage of the baseline fEPSP amplitude and presented as means \pm standard errors unless otherwise stated. Students' t-tests for unpaired data, accompanied by Levene's test for equal variance, were used to compare the last 10 min of stable responses following the stimulation protocols with various drug treatments.

Results

CB1 receptor inhibits long-term potentiation induced by moderate-frequency stimulation but not theta burst stimulation.

We found that a 10 Hz stimulation protocol (2 bursts of 100 pulses delivered at 10 Hz, 20 sec interburst interval), previously shown to elicit eCB-dependent iLTD without directly inducing LTP (Chevalleyre and Castillo, 2004) induced a transient depression followed by a persistent $117.9 \pm 2.1\%$ ($n=44$) potentiation of fEPSP amplitude in C57 Black6 wild type mice (Figure 1A,D). The potentiation, referred to as 10 Hz LTP, was seen in 33 of 44 experiments. In CB1(-/-) mice 10 Hz stimulation caused a much greater potentiation when compared to wild type mice ($138.0 \pm 4.6\%$, $n=24$, $p < 0.001$, Figure 1A,D), and was seen in 23 out of 24 experiments. The increased 10Hz LTP was mimicked by the application of CB1 antagonist SR141716 (1 μ M) in wild type mice ($144.3 \pm 12.0\%$, $n=9$, $p = 0.05$, Figure 1B,D). Application of 1 μ M SR141716 alone did not change the fEPSP amplitude (appendix C). Also, during the 10 Hz stimulation, fEPSP amplitudes from CB1 (-/-) mice and wild type mice in the presence of SR141716 were not elevated above control fEPSPs, suggesting the increased potentiation does not occur until after the stimulus train is complete (appendix D). LTP induced by the more robust theta burst stimulation (TBS) was not statistically different between wild type and CB1(-/-) mice ($148.4 \pm 5.8\%$, $n=7$; $147.0 \pm 11.9\%$, $n=4$, $p=0.90$, Figure 1C,D).

We also compared paired pulse ratio (PPR) measurements in wild type and CB1(-/-) animals, as changes in PPR can elude to changes in presynaptic neurotransmitter release probability. The basal PPR was slightly lower in the CB1(-/-) mice

Figure 1

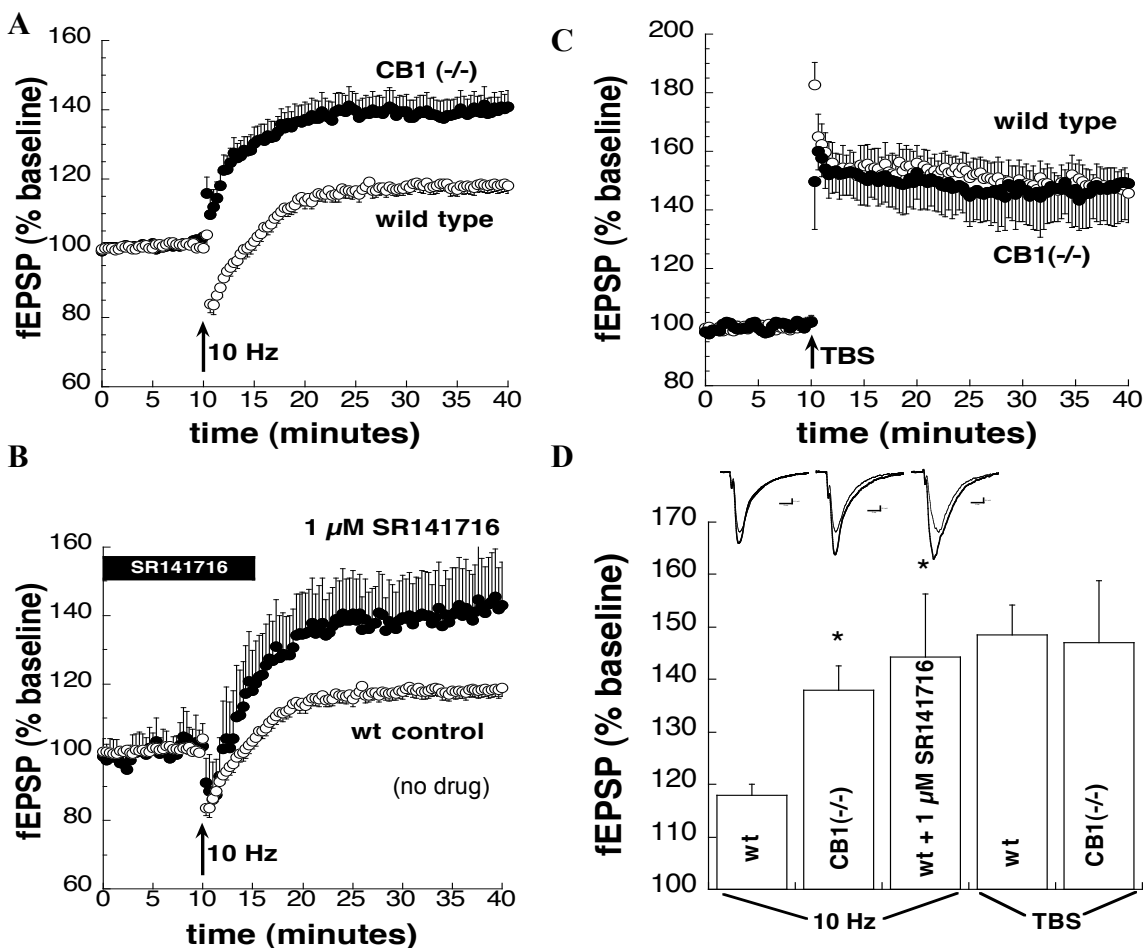


Figure 1: CB1 inhibits 10 Hz induced LTP, but not theta burst induced LTP

A) 10 Hz stimulation (2 trains of 100 pulses at 10 Hz, 20 s apart) induced a lasting $117.9 \pm 2.1\%$ of baseline potentiation of the fEPSP in wild type C57Black6 mice ($n = 44$) and in $CB1(-/-)$ mice it causes a $138.0 \pm 4.6\%$ potentiation ($n = 24$), ($p < 0.001$).

B) Enhanced 10 Hz LTP is achieved in wild type mice by application of the CB1 receptor antagonist SR141716 ($1 \mu\text{M}$). ($144.3 \pm 12.0\%$, $n = 9$ versus $117.9 \pm 2.1\%$, $n = 44$, $p = 0.05$).

C) TBS causes a lasting $148.4 \pm 5.8\%$ potentiation of the fEPSP in wild type C57Black6 mice ($n = 7$) and a $147.0 \pm 11.9\%$ potentiation of the fEPSP in $CB1(-/-)$ mice ($n = 4$). TBS LTP in the wild type and $CB1(-/-)$ mice are not significantly different ($p = 0.90$).

D) Bar graph showing average fEPSP increases in wild type, $CB1(-/-)$, and wild type with SR141716 application following 10 Hz and TBS stimulation. *Inset*: representative traces showing fEPSPs at baseline (thin trace) and 40 minutes post 10 Hz stimulation (bold trace) in wild type, $CB1(-/-)$, and wild type with $1 \mu\text{M}$ SR141716 respectively (scale bars 5 ms by 0.1 mV).

(1.65 ± 0.04 , $n=33$) compared to the wild type mice (1.75 ± 0.03 , $n=21$, $p=0.05$). This suggests that the CB1(-/-) mice have an increased presynaptic release probability and perhaps CB1 receptors cause a tonic level of presynaptic inhibition in wild type mice. Following 10 Hz stimulation, the PPR of wild type mice did not significantly change (1.71 ± 0.03 , $n=30$, $p=0.11$). However, in the CB1(-/-) mice the PPR following 10 Hz stimulation did significantly decrease (1.52 ± 0.05 post 10 Hz, $n=21$, $p<0.01$, Appendix E). Similarly, PPR following 10 Hz stimulation in the presence of $1 \mu\text{M}$ SR141716 also significantly decreased (baseline PPR = 1.78 ± 0.11 , post 10 Hz in SR141716 PPR = 1.62 ± 0.09 , $n=8$, $p = 0.02$), although the application of SR141716 alone did not change PPR (1.80 ± 0.12 , $n=8$, $p=0.39$). Together, this data supports an increased presynaptic release probability as one of the mechanisms responsible for the observed 10 Hz LTP.

WIN55,212-2 inhibits 10 Hz LTP

To further assess the involvement of CB1 in 10 Hz LTP, we tested the effect of the CB1 agonist WIN55,212-2 ($1 \mu\text{M}$) on basal recording and 10 Hz stimulation in wild type and CB1(-/-) mice. Application of agonist decreased the fEPSP amplitude in the wild type mice to $43.7 \pm 4.5\%$ of baseline ($n=6$) and increased paired pulse ratio from 1.76 ± 0.12 to 2.07 ± 0.10 ($n=6$, $p<0.01$) (Figure 2 A,B) in accordance with previous observations (Al-Hayani and Davies, 2000; Hajos and Freund, 2002; Hajos et al., 2001; Hoffman et al., 2005; Misner and Sullivan, 1999; Takahashi and Castillo, 2006). WIN55,212-2 had no effect on fEPSP amplitude ($99.0 \pm 4.4\%$, $n=9$, $p=0.97$) or paired pulse ratio (base PPR = 1.67 ± 0.06 , WIN PPR = 1.70 ± 0.08 , $n=9$, $p=0.43$; Figure 2A,B) in CB1(-/-) mice. In both wild type and CB1(-/-) mice WIN55,212-2 blocked 10 Hz LTP

Figure 2

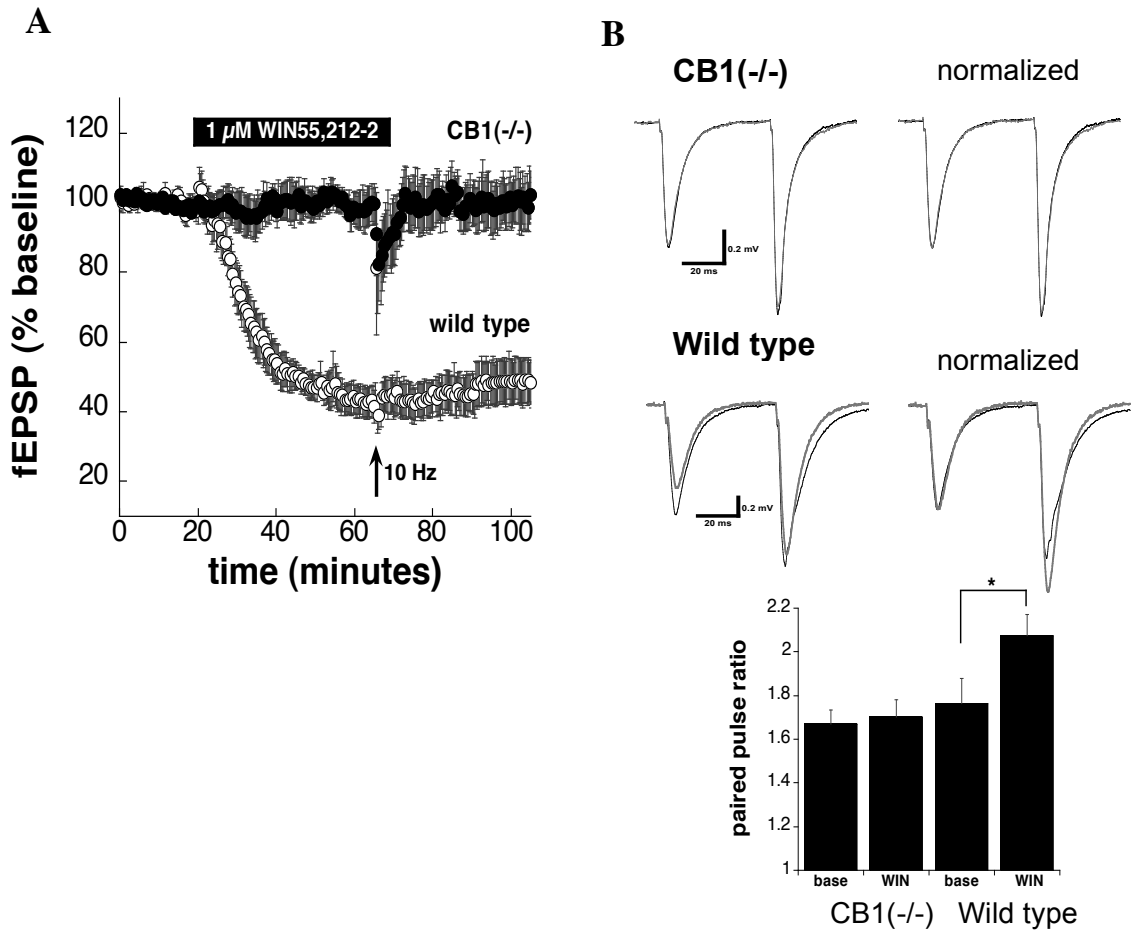


Figure 2: CB agonist WIN55,212-2 prevents 10 Hz LTP in wild type mice and CB1(-/-) mice.

A) Application of 1 μ M WIN55,212-2 causes a lasting depression of the fEPSP in wild type ($43.7 \pm 4.5\%$ of baseline, $n=6$), but not CB1(-/-) mice ($99.0 \pm 4.4\%$ of baseline, $n=9$). 10 Hz stimulation does not cause potentiation of the fEPSP above the baseline established in WIN55,212-2 in wild type and CB1(-/-) mice ($105.6 \pm 4.5\%$, $p=0.26$, $n=6$; $103.0 \pm 9.8\%$, $p=0.76$, $n=9$ respectively).

B) Representative traces showing the effect of 1 μ M WIN55,212-2 on fEPSP amplitude (left traces) and paired pulse ratio (right traces, normalized to the amplitude of the first pulse) in CB1(-/-) and wild type mice. Black traces represent baseline fEPSP before WIN application and gray traces show the fEPSP after 45 minutes of WIN application. *Inset* - average paired pulse ratio for CB1(-/-) and wild type animals, baseline and in 1 μ M WIN55,212-2. CB1(-/-) baseline ppr 1.67 ± 0.064 , WIN ppr 1.70 ± 0.080 , $n=9$, $p=0.43$; wild type baseline ppr $= 1.76 \pm 0.12$, WIN ppr $= 2.07 \pm 0.10$, $n=6$, $p=0.008$.

(wild type, $105.6 \pm 4.5\%$, $p=.026$, $n=6$; CB1(-/-), $103.0 \pm 9.8\%$, $p=.76$, $n=9$; Figure 2A). This data supports a role for CB1 in 10 Hz LTP, but also points to the possibility that an additional endocannabinoid receptor, which WIN55,212-2 also binds, may be modulating synaptic plasticity.

10 Hz LTP is NMDA receptor dependent

Our next step was to determine if 10 Hz LTP is NMDA receptor dependent. Similar to TBS induced LTP, 10 Hz LTP was completely blocked by the NMDA receptor antagonist APV (50 μ M) in both wild type and CB1(-/-) mice ($103.0 \pm 3.8\%$, $n=5$; $100.0 \pm 2.3\%$, $n=8$, Figure 3 A,B). Thus 10 Hz LTP, like traditional LTP, requires NMDA receptor activation and likely shares common expression mechanisms.

LTP threshold and LTD

Since both the magnitude and success rate of 10 Hz LTP was increased in the CB1(-/-) mice we wanted to assess whether there was a difference in the stimulus threshold required to induce fEPSP potentiation between CB1(-/-) and wild type mice. Initially we tried a 5 Hz stimulation protocol (2 bouts of 100 pulses at 5Hz, 20 s apart) and found no difference between wild type and CB1(-/-) mice, with each group showing a slight potentiation (CB1(-/-) $107.7 \pm 5.2\%$ $n=8$; wild type $112.2 \pm 3.1\%$ $n=9$; $p=0.46$, Figure 4A). Interestingly, both groups of animals exhibited the same transient depression that we observed following 10 Hz stimulation in wild type mice, suggesting that the mechanisms behind this depression are not CB1 mediated. Next, we tried a less intense 10 Hz stimulation that had 100 pulses instead of 200 (10 Hz(1), 100 pulses at 10 Hz, no

Figure 3

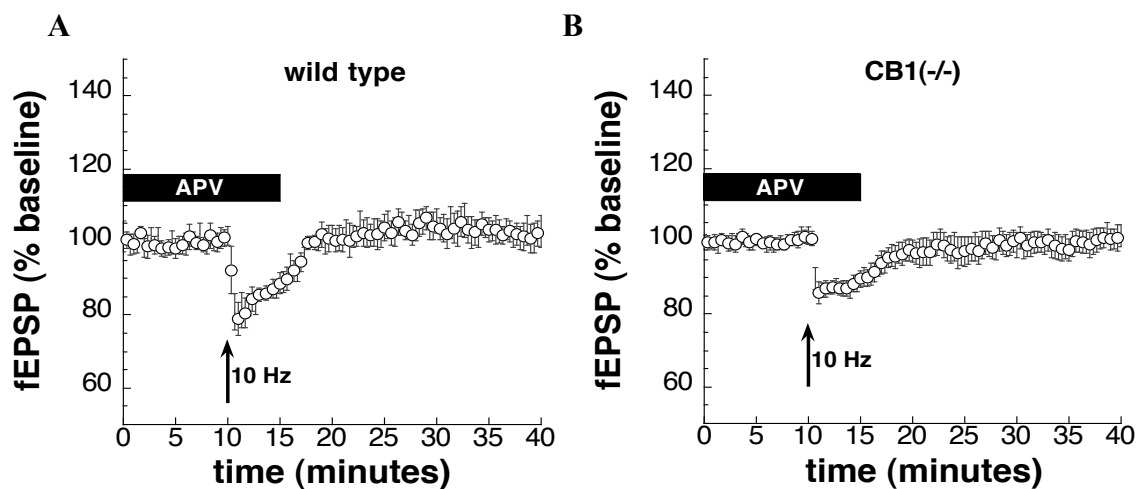


Figure 3: 10 Hz LTP is NMDA receptor dependent in both wild type and CB1(-/-) mice.

A) Application of the NMDA receptor antagonist APV ($50 \mu\text{M}$) for 20 minutes inhibits 10 Hz LTP in wild type mice ($103.2 \pm 3.8\%$ increase, $n = 5$, $p=0.45$ compared to baseline).

B) $50 \mu\text{M}$ APV also inhibits 10 Hz LTP in CB1(-/-) mice ($100.0 \pm 2.3\%$ increase, $n = 8$, $p=0.95$ compared to baseline). There is no significant difference between the wild type and knockout following 10 Hz stimulation in APV ($p = 0.45$).

Figure 4

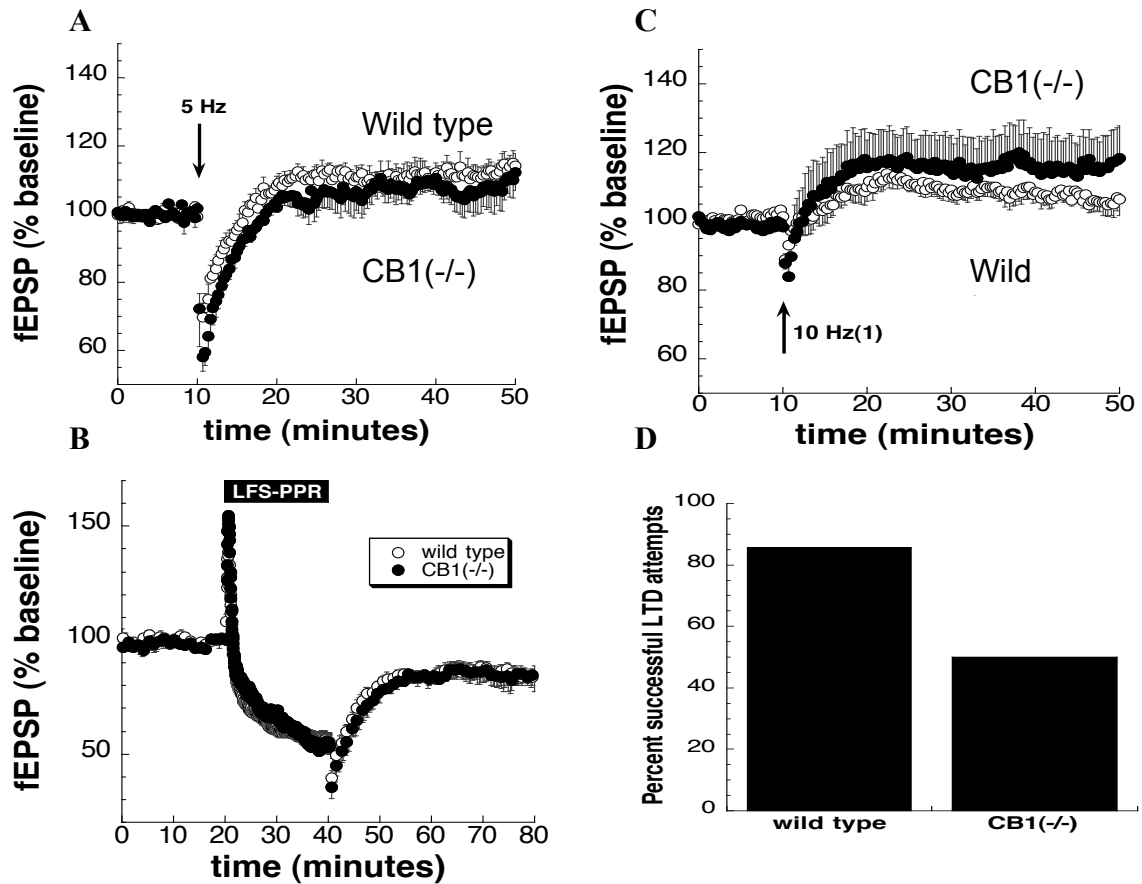


Figure 4: CB1 doesn't change potentiation with lower stimulus protocols but does affect the frequency of LTD induction.

A) 5 Hz stimulation (2 bouts of 100 pulses at 5 Hz, 20 s apart) does not cause LTP in either CB1(-/-) (filled circles) or wild type (open circles) mice and there is no difference between the groups following 5 Hz, CB1(-/-) $107.7 \pm 5.2\%$ n=8; wild type $112.2 \pm 3.1\%$ n=9; p=0.46.

B) A less intense 10 Hz stimulation protocol (100 pulses at 10 Hz, no repetitions) also does not cause significant differences between wild type and CB1(-/-) animals although the trend supports CB1 somewhat inhibiting potentiation (CB1(-/-) $116.8 \pm 9.3\%$ n=8; wild type $105.6 \pm 1.7\%$ n=8; p=0.27).

C) 1 Hz paired pulse stimulation (50 mS inter-pulse interval) for 20 min (1200 paired pulses) (LFS-PPR) depresses the fEPSP $15 \pm 2.4\%$ in C57Black6 wild type mice (n = 6) and $16 \pm 4.5\%$ in CB1(-/-) mice (n = 4). Only successful LTD experiments are plotted and there is no difference in LTD magnitude (p = 0.15).

D) The frequency of successful LTD induction was different between wild type and CB1(-/-) animals. In wild type mice LTD was successfully induced in 6 out of 7 attempts (86%) and in CB1 (-/-) mice LTD was successful in 4 out of 8 attempts (50%).

repetitions). Again, there was no significant difference between the wild type and CB1(-/-) mice and each group showed a slight potentiation (CB1(-/-), $116.8 \pm 9.3\%$, $n=8$; wild type, $105.0 \pm 2.2\%$, $n=6$; $p=0.25$, Figure 4B). None the less, the trend followed our earlier 10 Hz LTP data, with CB1(-/-) animals experiencing a greater increase than the wild type animals. Although the lower frequency stimulations do not reveal a CB1 mediated threshold effect the subtle differences we observe do support CB1s inhibition of potentiation.

Finally, we wanted to investigate if CB1 had an effect on the opposing form of synaptic plasticity, long-term depression (LTD). We used a low frequency paired pulse protocol (LFS-PPR), which consisted of 1200 paired pulses (50 mS interpulse interval) administered at 1 Hz to induce LTD. Although there were no differences in the level of LTD achieved during successful experiments (wild type, $85.5 \pm 2.4\%$, $n=6$; CB1(-/-), $84.8 \pm 4.5\%$, $n=4$; $p=0.88$), we did find that the frequency at which successful LTD (defined as at least a 5% depression sustained for 40 minutes post LFS-PPR) occurred was different. In wild type mice we had a 85.7% success rate (6 out of 7 successful attempts) in achieving LTD; however in CB1(-/-) mice our success rate was only 50.0% (4 out of 8 successful attempts, Figure 4C,D). This data supports a role for CB1 in facilitating LTD induction, which correlates with CB1s inhibition of long-term potentiation

Discussion

As the major result of this work we show that the CB1 receptor acts to inhibit LTP induced by moderate-frequency stimulation through the use of CB1 receptor

knockout mice and standard electrophysiological techniques in acute hippocampal slices. The 10 Hz stimulation protocol we used, which was previously described by Chevaleyre and Castillo (2004) to induce endocannabinoid release and cause iLTD without inducing LTP, in fact caused a lasting potentiation in C57Black6 wild type mice. This 10 Hz LTP is greatly increased with pharmacological blockade or genetic removal of the CB1 receptor. Our results are in contrast to Chevaleyre and Castillo's data in that they found 10 Hz stimulation did not potentiate fEPSPs; however, differences in the experimental conditions, such as species used and recording set up, may account for these inconsistencies. Also, we found that while 10 Hz induced LTP was increased in CB1 knockout mice, LTP caused by higher frequency theta burst stimulation was not altered. Additionally, it is important to note that the LTP induced by 10 Hz stimulation is likely controlled by the same mechanisms as traditional TBS or HFS induced LTP as it is NMDA receptor dependent.

We also found that the cannabinoid receptor agonist WIN55,212-2, which causes a significant depression of fEPSPs in wild type but not CB1(-/-) mice, completely prevented 10 Hz potentiation. While we predicted that WIN55,212-2 application would occlude potentiation in wild type animals we were surprised by the result in CB1 knockout mice. WIN55,212-2 blockade of 10 Hz LTP in CB1(-/-) mice supports the existence of another cannabinoid receptor that may have important roles in synaptic plasticity, and as evidence continues to accumulate in the favor of an additional cannabinoid receptor this finding could be of key importance (Begg et al., 2005; Lauckner et al., 2008; Mackie and Stella, 2006; Ryberg et al., 2007). Previous studies showing an inhibitory effect of WIN55,212-2 on synaptic plasticity have concentrated on

wild type animals and not CB1(-/-) mice so it is possible that past results could be partially attributed to the agonists effects on another cannabinoid receptor (Auclair et al., 2000; Levenes et al., 1998; Misner and Sullivan, 1999; Paton et al., 1998).

We next tested whether the threshold for LTP induction was shifted in CB1 (-/-) mice by using two less intense stimulation protocols. With 5 Hz stimulation we did not achieve significant potentiation in either wild type or CB1 knockout mice. We did note however that a transient depression, similar to that observed in wild type mice with 10 Hz stimulation, occurred in both the CB1 knockout and wild type animals. This leads us to believe that the depression following 10 Hz stimulation is not mediated by the CB1 receptor. Further work will be required to determine what is causing the transient depression as it was observed in all of our treatments with varying frequency and magnitude. A less intense 10 Hz protocol also did not reveal significant differences between wild type and CB1(-/-) animals, but the trend did support CB1 receptor's inhibition of LTP, as the small potentiation achieved following stimulation in CB1(-/-) mice was slightly larger than what was observed in wild type mice. Thus, although we did not find evidence for a clear CB1 mediated shift in LTP induction threshold our data still supports a role for CB1 in inhibition of potentiation.

Several other papers have found CB1 activation to inhibit LTP; however, there are differences in experimental conditions, mainly that these studies used higher frequency stimulation protocols, relied on pharmacological tools, and/or did not have use of the CB1 knockout mice (Collins et al., 1994; Misner and Sullivan, 1999; Nowicky et al., 1987; Stella et al., 1997; Terranova et al., 1995). Two more recent papers have found CB1 to inhibit LTP induced by lower frequency stimulation, without affecting high

frequency induced LTP (Hoffman et al., 2007; Slanina et al., 2005). Interestingly both these papers found TBS induced LTP to be inhibited but not 100 Hz high frequency stimulation (HFS). The TBS protocol used by Slanina et al (2005) was quite different from ours in that they used 5 bursts of 4 pulses at 100 Hz, 200 ms apart, repeated once after 5 seconds. Our protocol used 10 bursts of 5 pulses at 100 Hz, 200 ms apart, repeated once after 5 seconds. Thus, their TBS protocol was actually a less intense stimulation so their results may be closer to what we observed with the 10 Hz stimulation. There were also notable differences in experimental conditions, one major point being that they used rats instead of mice.

The experiments done by Hoffman et al (2007) also were completed in rats and used chronic Δ^9 -THC administration in living animals that were then used to prepare acute hippocampal slices. Although the theta burst protocol they used was the same as ours the difference in experimental setup could largely account for differences in results. Also, by administering Δ^9 -THC chronically they could be activating non-CB1 cannabinoid receptors, which also could affect plasticity. Overall, there is accumulating evidence to suggest that CB1 inhibits long-term potentiation and may have a larger effect on plasticity induced by lower levels of activity. This is hugely important as the high frequency stimulations used in many plasticity studies are less likely to occur naturally and lower frequency stimulations, such as theta rhythms, have been observed *in vivo*.

Just as we found CB1 receptor activation to inhibit LTP, we also found that it increased the success rate of LTD. In wild type mice we were able to successfully induce LTP in 86% of attempts; however, LTD induction was more difficult in CB1(-/-) mice, with success in only 50 % of attempts. This data supports our earlier findings and helps to

show just how important CB1 is as a modulator of synaptic plasticity. Auclair et al (2000) (Auclair et al., 2000) described a similar phenomenon using cortical recordings. They found that in synapses where tetanic stimulation equally induced either LTP or LTD application of WIN55,212-2 shifted plasticity towards LTD, whereas application of SR141716 shifted plasticity in favor of LTP.

Our results point to a very important role for endocannabinoids in modulating synaptic plasticity. We show evidence in support of CB1 modulating excitatory signaling, in particular by inhibiting LTP. Perhaps CB1 is directly inhibiting presynaptic glutamate release as our results cannot be explained by the known inhibition of GABA release typically associated with CB1 receptor activation. The differences we observed in paired pulse ratio (PPR) also support this idea as PPR was increased in the wild type mice relative to the CB1(-/-) mice. This suggests a decreased release probability when CB1 is present and active. Further work will be required to understand the mechanisms by which LTP is inhibited. It will also be important to look for what roles the non-CB1 receptor has in synaptic plasticity once it is well characterized.

CHAPTER 4: DISCUSSION

A moderate frequency induced LTP modulated by endocannabinoids was studied in rats and mice. We found that a 10 Hz stimulation protocol previously shown to cause endocannabinoid release and iLTD without potentiating excitatory inputs (Chevaleyre and Castillo, 2004), actually causes significant potentiation of fEPSPs in rat and mouse hippocampal slice recordings, with some key differences occurring between species.

10 Hz LTP requires cannabinoid receptor activation in rats

The results presented in the first manuscript describe how, in Sprague Dawley rats, 10 Hz induced LTP is different from previously described forms of LTP. Most notably it is distinct from traditional NMDA receptor dependent LTP in that it requires cannabinoid receptor activation but not NMDA receptor activation. We found that two different CB1 receptor antagonists prevented the 10 Hz LTP as did the cannabinoid receptor agonist WIN55,212-2. 10 Hz LTP was still viable when NMDA receptors were blocked, but higher frequency LTP, which is known to be NMDA receptor dependent, was completely prevented.

Our findings also support known mechanisms for endocannabinoid synthesis. When both NMDA receptors and group1 mGluR receptors are blocked, 10 Hz LTP is prevented. As both NMDA and group1 mGluR receptor activation serve to independently increase intracellular calcium, their dual inhibition could prevent sufficient rises in calcium levels, thereby blocking eCB synthesis (Cadas et al., 1996; Di Marzo et al., 1994; Stella and Piomelli, 2001; Stella et al., 1997).

Initially, we believed that 10 Hz LTP could be explained by CB1's known actions of inhibiting GABAergic signaling, which has been reported to occur following 10 Hz stimulation (Chevaleyre and Castillo, 2004). However, we were surprised to find that when GABAergic inputs were pharmacologically blocked 10 Hz LTP remained intact and was still prevented by cannabinoid receptor antagonists. This suggested a novel signaling pathway that does not require GABAergic signaling suppression is responsible for 10 Hz LTP. Possible alternative mechanisms for causing 10 Hz LTP include direct modulation of glutamatergic transmission by CB1 or a related receptor, or modulation of other classes of inputs such as dopaminergic or cholinergic inputs. It will be important to explore these avenues in future research.

10 Hz LTP in rats also exhibits age dependence, with older animals (42-98 days post natal) failing to achieve potentiation. Developmental changes in CB1 receptor distribution do not explain why this occurs as large changes in expression between adolescent animals (post natal day 21-35) and adults have not been reported (Belue et al., 1995; Morozov and F. Freund, 2003; Rodriguez de Fonseca et al., 1993). However, if another cannabinoid receptor is important for 10 Hz LTP, there could be age dependent changes in its distribution which would more aptly coincide with our phenomena. Overall, in rats we have described a novel form of LTP in hippocampus that is induced by moderate frequency stimulation and modulated by endocannabinoid signaling.

In mice, CB1 receptor activation inhibits long-term potentiation

We initially studied the 10 Hz LTP in mice to show its conservation between species and, based on our results in rats, we predicted it would be eliminated in CB1 (-/-)

mice. However, a second manuscript developed from the very intriguing results we found. Indeed, 10 Hz LTP was conserved in C57Blk6 mice and the potentiation was of a similar magnitude to what we observed in rats. The major difference we found was that in CB1 (-/-) mice 10 Hz stimulation roughly doubled the magnitude of LTP instead of preventing it as we had hypothesized. Application of a CB1 receptor antagonist mimicked these results in wild type mice. Also, the 10 Hz LTP in both wild type and CB1(-/-) mice was NMDA receptor dependent, unlike our observations in the rats. There were no differences between the wild type and CB1(-/-) animals following theta burst induced LTP. In general, this suggests that in C57Black6 mice the CB1 receptor acts to inhibit LTP induced by moderate frequency stimulation.

We also found that application of the CB1 agonist WIN55,212-2 blocked 10 Hz LTP in both wild type and CB1(-/-) mice. However, WIN55,212-2 application did not depress fEPSPs in the CB1(-/-) animals as it did in the wild type animals. This data supports the existence of another cannabinoid receptor that is also activated by WIN55,212-2 and is important for synaptic plasticity. Just as we found CB1 receptor activation to inhibit 10 Hz LTP, we also found that it increased the success of LTD. Perhaps CB1 is inhibiting presynaptic glutamate release as our results cannot be explained by the known inhibition of GABA release typically associated with CB1 receptor activation. Our comparison of the paired pulse ratio (PPR) between wild type and CB1(-/-) mice supports this idea as we observed a lower PPR in CB1(-/-) mice, indicating an increased release probability. Further work will be required to understand the mechanisms by which LTP is inhibited. It will also be important to look for what roles the non-CB1 receptor has in synaptic plasticity once it is better characterized.

Conclusion

The endocannabinoid system plays important roles as a modulator of synaptic plasticity and exhibits some very important differences between species. This work may help to explain some of the contradictions in the current literature, as well as illustrate the importance of carefully characterizing any model systems being used in eCB experimentation. Unfortunately, we cannot explain why eCBs seem to play opposing roles in rats and mice. The current literature detailing CB1's distribution shows a similar pattern between species, with few differences in CB1 expression in hippocampus (Haller et al., 2007; Morozov and F. Freund, 2003). Perhaps a more careful examination of the receptor localization and areas of endocannabinoid synthesis will help elucidate our findings. Also, identification and cloning of the novel cannabinoid receptor(s) will be of great importance as its localization and expression levels may differ between species.

Overall, it is apparent that eCB signaling plays crucial roles in the modulation of synaptic plasticity; especially, when moderate frequency stimulation paradigms are used. This points to the physiological importance of the eCB system, largely for learning and memory formation. Future work should focus on better understanding the physiological implications of these effects on plasticity, as well as better resolving the divergence between species. It will also be very important to uncover the possible roles of a novel cannabinoid receptor in synaptic modulation.

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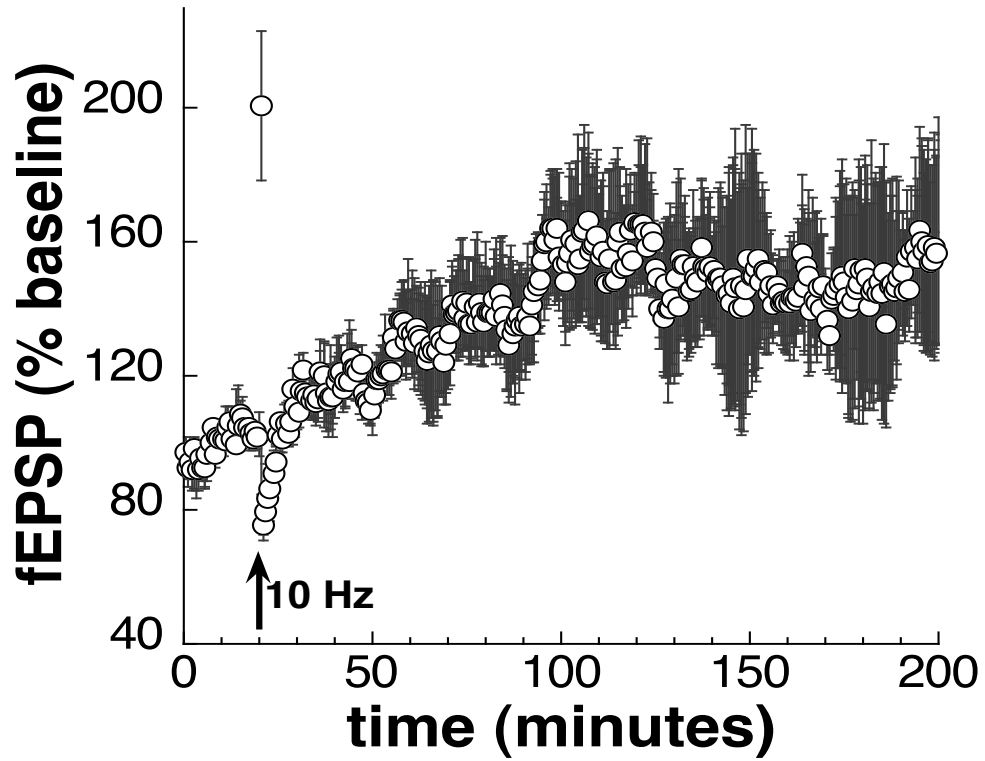
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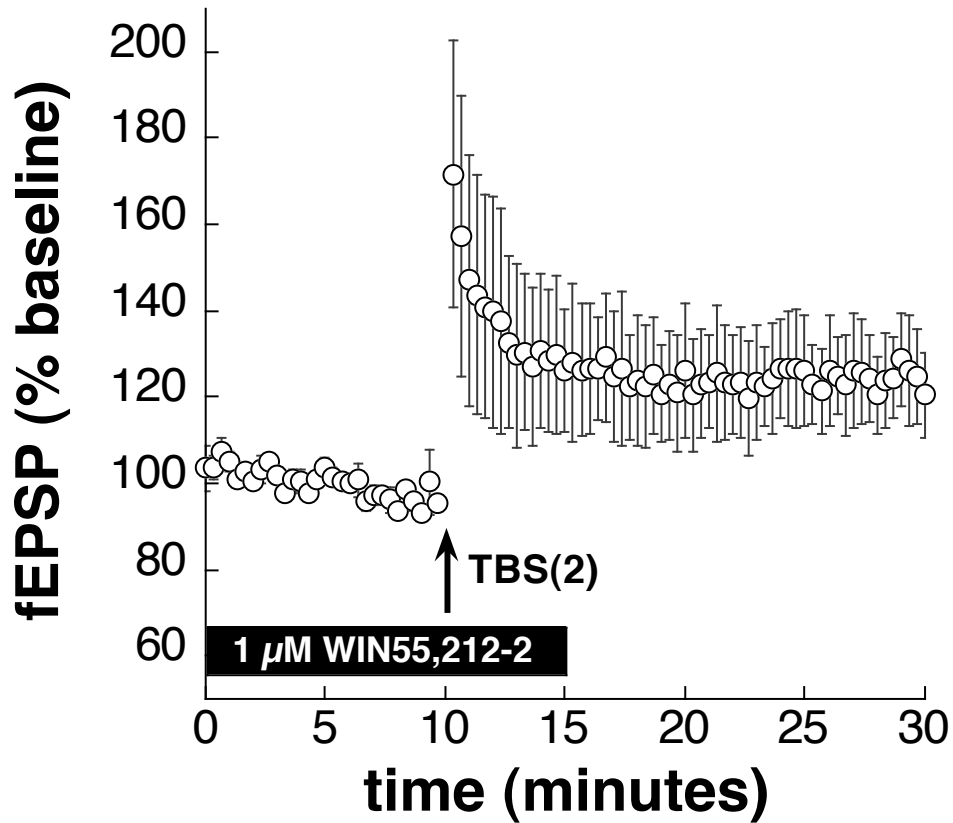
Appendix A: 10 Hz potentiation persists up to 3 hours.



Appendix A: 10 Hz potentiation persists up to 3 hours

fEPSP amplitude following 10 Hz stimulation shows a sustained potentiation for up to 180 minutes (n=3) in Sprague Dawley rats.

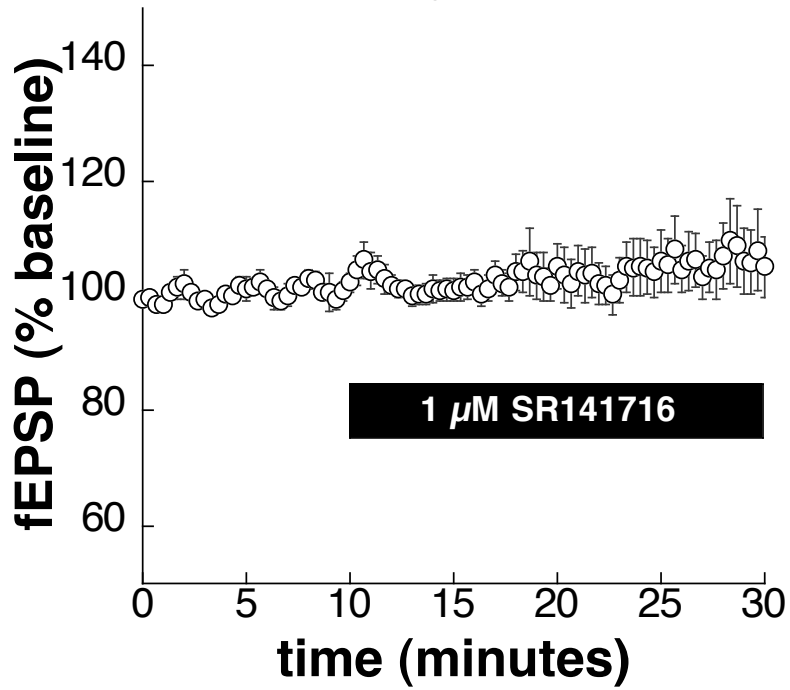
Appendix B: TBS LTP still occurs in WN55,212-2



Appendix B: TBS LTP still occurs in WIN55,212-2

Theta burst stimulation (TBS(2)) induces LTP in the presence of WIN55,212-2 in Sprague Dawley rats although it is reduced compared to control conditions ($128 \pm 11.8\%$ in WIN55,212-2, $n=3$, versus $157 \pm 7.9\%$ in control, $n=16$, $p<0.05$)

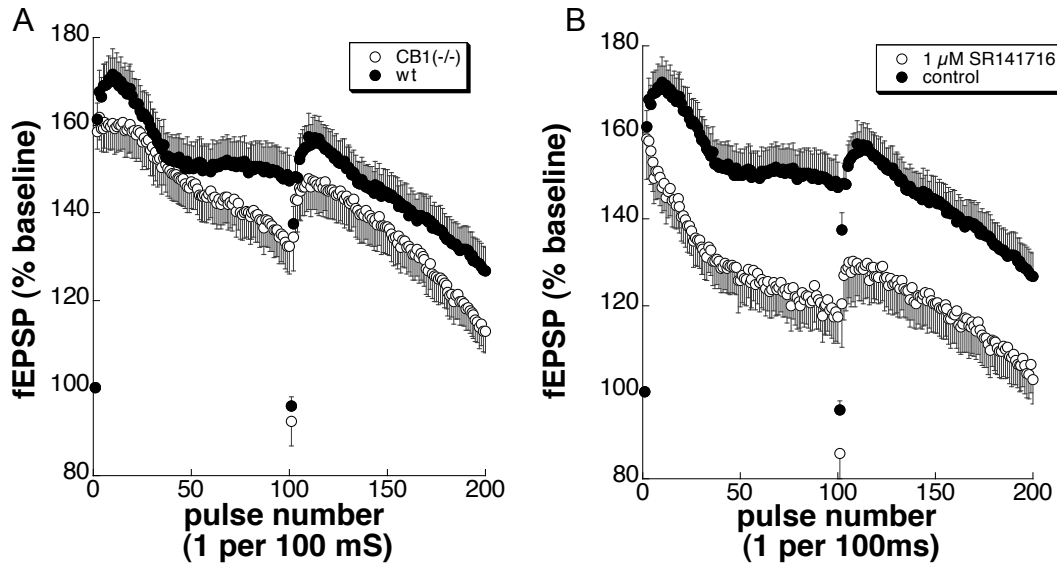
Appendix C: SR141716 application does not change fEPSP amplitude in C57Blk6 wild type mice.



Appendix C: SR141716 does not change fEPSP amplitude in C57Blk6 wild type mice

Application of 1 μM SR141716 does not change the fEPSP amplitude of responses in C57Blk6 wild type mice ($105.0 \pm 0.05\%$, $n=8$, $p=0.37$ compared to baseline).

Appendix D: CB1 receptor activation does not inhibit fEPSP amplitude during the 10 Hz stimulation trains

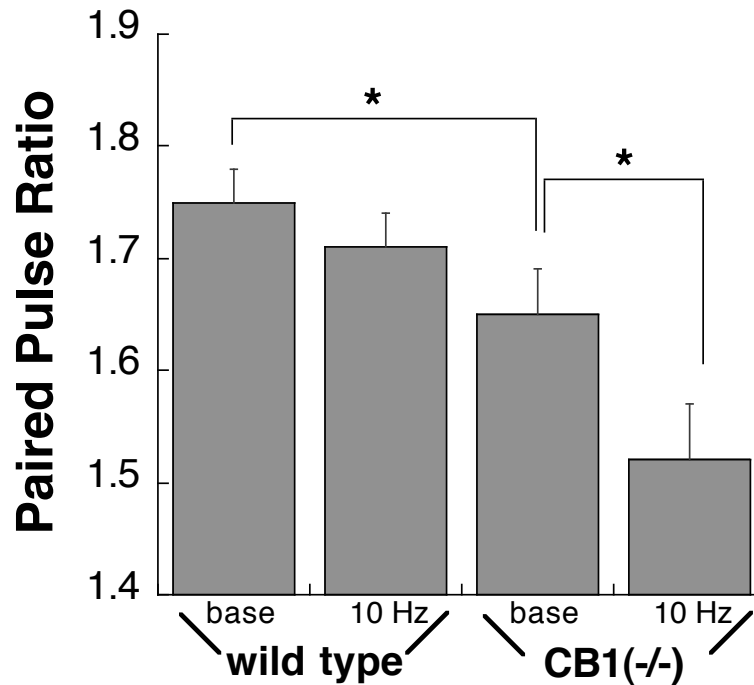


Appendix D : CB1 receptor activation does not inhibit fEPSP amplitude during the 10Hz stimulation trains.

A) Repetitive stimulation during the two 100 pulse 10 Hz bursts does not cause increased potentiation of the fEPSP in CB1(-/-) mice, instead the CB1(-/-) mice are actually less potentiated than the wild type mice.

B) Similar to what is seen in the CB1(-/-) mice, application of SR141716 also does not cause increased potentiation of fEPSP during the 10 Hz stimulation protocol, but instead SR141716 application depresses the fEPSP compared to control conditions.

Appendix E: Changes in paired pulse ratio in wild type and CB1(-/-) mice following 10 Hz LTP



Appendix E: Changes in paired pulse ratio in wild type and CB1(-/-) mice following 10 Hz LTP

The base paired pulse ratio (PPR) in CB1(-/-) mice is less than in wild type mice (1.65 ± 0.04 in CB1(-/-), $n=21$ versus 1.75 ± 0.03 , $n=33$ in wt, $p = 0.05$). Following 10 Hz stimulation PPR is not significantly change in wild type mice (1.71 ± 0.03 , $n=30$, $p=0.11$) but is decreased in CB1(-/-) mice (1.52 ± 0.05 , $n=21$, $p<0.01$).