Endocannabinoid function in hippocampal synaptic plasticity and spatial working memory

Farriss Blaskovits

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University of Ottawa Institute of Mental Health Research
Department of Cellular and Molecular Medicine
Faculty of Medicine

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Abstract

Cannabis has been used medicinally for millennia, but the cannabinoid (CB) field exploded with the identification of its endogenous receptors and endocannabinoids (eCBs). *In vitro* experimentation established that eCBs alter synaptic plasticity at presynaptic nerve terminals; however, the characterization of the eCB system (ECS) *in vivo* remains incomplete. This study aimed to determine the mechanism of *in vivo* eCB-mediated hippocampal synaptic plasticity and to analyze the effects this plasticity had on spatial working memory (SWM). With *in vivo* recordings of field excitatory postsynaptic potentials (fEPSPs) in anesthetized mice and rats as well as pharmacological manipulation of the ECS and glutamate receptor antagonism, it was found that eCBs, both anandamide (AEA) and 2-arachnidonyl glycerol (2-AG), caused LTD at hippocampal CA3-CA1 synapses. Induction of eCB-LTD occurs via a sequential activation of cannabinoid type-1 receptor (CB_1R) and NR2B-containing NMDA receptor (NR2BR) and is expressed through the endocytosis of AMPA receptors (AMPARs). Increased eCB tone also caused an impairment of SWM for over 24 hours in the Delayed Non-Match-To-Sample (DNMTS) T-maze. This study provides the first evidence that an acute administration of eCB degradative enzyme inhibitors not only produces an *in vivo* LTD at hippocampal CA3-CA1 synapses that requires CB_1R, NR2BR, and AMPAR, but also impairs SWM, a phenomenon also caused by an acute injection of exogenous CBs.

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List of Abbreviations and Chemical Names

2-AG	2-Arachnidonoylglyercol
ABHD6	α-β-hydrolase domain 6
AC	Adenylyl cyclase
ACC	Animal Care Committee
AEA	N-arachidonoylethanolamine or Anandamide
AM 251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl) pyrazole-3-carboxamide
AM 281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide
AM 404	*N*-arachidonoylaminophenol
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AMT	Anandamide membrane transporter
AP-5	(2*R*)-amino-5-phosphonovaleric acid; (2*R*)-amino-5-phosphonopentanoate
Arc	Activity-regulated cytoskeleton-associated protein
BCM	Bienenstock-Cooper-Munro
BDNF	Brain-derived neurotrophic factor
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
CaMKII	Ca^{2+}/calmodulin-dependent protein kinases II
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CB_1R	Type-1 cannabinoid receptor
CB_2R	Type-2 cannabinoid receptor
CCAC	Canadian Council on Animal Care
CNS	Central nervous system
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
D_2	Dopamine receptor 2
DAG	Diacylglycerol
DAGLα	Diacylglcerol lipase α
DHPG	(*S*)-3,5-Dihydroxyphenylglycine
DMSO	Dimethyl sulfoxide
DNMTS	Delayed Non-Match-To-Sample
DSE	Depolarization-induced suppression of excitation
DSI	Depolarization-induced suppression of inhibition
E4CPG	(*RS*)-α-Ethyl-4-carboxyphenylglycine
eCB	Endogenous cannabinoid or endocannabinoid
ECS	Endocannabinoid system
E-LTD	Early-phase long-term depression
E-LTP	Early-phase long-term potentiation
ER	Estrogen receptor
ERK	Extracelluar signal-regulated kinase
FAAH	Fatty acid amide hydrolase
fEPSP	Field excitatory postsynaptic potential
FMRP	Fragile X mental retardation protein

GABA	γ-aminobutyric acid
GDE1	Glycerophosphodiester phosphodiesterase 1
GFAP	Glial fibrillary acidic protein
GPCR	G protein-coupled receptor
GPR55	G protein-coupled receptor 55
HEK-293T	Human Embryonic Kidney 293 cells containing SV40 Large T-antigen
HU-210	(6a*R*,10a*R*)- 9-(Hydroxymethyl)- 6,6-dimethyl- 3-(2-methyloctan-2-yl)- 6a,7,10,10a-tetrahydrobenzo [c]chromen- 1-ol
Ifenprodil	4-[2-(4-benzylpiperidin-1-yl)-1-hydroxypropyl]
i.p.	Intraperitoneal
IP_3	Inositol trisphosphate
JZL 184	4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate
JZL 195	(4-nitrophenyl) 4-[(3-phenoxyphenyl)methyl]piperazine-1-carboxylate
L-LTD	Late-phase long-term depression
L-LTP	Late-phase long-term potentiation
LTD	Long-term depression
LTP	Long-term potentiation
Lyso-PLC	Lysophospholipase C
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
MK 801	Dizocilpine or [5R,10S]-[+]-5-methyl-10,11- dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine
mTOR	Mammalian target of rapamycin
NAPE	*N*-Acylphosphatidylethanolamine
NAT	*N*-Acetyltransferase
NMDAR	N-methyl-D-aspartic acid receptor
NO	Nitric oxide
NR2BR	NR2B subunit-containing NMDA receptor
NVP-AAM077	({[(1S)-1-(4-bromophenyl)ethyl]amino}-(2,3-dioxo-1,4-dihydroquinoxalin-5-yl)methyl)phosphonic acid
OHT	4-hydroxytamoxifen
PI3K	Phosphatidylinositide 3-kinase
PKA	cAMP-dependent Protein Kinase A
PKB	Protein Kinase B
PKC	Protein Kinase C
PLA1	Phospholipase A1
PLCβ	Phospholipase Cβ
PLD	Phospholipase D
PTSD	Post-traumatic stress disorder
Ro25-6981	[R-(R*,S*)]-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate
SD	Sprague-Dawley
SPM	Synaptic plasticity and memory

SR141716A	*N*-(Piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride
SR144528	N-[(1S)-endo-1,3,3-trimethylbicyclo [2.2.1]heptan2-yl]-5-(4-chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-1H-pyrazole-3-carboxamide
STDP	Spike timing-dependent plasticity
SWM	Spatial working memory
TBOA	DL-*threo*-β-Benzyloxyaspartic acid
TBS	Theta burst stimulation
THC	Δ^9-Tetrahydrocannabinol
TrkB	Neurotrophic type-2 tyrosine kinase receptor
TRVP1	transient receptor cation channel subfamily V member 1 or vanilloid receptor 1
URB597	[3-(3-carbamoylphenyl)phenyl] N-cyclohexylcarbamate
VDM 11	(5*Z*,8*Z*,11*Z*,14*Z*)-*N*-(4-Hydroxy-2-methylphenyl)-5,8,11,14-eicosatetraenamide
WIN 55,212-22	(R)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone
WT	Wild-type

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To the future and everything that will be achieved with my loved ones at my side

Chapter 1: Introduction

1.1 Cannabinoids, Endocannabinoids, and Cannabinoid Receptors

1.1.1 Overview

Marijuana is one of the most widely used illicit drugs in the world with the United Nations reporting close to 4% of the global population using the drug annually (World Drug Report, 2010). Heavy marijuana use causes addiction, decreased or impaired mobility, analgesia, and memory impairment in animals and a significant subset of humans (Kanayama et al., 2004; Solowij and Battisti, 2008; Lamarine, 2012; Schweinsburg et al., 2008). Marijuana's effects are due to Δ^9-tetrahydrocannabinol (THC), the psychoactive component in the *Cannabis sativa* plant. In the central nervous system (CNS), both exogenous cannabinoids such as THC, and endogenous cannabinoids (eCBs) biosynthesized by brain cells, act as neuromodulators by interacting with type-1 cannabinoid receptors (CB_1R) (Iversen, 2003). The type-2 cannabinoid receptor (CB_2R) is largely localized peripherally in the immune system whereas CB_1Rs are more prevalent in the CNS (Herkenham et al., 1990).

1.1.2 Classes of Cannabinoids

Cannabinoids can be divided into two main classes, exogenous cannabinoids (CBs) and eCBs. CBs can be further subdivided into plant-derived phytocannabinoids and man-made synthetic cannabinoids (Iversen, 2003). Early experiments with CBs allowed researchers to identify the pharmacological effects that are now known as the cannabinoid tetrad: hypomobility, hypothermia, catalepsy, and analgesia (Little et al., 1988). There is a wide range of potent synthetic CBs available that can interact with CB_1R, including HU-210 and WIN 55, 212-2 (Pop, 1999); however my work will focus on the eCB system (ECS).

eCBs are synthesized *in vivo*, where they are cleaved from the lipid membrane (Piomelli, 2003). eCBs are retrograde signaling molecules that are synthesized and released from the postsynaptic neuron on demand, in an activity-dependent manner, and then act on presynaptic CB_1R to decrease neurotransmitter release (Heifets and Castillo, 2009). The two major eCBs are anandamide (AEA) and 2-arachidonyl glycerol (2-AG) (Hillard, 2000).

1.1.3 Cannabinoid Receptors

There are currently three known CB receptors; CB_1R, CB_2R, and an atypical CB receptor, GPR55 (Felder and Glass, 1998; Henstridge et al., 2011). CB_1R is one of the most abundant G protein-coupled receptors (GPCRs) in the brain, and as such, CB_1R exhibits characteristics that are typical of this class of signal transducers (Twitchell et al., 1997). The CB_1R is widely accepted as a member of the $G_{i/o}$ class of GPCRs (Howlett, 2004), and as such activation of CB_1R causes a hyperpolarization of the presynaptic membrane through interactions with cation channels that reduce neurotransmitter vesicle release, and inhibits adenylyl cyclase (AC) activity resulting in a decrease of cAMP (Twitchell et al., 1997; Wilson et al., 2001) that ultimately deactivates cAMP-dependent protein kinase (PKA) (Chevaleyre et al., 2007). Some evidence implicates $G_{q/ll}$ G proteins in CB_1R-dependent signaling based on a WIN55,212-2-dependent and pertussis toxin-insensitive rise in intracellular Ca^{2+} (Lauckner et al., 2005), however other groups have also shown that CB_1R-dependent rises in intracellular Ca^{2+} are pertussis toxin-sensitive (Netzeband et al., 1999). Additionally, $G_{i/o}$ G proteins have been shown to have a minor role in phospholipase C (PLC) activation (Mizuta et al., 2011), a signaling mechanism normally attributed to $G_{q/ll}$ G proteins. Thus, the role of $G_{i/o}$ G proteins in CB_1R-dependent intracellular signaling is well established, but the role of $G_{q/11}$ remains controversial.

CB_2R is the second type of typical CB receptor. CB_2R shares 44% of the total sequence homology with CB_1R, and 68% sequence homology throughout the transmembrane domain (Munro et al., 1993). CB_2R is largely found in the immune system, where the largest numbers are associated with B lymphocytes, NK cells, and monocytes (Cabral et al., 2008). Puffenbarger et al. have shown that CB_2R is expressed in the CNS on activated microglia and the interaction of THC and methanandamide with the microglial CB_2R mediates the downregulation of inflammatory cytokine release (Puffenbarger et al., 2000). As such, CB_2R expressed on microglia throughout the CNS plays a role in neuroinflammatory disorders without a pertinent role in non-pathologic brain function (Cabral et al., 2008).

GPR55 has only recently been characterized and although GPR55 mRNA has been found in the striatum, hypothalamus, and brain stem, there is no current evidence of GPR55 protein in the CNS (Henstidge et al., 2011). Interest in this putative CB receptor arose when studies showed that eicosanoids palmitoylethanolamide and oleamide produced biological effects that were not mediated by the typical CB receptors (Godlewski et al., 2009). It has only 14% sequence homology with the typical CB receptors, CB_1R and CB_2R, and has a largely hydrophilic ligand-binding site as compared to the hydrophobic pocket binding site found in the other CB receptors (Henstridge et al., 2011). As such, GPR55 is currently not explored in eCB-mediated synaptic plasticity studies.

1.1.4 Neuronal and Glial CB Receptors

In cortical regions of the brain CB_1Rs are largely located on GABAergic and glutamatergic presynaptic neuron terminals, with the receptor protein levels estimated at 10-20 times higher on GABAergic neurons (Kawamura et al., 2006). Despite this fact, glutamatergic

CB_1Rs can have a more profound neuroplastic effect than their GABAergic CB_1R counterparts based on their discrete brain locations, and enhanced ability to recruit G proteins and to influence the presynaptic release of excitatory neurotransmitters (Ruehle et al., 2012).

In the late 1980s evidence began to accumulate indicating that glial cells played a more active role in neuronal signaling than previously thought. Functional neurotransmitter receptors were identified on astrocytic membranes, and activation of these receptors with neurotransmitters such as glutamate caused release of Ca^{2+} from intracellular stores (Enkvist et al., 1989). Furthermore, application of glutamate to cultured astrocytes elicited Ca^{2+} oscillations and propagating Ca^{2+} waves within the astrocytic network that were indicative of Ca^{2+}-based excitability (Cornell-Bell et al. 1990). Neuronal stimulation of CA3 pyramidal cells caused Ca^{2+} mobilization in both the neurons themselves and astrocytes in the strata surrounding the pyramidal layer (Dani et al., 1992). These experiments prompted researchers to explore other neurotransmitter families, and a functional expression of CB_1R on astrocytes was found. Bari et al. identified the presence of eCBs and their regulatory enzymes in gliosomes, and found that applications of CB_1R, but not CB_2R or transient receptor cation channel subfamily V member 1 (TRVP1) agonists increased gliosomal glutamate release (Bari et al., 2010). Navarrete and Araque recorded astrocytic intracellular Ca^{2+} levels and found that CB_1R agonist applications caused intracellular astrocyte Ca^{2+} increases that were attenuated with the CB_1R antagonist AM 251, and eCBs released due to pyramidal cell depolarization elicited similar effects (Navarrete et al., 2008). More recently, my colleagues provide the first morphological evidence showing the presence of CB_1R on astrocytes using double immunostaining at the electron microscopic level (Han et al., 2012).

1.1.5 Endocannabinoids

The discovery of CB receptors was accompanied with a search for their endogenous ligands and by 2000 the two major eCBs, AEA and 2-AG, were identified (Hillard, 2000). These eCBs accomplish most of the signaling necessary for the proper functioning of the ECS (see Figure 1-1). This is demonstrated by the fact that 2-AG (Stella et al., 1997) and AEA (Di Marzo et al., 1994) levels increase in an activity-dependent manner, and increased levels of both eCBs elicit synaptic plasticity (Mackie et al., 1993; Felder et al., 1993; Péterfi et al., 2012). 2-AG is a crucial eCB found at the intersections of many different lipid and secondary messenger pathways. Furthermore, 2-AG is present at levels 200 times higher than AEA (Felder and Glass, 1998), suggesting an important role in housekeeping functions. For instance, when 2-AG is broken down arachidonic acid is produced, a compound needed in sufficient quantities for the synthesis of AEA (Piomelli, 2003). Therefore, interfering with 2-AG synthesis can greatly affect basal neuronal function.

In more recent years, eCBs such as palmiethanolamide and olemide have been identified but their cannabimimetic natures remain controversial (Fowler, 2004; Lambert et al., 1999). Palmiethanolamide is devoid of an affinity for CB_1Rs and CB_2Rs in culture and its effects appear to be mediated through the newly discovered GPR55 (Lambert et al., 1999). Oleamide has been shown to bind CB_1Rs as a full agonist in human CB_1R RNA-transfected HEK-293T cells and occludes the AEA-induced physiological tetrad *in vivo* (Leggett et al., 2004); however these experiments use an overexpression of oleamide. Unlike AEA and 2-AG, *in vivo* oleamide levels are not increased due to neural activity (Buczynski and Parsons, 2010), and thus it appears that oleamide plays little role in neuromodulatory function and is mainly effective in the presence of AEA (Mechoulam et al., 1997).

1.1.6 Endocannabinoid Synthesis

AEA synthesis can occur through numerous pathways including the more prominent N-acylphosphatidylethanolamine-specific Phospholipase D (NAPE-PLD) pathway (Di Marzo, 2011) and another involving α,β-Hydrolase-4 (ABHD4) and glycerophosphodiesterase-1 (GDE1) catalyzing the formation of AEA through a multistep process with numerous transient intermediates (Piomelli, 2003). The former process begins with the activity-dependent cleavage of NAPE by PLD to yield AEA and phosphatidic acid. The brain contains very little NAPE and stores must be replenished by N-acyltransferase (NAT) for the continued formation of AEA through this pathway to occur. NAT activity is Ca^{2+}-dependent and enhanced by cAMP-dependent phosphorylation of NAT through PKA (Di Marzo et al., 1994). However, since the formation of AEA and its precursor NAPE are both Ca^{2+}-dependent, the two processes are thought to occur in parallel with a lack of precursor rarely encountered (Piomelli, 2003). Given the intricacy of the various AEA synthesis pathways, pharmacological targeting of AEA synthesis has proven challenging as alternate pathways are able to recover AEA synthesis when one is manipulated. Indeed, brain homogenates of GDE1 and NAPE-PLD double knockout embryonic mice show reduced AEA levels, but small amounts of AEA were still able to be produced (Simon and Cravatt, 2010) (see Figure 1-2).

Similar to AEA formation, there are multiple pathways for 2-AG synthesis; however, the majority of 2-AG synthesis is accomplished through the PLCβ pathway. In this pathway, PLCβ cleaves phosphotidylionsitol from the membrane to generate 1,2-diacylglycerol (DAG). Diacylglycerol lipase α (DAGLα) then acts on DAG to generate 2-AG (Piomelli, 2003). An alternative route speculates that Phospholipase A1 (PLA1) cleaves phosphatidylinositol into a lyso-phosphatidylinositol product such as 2-arachnidonoyl-lysopholipid. Lyso-PLC then

hydrolyses 2-arachnidonoyl-lysophospholipid to generate 2-AG; however, this pathway is hypothetical as there has been no direct evidence showing that this mechanism can increase levels of 2-AG in the brain (Piomelli, 2003) (see Figure 1-2).

1.1.7 Endocannabinoid Degradation

eCBs are lipid-based molecules that can diffuse across the lipid membrane following their release from the postsynaptic neuron; however, there are still carriers present in both neurons and glia that facilitate this diffusion (Piomelli, 2003). For instance, AEA is transported into the cell by an uncharacterized AEA membrane transporter (AMT) (Fowler, 2013). VDM 11 is a drug that interferes with AMT, allowing AEA to remain in the synapse longer and amplify its biological effects (de Lago et al., 2005). eCB degradation is tightly regulated by enzymatic activity. Fatty acid amide hydrolase (FAAH) is a membrane-bound serine hydrolase that breaks down AEA, and 2-AG is broken down by the cytosolic serine hydrolase monoacylglycerol lipase (MAGL) (Castillo et al., 2012) (see Figure 1-3). JZL 184 inhibits MAGL (Long et al., 2009b) causing an isolated increase in extracellular 2-AG. Other enzymes, such as ABHD6, have also been shown to degrade 2-AG, but MAGL is the only one that is located presynaptically and is understood to be responsible for the degradation of the retrograde signal (Blankman et al., 2007). JZL 195 inhibits both FAAH and MAGL, causing a global increase of eCB in the extracellular space which in turn allows researchers to analyze the ECS as a whole (Long et al., 2009a).

1.1.8 Endocannabinoid Function in the Brain

As mentioned above, eCBs released from the postsynaptic neuron can travel retrogradely across the synaptic cleft and interact with presynaptic CB_1Rs to elicit their various effects

(Castillo et al., 2012). CB_1Rs have been identified in high levels in the frontal cerebral cortex, basal ganglia, cerebellum, hippocampus, hypothalamus, and anterior cingulate cortex (Iversen, 2003). The high CB_1R densities in the basal ganglia and cerebellum account for eCB-mediated effects on psychomotor function (El Manira and Kyriakatos, 2010). Indeed, both CB_1R- and D_2-deficient mice show movement impairments characteristic of Parkinson's disease (Katona and Freund, 2008). The presence of CB_1Rs in the hippocampus has further implications in various forms of memory including spatial working memory (SWM) (Robinson et al., 2008), fear memory extinction and consolidation (Lin et al., 2013; Takahashi, 2011; Ruehle et al., 2012), and several other forms of memory that will be discussed below.

1.2 Synaptic Plasticity

1.2.1 Synaptic Physiology Overview

The synapse is a junction between the presynaptic neuron and, in most cases, the postsynaptic neuron. An incoming electrical signal in the form of an action potential is converted at the presynaptic active zone to a chemical signal in the form of neurotransmitter release. When an action potential enters a presynaptic terminal it causes the opening of voltage-gated Ca^{2+} channels (Regehr, 2012). Neurotransmitters packaged in synaptic vesicles are released via exocytosis into the synaptic cleft, in a Ca^{2+}-dependent manner upon this Ca^{2+} entry, and travel to the postsynaptic neuron where they bind with various transmitter-specific receptors. There are hundreds of types of synapses in the mammalian brain that all function with the same fundamental mechanism but differ in various aspects including neurotransmitter type, vesicular release probabilities, postsynaptic receptor composition, Ca^{2+} buffers, size of releasable synaptic vesicle pool, among others (Südhof, 2012).

Synaptic plasticity is characterized by changes in the strength of synaptic transmission and modifications in the synaptic connections of neurons (Misner and Sullivan, 1999). This plasticity is integral to the brain's ability to respond to incoming stimuli and store information (Regehr, 2012; Goda and Stevens, 1996). Ca^{2+} plays an important role in many forms of presynaptic use-dependent synaptic plasticity, and many forms of plasticity occur in tandem to have a net effect on synaptic strength (Regehr, 2012). Postsynaptic receptor saturation and changes to receptor sensitivity can also contribute to changes in synaptic plasticity (Regehr, 2012). More durable forms of synaptic plasticity can also be accompanied by changes to the morphology of neurons, such as dendritic spine enlargement or shrinkage in cases of long-term potentiation or long-term depression respectively (Murakoshi and Yasuda, 2012).

1.2.2 Short-term Plasticity

Short-term plasticity falls within a time window of tens of milliseconds to minutes and can fall into 3 main categories: depression, facilitation, and augmentation (Regehr, 2012). Given the short duration of this form of plasticity, it is not characterized by new protein synthesis or the growth of new neuronal connections (Mayford et al., 2012). This form of plasticity is most commonly use-dependent - with short-term synaptic depression being due to the depletion of the readily releasable pool of neurotransmitter vesicles, short-term facilitation due to a durable Ca^{2+} signal, and augmentation due to high frequency presynaptic activity (Regehr, 2012). Indeed, presynaptic forms of short-term plasticity are largely regulated by Ca^{2+}-transients, examples of which include the interplay between Ca^{2+}-transients and pools of release-ready synaptic vesicles, the modulation of Ca^{2+} channel activation, and changes to synaptic release machinery due to Ca^{2+}-dependent kinases (Südhof, 2012).

1.2.3 Long-term Plasticity

Contrary to short-term plasticity, long-term plasticity usually ranges from hours to days (Abraham and Williams, 2008). Long-term synaptic plasticity occurs through either the enhanced signal transmission, long-term potentiation (LTP), or the decreased signal transmission, long-term depression (LTD), between neurons. Long-term forms of plasticity commonly involve glutamate, which can bind to both ionotropic receptors, such as NMDA receptors (NMDARs) and AMPA receptors (AMPARs), or metabotropic glutamate receptors (mGluRs) (Watkins and Jane, 2006). NMDARs are commonly referred to as coincidence detectors given that they require both membrane depolarization and agonist binding of glutamate and glycine to relieve their Mg^{2+} block; however, once the receptor has been activated it allows for the influx of Na^{+} and Ca^{2+}, and the efflux of K^{+} (Zito and Scheuss, 2009). Unlike NMDARs, mGluRs are not ion channels but activate biochemical cascades via G protein coupling (Niswender and Conn, 2010). Group I mGluRs, $mGluR_1$ and $mGluR_5$, are often located postsynaptically where their activation leads to neuronal depolarization and increased excitation through $G_{q/11}$ activation. This results in the activation of various kinase pathways, including the Mitogen-activated protein kinase/Extracelluar signal-regulated kinase (MAPK/ERK) and Mammalian target of rapamycin (mTOR)/p70 S6 kinase pathways that are important in synaptic plasticity. Groups II and III mGluRs are primarily located presynaptically where they inhibit neurotransmitter release at excitatory, inhibitory, and other synapses through an interaction with $G_{i/o}$ proteins causing the inhibition of AC and the direct regulation of ion channels (Niswender and Conn, 2010).

Long-term synaptic plasticity has several critical features, including synapse specificity, cooperativity, and associativity (Mayford et al., 2012), and can be largely explained by the

behavior of NMDARs. Associativity can be demonstrated by the fact that the perforant path from the contralateral cortex of the hippocampus can only be potentiated if the ipsilateral perforant path is stimulated simultaneously (Levy and Steward, 1979). There also appears to be interplay between various retrograde signaling molecules, which can either cause LTP or LTD depending on their concentrations, and synaptic activity (Castillo et al., 2012). For instance, eCB-mediated LTD and nitric oxide (NO)-mediated LTP in the ventral tegmental area are both generated by postsynaptic glutamate receptors, but calcium chelators in the postsynaptic neuron prevent the expression of LTP and not LTD (Pan et al., 2008; Nugent et al., 2007).

This study focuses on eCB signaling in the hippocampus, and synaptic plasticity in this region is largely a NMDAR-dependent process. Mulkey and Malenka showed that LTD at CA1 pyramidal cells can be homosynaptic that it was not dependent on presynaptic NMDARs as past experiments had indicated, and that it was due to a moderate rise in postsynaptic inward Ca^{2+} currents through NMDARs (Mulkey and Malenka, 1992). More recent experiments have shown that NMDAR-dependent hippocampal LTD is mediated by extrasynaptic NMDARs that likely contain NR2B subunits (Liu et al., 2013). LTP is accompanied by the formation of new dendritic spines, and recent evidence has shown that these spines are stabilized due to NMDAR activation and consequent interactions of the NR2B subunit with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Hill and Zito, 2013).

1.2.4 Expression and Protein Synthesis

The expression of LTP and LTD can be broken down into early and late phases. For instance, hippocampal early phase LTP (E-LTP) lasts 1-3 hr and does not require protein synthesis; whereas late phase LTP (L-LTP) lasts at least 24 hr, is activated by PKA, and requires

protein synthesis (Mayford et al., 2012). It is widely acknowledged that the early phase is dependent on modification of proteins already present at the synapse (Mayford et al., 2012), such as activity-dependent AMPAR and NMDAR phosphorylation and consequent mobilization (Zuki et al., 2009). The late phase is marked by local protein synthesis in the dendrites and around synaptic sites (Torre et al., 1992) resulting in changes that are locked-in by structural modifications (Zuki et al., 2009). Indeed, the maintenance of long-term plasticity in both L-LTP and L-LTD is largely accomplished through structural changes in spine morphology (Malenka and Bear, 2004; Hill and Zito, 2013), as well as protein synthesis (Abraham and Williams, 2008).

A great deal of work has contributed to our current understanding of protein synthesis in the expression of LTD in particular. Huber and his colleagues showed that the mGluR agonist DHPG-induced LTD in hippocampal slices is sensitive to the protein translation inhibitor anisomycin but not the transcription inhibitor actinomycin-D, implicating a role for protein synthesis and preexisting mRNA in the maintenance of mGluR-LTD (Huber et al., 2000). Also supporting the concept that preexisting dendritic polyribosomes and mRNA contribute to LTD is work done by Linden showing that the application of both anisomycin and actinomycin-D in cerebellar slices blocked LTD, but that anisomycin blocked LTD more quickly than actinomycin-D (Linden, 1996). My colleagues also showed a role for protein synthesis in CB-mediated LTD. An intraperitoneal (i.p.) injection of anisomycin prevented the expression of CB-LTD (Han et al., 2012). Interesting, RNA transcription inhibitor actinomycin-D also prevented the expression of CB-LTD, possibly indicating a more prominent role for newly transcribed mRNAs in CB-LTD versus mGluR-mediated LTD (Han et al., 2012).

A detailed profile of the proteins that are regulated in this manner is not currently available. However, some important proteins, including fragile X mental retardation protein

(FMRP), which controls activity-dependent dendritic mRNA localization, stability, and translational efficiency (Zuki et al., 2009), as well as some targets of FMRP including CaMKII and activity-regulated cytoskeleton-associated protein (Arc) (Kanai et al., 2004).

1.2.5 Presynaptic versus Postsynaptic Mechanisms

A great deal of research has shown that the expression mechanisms of synaptic plasticity can be initiated both presynaptically and postsynaptically. Changes in neurotransmitter release probability typically explain presynaptic mechanisms of plasticity, whereas increasing receptor number and function are associated with postsynaptic mechanisms (Abbott and Nelson, 2000). A classical understanding of long-term plasticity states that induction begins with the influx of Ca^{2+} through postsynaptic NMDARs - with large increases in Ca^{2+} contributing to LTP and smaller increases initiating LTD (Lisman, 1994). This initial induction step causes increases in intracellular Ca^{2+} levels that activate kinases and phosphatases and ultimately lead to AMPAR endocytosis, specifically AMPARs containing the GluR2 subunit (Fetterolf and Foster, 2011). The trafficking of AMPARs also forms the foundation of our understanding of silent synapses; NMDAR-dominant synapses that were previously termed to be 'silent' undergo insertion of AMPARs into the membrane following postsynaptic depolarization and consequently become 'commmunicative' or more responsive to incoming glutamateric signals (Isaac et al., 1995).

Most long-term forms of GABAergic plasticity involve changes in release from the presynaptic terminal (Castillo et al., 2011). Many of these mechanisms are defined by endogenous retrograde messengers including but not limited to brain-derived neurotrophic factor (BDNF), eCB, and NO (Castillo et al., 2011), but bath applications of CBs also showed the ability to decrease synaptic transmission in the hippocampus in a presynaptic manner (Misner

and Sullivan, 1999). The Bienenstock-Cooper-Munro (BCM) model of metaplasticity, defined as the ability for the history of a synapse's activity to influence the magnitude and direction of the change in that synapse's efficacy, also argues for a presynaptic mechanism of synaptic plasticity (Martin et al., 2000). In this model, Bienenstock, Cooper, and Munro propose a temporal mechanism of synaptic competition rather than the spatial concept that was previous held - specific incoming firing patterns determine synaptic specificity rather than their position within the neuronal network. Thus, the firing of the presynaptic neuron will determine whether the postsynaptic neuron will respond more or less readily to the stimulus (Bienenstock et al., 1981).

1.2.6 Induction Methods of Long-term Depression

In this study, my focus on eCBs has concentrated attention on LTD given that eCBs have been shown to travel retrogradely to inhibit neurotransmitter release (Castillo et al., 2012). The most widely used LTD induction methods currently used are pharmacological, electrical, and photostimulation-based. Researchers will choose protocols that are most suited to the system they are investigating. For instance, young rat brains appear to be more susceptible to plastic changes and respond more readily to pharmacological manipulation and lower frequency electrical stimulation (Fitzjohn et al., 1999).

Pharmacologically induced LTD has an important role in G protein mediated forms of synaptic plasticity. DHPG has been widely used to induce LTD; DHPG is a group I mGluR agonist that acts on both $mGluR_1$ and $mGluR_5$ and as such activates $G_{q/11}$ proteins that activate PLC to enhance the DAG-inositol trisphosphate (IP_3) intracellular cascade that ultimately increases intracellular levels of Ca^{2+}, and activates protein kinase C (PKC). Similarly with many LTD-inducing protocols, the robustness of DHPG-induced LTD is significantly affected by brain

age and region. *In vitro* applications of DHPG in the adult rat hippocampus elicits small LTD that is enhanced when extracellular Mg^{2+} is removed, indicating a cooperative role for mGluRs and NMDARs in LTD induction in the adult brain (Fitzjohn et al., 1999). When DHPG is applied to the hippocampus of rats at 12-18 days of age, a large LTD is induced that does not require any other accompanying treatments. DHPG can also induce LTD in corticostriatal and neostriatal regions, although the pathways involved differ. In the neostriatum, DHPG-induced LTD is dependent on CB_1R and the subsequent synthesis of NO downstream (Sergeeva et al., 2007); however, in the corticostriatum there is a switch around age P16 in rats that changes DHPG-LTD from NMDAR-sensitive to CB_1R-sensitive (Chepkova et al., 2009).

Various stimulation protocols have been used to induce LTD, and depend on the age of the animal and the most recent neuronal activity. Low frequency stimulation is commonly used to induce LTD including classical protocols such as 900 pulses at 3 Hz which can decrease EPSP amplitudes to 40–60% of the maximal amplitude, or amended protocols such as low-intensity low frequency stimulation that seeks to mimic the slow wave activity seen in sleeping animals (Yang et al., 2012). Spike timing-dependent plasticity (STDP) is another stimulation-based method to induce LTD that relies on the temporality of firing (Rodríguez-Moreno and Paulsen, 2008). How pre- and postsynaptic neurons fire in relation to each other determines the direction of plasticity that is induced. For instance, if the postsynaptic cell fires immediately before the presynaptic neuron then LTD will occur. In this way the relationship between NMDAR activation and the timing of backpropagating action potentials from the postsynaptic dendrites allows for neural network coordination to prevent excessive and possibly excitotoxic firing (Abbott and Nelson, 2000). Counterintuitively, high frequency stimulation has also been shown to induce LTD, but at $GABA_A$-containing synapses. Calliard and his collegues used three tetanic

stimuli of 100 Hz to show that neuronal depolarization causing the coactivation of $GABA_A$ and NMDARs leads to LTD of GABAergic signaling in 2-4 days postnatal rats (Calliard et al., 1999).

Photo-stimulation is the newest method used in LTD paradigms both *in vivo* and *in vitro*. Electrode-driven stimulation is useful for temporal precision but it does not allow researchers to target specific neurons, and in certain recording environments the electrode is open to deterioration causing greater experimental variability (Xiong and Jin, 2012). Optical techniques arose out of a need for greater spatial specificity and involve chemically modifying proteins to render them light responsive, introducing light-sensitive proteins into cells, and uncaging chemically modified neurotransmitters (Aravanis et al., 2007). Thus far techniques using channelrhodopsin-2 which is sensitive to blue 473 nm light and light-gated receptor that is regulated by wavelengths from 380-500 nm, have been employed in concert with pharmacological and electrical protocols to induce LTD (Higley and Sabatini, 2010; Izquierdo-Serra et al., 2013). However, researchers are experimenting with various laser pulse paradigms to produce similar effects as low frequency stimulation, such as 900 pulses at 2 or 4 Hz, and manipulating the rate of Ca^{2+} influx using the light-gated glutamate receptors (Xiong and Jin, 2012; Izquierdo-Serra et al., 2013). These attempts have been promising, but have yet to produce reliable methods to elicit LTD with light alone.

The protocol (see Methods section for details) for this study was chosen with the intent to keep the conditions as physiologically relevant as possible. Therefore, low frequency stimulation consistent with that seen in the spontaneous firing of hippocampal neurons (Mazzoni et al., 2007) and *in vivo* recordings were used alongside pharmacological manipulation of the ECS. In this way the experiments were more readily able to represent physiological conditions as the higher

degree of electrical stimulation that is normally used to induce the accumulation of eCBs (Ohno-Shosaku et al., 2012) could elicit many far-reaching and unpredictable consequences on network function (Zoppi et al., 2011). Adult rodents were used due to our lab's interest in the developed mammalian brain.

1.3 CB- and eCB-mediated Synaptic Depression

1.3.1 Overview

Various experimental techniques can be employed for electrophysiological study on CB- and eCB-mediated synaptic plasticity. Given that eCBs are released in an activity dependent manner, electric stimulation protocols can be used to elicit eCB-dependent short-term synaptic plasticity. Induction protocols range from 100 pulses at 1 Hz to 100 Hz, or the more structured theta burst stimulation (TBS) (Heifets and Castillo, 2009). Upon stimulation to excitatory or inhibitory synapses, the glutamate or GABA released can stimulate postsynaptic group I mGluRs to produce and release eCBs into the synaptic cleft. The eCBs then signal retrogradely to cause presynaptic inhibition of excitatory or inhibitory neurotransmitter release by activating presynaptic CB_1Rs (Varma et al., 2001). This phenomenon is referred to as depolarization-induced suppression of excitation (DSE) or inhibition (DSI). Varma and her colleagues found that the mGluR antagonist MCPG applied to hippocampal slices of wild-type mice prevented and the mGluR agonist DHPG enhanced the DSE - effects that were blocked in both CB_1R -/- mice and with CB_1R antagonist application (Varma et al., 2001).

However, CB- and eCB-mediated plasticity can also be induced pharmacologically. My colleagues showed that exogenous applications of CBs could induce LTD at CA3-CA1 hippocampal synapses (Han et al., 2012). The ECS can itself be manipulated to induce plasticity

by increasing levels of eCBs which can be accomplished by direct injections of the eCBs themselves (Fernadez-Solari et al., 2009), pharmacological inhibition of eCB degradative enzymes (de Lago et al., 2005; Long et al., 2009a; Long et al., 2009b), or the generation of mouse knockout models for eCB degradative enzymes (Maldonado et al., 2013). In the cases of stimulation or pharmacologic/genetic manipulation, increased extracellular levels of CBs or eCBs can induce various types of synaptic plasticity, some of which are outlined below.

1.3.2 Transient versus long-term plasticity

Early *in vitro* experiments on brain slices showed that synaptic plasticity, particularly DSE, in hippocampal neurons was mediated exclusively by CB_1R on the glutamatergic nerve terminals in a short-term manner (Wilson and Nicoll, 2001). Yoshida and his colleagues yielded similar results in cerebellar Purkinje cells where DSI was occluded by the CB_1R agonist WIN55,212-2 and prevented by the CB_1R antagonist SR141716A (Yoshida et al., 2002). In both experiments, the CB_1R-dependent DSI or DSE was on the time scale of tens of seconds and rarely exceeded 5 min (Wilson and Nicoll, 2001; Yoshida et al., 2002).

Members of our lab have also contributed to the characterization of CB-meditated hippocampal synaptic plasticity by exploring the mechanism *in vivo*. They found that an i.p. injection of CBs was able to depress synaptic transmission in the CA3-CA1 region of the rat hippocampus for more than 12 h (Han et al., 2012). This lengthy depression of synaptic transmission was characterized as LTD, contrary to results obtained in the previous *in vitro* experiments. To begin this characterization, CB_1R antagonists were injected 10 min after the injection of CBs and failed to prevent the depression of synaptic transmission. Given the duration of LTD being in the magnitude of hours to days rather than minutes (Costa-Mattioli et

al., 2009), the fact that there was no reversion of the depression provided strong evidence for its long term nature. Indeed, CB_1Rs are internalized upon agonist activation, peaking at 83% internalization compared to controls 5-16 h following the application of CB_1R agonists to cultured hippocampal neurons (Coutts et al., 2001), partially explaining the failure of antagonists applied after the induction of depressed synaptic transmission to reverse the process. mRNA transcription and protein synthesis, other hallmarks of long-term synaptic plasticity (Manahan-Vaughan et al., 2000; Puighermanal et al., 2009), were also required for the late-phase expression of CB-mediated depression of synaptic transmission providing further evidence for the long-term nature of the process *in vivo* and allowing my colleagues to reasonably conclude that CBs induce LTD at CA3-CA1 synapses *in vivo* (Han et al., 2012).

1.3.3 Role of glutamate receptors

My colleagues also showed the role of glutamate receptors in CB-LTD. Injections of the glutamate reuptake inhibitor TBOA caused *in vivo* hippocampal LTD comparable to CB-LTD. This was mediated by NMDARs as the NMDAR antagonist AP-5 prevented CB-LTD, whereas the mGluR antagonist E4CPG had no effect on CB-LTD. Furthermore, CB-LTD was shown to be dependent on NR2B-containing NMDARs (NR2BR) as NR2B subunit-specific antagonists Ro25-6981 and ifenprodil, but not NR2A-specific antagonist NVP-AAM077, prevented CB-LTD (Han et al., 2012).

In the same published findings by my colleagues, AMPARs were also implicated in CB-LTD. Injections of a $TatGluR2_{3Y}$ peptide, an inhibitor of AMPAR endocytosis, 2 h prior to CB injections prevented CB-LTD (Han et al., 2012). These findings were validated molecularly

when decreased surface expression levels of GluR1/GluR2 AMPAR subunits were found in CA1 synaptosomes following injections of CBs.

1.3.4 Role of Astrocytic CB_1R

As mentioned previously, astrocytes have been increasingly attributed with the ability to communicate with neurons, to control neuronal activity, and vice versa (Navarrete and Araque, 2008). Neuron-astrocyte communication has been shown to occur via increases in intracellular Ca^{2+} levels in astrocytes in response to both presynaptic neurotransmitters interacting with neurotransmitter specific receptors on glial cells and the direct afferent excitation of glial cells (Pirttimaki et al., 2011). Following rises in intracellular Ca^{2+} levels, astrocytes can release gliotransmitters to regulate neuron activity (Santello and Volterra, 2008). This process is exemplified by the functional CB_1R and ECS enzymatic machinery expressed by astrocytes (Bari et al., 2010); activation of these receptors with application of AEA, 2-AG, and WIN55,212-2 elicits a release of glutamate from the astrocytes (Navarrete and Araque, 2008). In the same work by Navarrete and Araque it was shown that stimulation-induced release of eCBs from pyramidal cells can similarly increase astrocytic Ca^{2+} levels and induce the release of astrocytic glutamate, indicating a more physiological role for CB_1R on astrocytes (Navarrete and Araque, 2008).

Work done by our lab demonstrated that CB-LTD was also mediated by CB_1R on astrocytes, but not by CB_1R on nerve terminals, *in vivo*. Mice with selective deletions of CB_1R on glutamatergic and GABAergic neurons showed the same depression of synaptic transmission upon an i.p. injection of CBs as compared to their wild-type littermates. Mice with a CB_1R deletion specific to astrocytes, on the other hand, showed an absence of CB-LTD.

1.3.5 eCB-mediated LTD

There are currently two known eCB-LTD mechanisms: the classically understood retrograde mechanism with a presynaptic locus of induction and expression, and a more novel mechanism whose expression and induction are postsynaptic. In both cases the eCBs are synthesized and released from the postsynaptic terminal; however, in the first mechanism the eCBs cross the synaptic cleft to work presynaptically whereas in the second the eCBs signal back to activate postsynaptic receptors (Chevaleyre et al., 2006).

Traditionally, eCBs have shown the ability to regulate synaptic plasticity in the hippocampus by decreasing the release of the inhibitory and excitatory neurotransmitters GABA and glutamate from presynaptic terminals. This was shown using 2-AG baths of zebra finch brain slices, a treatment that decreased hippocampal synaptic strength as evidenced by the decreased frequency of postsynaptic excitation (Thompson and Perkel, 2010). Recent studies involving the treatment of mice with 2-AG and various synthetic CBs demonstrated that THC and 2-AG elicited the largest reduction in presynaptic neurotransmitter release in the hippocampus as compared to a range of other synthetic CBs. Furthermore, these reductions in neurotransmitter release were attenuated in CB_1R knock-out mice (Andó et al., 2011). Paired-pulse experiments were also performed to determine if eCB-LTD is dependent on presynaptic mechanistic changes. It was found in the dorsal striatum that eCB applications increased the paired-pulse ratio (Gerdeman et al., 2002), and in the hippocampus that an electrically-induced increase in eCB tone augmented the failure rate and paired-pulse ratio (Chevaleyre and Castillo, 2003) providing further evidence for the presynaptic locus of eCB-LTD expression.

Recently, another form of eCB-LTD was discovered in the cerebellum. Cerebellar LTD has been shown to require the convergence of large postsynaptic Ca^{2+} influx, postsynaptic

$mGluR_1$ activation, and NO (Daniel et al., 1998). Given that eCB synthesis requires a postsynaptic influx of Ca^{2+} and mGluR activation, and that the ECS has been associated with NO pathways (Sergeeva et al., 2007), researchers hypothesized that a link existed between eCBs and cerebellar LTD. Safo and Regehr found that the CB_1R antagonist AM 251 blocked cerebellar LTD as did loading the postsynaptic neuron with DAGL inhibitors. These researchers also showed that cerebellar eCB-LTD did not alter the paired-pulse facilitation ratio, indicating a postsynaptic mechanism (Safo and Regehr, 2005). Although the precise mechanism has not been completely elucidated, there are two presiding theories as to how eCBs binding presynaptic CB_1Rs could produce a postsynaptically expressed eCB-LTD. Safo and Regehr suggested that presynaptic CB_1R activation causes increased NO synthase activity based on experiments showing that NO synthase inhibitors blocked cerebellar LTD. They suggested that the synthesized NO would then diffuse across the synapse to bring about LTD (Safo and Regehr, 2005). However, the role of NO in eCB-mediated changes in synaptic transmission remains controversial, so an alternate theory was developed hypothesizing that cerebellar inhibitory terminals contain an additional neurotransmitter that suppresses LTD until eCB retrograde signaling inhibits its release (Chevaleyre and Castillo, 2003).

It remains unclear how eCB-mediated plasticity is carried out *in vivo* and what the relative contributions of the various eCBs are. This study endeavored to elucidate the mechanisms involved in *in vivo* eCB-mediated plasticity and in doing so discovered an additional mechanism hypothesized to be similar to *in vivo* CB-mediated hippocampal LTD (Han et al., 2012) – CB_1R-dependent rises in extracellular glutamate activate extrasynaptic NMDARs, leading to the internalization of AMPARs and the postsynaptic expression of LTD.

1.4 Working Memory

1.4.1 Overview

Working memory has been defined as a set of memory processing mechanisms that interact to maintain the short-term storage of information and can be actively manipulated (Becker and Morris, 1999). Two classical theories have been developed to explain working memory, one derived from research in animals and another from work in human subjects. Both theories maintain that information held for working memory is short term, the material has a specific temporal context, and that rehearsal is necessary for the active maintenance of information in working memory (Becker and Morris, 1999). Honig's theory stated that working memory and reference memory are separate; that working memory is the information an animal needs in order to successfully perform a single task trial whereas reference memory remains constant throughout all trials of a behavioral task (Cohen et al., 1986). Baddeley and Hitch's theory, based on experiments in human, stated that various interconnected systems are required to store information, and emphasized the importance of verbal- and visual-processing regions of the brain in the maintenance of working memory. Furthermore, Baddeley and Hitch did not distinguish between working and reference memory (Logie et al., 1990). These theories have been combined and restructured to the current definition of working memory as the capacity to maintain a limited amount of information through active rehearsal (Becker and Morris, 1999). The length of time memory is held is not needed to define working memory, more relevant factors include the amount of information and how amenable the information is to active rehearsal (Jeneson and Squire, 2011). Indeed, a limited amount of information can be held in a working manner indefinitely if attention is constantly directed toward the memorandum (see Figure 1-4) (Drachman et al., 1966). It is not uncommon to use experimental conditions that

require animals to hold information in their mind from 30 seconds to 2 minutes (Clark et al., 2001), or even 5 minutes (Duva et al., 1997).

1.4.2 Synaptic plasticity and memory

Synaptic plasticity, known at the time simply as changes in neural activity, was first hypothesized to encode memory and behavior by Donald Hebb and his contemporaries. Hebb is most famously attributed with the phrase "cells that fire together wire together", a concept that he developed in the 1930s but was refined to have behavioral implications by the 1940s (Brown and Milner, 2003). A great deal of work was done with the visual system and it was believed that visual recognition depended on the excitation of specific groups of neurons, implying that an exponentially large population of neurons would be required as an animal's catalogued recollections compounded. Hebb and Hull proposed that groups and chains of cells linked by a common incoming stimulus, in this case the eye movements associated with recognition, served as neural representations of images and concepts (Brown and Milner, 2003). Hull went further to say that it was due to alteration in afferent neural connections but did not explain the methodology (Hull, 1943).

Indeed, to this day it is still proposed that long-lasting alterations in the efficacy of connections between neurons are the cellular basis of learning and memory (Neves et al., 2008). Morris and his colleagues formalized the synaptic plasticity and memory (SPM) hypothesis in the late 1980s, which states that activity-dependent plasticity at appropriate synapses during memory formation is both necessary and sufficient for the storage of memory particular to the brain area in which the plasticity occurred (Martin et al., 2000). Some of the first studies to show that learning and changes in behavior are a consequence of changes in synaptic strength due to

modulations in neurotransmitter release required the use electrophysiological recordings from individual neurons. Intracellular recordings by Castellucci and Kandel showed that the synaptic depression seen in *Aplysia* abdominal ganglia upon the habituation of the gill-withdrawal response was due to decrease in neurotransmitter release rather than postsynaptic receptor sensitivity changes (Castellucci and Kandel, 1974). Thus, they were able to demonstrate that the habituation, or decrease in behavioral response, was due to underlying changes in synaptic strength.

Pharmacological manipulation of glutamate receptors and the requirement for protein synthesis and directed mobilization further substantiate the role of long-term synaptic plasticity in memory. As mentioned above, AMPARs and NMDARs have been shown to play an integral role in synaptic plasticity via LTP and LTD induction and expression (Fitzjohn et al., 1999; Fetterolf and Foster, 2011; Lisman, 1994; Isaac et al., 1995). Following logically, any disruption to normal glutamate receptor function should interfere with proper memory formation, and that is indeed what researchers have found. Dalton and her colleagues found that blocking AMPAR endocytosis with the TatGluR2$_{3Y}$ peptide impairs the formation and retrieval of extinction learning in a fear conditioning paradigm, results that were mimicked with NR2BR inhibitor Ro25-6981 (Dalton et al., 2008). Even interfering with the balance between protein synthesis and degradation, without inhibiting protein synthesis altogether, can have negative consequences for memory. For instance, shifting the balance in protein metabolism during the labilization stage of memory reconsolidation via the inhibition of CaMKII was shown to have a detrimental effect on the maintenance of reconsolidated memories (Da Silva et al., 2013).

1.4.3 Memory and the hippocampus

The hippocampus is a structure of the limbic system that is central to memory processing, largely spatial memory. Early experiments by Olton and Papas demonstrated a role for the hippocampus in spatial memory by showing that fimbria-fornix lesions decreased spatial working memory (SWM) performance in a 17-arm radial maze, but had no effect on spatial reference memory (Olton and Papas, 1979).

However, for memory to be processed and encoded in the hippocampus we now know that neuronal synapses must exhibit plasticity (Collingridge and Bliss, 1993). Some of the first evidence for the hippocampus as a cellular-based spatial map was done by O'Keefe and Dostrovsky using single unit recordings from freely moving rats. These researchers were able to provide evidence suggesting that specific cells within the hippocampus could register information about the space surrounding the animal, neurons now known as place cells (O'Keefe and Dostrovsky, 1971). CA3-CA1 synapses within the hippocampus in particular, connecting glutamatergic axons in the CA3 region with dendrites of CA1 pyramidal neurons, are integral to memory formation (Hunsaker MR et al., 2008). Altering these neuronal networks will cause disruptions to plasticity, establishing the memory impairment that is characteristic of cannabis use (De Oliveira Alvares et al., 2008). An effective way to accomplish these disruptions in plasticity is through the pharmacological manipulation of glutamate receptor function and trafficking. Dong and his colleagues manipulated LTD function with intra-hippocampal injections of NR2BR antagonist Ro25-6981 and AMPAR endocytosis inhibiting peptide TatGluR2$_{3Y}$ (Dong et al., 2013). They found that both drugs prevented spatial reversal learning in the Morris water maze task, a form of learning that must occur after initial spatial learning by changing the location of the water maze's hidden platform (Dong et al., 2013).

1.4.4. Memory and eCBs

One of the major biological implications of eCB accumulation is memory impairment. A great deal of work has been done with long-term memory (Clarke et al. 2008), anxiety memory (Busquets-Garcia et al., 2011), and fear memory (Takahashi, 2011; Ruehle et al., 2012). Clarke and his colleagues revealed that intra-hippocampal post-training injections of VDM 11, an inhibitor of AEA reuptake, impairs long-term object recognition memory given that rats explored both novel and familiar objects equally 24 hr following the training session (Clarke et al., 2008). Busquets-Garcia and his colleagues found that acute injections of either the FAAH inhibitor URB597 (which increases levels of AEA) or MAGL inhibitor JZL184 (which increases levels of 2-AG) both produced anxiolytic effects in the elevated plus maze. The effect was more pronounced with increased levels of 2-AG, and interestingly, the 2-AG-dependent anxiolytic effect was mediated by CB_2R as the CB_2R antagonist SR144528 abolished the effect but CB_1R antagonist rimonabant did not (Busquets-Garcia et al., 2011). Lin and his colleagues showed that intra-hippocampal injection of AEA reuptake inhibitor AM404 decreased fear memory in a contextual fear conditioning paradigm, an effect that was prevented with co-injections of CB_1R antagonist AM281 and AM404 and enhanced with injections of AM281 alone (Lin et al., 2011). Lin also shows that AM404, and consequently AEA, inhibited fear memory through an LTP-independent mechanism, possibly indicating a role for LTD in fear memory formation (Lin et al., 2013). It was also found that intra-hippocampal infusions of AEA prior to training or testing in a step-down inhibitory avoidance task had no effect on acquisition or retrieval respectively as compared to vehicle infusions, however infusions of AEA increased the consolidation of the fear response (De Oliveira Alvares et al., 2008).

Studies of how eCBs affect SWM are beginning as well, with very few publications to date dealing with spatial memory performance and eCBs. A Morris water maze study done by Robinson and her colleagues showed that systemic injection of CB_1R antagonist SR141716A 30 min after acquisition training each day impaired SWM, whereas intra-hippocampal injections of SR141716A had a facilitory effect on SWM (Robinson et al., 2008). The researchers account for this discrepancy due to a net effect of SR141716A on CB_1R throughout multiple brain regions versus a more confined effect within the hippocampus – the intrahippocampal injections being consistent with other studies that show CB_1R knockouts exhibit improved memory in object-recognition tasks (Reibaud et al., 1999). Logically, if the ECS has a detrimental effect on memory the removal of the functional neuronal receptor, through the use CB_1R antagonists and knockouts, would prevent the amnesic effect and improve. Water maze performance of FAAH knockout mice was also studied and it was found that compared to wild-type controls, FAAH knockouts showed increased acquisition and decreased extinction times (Varvel et al., 2007). The decreased extinction times are as expected given that it has been shown that CB injections caused increased extinction rates (Ashton et al., 2008). In his study, Varvel and his colleagues indicate there was an anxiogenic effect in the FAAH knockouts seen as faster swim speeds and shorter path lengths (Varvel et al., 2007). The researchers also acknowledge that they moved the platform to the side of the tank opposite the researcher's entrance, and therefore the increased acquisition seen in FAAH mice could be a result of an anxiety-driven desire to evade the researchers. Thus, due to the discrepancies and small amount of behavioural research done with eCBs in a spatial memory paradigm it was useful to explore this memory for the current study.

1.5 Aims

Given the novel findings of our lab using CBs (see Figure 1-5), we aimed to characterize the ECS in the hippocampus. I began by using a course of action similar to that previously used by members of my lab, with a goal of determining how closely the exogenous system is able to mimic the effects seen with the ECS *in vivo*.

The first objective of this study was to determine if eCB-mediated LTD can be seen at CA3-CA1 hippocampal synapses in CB_1R-dependent manner. In order to achieve this I performed field excitatory postsynaptic potential (fEPSP) recordings from CA3-CA1 synapses and prevented the degradation of eCBs with acute injections of inhibitors (de Lago et al., 2005; Long et al., 2009a; Long et al., 2009b) to determine if increased levels of eCBs would affect synaptic transmission. CB_1R antagonist pre- and post-treatments were performed to determine if CB_1Rs were necessary for eCB-mediated depression of synaptic transmission. To determine if the process was long term I inhibited protein synthesis, given that work done by Abraham and his colleagues, as well as my colleagues, has shown the protein dependence of long term plasticity (Abraham and Williams, 2008; Han et al., 2012).

The second aim of this study was to determine the role of glutamatergic ionotropic receptors in eCB-LTD. Hippocampal synaptic plasticity is largely regulated by NMDARs (Mulkey and Malenka, 1992). Furthermore, in an earlier study by my colleagues it was shown that glutamate interacted with NR2BRs to induce the endocytosis of AMPARs, providing a mechanism for eCB-LTD in the hippocampus (Han et al., 2012). I performed field potential recordings from CA3-CA1 synapses during which I inhibited NR2BRs and prevented AMPAR endocytosis in the presence of increased levels of eCBs to determine if eCB-LTD was mediated in a similar manner.

Finally, I hypothesized that eCB-LTD would have functional implications in the processing of SWM given that such memory is reliant on proper hippocampal synaptic plasticity and neurotransmission (Jeneson and Squire, 2011; Bliss and Collingridge, 1993). To explore this hypothesis I performed delayed non-match-to-sample (DNMTS) T-maze experiments in the presence of increased levels of eCBs to determine if there was a SWM deficit under such conditions.

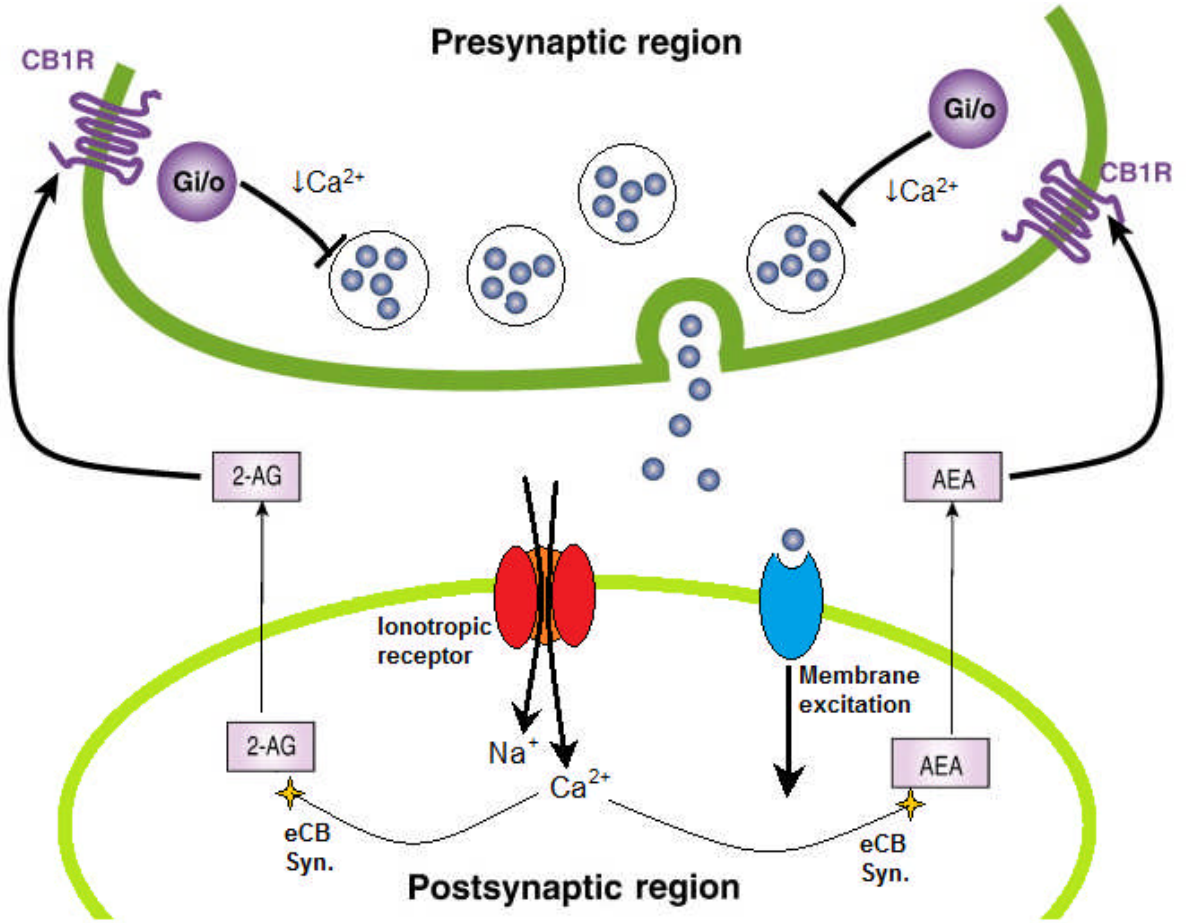

Figure 1-1. Overview of eCB signaling mechanism.
Upon the release of neurotransmitter from the presynaptic terminal, the neurotransmitter will travel across the synaptic cleft and interact with their specific receptor on the postsynaptic membrane. When there are sufficient amounts of neurotransmitter to cause the depolarization of the postsynaptic membrane a large influx of cations will occur, of particular interest to the ECS is Ca^{2+}. Ca^{2+} entry will activate various kinase pathways that lead to the synthesis and release of eCBs. eCBs then travel retrogradely to the presynaptic membrane where they interact with $G_{i/o}$-coupled CB_1Rs to inhibit voltage-gated Ca^{2+} channels, inward rectifying K^+ channels, and AC leading to decreased levels of cAMP. These actions ultimately lead to the hyperpolarization of the presynaptic neuron, preventing the release of neurotransmitter – effectively decreasing synaptic transmission.

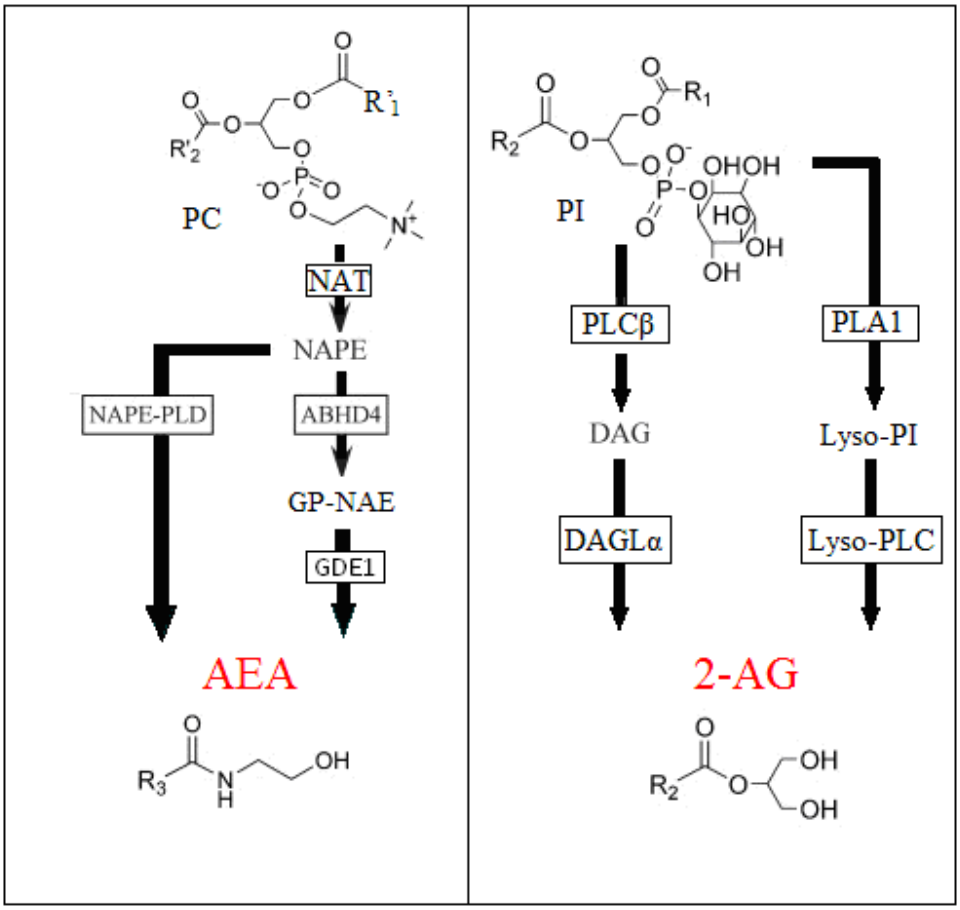

Figure 1-2. The main synthetic pathways for the two major eCBs.
AEA's two main synthetic pathways include the PLD pathway and the ABHD4/GDE1 pathway. In both pathways phosphotidylcholine (PC) is broken down into N-acylphosphatidylethanolamine (NAPE). From there NAPE can either be broken down further by NAPE-dependent phospholipase D (NAPE-PLD) to directly make AEA, or NAPE can be worked on in a step-wise process by α,β-Hydrolase-4 (ABHD4) and then glycerophosphodiesterase-1 (GDE-1) to generate AEA. 2-AG also has two main synthethic pathways. Phosphotidylinositol (PI) can either be hydrolyzed by phospholipase β (PLCβ) or phospholipase A1 (PLA1). If broken down by PLCβ, then diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) are generated, after which DAG is broken down further by diacylglycerol lipase α (DAGLα) into 2-AG. Another more speculative pathway suggests that PI is broken down by PLA1 to generate lyso-PI which is broken down again by Lyso-PLC to generate 2-AG.

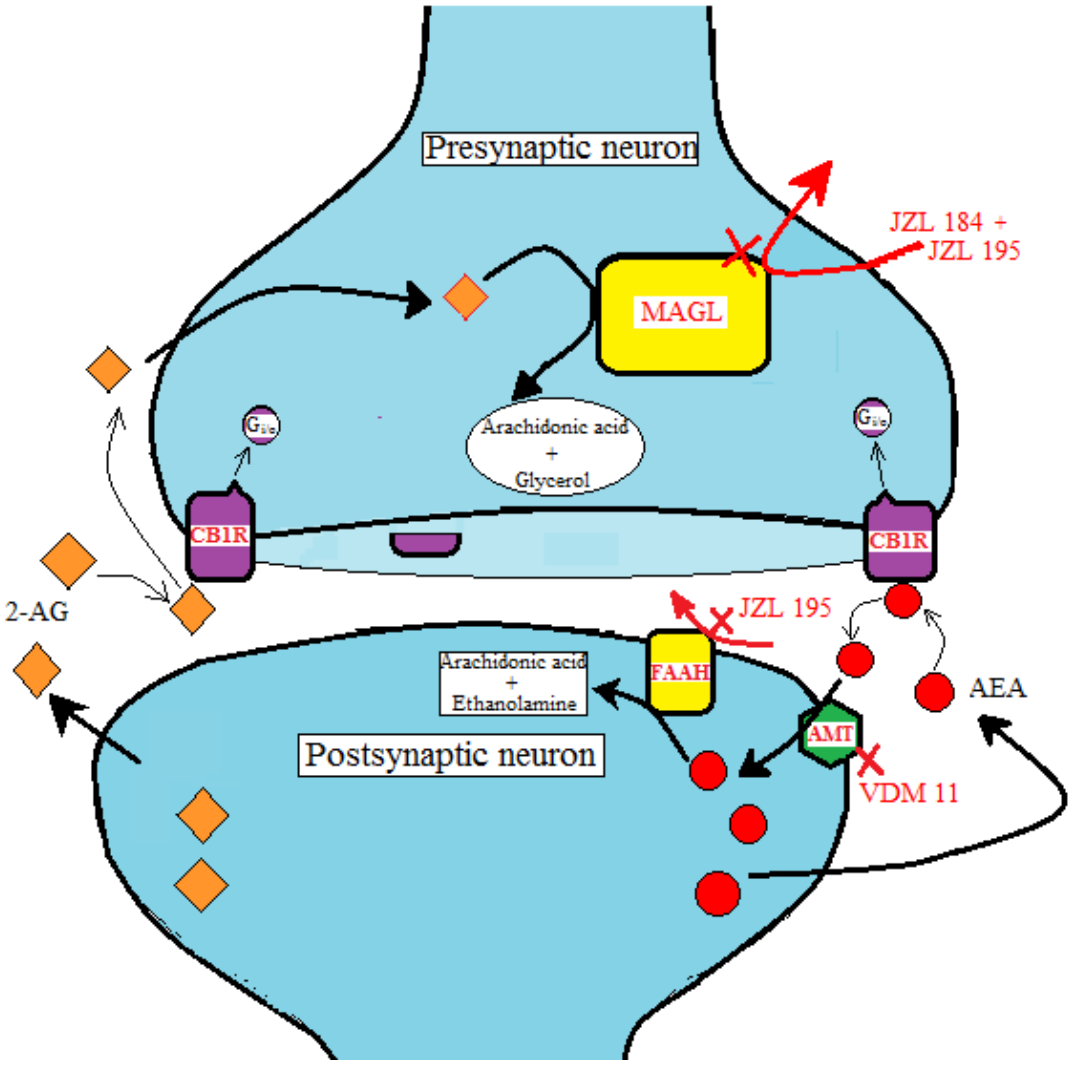

Figure 1-3. Degradative pathways of the two major eCBs.
Once eCBs are synthesized and released in an activity-dependent manner they travel retrogradely where they interact with CB_1Rs to elicit their characteristic effects. After dissociation from CB_1R, they are inactivated by various enzymes. The most well characterized pathways are as follows: AEA moves from the synaptic cleft to the postsynaptic membrane where the anandamide membrane transporter (AMT) aids AEA's reentry into the postsynaptic neuron where it is broken down into arachnidonic acid and ethanolamine by membrane-bound fatty acid amide hydrolase (FAAH), and 2-AG diffuses across the presynaptic membrane where it is broken down by monoacylglycerol lipase (MAGL) into arachnidonic acid and glycerol. The different locations of the degradative enzymes have lead researchers to hypothesize a larger role for 2-AG in eCB-mediated changes in synaptic plasticity given the enzyme's proximity to the site of action. JZL 195 is a drug that inhibits both MAGL and FAAH, leading to increased levels of both 2-AG and AEA. JZL 184 is a drug that specifically inhibits MAGL and increases levels of 2-AG only. VDM 11 inhibits AMT, decreasing AEA's ability to cross the postsynaptic membrane and therefore preventing its degradation, leading to increased levels of AEA only (pharmacological sites of action are represented by red crosses).

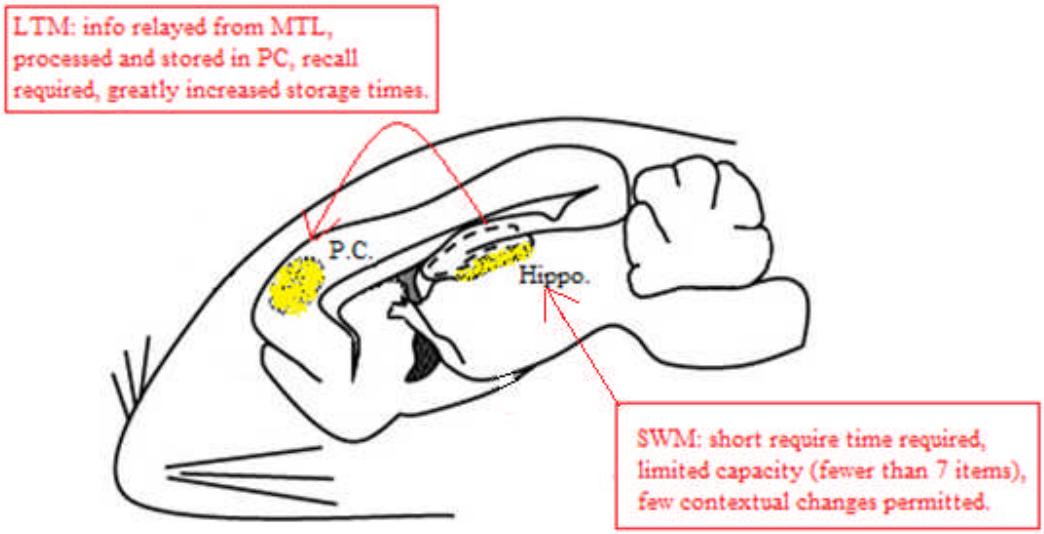

Figure 1-4. Illustration of the locations of two forms of memory.
Spatial working memory (SWM), largely processed in the hippocampus (hippo.) and prefrontal cortex (PC), is only able to retain a few simple cues for a short time frame or for the duration that the animal actively thinks about the cues in question. In a review by Jeneson and Squire they explain that it is generally accepted that approximately 7 cues are considered the limit for shorter term working memory, and that researchers rarely use protocols with retention times longer than 2 min to study working memory. The hippocampus is ideally located at a crossroads between multiple cortical regions, and as such can alter and allow for the selective storage of memoranda for much longer periods of time. It is currently held that once memory passes through the medial temporal region and begins to be processed in the cortical regions, specifically the PC, it can then be stored as long term memory (LTM), implicating the PC in LTM as well as SWM (Jeneson and Squire, 2011).

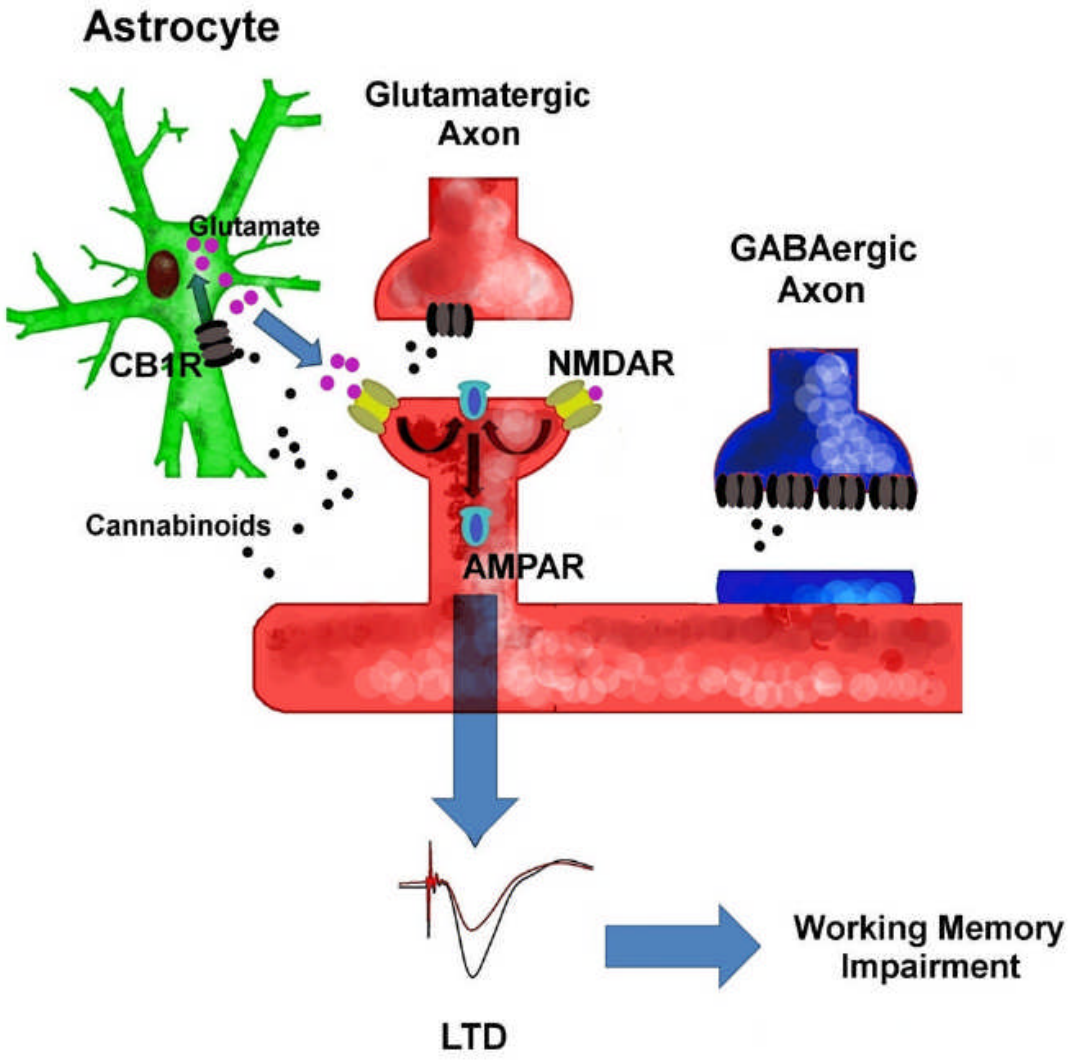

Figure 1-5. Mechanism of CB-LTD at hippocampal CA3-CA1 synapses *in vivo*.
The mechanism for CB-LTD at hippocampal CA3-CA1 synapses *in vivo* developed by work done my colleagues is as follows: exogenously introduced CBs interact with astrocytic CB_1Rs to cause an increase in glutamate, which then interacts with extrasynaptic NR2BRs to induce the expression of LTD via the internalization of GluR2-containing AMPARs (Han et al., 2012). This mechanism of CB-LTD was then found to cause functional deficits in spatial working memory.

Chapter 2: Materials and Methods

2.1 Animal Protocol

Adult male C57BL mice weighing 25-30 g and adult male Sprague-Dawley (SD) rats weighing 250-350 g (Charles River, Saint-Constant, QC, Canada) were used. Animals were housed 4 (mice) or 2 (rats) per cage at standard experimental conditions (12 h light/dark cycle, lights on at 7:00 AM, temperature of 21 ± 1°C, 40-50% relative humidity) with access to food and water *ad libitum*, unless the rodents were used for T-maze experiments (see Section 2.4.1). All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care (CCAC) as approved by the Animal Care Committee (ACC) of the University Of Ottawa Institute Of Mental Health Research.

2.2 Drugs and Vehicles

JZL 195 i.p. (Sigma-Aldrich, St. Louis, USA) at 20 mg/kg in mice (Long et al., 2009a; Wise et al., 2012) and rats (Wiskerke et al., 2012; Long et al., 2009a) was dissolved in DMSO: Tween 80: 0.9% saline (1:1:8). JZL 184 i.p. (Cayman Chemical, Burlington, ON) at 20mg/kg in mice (Ghosh et al., 2013; Busquets-Garcia et al., 2011; Long et al., 2009b) was dissolved in DMSO: Tween 80: 0.9% saline (1:1:8). VDM 11 i.p. (Cayman Chemical, Burlington, ON) at 5 mg/kg in mice (de Lago et al., 2005; Storr et al., 2008; van der Stelt et al., 2006) and rats (Gamaleddin et al., 2011) was dissolved in ethanol: Tween 80: 0.9% saline (1:1:10). AM 281 i.p. (Tocris Biosciences, Ellisville, USA) at 3 mg/kg in rats (Cui et al., 2001; Han et al., 2012) was dissolved in DMSO: Tween 80: 0.9% saline (2:1:37). Anisomycin i.p. (Sigma-Aldrich, St. Louis, USA) at 75 mg/kg in mice (Fan et al., 2010; Wanisch and Wotjak, 2008) was dissolved in 0.9%

saline. Ro25-6981 i.p. (Sigma-Aldrich, St. Louis, USA) at 6 mg/kg in mice (Jiang et al., 2010; Iijima et al., 2010; Tallaksen-Greene et al., 2010) was dissolved in 0.9% saline. Both TatGluR2$_{3Y}$ and TatGluR2S i.p. (GL Biochem, Shanghai, China) at 10 μmol/kg in mice (Yang et al., 2011) were dissolved in 0.9% saline. All doses were chosen based on dosages previously used by my colleagues (Han et al., 2012) as well as previous literature (as indicated above) so that the dosages fit with both electrophysiological and behavioural precedents.

2.3 Electrophysiology

2.3.1 *In vivo* setup

In vivo fEPSP recordings in both anaesthetized mice and rats were recorded according to the following protocol. Adult male C57BL mice (25-30 g) and adult male Sprague-Dawley rats (250-350 g) were anaesthetized using 40% urethane (3 ml/kg, i.p.). The rodent's temperature was regulated throughout the course of the experiment with a temperature therapy mat and T/PUMP at 37°C (Gaymar, Orchard Park, NY). The rodent's skull was then fixed to a stereotaxic apparatus (Model 1730, David Kopf Instruments), a hole was drilled into the skull according to the coordinates, and two electrodes were inserted into the hippocampus. The recording electrode (glass micropipette with tip diameter ≈ 2 μm, Stoelting, Illinois, USA) was inserted in the CA1 area, and the stimulating electrode (concentric bipolar electrode, SNEX-200X 70 mm, Rhodes Medical Instruments Inc., Summerland, CA) in the tracts between the CA3 and CA1 regions. The following coordinates were used for mice: CA1 area, B/P -2.30 mm, M/L -1.80 mm, D/V 1.25 mm, and CA3 area, B/P +1.94 mm, M/L +2.30 mm, D/V -1.73 mm; the coordinates used for rats were as follows: CA1 area, B/P -4.20 mm, M/L -2.80 mm, D/V 2.46 mm, and CA3 area, B/P +3.50 mm, M/L +2.90 mm, D/V -2.40 mm (B/P is posterior to bregma, M/L is mediolateral,

D/V is dorsoventral, and the atlases used were: mouse – Franklin and Paxinos, 1997; rat – Paxinos and Watson, 1998) (Figure 2-1). The stimulating electrode was positioned manually to obtain the best fEPSP wave (negative extracellular wave, approx. 6-18 ms from stimulus to trough, approx. 12 ms wave duration, baseline amplitude of 0.8-1.2 V, gain = 1000x), whereas the recording electrode containing 2 M NaCl (resistance 2-4 MΩ) was first manually lowered to the brain surface and then lowered and adjusted with a hydraulic Micropositioner (Model 2650, David Kopf Instruments) to ensure accuracy.

The recordings were performed with a Model MDA-3 AC Differential Amplifier (BAK Electronics, Inc.). Single pulses of 0.067 Hz were applied to evoke fEPSPs, and stimulus pulse intensities were typically 100-300 μA with a duration of 500 μs. A stimulus intensity yielding approx. 60% of the maximal response was selected for baseline measurements using a Stimulator (Model S88, Grass Telefactor) and a Stimulus Isolator (Model A385, World Precision Instruments). Spike2 software was used to record the data. To conclude the experiment, a high frequency stimulation protocol (HFS, 100 Hz train for 1 s x 3 times spaced 20 s apart) was performed (Figure 2-2); if HFS produced LTP then the neurons were assumed to be in good condition and to have not endured damage or fatigue that would have appeared as LTD. If no LTP occurred then the results would have to be rejected as no conclusion could be made as to the consequence of pharmacological treatment alone.

2.3.2 Intraperitoneal Treatments

After the ideal electrode placement was obtained, baseline fEPSPs were then recorded for at least 30 min for all experiments. For the first series of experiments JZL 184 (20mg/kg, i.p.), VDM 11 (5 mg/kg, i.p.), JZL 195 (20 mg/kg, i.p.), or vehicle were acutely administered in

C57BL mice after baseline was established, and then fEPSPs were recorded for an additional 120 min, followed by the HFS control (Figure 3-1).

CB_1R antagonist AM 281 (3 mg/kg, i.p.) or its vehicle was injected 10 min before or 10 min after JZL 195 (20 mg/kg, i.p.) treatment in SD rats. Baseline was attained prior to the injection of any drug. The fEPSPs were then recorded for 120 min, followed by the HFS control (Figure 3-3).

Anisomycin (75 mg/kg, i.p.), a protein translation inhibitor, or vehicle was administered in C57BL mice. Baseline was established, and 2 h after Anisomycin pretreatment JZL 195 (20 mg/kg, i.p.) treatment was given. The fEPSPs were then recorded for another 120 min, followed by the HFS control (Figure 3-3).

Ro25-6981 (6 mg/kg, i.p.), NR2B subunit-specific NMDAR antagonist, or vehicle, were given 10 min prior to JZL 195 (20 mg/kg, i.p.) treatment in C57BL mice. The fEPSPs were then recorded for an additional 120 min after which the HFS control was given (Figure 3-4).

$TatGluR2_{3Y}$, inhibitor of AMPAR endocytosis, and its control peptide TatGluR2S (10 μmol/kg) were injected in C57BL mice. Baseline conditions were established, and 1.5 h after $TatGluR2_{3Y}$/TatGluR2S pretreatment JZL 195 (20 mg/kg, i.p.) treatment was given. The fEPSPs were recorded for an additional 120 min, and the HFS control was then given (Figure 3-5).

2.4 Delayed Non-Match-To-Sample T-Maze

2.4.1 Overview

I used an established DNMTS T-maze protocol that has been employed by other researchers (Kelsey and Vargas, 1993) as well as our lab (Han et al., 2012). One week after arriving, SD rats were handled once a day for 2 days at which time their chow was removed and

the reward cereal was introduced (crushed Whole O's from Nature's Path Foods, Inc.; 1-2 g). During the training and testing the rats were given 5g/100g body weight of chow inside their cages to maintain their body weight above 85% of their original weight. A wooden T-shaped box was used (75 cm-long start alley attached to two 30 cm-long goal arms, the width was 15 cm and the height was 30 cm) with inside walls and doors painted black. The experiment consisted of 2 days of pretest training, 6 days of acquisition training, and 5 days of performance testing (Figure 2-3).

2.4.2 Training Procedure

Pretest training was performed in order to acquaint the rats with the T-maze environment before the start of the acquisition training. On day 1 of the pretest training, cereal was placed in small glass dishes in both arms, the guillotine doors were left open, and the rats were allowed to explore until the cereal was eaten out of both arms. On the next pretest training day, the rats were allowed to leave the start box and enter a goal box where the door was closed and they remained until they finished the cereal; 5 trials were performed for each rat to allow them to be habituated to the guillotine doors. Between each trial the rat was replaced in their home cage.

Acquisition training began the following day and rats were brought in pairs to the maze room where they were trained for 10 trials per day, each composed of a sample run and a choice run (Figure 2-4). During the initial sample run access to one goal box was blocked. The rat was placed in the start box for 5 s; the start box door was then opened to allow the rat to enter the goal box where the door was closed while they finished the cereal. The rat was then removed from the goal box, placed back in the start box for 5 s; where upon the 3 doors were raised. The rat received cereal if it entered the goal box opposite to the goal box from the sample run, at

which point it was closed in the arm until the cereal was finished. If the rat did not enter the correct box it was kept in the incorrect goal box for 25 s. The rat was then returned to the home cage for a 3 min inter-trial interval while the other rat performed a trial. The sample run goal box sides were picked using a random sequence generator with the requirement that no more than 3 trials in a row could start with the same arm. The maze was wiped down with 30% ethanol between each rat.

2.4.3 Testing Procedure

The testing began the day following the last acquisition day. Rats were tested 10 trials a day for 5 days as per the same protocol used for the acquisition training with the exception of a 30 s retention time between runs instead of 5 s. For the single dose experiments, an injection of JZL 195 (20 mg/kg, i.p.) or vehicle was given 10 min prior to the first sample run on the first day, and no additional injections were administered. For the multi-dose experiments, JZL 195 (20 mg/kg, i.p.), VDM 11 (5 mg/kg, i.p.), or their vehicles were injected 10 min prior to the first sample run on each of the 5 testing days (Figure 2-3).

2.5 Statistical Analysis

SPSS 9.0 for Windows was used for all statistical analyses. Electrophysiology results were reported as mean ± SEM. Statistical analysis of the data was performed using a one-way ANOVA, followed by Bonferronni post-hoc test. Statistical significance was set at $p < 0.05$ (Figures 3-2B, 3-3B, 3-4B, 3-5B, 3-6B).

DNMTS T-maze results were reported as daily mean ± SEM percent correct choice. Two-way repeated-measures ANOVA was used to determine changes over time as well as

comparisons between groups (Figure 3-8, 3-9, 3-10). All were followed by Bonferronni post-hoc test. Statistical significance was set at $p < 0.05$.

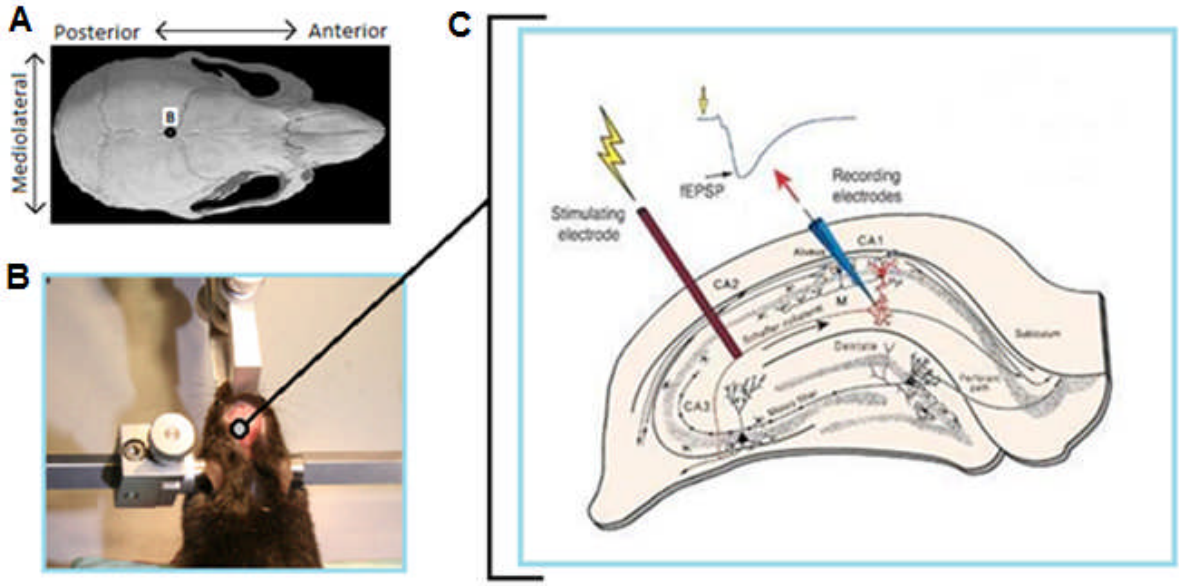

Figure 2-1. Stereotaxic setup and electrode placement.
(A) A depiction of the axes used to determine coordinates on the mouse skull. B denotes the bregma, and coordinates are written in relation to this point (eg. M/L -1.80 mm can be written as: 1.80 mm posterior to the bregma along the mediolateral axis). **(B)** Shows a mouse's head placement within the stereotaxic frame. The grey circle depicts, on average, the location and size of the hole that is drilled in the skull to allow for the insertion of the 2 electrodes. **(C)** Shows a brain slice with the locations of the stimulating and recording electrodes within the brain. The recording electrode (blue) is situated in the CA1 region of the hippocampus where it records current sinks as waveform data or fEPSPs, the stimulating electrode in situated in the tracts between the CA3 and CA1 regions of the hippocampus.

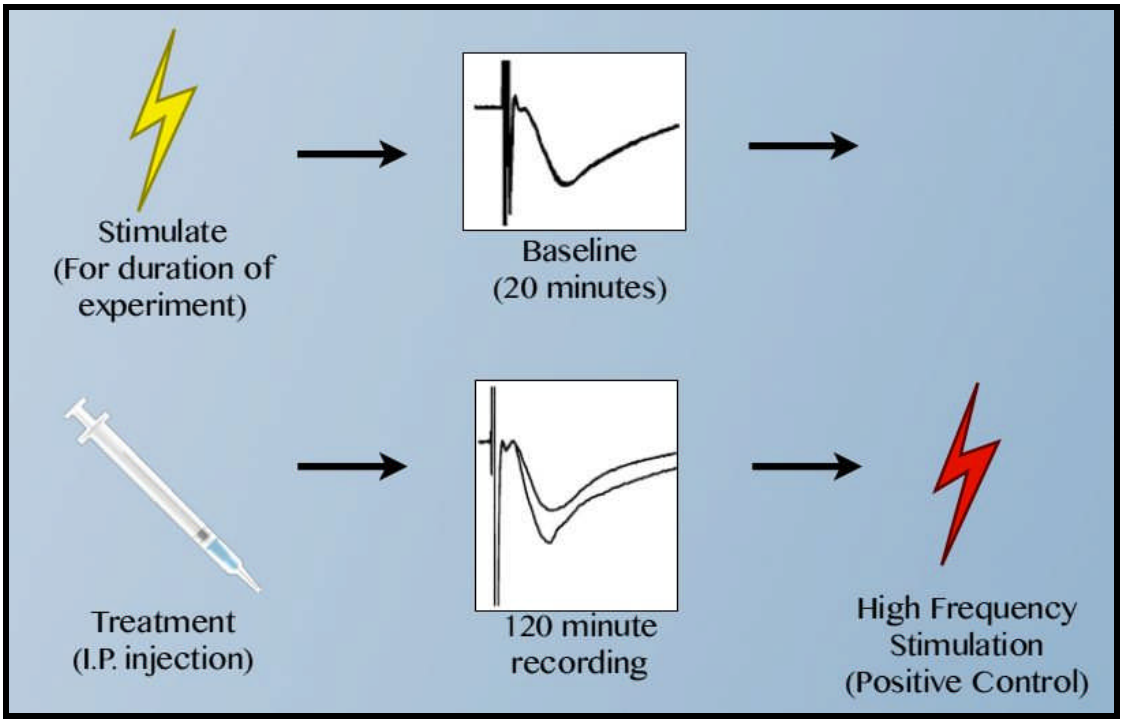

Figure 2-2. Schematic of electrophysiological experiment timeline.
Stimulation is given to begin the experiment and continues throughout the experiment at 15 sec intervals. The evoked fEPSPs are then recorded until fEPSPs of a stable amplitude (varying no more than 5% from the mean) are recorded for at least 20 min. An acute systemic injection of one of various drugs is then administered after attaining baseline. The recording then continues following the injection for another 120 min. After the 120 min a HFS control is given to ensure that the neurons are still able to undergo potentiation. If the fEPSPs show no potentiated response then the experiment is rejected, as any depression of synaptic transmission that was seen could have been due to neuronal death or fatigue rather than the treatment.

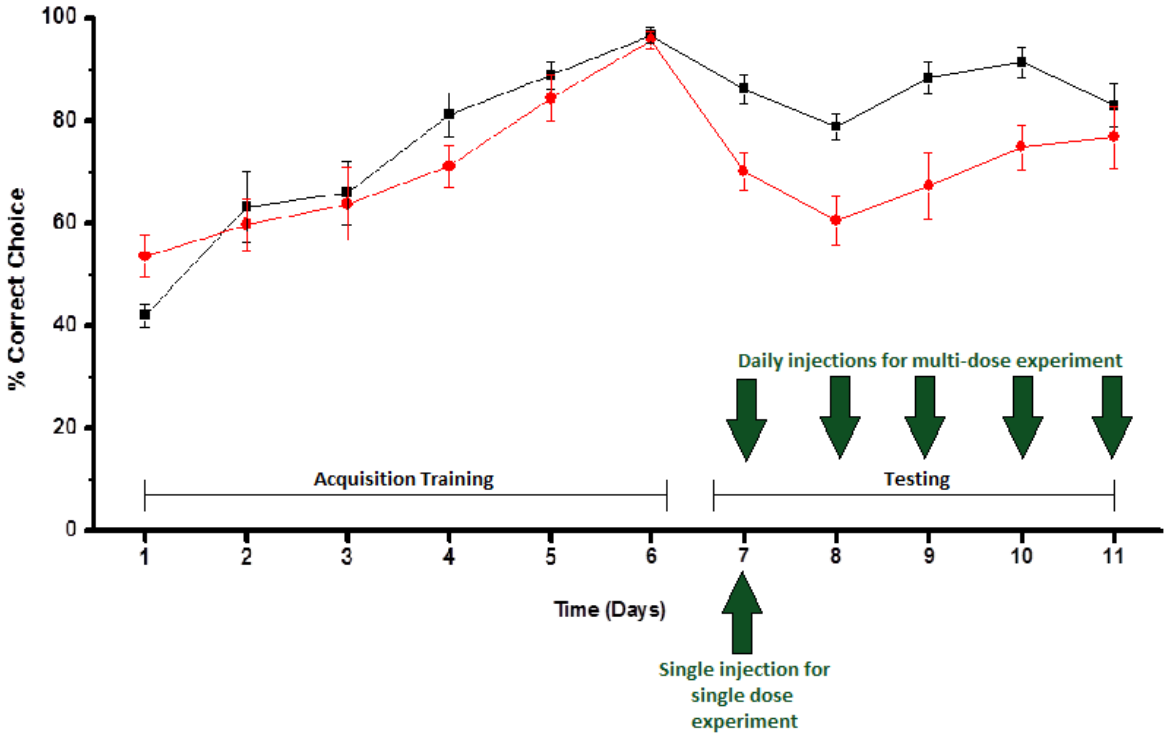

Figure 2-3. Schematic of the Delayed Non-Match-To-Sample T-maze experiment timeline. The first 6 days of the T-maze experiment consist of acquisition training, throughout which the rats are expected to learn the task to between 90% and 100% accuracy (only 4 rats throughout all the experiments had to be excluded due to poor performance). After the 6th day of training the testing phase begins. Testing lasts for 5 days. If a multi-dose experiment is undertaken then an injection of the drug is given 10 min before the first trial each of the 5 days. If a single dose experiment is being run, then only one injection is given on the first day of testing 10 min prior to the first trial.

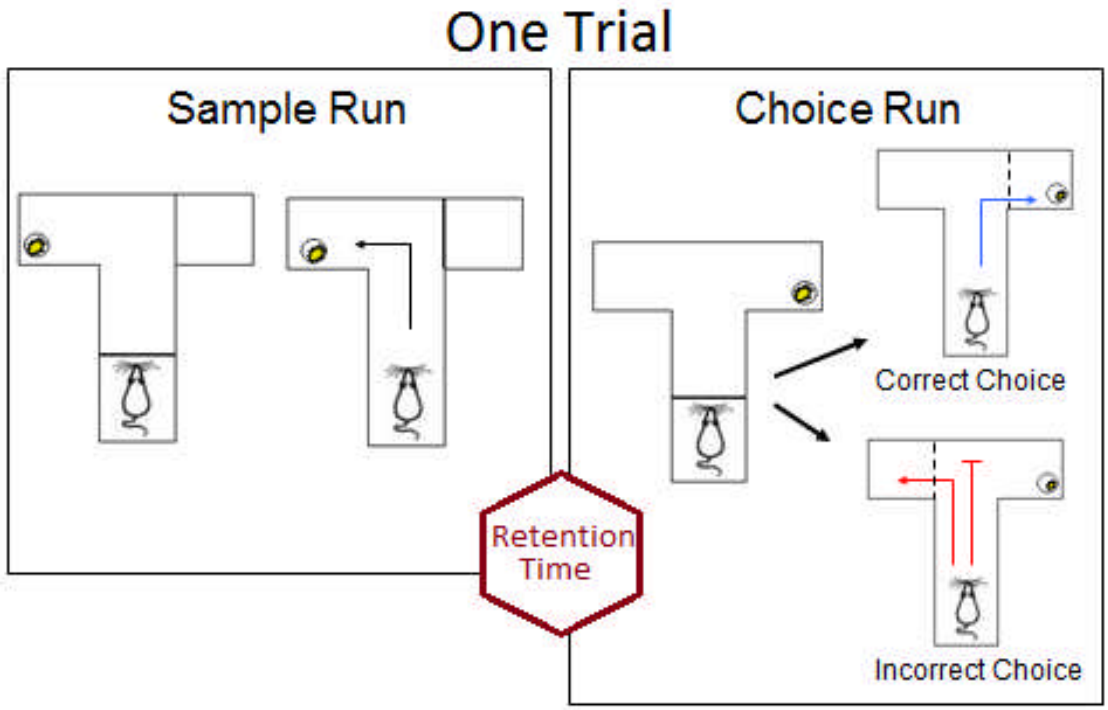

Figure 2-4. Each trial consists of 2 runs: the sample run and the choice run.
Ten trials are run per rat each day of acquisition training and testing. Each trial consists of 2 runs: the sample run, and the choice run. For the sample run, food is placed in one of the arms of the T-maze and the other arm is closed off by a guillotine door. The rat starts in the start box (the base of the T), enters the open arm, and is closed in while finishing the reward food. After finishing, the rat is removed and made to wait the required retention time before the second choice run (for acquisition training the retention time is 5 sec, for testing it is 30 sec). The rat then starts its choice run in the start box. Once allowed to traverse the T-maze, it should choose the arm opposite to the one that contained the food in the sample run. If the rat chooses correctly it will be sealed in the arm, allowed to finish the reward food, and immediately removed from the T-maze. If the rat chooses incorrectly or pauses for an extended period at the junction between the two arms it will be sealed in the incorrect arm without food for 25 sec and then removed.

Chapter 3: Results

3.1 Inhibitors of endocannabinoid degradation induce in vivo LTD at CA3-CA1 synapses

In vivo recordings of fEPSP from CA3-CA1 synapses in anesthetized mice showed that an i.p. injection of JZL 195 (20 mg/kg), a potent inhibitor of both MAGL and FAAH that globally increases the brain concentration of eCB, decreased fEPSP amplitude to approx. 40% of baseline ($F_{3,11} = 40.061$, $p < 0.01$) (Figure 3-1A). An i.p. injection of JZL 184 (20 mg/kg), an inhibitor of MAGL that specifically increases 2-AG concentrations, decreased fEPSP amplitude to approx. 70% of baseline levels ($F_{3,11} = 45.260$, $p < 0.01$) (Figure 3-1B). The inhibition of AEA re-uptake via i.p. injections of VDM 11 decreased fEPSP amplitude to 70% of baseline ($F_{3,11} = 13.084$, $p < 0.01$) (Figure 3-1C). It appears that the two eCBs have a culminative effect in decreasing hippocampal synaptic transmission, given that both cause half of the synaptic depression seen with the global inhibition of eCB degradation (Figure 3-2).

3.2 CB_1R antagonist pretreatment blocks eCB-LTD

In vivo recordings of fEPSP from CA3-CA1 synapses in anesthetized rats showed that CB_1R antagonist AM 281 (3 mg/kg; i.p.) injected 10 min prior to JZL 195 (20 mg/kg) completely blocked the depression of synaptic transmission seen when JZL 195 was injected alone ($F_{3,15} = 3.647$, $p = 0.054$). AM 281 injected 10 min after JZL 195 was not able to reverse the depression and synaptic transmission decreased to approx. 70% of baseline levels ($F_{3,15} = 30.466$, $p < 0.01$) (Figure 3-3).

3.3 Inhibition of de novo protein synthesis prevents the expression eCB-LTD

In vivo recordings of fEPSP from CA3-CA1 synapses in anesthetized mice showed that protein synthesis inhibitor anisomycin (75 mg/kg; i.p.) injected 2 h prior to JZL 195 (20 mg/kg) completely blocked the depression of synaptic transmission seen when JZL 195 was injected alone ($F_{1,5} = 0.0084$, $p = 0.938$) (Figure 3-4).

3.4 Inhibition of NR2B-containing NMDARs prevents the production of eCB-LTD

In vivo recordings of fEPSP from CA3-CA1 synapses in anesthetized mice showed that an i.p. injection of NR2BR antagonist Ro 25-6981 (6 mg/kg) 10 min prior to a JZL 195 i.p. injection (20 mg/kg) was able to prevent the LTD elicited with treatment of the inhibitor of eCB degradation alone ($F_{1,7} = 1.184$, $p = 0.331$). In the absence of the NMDAR subunit-specific antagonist in the vehicle, JZL 195 was still able to cause eCB-LTD to approx. 60 % of baseline levels ($F_{1,7} = 51.202$, $p < 0.01$) (Figure 3-5).

3.5 Inhibition of AMPAR endocytosis prevents the expression of eCB-LTD

In vivo recordings of fEPSP from CA3-CA1 synapses in anesthetized mice showed that treatment of mice with TatGluR2$_{3Y}$ peptide (10 µmol/kg) 1.5 h before the injection of global eCB metabolism inhibitor JZL 195 (20 mg/kg) prevented the expression of eCB-LTD in this region ($F_{3,15} = 1.229$, $p = 0.466$). However, pretreatment with TatGluR2S (10 µmol/kg), the scrambled analog, did not prevent the expression of eCB-LTD which still decreased to approx. 60 % of baseline levels ($F_{3,15} = 145.467$, $p < 0.01$) (Figure 3-6).

3.6 A single dose of eCB degradation inhibitor impairs spatial working memory

DNMTS T-maze experiments involving 6 days of acquisition training and 5 testing days were performed. All groups in each experiment adequately learned the task as percent correct choice, graphed as mean ± SEM, for days 4-6 of the acquisition period were significantly increased as compared to day 1 and reached 90%-100% correct choice (Figure 3-7). A single injection of the inhibitor of eCB degradation JZL 195 (20 mg/kg) was given on the first day of the testing period and caused a significant decrease in SWM performance as compared to the vehicle control ($F_{1,15}$ = 7.692, $p < 0.05$) (Figure 3-8). The impairment in SWM performance extended to 24 h following the injection, however by 48 h the SWM performance had returned to baseline levels.

3.7 Daily injections of an inhibitor of eCB degradation impairs spatial working memory

DNMTS T-maze experiments involving 6 days of acquisition training and 5 testing days were performed. Again, all groups in each experiment adequately learned the task as percent correct choice, graphed as mean ± SEM, for days 4-6 of the acquisition period were significantly increased as compared to day 1 and reached 90%-100% correct choice (Figure 3-7). Daily injections of the inhibitor of eCB degradation JZL 195 (20 mg/kg) throughout the testing period caused a significant decrease in SWM performance as compared to the vehicle control ($F_{1,16}$ = 6.925, $p < 0.05$) (Figure 3-9). SWM impairment persisted through most days of testing, however by day 5 there was no longer significance between drug and vehicle groups. There was no significant change to SWM performance on any day following daily injections of AEA reuptake inhibitor VDM 11 (5 mg/kg) ($F_{1,15}$ = 0.245, $p = 0.628$) (Figures 3-10).

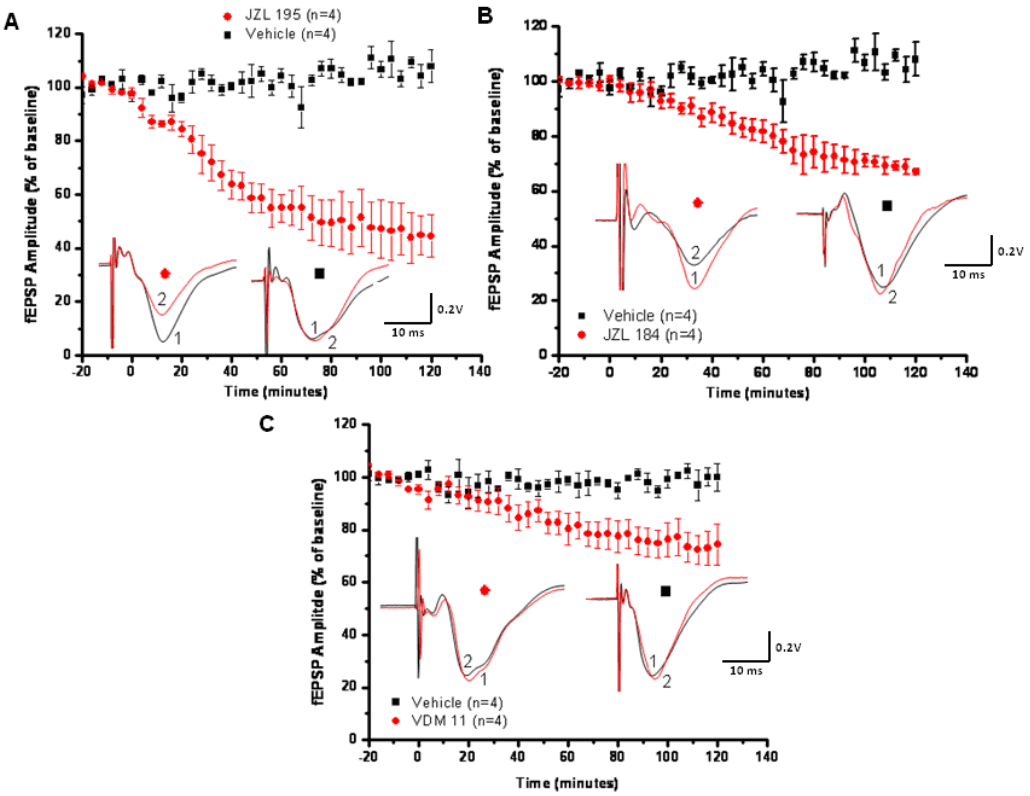

Figure 3-1. Inhibition of eCB degradation induces *in vivo* depression of synaptic transmission at CA3-CA1 synapses.

Plots of normalized fEPSP slopes in anesthetized mice are shown as means ± SEMs. Representative fEPSP traces before (1) and after (2) JZL 195, JZL 184, VDM 11 (red circles), and each drug's vehicle (black squares) injection are shown. n = 4. **(A)** Injection of an inhibitor of eCB degradation (JZL 195) caused a decrease in hippocampal synaptic transmission to 40 % of baseline levels for >2 h. ($F_{3,11}$ = 40.061, $p < 0.01$) **(B)** An injection of the MAGL inhibitor JZL 184, a drug that specifically elevates 2-AG, at 0 min depressed CA1 synaptic transmission to 70 % of baseline levels and lasted for > 2 h. ($F_{3,11}$ = 45.260, $p < 0.01$) **(C)** An injection of the AMT inhibitor VDM 11, a drug that elevates levels of AEA, at 0 min depressed synaptic transmission to 70 % of baseline levels in CA3-CA1 recordings lasting > 2 h. ($F_{3,11}$ = 13.084, $p < 0.01$).

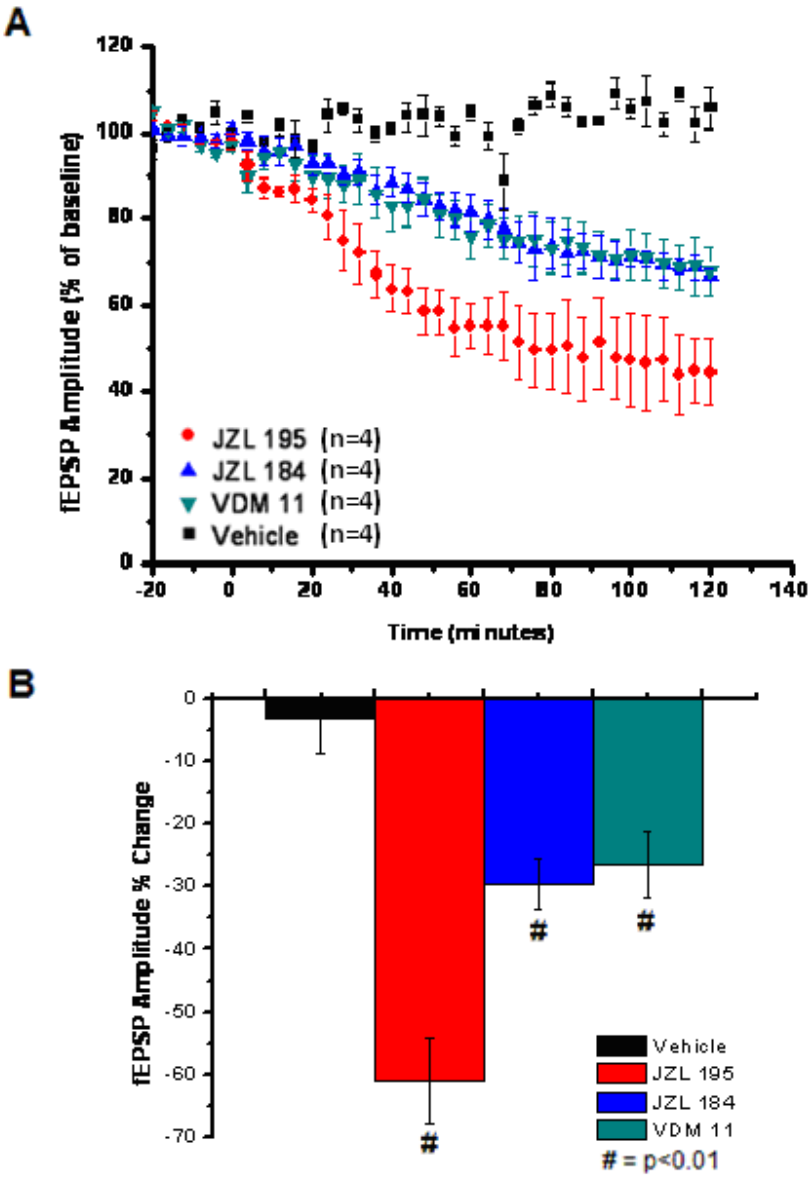

Figure 3-2. Combined plot and histogram for inhibitors of eCB degradation.
(A) Combined normalized plots from Figure 3-1 showing means ± SEMs. JZL 195 (red circles), JZL 184 (blue triangles), VDM 11 (green triangles), and vehicle (black squares) injected following baseline. n=4. **(B)** Histogram summarizing the average percent changes of fEPSP slope between vehicle (black) and drug treatment [VDM 11 (green), JZL 184 (blue), or JZL 195 (red)]. All changes due to drug treatment were significantly different from vehicle control (# = $p < 0.01$).

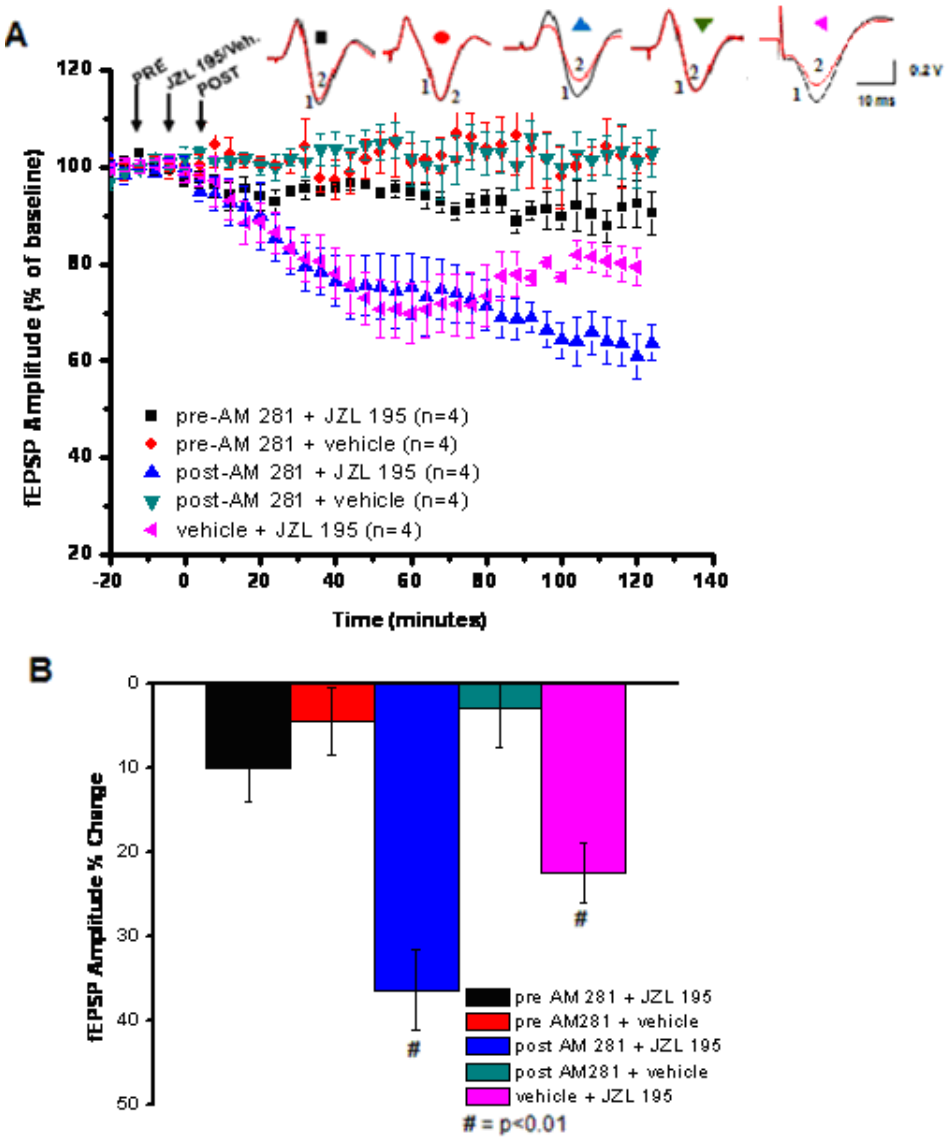

Figure 3-3. CB_1R antagonist pretreatment blocks eCB-LTD.
(A) Plots of normalized fEPSP slopes in anesthetized rats shown as means ± SEMs. Injection of AM 281 10 min before (pre) the injection of JZL 195 (black squares) at 0 min blocked the depression of synaptic transmission seen with inhibition of eCB degradation alone ($F_{3,15} = 3.647$, $p = 0.054$). This did not occur with injection of AM 281 10 min after (post) the injection of JZL 195 (blue triangles) where CA1 depressed synaptic transmission persisted to 70 % of baseline levels and lasted for > 2 h. ($F_{3,15} = 30.466$, $p < 0.01$). Representative fEPSP traces for baseline (1) and after treatments and 100-110 min into recording (2) are shown. n = 4. **(B)** Histogram summarizing the average percent changes of fEPSP slope between treatment groups. Decrease in fEPSP slope due to injection of JZL 195 followed by AM 281 (blue) and injection of vehicle prior to JZL 195 (pink) were significantly different from other treatment groups (green) (# = $p < 0.01$).

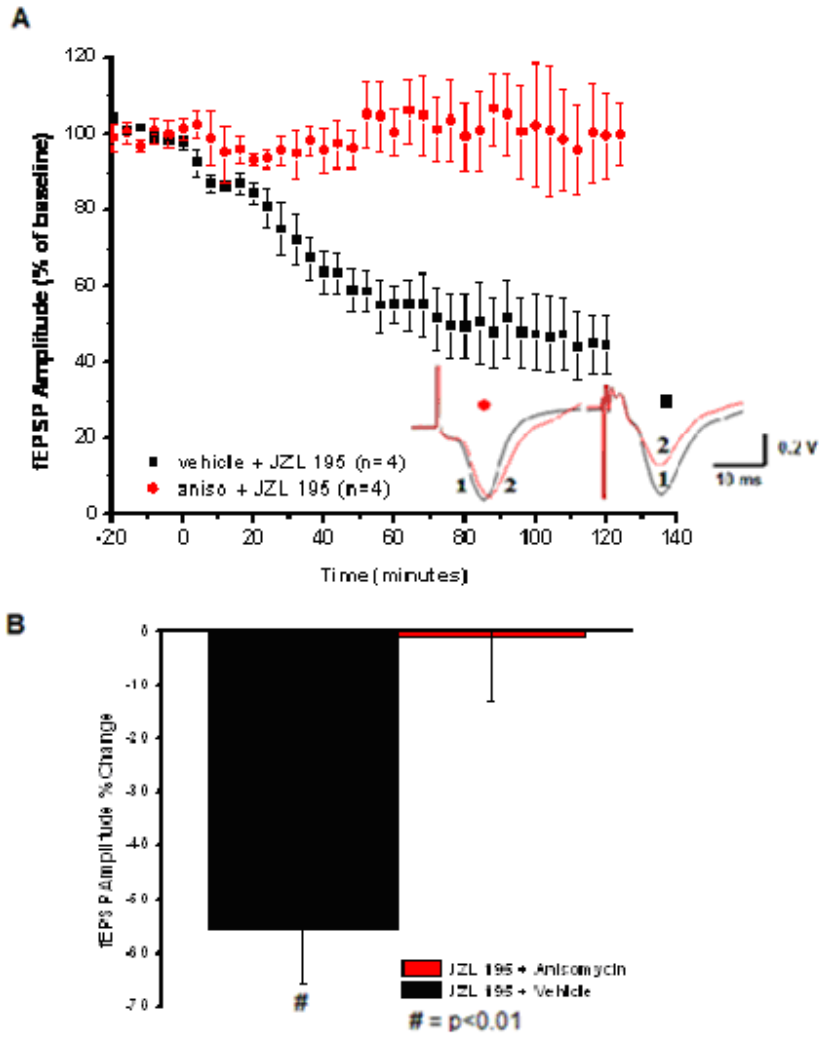

Figure 3-4. Inhibition of protein synthesis prevents eCB-mediated depression in synaptic plasticity.

(A) Plots of normalized fEPSP slopes in anesthetized mice shown as means ± SEMs. Injection of anisomycin 2 h before the injection of JZL 195 (red circles) at 0 min blocked the depression of synaptic transmission seen with inhibition of eCB degradation alone ($F_{1,5}$ = 0.0084, p = 0.938). This did not occur with injection of vehicle before the injection of JZL 195 (black squares) where CA1 depressed synaptic transmission persisted to 60 % of baseline levels and lasted for > 2 h. Representative fEPSP traces for baseline (1) and after treatments and 100-110 min into recording (2) are shown. n = 4. **(B)** Histogram summarizing the average percent changes of fEPSP slope between treatment groups. Decrease in fEPSP slope seen with injection of vehicle prior to JZL 195 was significantly different from other treatment group (black) (# = $p < 0.01$).

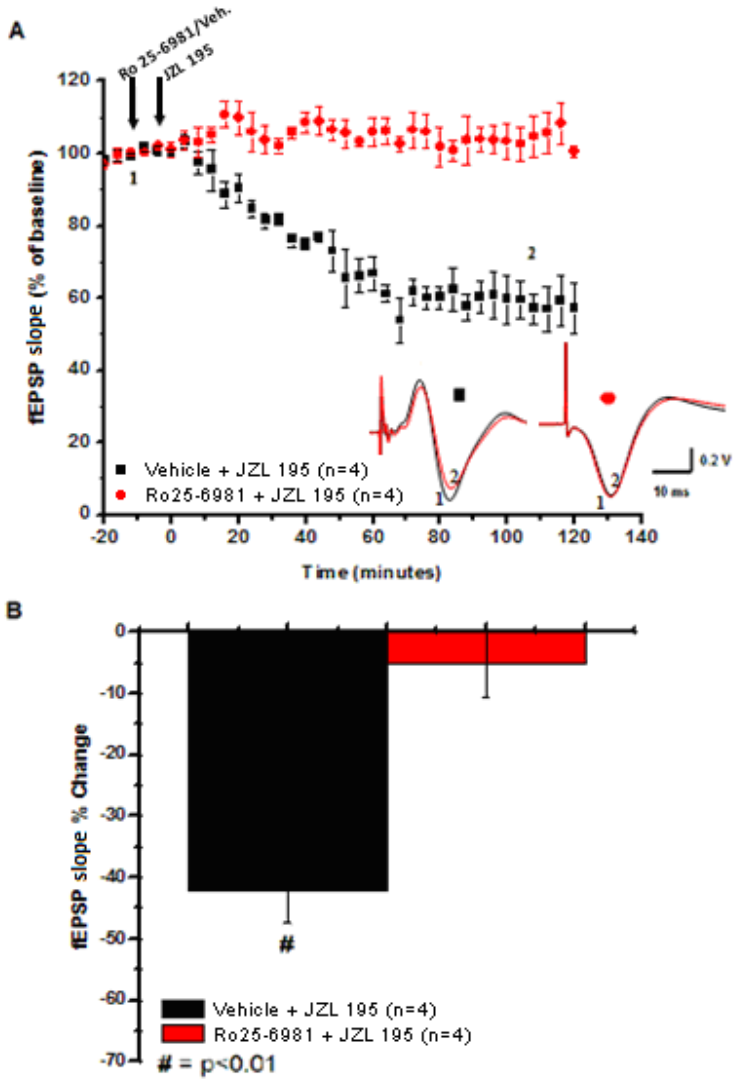

Figure 3-5. Inhibition of NR2BRs prevented the production of eCB-LTD.
(A) Plot of normalized fEPSP slopes in anesthetized mice shown as means ± SEMs. Injection of NR2BR specific antagonist (Ro 25-6981; 6 mg/kg; i.p.; red circles) 10 min before the injection of JZL 195 at 0 min blocked the depression of synaptic transmission seen with vehicle preinjection (black squares) ($F_{1,7}$ = 1.184, p = 0.331). Injection of JZL 195 depressed CA1 synaptic transmission to 70 % of baseline levels and lasted for > 2 h. ($F_{1,7}$ = 51.202, p < 0.01). Representative fEPSP traces for baseline (1) and after treatments and 100-110 min into recording (2) are shown. n = 4. **(B)** Histogram summarizing the average percent changes of fEPSP slope. Decrease in fEPSP slope due to injection of vehicle 10 min prior to JZL 195 (black) was significantly different from groups pretreated with NR2BR antagonist (red) (# = p < 0.01).

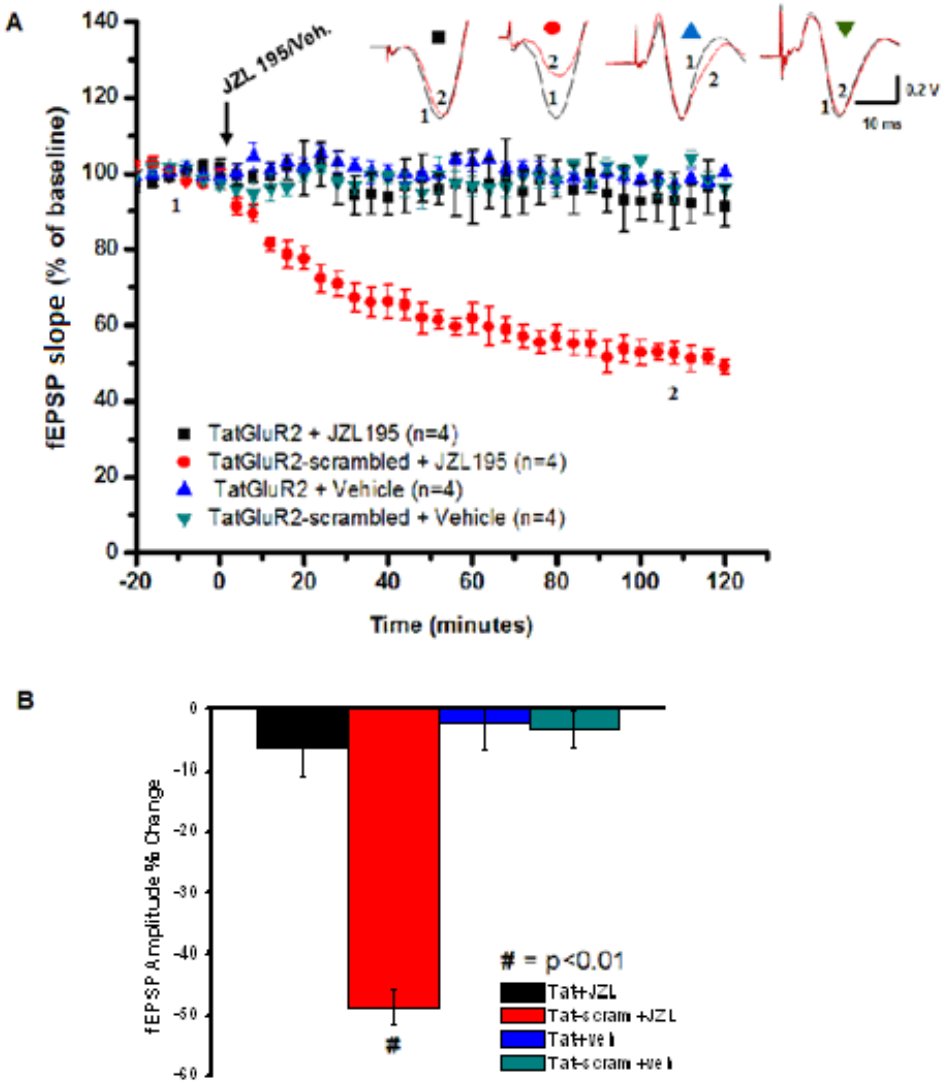

Figure 3-6. Inhibition of AMPAR endocytosis prevents the expression of eCB-LTD.
(A) Plots of normalized fEPSP slopes in anesthetized mice shown as means ± SEMs. Injection of TatGluR2$_{3Y}$ peptide 1.5 h before the injection of JZL 195 (black squares) at 0 min blocked the depression of synaptic transmission seen with pre-treatment with the control peptide ($F_{3,15}$ = 1.229, p = 0.466). Injection of JZL 195 with TatGluR2S depressed CA1 synaptic transmission to 60 % of baseline levels (red circles) and lasted for > 2 h. ($F_{3,15}$ = 145.467, p < 0.01). Representative fEPSP traces for baseline (1) and after treatments and 100-110 min into recording (2) are shown. n = 4. **(B)** Histogram summarizing the average percent changes of fEPSP slopes. Decrease in fEPSP slope seen with pre-treatment of TatGluR2S (red) was significantly different from control groups (black, blue, and green) (# = p < 0.01).

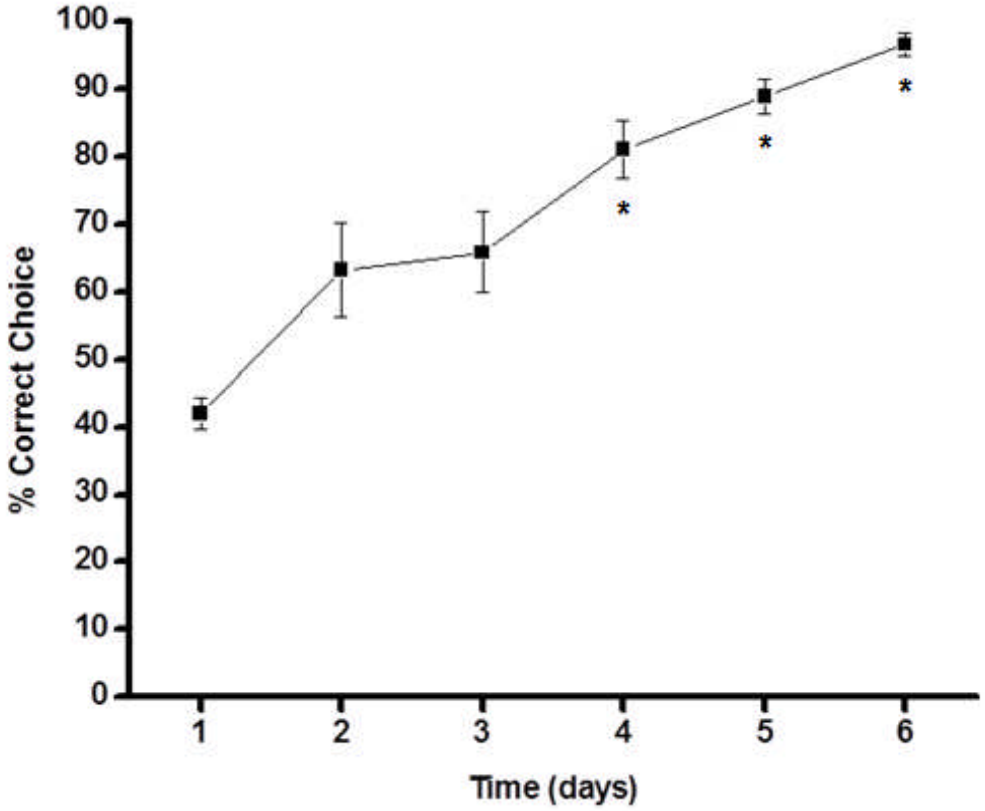

Figure 3-7. Acquisition of spatial working memory (SWM).
Representative plot showing that all groups in each experiment adequately learned the task as percent correct choice, graphed as mean ± SEM, for days 4-6 of the acquisition period were significantly increased as compared to day 1 (* = $p < 0.01$) and reached 90%-100% correct choice by day 6. n = 9.

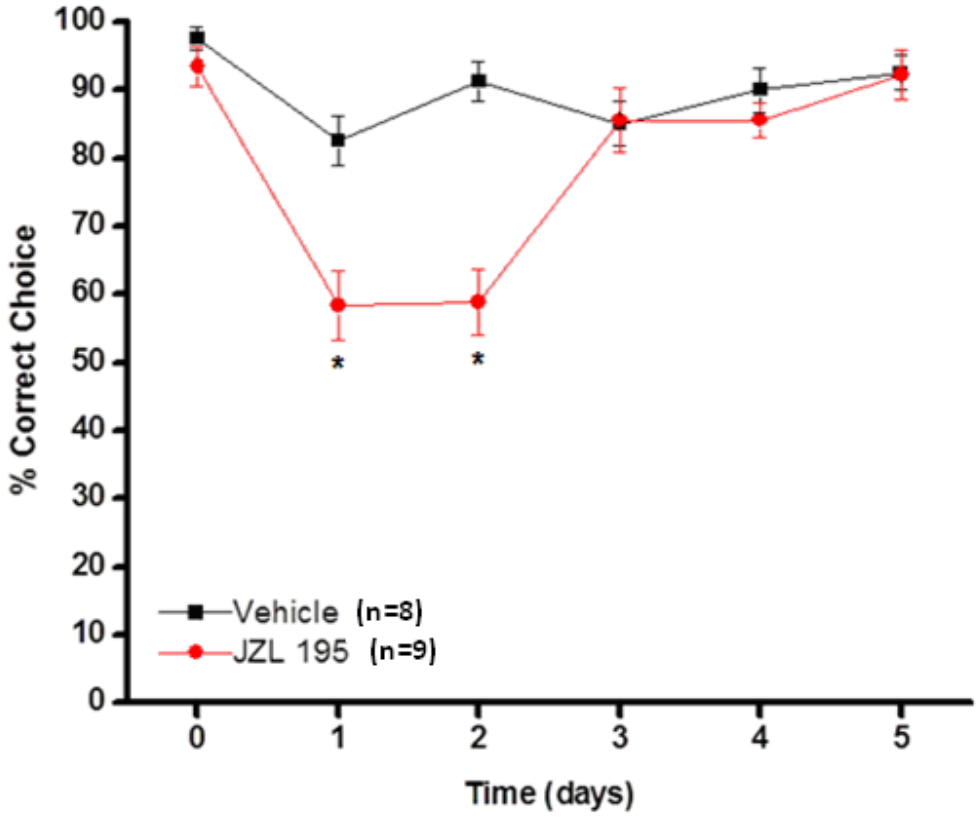

Figure 3-8. SWM impairment following a single systemic injection of eCB degradative inhibitor.

Plot of percent correct choice means ± SEM. A single injection of JZL 195 (20 mg/kg; red circles) prior to the start of the first trial on the first testing day caused a significant decrease in SWM performance as compared to vehicle control (black squares) for 48 h ($F_{1,16} = 10.44$, $p < 0.01$). Day 0 is the final day of acquisition training, with the injection given on day 1. n = 8 or 9. (* = $p < 0.01$).

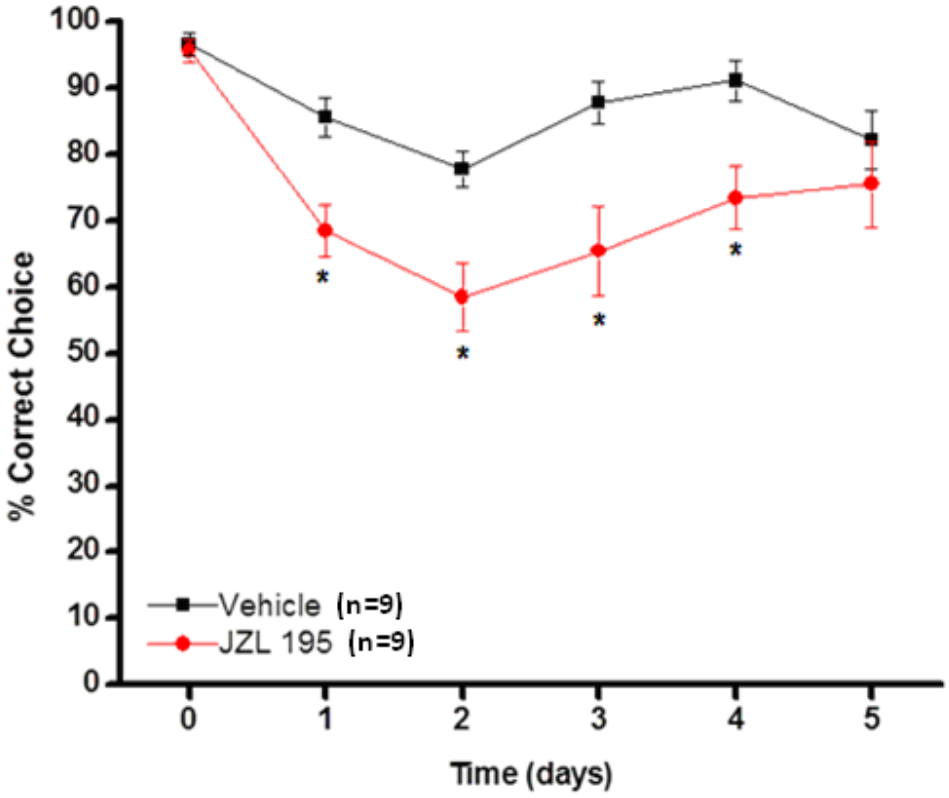

Figure 3-9. SWM impairment following daily systemic injections of eCB degradation inhibitor.

Plot of percent correct choice means ± SEM. Daily injections prior to the start of the first trial of global inhibitor of eCB degradation JZL 195 (20 mg/kg; red circles) caused a significant decrease in SWM performance as compared to vehicle control (black squares) for the first 4 of 5 testing days ($F_{1,16} = 10.44$, $p < 0.01$). n = 9. Day 0 is the last day of acquisition training, with the first injection given on day 1. (* = $p < 0.01$).

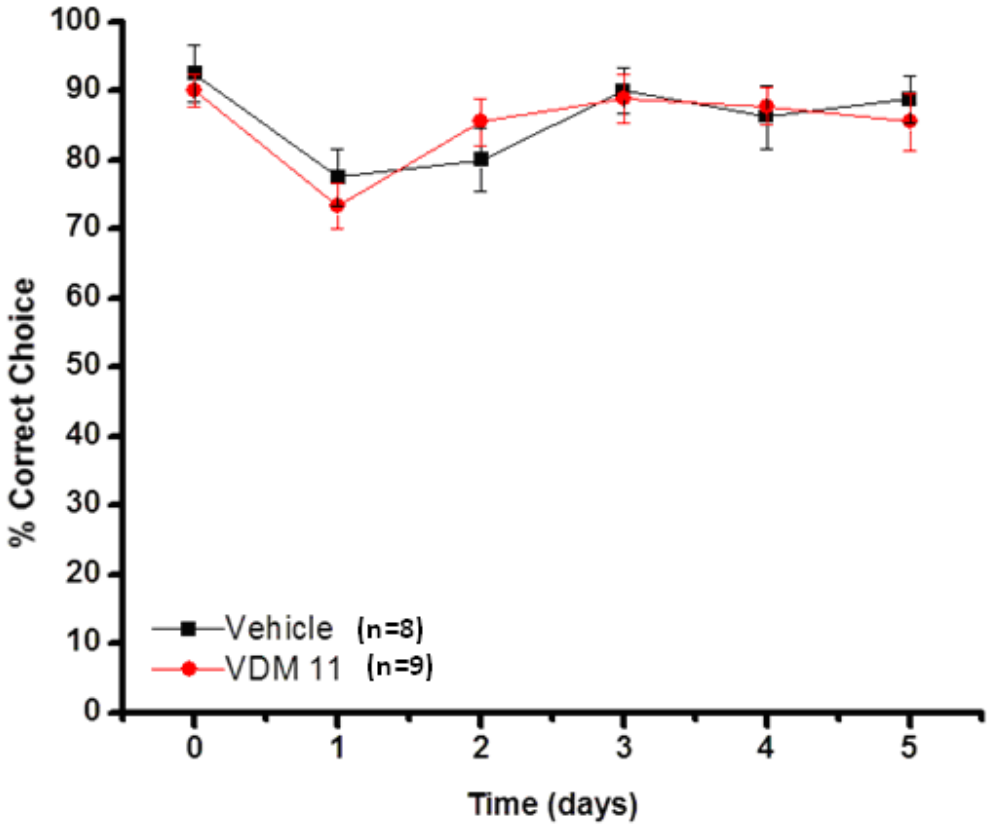

Figure 3-10. Systemic injections of AEA reuptake inhibitor does not impair SWM.
Plot of percent correct choice means ± SEM. Daily injections prior to the start of the first trial of inhibitor of AEA reuptake VDM 11 (5 mg/kg; red circles) had no significant effect on SWM performance as compared to vehicle control (black squares) ($F_{1,15} = 0.350$, $p = 0.620$). n = 8 or 9. Day 0 is the last day of acquisition training, with the first injection given on day 1.

Chapter 4: Discussion

4.1 Inhibition of eCB degradation causes depression of synaptic transmission

I began the experiments by performing individual i.p. injections of 3 drugs: JZL 184, VDM 11, and JZL 195. JZL 184 is a potent and selective inhibitor of MAGL, the enzyme responsible for breaking down 2-AG into arachidonic acid and glycerol; JZL 184 decreases the degradation of 2-AG, allowing it to accumulate in the extracellular space and amplify its effects (Long et al., 2009b). VDM 11 is an AEA transport inhibitor and allows AEA to accumulate in the extracellular space (de Lago et al., 2004). The net increase in AEA or 2-AG with these 2 drugs means that eCB-specific functions could be investigated individually. JZL 195 is a potent inhibitor of both MAGL and FAAH, causing a global increase of eCBs in the brain (Long et al., 2009a).

I have shown that JZL 184 and VDM 11 are able to decrease synaptic transmission to approximately 70% of baseline levels (see Figures 3-1, B and C), and that JZL 195 administration causes a decrease in synaptic transmission to approximately 40% of baseline levels (see Figure 3-1 A). Given the large decrease in synaptic transmission seen with JZL 195 administration, it was useful as a positive control when used in combination with other drugs treatments.

These results indicate that both 2-AG and AEA could work together to influence eCB-mediated metaplasticity in a cumulative manner. This deviates slightly from some researchers' consideration of 2-AG being the main eCB necessary to induce changes in synaptic plasticity due to the presynaptic location of its degradative enzyme and higher levels in brain lipid fractionates as compared to AEA (Castillo et al., 2012; Stella et al., 1997; Kim and Alger, 2004). Contrary to

this, other researchers have shown that both AEA and 2-AG play a role in eCB-mediated changes to synaptic transmission *in vitro* (Bajo et al., 2009; Di et al., 2005; Gerdeman et al., 2002). I was able to confirm this finding *in vivo*. Both AEA and 2-AG are synthesized on demand due to excitation of the postsynaptic neuron (Heifets and Castillo, 2009), patterns of activity which are frequently required to cause long-term changes in synaptic transmission (Rodríguez-Moreno and Paulsen, 2008). Taken together, it is reasonable that AEA and 2-AG are cumulatively responsible for eCB-mediated depression of synaptic transmission at CA3-CA1 hippocampal synapses.

4.2 Evidence for the mediation of CB_1R in eCB-depressed synaptic transmission

To determine if the plasticity I observed was mediated by CB_1Rs, I injected CB_1R antagonist AM 281 either 10 minutes before or 10 minutes after the inhibitor of eCB degradation, JZL 195. If the injection of AM 281 before JZL 195 blocked the eCB-mediated depression of synaptic transmission then I would have evidence of CB_1R involvement in eCB-mediated depression of synaptic transmission *in vivo*. Indeed, the pretreatment of AM 281 blocked, whereas the post-treament of AM 281 failed to block, eCB-mediated depression of synaptic transmission (see Figure 3-3). These results indicate that CB_1Rs play role in this form of synaptic plasticity. Furthermore, as mentioned previously, the evidence that post-treatment of AM 281 failed to block eCB-mediated depressed synaptic transmission is supported by the fact that once activated CB_1R are internalized for upwards of 16 h (Coutts et al., 2001). This also suggests that eCB-mediated depression of synaptic transmission is non-transient in nature. However, given that no experiments have been performed *in vitro* to indicate that post-treatment of AM 281 reverses eCB-mediated DSI as there is with CB-mediated DSI (Kawamura et al.,

2006), little can be said as to the long-term nature of eCB-mediated depression of synaptic transmission based on this experiment at this time.

Rats were used in this experiment due to adverse effects encountered following the injection of these two drugs in mice. I validated the results by performing the appropriate controls in rats, such as injection of JZL 195 alone (see Figure 3-3). Mice have been shown to be more pharmacologically sensitive than rats in various other precedents (Kinch et al., 2012; Csanády et al., 2011), thus it is not unusual that I encountered similar sensitivities in these experiments.

4.3 Evidence for the long-term nature of eCB-mediated depressed synaptic transmission

In an effort to identify whether this eCB-mediated depression of synaptic transmission is transient or long-term, I used the inhibitor of protein translation anisomycin. This technique was previously used by members of my lab to determine if *in vivo* CB-mediated hippocampal depression of synaptic transmission was LTD (Han et al., 2012). They found that anisomycin was able to reverse the depressed synaptic transmission 30 min after the injection of HU 210. This result was supported by studies showing that new protein synthesis was required for L-LTD and that preexisting proteins were sufficient to support E-LTD (Manahan-Vaughan et al., 2000; Kauderer and Kandel, 2000). However, in the present study when mice were pretreated with anisomycin prior to an injection of JZL 195 there was a complete blockade of eCB-mediated depressed synaptic transmission (see Figure 3-4). The absence of an initial decrease in synaptic efficacy indicates that both the early-phase and the late-phase expression of the depressed synaptic transmission induced by an acute eCB accumulation require new protein synthesis.

To determine the necessity of protein synthesis for eCB-mediated LTD it would also be useful to target the proteins that are specifically upregulated during the maintenance of L-LTD. Changes in synaptic growth are another way to characterize late-phase long-term plasticity, and as such interfering with specific proteins involved in the morphological changes at synapses during LTD could help rectify the obstacle of complete inhibition of protein synthesis in the endogenous model (Pang and Lu, 2004). BNDF is one such protein that has been shown to increase in concert with long-term synaptic plasticity (Lu, 2003). BDNF is secreted in an activity-dependent manner, and through its Neurotrophic type-2 tyrosine kinase (TrkB) receptor, leads to the activation of protein kinase B (PKB) and phosphatidylinositol 3-kinase (PI3K). These kinases then induce mTOR signaling that ultimately leads to the phosphorylation of mRNA binding proteins and initiates translation of other plasticity proteins (Tang et al., 2002). Various inhibitors of this pathway such as TrkB inhibitor cyclotraxin-B (Cazorla et al., 2010) or mTOR inhibitor rapamycin (Sehgal and Bansbach, 1993) could be employed to prevent LTD specific proteins and test the protein-dependence of eCB-LTD in a more precise manner.

4.4 NMDARs and their subunit specificity in eCB-mediated induction of LTD

Once the individual contributions of the eCBs had been determined and the process was confirmed as non-transient, I asked if glutamate, through NMDARs, was involved in this process for two reasons. First, the recent work by members of my lab showed that an acute injection of CBs is able to induce an *in vivo* LTD through a cascade of events involving the activation of astrocytic CB_1R, subsequent glutamate release from astrocytes, activation of postsynaptic NMDAR and the internalization of AMPARs (Han et al., 2012). Second, other research has

shown that glutamate is released from astrocytes following eCB interactions with astroglial CB_1R (Bari et al., 2010). NMDARs are one of the neuron's main glutamate receptors and they have been shown to regulate synaptic plasticity through the internalization of AMPARs (Collingridge et al., 2010). Given that our previous work demonstrated a role for NR2BRs in CB-LTD, this investigation began with an NR2BR antagonist. The NR2BR specific antagonist Ro25-6981 was injected 10 min prior to the inhibition of eCB degradation with an injection of JZL 195, and prevented eCB-LTD (see Figure 3-5). These results indicate a role for NR2BRs in eCB-mediated plasticity, as was the case with CB-LTD. Research has shown that the majority of NR2BRs are located extrasynaptically (Köhr G, 2006), therefore it seems plausible that glutamate from a source other than the presynaptic neuron could be responsible for eCB-mediated synaptic plasticity.

4.5 Source of glutamatergic release and location of extrasynaptic NMDARs

It will also be important to determine the source of glutamate involved in eCB-mediated synaptic plasticity. I hypothesize that extrasynaptic NMDARs mediate this plasticity, but even so the glutamate could come from synaptic spillover (Rusakov and Kullmann, 1998), microglia (Domercq et al., 2007), or heterosynaptically (Asztely et al., 1997) rather than from astrocytes, the hypothesis proposed based on the mechanism of CB-LTD described by my colleagues (Han et al., 2012). My colleagues proposed a mechanism whereby activation of astrocytic CB_1Rs causes the increase of extracellular glutamate leading to the activation of extrasynaptic NMDARs and the expression of CB-LTD. The similar results between the experiments my colleagues have performed with CBs and my own with eCBs indicate that astrocytic CB_1Rs might indeed cause the releases of astrocytic glutamate in the eCB-mediated model of synaptic

plasticity. However, further experiments will need to be completed to draw any conclusions. Mutant mice with cell-type specific knockout of CB_1R will be especially useful to determine if CB_1Rs located on astrocytes, microglia, excitatory neurons, inhibitory neurons, or multiple cell types are necessary for eCB-mediated hippocampal synaptic plasticity. Our lab is now close to the establishment of these mutant mouse lines.

Not only is the location of CB_1R important, but where the extrasynaptic NMDARs are located will also be useful to determine. Extrasynaptic NMDARs can be located on the sides of dendritic spines, on the dendritic surface itself, or at presynaptic terminals (Petralia, 2012). Indeed, at some synapses, glutamate can act on presynaptic NR2BRs to cause an increase in glutamate that could in turn activate NMDARs located on the postsynaptic neuron to induce AMPAR endocytosis in a feedforward manner (Rossi et al., 2012). Paired-pulse facilitation experiments will be useful to determine if there is a presynaptic mechanism of glutamate release that could serve as a source of glutamate. If there is no change to the paired-pulse ratio using this technique, then it can be concluded that the extrasynaptic NMDARs involved in eCB-mediated alterations in synaptic transmission are located postsynaptically, likely adjacent to points of contact with glia, axons, or other dendrites (Petralia, 2012).

4.6 Usefulness of knock out models for exploring the mechanism of eCB-LTD

To clarify some of the questions my study poses, work is being performed in parallel to my own by collaborators in China. Their work with mouse knockout models will supplement my experiments and strengthen my results with the addition of novel paradigms. The mouse lines that are being developed are inducible Cre recombinase knockouts, and as such are more

temporally and spatially accurate than the traditional knockout model (Eisener-Dorman et al., 2009). In the inducible system, a Cre recombinase is fused with the mutated hormone-binding domains of the estrogen receptor (CreER) so that 4-hydroxytamoxifen (OHT) must be introduced to activate the CreER recombinase to excise the LoxP sites (Feil et al., 2009). This technique will be used in combination with tissue-specific markers so that DAGLα, FAAH, MAGL, CB_1R, and CB_2R can be knocked out individually in microglia, astrocytes, as well as GABAergic and glutamatergic neurons. The knockouts will be more precise than pharmacological targeting of desired enzymes, and consequently an excellent addition to my experiments using drugs to inhibit eCB degradation. The cell-specific resolution of the knockouts will also strengthen the evidence I have attained.

It will also be helpful to study knockout models for synthetic enzymes as the techniques we have used thus far are solely overexpression. The use of several cell- and neuron-specific knockouts of CB_1R will be an excellent substitute in the interim since the convoluted synthetic pathways have not yet been explored for knockout potential.

4.7 GluR2-containing AMPAR endocytosis in eCB-mediated LTD

As mentioned previously, NMDARs have been shown to regulate synaptic plasticity through the trafficking of AMPARs (Collingridge et al., 2010), with NR2BRs shown to play a predominant role in the LTD process in juvenile rats (Yu et al., 2010). Evidence suggests that this is achieved by clathrin-medaited endocytosis. Binding of the clathrin adaptor protein AP2 to the NSF site on the AMPAR GluR2 subunit destabilizes the AMPAR in the postsynaptic membrane, causing AMPAR endocytosis, and mimicking NMDAR-LTD (Lee et al., 2002).

To determine if this NMDAR-mediated eCB-LTD was a function of AMPAR endocytosis I administered either TatGluR2$_{3Y}$ or TatGluR2S 1.5 h before the treatment of JZL 195. TatGluR2$_{3Y}$ has been shown to block the internalization of the GluR2 AMPAR subunit (Wong et al., 2007), and given that GluR2-containing AMPARs in particular have been shown to play an integral role in AMPAR trafficking this was an appropriate way to proceed (Fetterolf and Foster, 2011). Injection of TatGluR2$_{3Y}$, but not the scrambled analog, was able to block the LTD elicited by JZL 195 (see Figure 3-6). These experiments provided further evidence for the mechanism involved in eCB-LTD. It would appear that glutamate, possibly released from nearby astrocytes, activates NR2BRs ultimately leading to the internalization of GluR2-containing AMPARs.

It is known that the AMPAR GluR2 subunit is located in the postsynaptic neurons at CA3-CA1 synapses (Tsuzuki et al., 2001; Bolshakov and Buldokova, 2001). Therefore, our results, together with other lines of evidence discussed so far, suggest a novel type of eCB-LTD involving activation of astroglial CB_1R, activation of postsynaptic NMDAR, and subsequent AMPAR endocytosis. If this novel eCB-LTD is confirmed by future studies in our mutant mouse lines of cell-type specific knockout of CB_1R and eCB degradative enzymes, the results would suggest the existence of constitutive levels of eCB in the extracellular space that can be quickly accumulated upon administration of eCB degradation inhibitors. This idea would add to the well-established notion that both AEA and 2-AG in neurons are synthesized on demand due to excitation of the postsynaptic neuron (Heifets et al., 2009)

4.8 Implications of hippocampal eCB-mediated LTD on SWM

Next, I wanted to determine if there was a behavioral implication to the hippocampal eCB-LTD that I observed. Spatial memory is largely processed in the hippocampus (Ge et al., 2010) and an exploration of how this type of memory was affected by the acute accumulation of eCB would show if there was a behavioral consequence associated with the electrophysiological phenomena I observed. To do this, I chose to use the DNMTS T-maze given that unlike other memory tests employed by members of my lab such as conditioned-place preference there is less of a traumatic factor, thus decreasing influence of other medial brain structures such as the amygdala (Jeneson and Squire, 2011).

The effects of daily systemic injections of eCB degradation inhibitors were examined by the administration of an i.p. injection of either JZL 195 or VDM 11 10 min prior to the start of the first trial each testing day. It was found that the global inhibition of eCB degradation by JZL 195 significantly impaired SWM (see Figure 3-9). However, i.p. injections of AEA reuptake inhibitor VDM 11 had no significant effects on SWM (see Figure 3-10). This adds weight to the electrophysiological results attained showing that inhibition of eCB degradation with JZL 195 caused the greatest decrease in synaptic transmission, with VDM 11 producing a much smaller decrease in synaptic transmission. It appears that large decreases in synaptic transmission, and possibly the cooperation of AEA and 2-AG (Long et al., 2009a), are required for the presentation of a SWM deficient phenotype.

Single dose experiments using JZL 195 were performed to determine if the persistent impairment of SWM performance seen in the previous experiments was due to a multiple dose effect of the drug or if a one-time injection of the eCB degradation inhibitor could achieve a long-term memory deficit. It was found that one dose of JZL 195 was sufficient to cause a

memory deficit that persisted for 2 days (see Figure 3-8), half the length of time seen with daily injections of the drug. It has been shown that chronic injections of CB_1R agonist can have lasting adverse effects on memory (Hampson et al., 2011; Sciolino et al., 2011); however this study is novel in showing that a single acute injection of inhibitors of eCB degradation can have long-term effects on SWM.

4.9 Role of glutamate receptors in eCB-mediated deficits in SWM

Initial pilot studies using systemic injections of the LTD-blocking peptide $TatGluR2_{3Y}$ prior to JZL 195 treatment were performed and yielded inconsistent results. Both groups showed varied SWM impairment with no significant difference between them (data not shown) despite the fact that it is well established that AMPAR dynamics mediate hippocampal-dependent memory (Dong et al., 2013; Takahashi, 2011). This was likely due to detrimental effects on SWM performance caused by inhibition of AMPAR endocytosis throughout the entire brain. Performance differences in memory tasks using systemic versus intrahippocampal treatments have been shown previously (Robinson et al., 2008). Furthermore, eCBs mediate different synaptic plasticity mechanisms based on the location of increased eCB levels. For instance, 5-HT firing in the raphe nuclei was increased and norepinephrine levels increased in the locus coeruleus when the hydrolysis of AEA was blocked (Gobbi et al., 2005), contrary to the effects seen when eCB levels are increased in the hippocampus (Péterfi et al., 2012). To rectify these discrepant results it would be useful to perform similar experiments but with intrahippocampal injections of the $TatGluR2_{3Y}$ peptide using cannula implants. In this way the inhibition of AMPAR endocytosis will be more localized, and the results more representative of hippocampal function alone.

To clarify the role of AMPARs in eCB-mediated SWM deficits, AMPAR inhibitors such as CNQX could also be used (Lee et al., 2010). Also, given that my electrophysiology results indicate a role for NR2BRs in the induction of eCB-LTD, NMDAR antagonists could also be used to determine if the hippocampal eCB-LTD defined in the current study underlies eCB-mediated deficits in SWM.

4.10 Knockout models and varying protocols in SWM tasks

Researchers suggest that working memory can be regulated by different regions of the brain depending on the length of time the information needs to be held in the subject's mind (Jeneson and Squire, 2011). It could be that given the length of retention time used for our T-maze, the prefrontal cortex could play a more involved role in the process. To rectify the implications of time-dependency on eCB-mediated hippocampal memory impairment it would be helpful to perform a shorter memory task such as the Morris water maze (Stern and Alberini, 2012).

As in the electrophysiological experiments, knockout mouse lines will help characterize this complex system. This would include the role that astrocytes play in eCB-LTD through the use of conditional knock-outs in both Morris water maze and DNMTS T-maze testing. The use of knockout models of the various eCB degradative enzymes will be particularly advantageous for behavioural experiments because they remove the need for acute i.p. injections of JZL 195. Researchers have shown that acute i.p. injections can stress mice, as seen by significant increases in heart rate and body temperature compared to baseline levels that last more than 30 min post injection (Meijer et al., 2006). Given that stress can have adverse effects on behavioural

performance (Devilbiss et al., 2012; Dalm et al., 2012), removing a measure of stress will increase the validity of behavioural results.

4.11 Translational capabilities of ECS manipulation

Our continued work elucidating the CB_1R endogenous signaling pathway could allow the ECS to be pharmacologically manipulated to great clinical advantage. Imbalances in eCB levels and function have been shown to play a role in depression, post-traumatic stress disorder (PTSD), Parkinson's disease, Huntington's disease, and Alzheimer's disease to name a few (Orgado et al., 2009; Pertwee, 2012; Bilkei-Gorzo, 2012; Piro et al., 2012; Micale et al., 2013).

Increased CB_1R density has been found in the prefrontal cortex of depressed suicide patients (Hungund et al., 2004), but decreased CB_1R density was reported in the cortex of patients suffering from major depressive disorder (Koethe et al., 2007). Contradicting results have also been obtained in PTSD patients where higher peripheral concentrations of AEA and 2-AG were reported (Hauer et al., 2013), but lower levels of AEA accompanied by higher CB_1R availability compared to healthy controls were found in the brain (Neumeister et al., 2013). To rectify ambiguous data such as these, it will be helpful to first fundamentally understand eCB signaling mechanisms throughout the brain with studies similar to my own.

Additionally, evidence suggests that eCB tone increases in proximity to plaques and tangles as neurodegenerative disorders progress, causing neuronal silencing and dysfunction (Mulder et al., 2011; Pisani et al., 2005). The translational ability of our *in vivo* experiments, as opposed to past *in vitro* experiments, are an essential stepping stone towards the ultimate goal of influencing the ECS for clinical purposes – developing drugs that can regulate the particular type of ECS dysfunction associated with various neurological disorders.

4.12 Conclusion

The goal of this study was to characterize the *in vivo* mechanism responsible for eCB-induced synaptic plasticity at CA3-CA1 hippocampal synapses. I showed that 2-AG and AEA play a cumulative role in eCB-mediated depression of synaptic transmission. This depression of synaptic transmission is likely LTD due to the prolonged activation of CB_1R and the requirement for protein synthesis. I also showed that the induction of eCB-LTD occurs due to the activation of NR2BR, and is expressed following the internalization of GluR2-containing AMPARs. Thus, our results suggest a novel type of eCB-LTD involving the activation of astroglial CB_1R, postsynaptic NMDAR, and consequent internalization of AMPARs. Furthermore, the rapid changes in synaptic transmission following acute drug injections that was found suggest constitutive levels of eCB in the extracellular space that accumulates following the inhibition of eCB degradation enzymes. The increased levels of both AEA and 2-AG that cause eCB-LTD have further functional implications as shown by eCB-mediated long-term SWM impairments.

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