Acute cannabinoid treatment *in vivo* causes an astroglial CB_1R-dependent LTD at excitatory CA3-CA1 synapses involving NMDARs and protein synthesis

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Abstract

Cannabinoids have been shown to alter synaptic plasticity but the mechanism by which this occurs at hippocampal CA3-CA1 synapses *in vivo* is not yet known. Utilizing *in vivo* electrophysiological recordings of field excitatory postsynaptic potentials (fEPSP) on anesthetized rats and mice as well as three lines of conditional knockout mouse models, the objective was to show a two-part mechanistic breakdown of cannabinoid-evoked CA3-CA1 long-term depression (LTD) in its induction as well as early and later-phase expression stages. It was determined that this cannabinoid-induced *in vivo* LTD requires cannabinoid type-1 receptors (CB_1Rs) on astrocytes, but not CB_1Rs on glutamatergic or GABAergic neuronal axons/terminals. Pharmacological testing determined that cannabinoid-induced *in vivo* LTD also requires activation of NMDA receptors (NMDAR) and subsequent postsynaptic endocytosis of AMPA receptors (AMPAR). There exists a clear role for NR2B-containing NMDARs in a persistent, transitory form, potentially related to prolonged or delayed glutamate release (possibly as a result of the astrocytic network). A key determination of the expression phase is the involvement of new protein synthesis (using translation and transcription inhibitors) – further evidence of the long-term action of the synaptic plasticity from a single cannabinoid dose.

Preface

Published paper presented in this thesis:

1. Han, J.,* **Kesner, P**.,* Metna-Laurent, M.,* Duan T., Xu, L., Georges, F., Koehl, M., Abrous, D.N., Mendizabal-Zubiaga, J., Grandes, P., Liu, Q., Bai, G., Wang, W., Xiong, L., Ren, W., Marsicano, G., Zhang, X. (2012). Acute Cannabinoids Impair Working Memory Through Astroglial CB_1 Receptor Modulation of Hippocampal LTD. ***Cell***, 148: 1039-1050. (* the first three authors contributed equally to this research).

This paper will be referenced throughout the thesis, however, due to the amount of researchers contributing to this study and the additional work I have done that is not included in this paper, this thesis will focus on the work that I contributed and the supplemental work that has been part of my Master's project.

My figures in this paper include: Fig. 1B, 1C, 2A, 2B, 3B, 7, S1A, S1B

List of Figures

List of Abbreviations and Chemical Names

2-AG	2-Arachidonoylglycerol
ACC	Animal care committee
AM 281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
AMPAR	AMPA receptor
BDNF	Brain-derived neurotrophic factor
CA1	Cornu Ammonis area 1
CA3	Cornu Ammonis area 3
cAMP	Cyclic adenosine monophosphate
CB	Cannabinoid
CB_1R	Type-one cannabinoid receptor
CB_2R	Type-two cannabinoid receptor
DAG	Diacylglycerol
ddH_2O	Double-distilled water
DG	Dentate gyrus
DHPG	3,5-Dihydroxyphenylglycine
DMTP	Delayed-matching-to place
DNMTST	Delayed nonmatching-to-sample T-maze
E4CPG	α-Ethyl-4-carboxyphenylglycine
eCB	Endocannabinoid
EPSP	Excitatory postsynaptic potential
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
fEPSP	Field excitatory postsynaptic potential
FRT	Flp recombinase recognition target site
GABA	γ-Aminobutyric acid
GFAP	Glial fibrillary acidic protein
Glu	Glutamate
GRIP	Glutamate receptor interacting protein

Ifenprodil	(1*R**,2*S**)-*erythro*-2-(4-Benzylpiperidino)-1-(4-hydroxyphenyl)-1-propanol hemitartrate
IEG	Immediate early genes
IP3	Inositol trisphosphate
LFS	Low frequency stimulation
LTD	Long-term depression
E-LTD	Early phase expression – Long-term depression
L-LTD	Late phase expression – Long-term depression
LTP	Long-term potentiation
LV	Lateral ventricle
MAGL	Monoacylglycerol lipase
MAP-2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
mGluR	Metabotropic glutamate receptor
mTOR	Mammalian target of rapamycin
NAc	Nucleus accumbens
Neo	Neomycin
NMDA	N-Methyl-D-aspartic acid
NMDAR	NMDA receptor
p38	p38 mitogen-activated protein kinases
PCR	Polymerase chain reaction
pFRTZ	Flp recombinase target sites
PGK	Phosphoglycerate kinase
PICK1	PRKCA-binding protein
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Ro25-6981	(α*R*,β*S*)-α-(4-Hydroxyphenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol maleate
shRNA Cx43	Short hairpin RNA of connexin 43
SWM	Spatial working memory
THC	Tetrahydrocannabinol

TPS	Theta-patterned stimulus
VTA	Ventral tegmental area
WM	Working memory

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Chapter 1: Introduction

1.1 Cannabinoids and CB1Rs

1.1.1 Overview

Cannabis or marijuana is a plant that is believed to be among the most used illicit substances worldwide (Lorenzetti, 2010). This is due mainly to the fact that it contains tetrahydrocannabinol (THC), which is the major psychoactive component and provides the "high" affect for recreational users. Cannabinoids are neuromodulators that are known to activate type-one cannabinoid receptors (CB_1Rs) on the presynaptic terminals of inhibitory and excitatory neurons as well as on astrocytes, and recently even on mitochondria (Bénard et al., 2012). Type-two cannabinoid receptors (CB_2Rs) also exist but are found mainly in the immune system (Guo and Ikeda, 2004).

1.1.2 Types of cannabinoids

Cannabinoids are subdivided into several groups including endocannabinoids (eCB) (developed in the brain and act in the nervous system), phytocannabinoids (from the Cannabis plant – these are related to THC) and synthetic cannabinoids (Lambert and Fowler, 2005). Endocannabinoids are known as retrograde signaling molecules in that they are released from the postsynaptic neuron, travel back across the synapse and activate CB_1Rs on the presynaptic terminals (Heifets and Castillo, 2009). Although these chemicals are functionally very similar, this paper will focus on THC and the synthetic cannabinoid HU210. HU210 was first synthesized by Dr. Mechoulam's group at the

Hebrew University (where it gets its name) and is approximately100 times more potent than THC (Mechoulam et al., 1988; Devane, 1992).

1.1.3 CB_1R and CB_2R

As mentioned earlier, the two types of cannabinoid receptors are CB_1R and CB_2R. Both receptor types are linked to G-proteins. These G-proteins can be of the $G_{i/o}$ family and thus inhibit the adenylate cyclase pathway. However, the signaling pathways downstream of the CB_1Rs are much more complex and can involve everything from mTOR, MAPK/ERK, Ca^{2+} channels, and p38 among others (Demuth and Molleman, 2006; Pagotto et al., 2006; Puighermanal et al., 2009) (see Fig. 1-1).

1.1.4 Neuronal and astrocytic receptors

The CB_1Rs discussed, exist on various neuronal populations (including glutamatergic and GABAergic) but also are found on astrocytes (Rodriguez et al., 2001). In numerous papers, Araque's group has shown that CB_1R activation on astrocytes can lead to modulation of neuronal transmission (to be discussed later) (Navarrete and Araque, 2008; Navarrete and Araque, 2010). Recently, our electron microscopic evidence has been presented, and visually shows (via double immunostaining) the presence of CB_1Rs on astrocytes (Han et al., 2012). Beyond this work, astrocytic CB_1Rs have been researched by the labs of Guzman, Maccarrone, Stella and Pickel among others (Sanchez et al., 2001; Bari et al., 2011; Witting et al., 2004; Stella, 2010; Rodriguez et al., 2001).

1.1.5 Cannabinoid function throughout the brain

Cannabinoid function has been seen in many different areas of the brain including the cerebellum, amygdala, hippocampus, neocortex and striatum among others (Levenes et al., 1998; Azad et al., 2003; Wise et al., 2009; Sjostrom et al., 2003; Singla et al., 2007) and they have been associated with functions including cognition, emotions, mood, etc. (Abush and Akirav, 2012; Martin et al., 2002; Witkin et al., 2005). Because cannabinoids act in the hippocampus, they can induce various types of memory impairment. One example included Cammarota's group showing that the activation of CB_1Rs impairs object recognition memory (Clarke et al., 2008). Influx of psychoactive chemicals such as cannabinoids is understood to be an experience-related activity and is associated to learning and memory by means of altering synaptic plasticity (Citri and Malenka, 2008).

1.2 Synaptic plasticity

1.2.1 Synaptic physiology overview

Synapses require many necessities similar to other developing areas. There is a large amount of molecular machinery that is first required in order to create synapse specificity. This specificity in essence allows for coordinated signals via neurotransmitters in specific areas of the brain (Benson et al., 2001). Beyond this, there is tight regulation of numerous other elements including development of the correct morphological and functional properties, accurate synaptic density requirements as well as simply the proper assembly of synaptic machinery (Margeta and Shen, 2010). Upon fulfillment of the appropriate synaptic development, the primary role of the synapse is to perpetuate and mediate neurotransmission.

In the most basic sense, neurotransmission involves complex presynaptic proteins and machinery (i.e. the process by which electrical depolarization causes an increase in intracellular calcium levels) whose general goal is to cause the appropriate release of neurotransmitter into the cleft. These neurotransmitters bind to specific receptors on the postsynaptic density which in turn propagate the signal back into an electrical one (a postsynaptic potential) (Margeta and Shen, 2010). The alteration (strengthening or weakening) of the various forms of synaptic neurotransmission is known as synaptic plasticity (Sjostrom et al., 2008).

1.2.2 Long-term synaptic plasticity

Synaptic strength can be altered by various activity- or experience-dependent means. The two forms of long-term synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD). These phenomena vary greatly depending on the location of the circuits they affect as well as the function of neuromodulators and other effectors. These forms of synaptic plasticity commonly involve excitatory synapses – i.e. glutamatergic synapses (Citri and Malenka, 2008) – although are not exclusive to them (Gaiarsa et al., 2002). Glutamate can bind to ionotropic receptors (i.e. NMDARs, AMPARs, etc.) or metabotropic glutamate receptors (i.e. mGluRs). In terms of NMDAR-dependent synaptic plasticity, once the postsynaptic neuron is sufficiently depolarized, the Mg^{2+} block can be removed from the NMDAR and when it binds glutamate, Ca^{2+} as well as Na^{+} flows into the cell (Mayer et al, 1984). LTD has also been seen to be induced by the binding of glutamate to mGluRs in many areas of the brain (Gladding et al., 2009). This induction

process essentially refers to the mechanisms by which long-term synaptic plasticity can be triggered.

1.2.3 Induction mechanisms

Induction mechanisms usually involve increases or decreases in intracellular Ca^{2+} concentrations (pre or postsynaptically) and activation of kinases or phosphatases (Anwyl, 2006; Puighermanal et al., 2009). LTP is understood to require a large influx of calcium whereas LTD is induced by a small influx (below a required threshold) (Malenka et al., 1996; Reyes and Stanton, 1996). It has been shown by many groups that repetitive low frequency stimulation (LFS) (i.e. 1 Hz for 10-15 minutes – approx. 900 stimuli) induces LTD at CA3-CA1 synapses (Citri and Malenka, 2008). LTD can occur in homosynaptic or heterosynaptic forms. Homosynaptic involves the presynaptic neuron acting on the postsynaptic one, and is thus activity dependent (modulatory events are occurring at the same synapse that activation is occurring), where the heterosynaptic form involves interneurons modulating the synapse and therefore is not directly activity-dependent (Escobar and Derrick, 2007).

1.2.4 NMDAR subunits

The main interest for this paper is the CA3-CA1 synapses of the hippocampus. Based on many studies of these synapses, it was shown that there existed NMDAR-dependent LTD and LTP where the induction required NMDAR activation (Kelleher et al., 2004). NMDARs contain two NR1 subunits (which in turn have eight potential variants), as well

as two NR2 subunits (A through D), or an NR2 and NR3 combination (Traynelis et al., 2010). The NR1 subunits as well as the NR3 (A through B) contain the sites for binding glycine, while the NR2 have the sites for glutamate binding – both agonists are required for activation of the NMDAR (Furukawa et al., 2005). Studies have shown that the key functional unit of NMDARs is the NR1-NR2 heterodimer. The interface of this dimer is involved in allosteric alteration of gating, opening of the ion path, and as previously mentioned, contains the agonist-binding domains (Furukawa et al., 2005).

The role of the various NR2 subunits has been studied over the years. Studies were done to elucidate the involvement of these subunits in inducing LTP and LTD. It had been at one time shown that selectively blocking the NR2A subunit can prevent LTP while blocking the NR2B subunit prevents LTD (Liu et al., 2004). However, various experiments performed have challenged this concept. Malenka's group showed that putative NR2B antagonists did not fully prevent LTD induction and thus more complex requirements for induction are implied (Morishita et al., 2007). Another study by Sheng's group looking at the cytoplasmic tails showed that NR2B is important in the induction of certain forms of LTP and while the NR2A subunit is not; its tail may contain components important in interfering with LTP (Foster et al., 2010).

Beyond the role of these subunits, it has been shown that forms of LTP and LTD can be induced independently of NMDARs, i.e. via mGluRs (as discussed below) (Balschun et al., 1999; Citri and Malenka, 2008). Another potential mechanism that may be related to the bidirectional selectivity for long-term potentiation or depression is the dual MAP kinase pathways. The evidence shown by researchers states that the forms of long-term plasticity, LTP and LTD, may be mediated by the p42/p44 MAPK and p38

MAPK pathways respectively. The 2000 Belardetti study concluded that the p38 MAPK pathway was involved in the induction of mGluR-dependent LTD at CA3-CA1 synapses (Bolshakov et al., 2000). The study published by Jin and Feig in 2010 looked at the roles of NR2 subunits in relation to MAPK pathways and synaptic plasticity. Their work concluded that NR2A subunits can induce a form of LTP that is also dependent on a downstream MAPK pathway, but also that NR2B-containing NMDARs can induce LTP via a pathway that does not contain MAPK (Jin and Feig, 2010). Finally, in Izumi and Zorumski's 2012 paper, they present the idea that hippocampal LTD may involve a mechanism that is dependent on mGluRs, NMDARs and CB_1Rs. This paper presents a unique paradigm that instead of these receptors working independently to induce LTD, they work in concert with one another. Their proposed mechanism states that NMDARs and mGluRs are activated early in the induction phase, and then followed by the endocannabinoid activation of CB_1Rs – which collectively cause a long-lasting depression of the synaptic neurotransmission (Izumi and Zorumski, 2012).

1.2.5 Expression and protein synthesis

Based on the work of Kandel and others, it has been shown that in many areas of the brain including the hippocampus and cerebellum, the expression of both LTP and LTD can be sub-divided into two main phases: early and late phase (Reymann and Frey, 2007; Linden, 1996). Early phase expression (E-LTP or E-LTD) begins immediately post-induction, and does not require protein synthesis (is unaffected by translational and transcriptional inhibition), while late phase expression (L-LTP or L-LTD) is associated with long-term maintenance and requires protein synthesis (Manahan-Vaughan et al.,

2000; Linden, 1996; Kelleher et al., 2004). These processes allow for precision flexibility and seem to be involved in information storing (Kauderer et al., 2000). This protein/mRNA synthesis can occur in the soma or dendrites (Huang et al., 2005). While late-phase expression of LTP has been heavily studied, less is known about late-phase expression of LTD.

1.2.6 Presynaptic versus postsynaptic expression

A great deal of evidence states that the mechanisms for the expression of synaptic plasticity can be postsynaptic or presynaptic. One of the most understood mechanisms by which LTD is implemented is AMPAR endocytosis. This is a process that involves phosphorylation of the glutamate receptor subunit (i.e. GluR2 or GluR1) followed by clathrin- or dynamin-mediated internalization of AMPARs (Anwyl, 2006). There is also a requirement for protein complexes including β-arrestin, adaptor proteins, etc. This can involve a mechanism such as tyrosine phosphorylation on the endocytic motif of the GluR2 subunit. This causes a removal of the scaffolding protein GRIP and a subsequent binding of PICK1 (which recruits a clathrin-adaptor protein) (Wang, 2008; Xia et al., 2000; Kim et al., 2001). Thus there is a postsynaptic decrease in AMPAR functioning or density.

Expression is also associated with the concept of synaptic tagging and capture. This theory states that at activated synapses, specific mRNA and/or proteins responsible for the maintenance of L-LTD are captured by an unknown molecular mechanism. This allows a synapse to capture previously made proteins or subsequently made ones

(Kelleher III et al., 2004). This indicates that the early phase of synaptic plasticity may have a role in contributing to the preparation for the protein synthesis in late phase.

1.3 Other methods to induce LTD

1.3.1 DHPG-induced LTD

Currently, there are two main ways that LTD is induced in experimental conditions, these being electrical and pharmacological, with methods like optogenetic, light-gated channelrhodopsin potentially in their infancy (Schoenenberger et al., 2011). One type of LTD seen throughout the brain is an mGluR-dependent form (mGluR-LTD). One way in which this is done is via pharmacological agents such as the group I mGluR agonist DHPG (low frequency stimulation-induction is discussed below) (Gladding et al., 2009). It is understood that because mGluRs are G-protein coupled receptors, the influx of calcium is not required for mGluR-LTD. They exert their function and cause LTD via second messenger cascades (typically involving phospholipase C, protein kinase C, MAPK, p38 among others) which may lead to involvement of endocytosis of AMPARs (as in NMDAR-LTD) (Citri and Malenka, 2008; Gladding et al., 2009).

Because DHPG is a group I mGluR agonist, it can activate both $mGluR_1$ and $mGluR_5$. Although researchers agree that these group I receptors are key, which is of more importance has been subject to debate. Some believe that the receptors involved are dependent on the brain area, where in certain areas (i.e. VTA and cerebellum) $mGluR_1$ are key, while in other areas (i.e. hippocampus and cortex) $mGluR_5$ play the vital role (Citri and Malenka, 2008; Anwyl, 2006). In one study, researchers showed that mGluR5 knockout mice did not show DHPG-LTD in the CA1 region implicating the key role

these receptors play (Huber et al., 2001). In the end, it is currently understood that although the clear link has been made between $mGluR_5$ and DHPG-LTD, the $mGluR_1$ may play a partial role as well (Gladding et al., 2009).

1.3.2 LFS-induced LTD

Electrically-stimulated induction of LTD is used commonly in studies. Most notably, it is induced via low-frequency stimulation (LFS) (although high frequency stimulation (HFS) has also been used). The proposed significant occurrence related to the LFS is the entry of calcium via the NMDARs in the postsynaptic neuron (Bear, 1996). The exact protocols that produce this effect differ depending on brain region as well as experimental paradigm and the receptor dependencies (i.e. NMDARs versus mGluRs) and are far too numerous to go through in this paper, thus the focus here will be on the CA3-CA1 synapses. Overall, the frequencies used for induction of non-NMDAR-dependent LTD are normally in the range of 1-10 Hz or in regards to the HFS protocols, 50-300 Hz for hundreds of milliseconds to several seconds (Anwyl, 2006). The NMDAR-dependent LTD is normally induced by repetitive (i.e. 900) stimuli at 0.5-5 Hz (Malenka and Bear, 2005).

In regards to the synaptic input into the CA1 region, and dealing with LFS protocols, even in this regard there exist numerous types. They are usually dependent on the age of the animal and more importantly the recent activity that has occurred in the synapse (Malenka and Bear, 2005; Anwyl, 2006). In Bear's 1996 review, he outlined numerous potential dependencies that are required for LFS-induced LTD. These

included: input specificity, frequency dependence, often the requirement of the activation of NMDARs (but not always), age-dependency, that they are saturable and reversible (Bear, 1996). Ultimately, it comes down to discovering the optimal protocol for the animal, brain region and synaptic activity in order to properly perform LFS-induced LTD.

In this study, the protocol was chosen to fit the goal of the research – the slow alterations in synaptic plasticity upon treatment with a cannabinoid. Thus the low frequency stimulation and *in vivo* conditions were selected in order to maintain physiological relevance, the pharmacological induction was performed to imitate a natural exposure to the drug (opposed to electrical stimulation), adult rodents were used due to the interest in observing the effect in developed brains (and the potential for human translation in the future). Using these concepts, previous literature, the guidance of my colleagues and their past experimentation and knowledge, the protocol (described in the Methods section) was chosen.

1.4 Astrocytes

1.4.1 Function and network

An important component of this study is the involvement of astrocytes. Although the current view of astrocytes is very complex, especially in their relationship to neuronal function, the classical understanding was far simpler. This can be seen even in the nomenclature given to these types of cells in the mid 1800s: neuroglia, which essentially translates into “nerve glue” (Zigmond et al., 1999). Astrocytes create this structural support via their long processes and their complex network. Yet beyond the structural

network, astrocytes are known to be connected with one another by gap junctions, in this way forming a syncytium (Zigmond et al., 1999) (see Fig. 1-2). This glial syncytium helps forward the notion that beyond structural importance, astrocytes have a functional role in regulating energy supply, neuronal function, blood flow, etc. (Giaume et al., 2010). This understanding of astrocytes as structural and functional support of the neurons persisted until numerous papers published around the 1990s began to present new data (Santello et al., 2012). Since then, a large deal of research has been designated to furthering the understanding of the astrocyte in relation to neurons, playing a far more active role than previously comprehended.

1.4.2 Astrocyte-neural interactions

More and more work has begun to show that astrocytes play an active role in both the modification of neuronal activity and the modulation of synaptic plasticity as well (Achour and Pascual, 2010). A great deal of this work focuses on the calcium-dependent release of transmitters including glutamate (known collectively as gliotransmitters) from an astroglial origin (Fiacco et al., 2009). Astrocytes themselves share a significant deal of G-protein coupled receptors as well as various transporters with neurons and thus it is understood that they have the ability to bind transmitters, alter their own intracellular calcium concentration and even release gliotransmitters (Santello et al., 2012; Fiacco et al., 2009). From all this work, a new view of the synapse has begun to develop and has been coined the “tripartite synapse” (Araque et al., 1999). In this view, the classic presynaptic-postsynaptic neuronal interaction is now accompanied by the astrocytic

element, providing a new view on how synaptic transmission and plasticity may be modulated.

1.4.3 Astrocytes in cannabinoid research

Thus far, the involvement of astrocytes in cannabinoid research is in its infant stages, but the possibilities seem large and intricate. As cannabinoids in the central nervous system primarily act via CB_1Rs, the work in this area has been done by studying the effects of CB_1R agonists on various processes.

Araque's group has been one of the key proponents of this inter-relationship. In both their 2008 and 2010 papers, they show how endocannabinoids can activate the CB_1Rs on astrocytes, which in turn lead to phospholipase C (PLC) dependant calcium mobilization (via a $G_{q/11}$ protein coupling), eventually inducing the release of glutamate. They go further than this, using *in vitro* whole-cell patch-clamp techniques in brain slices to show that this process leads to alteration of synaptic transmission in pyramidal neurons and this plasticity is NMDAR-dependent (Navarrete and Araque, 2008; Navarrete and Araque, 2010).

In 2001, Guzman's group showed that the activation of CB_1Rs on astrocytes by cannabinoids including THC, anandamide and HU210, caused the hydrolysis of sphingomyelin – which may play a significant role in the regulation of cell physiology (Sanchez et al., 2001). This phenomenon did not occur with CB_1Rs on other cells. This provided the world with information that cannabinoids play a key role as second messengers in the determination of some cell fates, but also that astrocytic CB_1Rs may be coupled to proteins that are not of the $G_{i/o}$ class (Sanchez et al., 2001).

Maccarrone's lab has provided some seminal work which furthers the understanding of astrocytic CB_1Rs and the release of glutamate (furthering the novel work by Araque's group). Using a gliosome assay (gliosomes being essentially the end processes of glial cells – including astrocytes) that is developed out of a specific Percoll fractionation protocol, the investigators were able to move into the *in vivo* observations and note that upon activation by CB_1R agonists, the intracellular levels of IP3 and calcium were increased in the astrocytes (Bari et al., 2011). Thus, as in Araque's work, these researchers showed a phospholipase C dependant release of glutamate by endocannabinoid activation of CB_1Rs, yet in these works, they showed these results *in vivo* (Bari et al., 2011).

In the work done by Stella and colleagues, they took a different approach and showed that microglial cells actually produce the endocannabinoid 2-AG and do so through $P2X_7$ receptors (Witting et al., 2004). Pickel's group looked at CB_1R localization in association with μ-opioid receptors – one of their observations being that CB_1Rs on glial processes do not have accompanying μ-opioid receptors (Rodriguez et al., 2001). Our lab has recently shown the possible involvement of astrocytes in cannabinoid-evoked LTD in the ventral tegmental area (VTA) (Liu et al., 2010). Our current work in Han et al., 2012, decided to look at the work done in the relationship of astrocytes to cannabinoid function in the hippocampus and examine an *in vivo* mechanistic and behavioural model.

1.5 Cannabinoid-evoked Synaptic Depression

1.5.1 Background

Cannabinoids have been known to evoke LTD in various locations in the brain. This has been observed in both the endogenous system and with exogenous treatment. Castillo's group observed LTD in the dentate gyrus (DG) due to the effects of anandamide (Chavez et al., 2010). Malenka's lab has done work flushing out mechanistic requirements of endocannabinoid mediated LTD in the striatum (Singla et al., 2007). Zorumski studied hippocampal LTD created by the cannabinoid system (Izumi and Zorumski, 2012). Abush and Akirav have written extensively on the relationship between cannabinoids, synaptic plasticity and memory (Abush and Akirav, 2010).

It has been shown by numerous groups that synaptically stimulated LTD is abolished by pre-treating with a CB_1R antagonists in regions of the brain including the neostriatum, frontal cortex, etc. (Anwyl, 2006). CB_1R agonists have been shown to induce mGluR-dependent LTD in the neostriatum, nucleus accumbens (NAc) and prefrontal cortex (Chevaleyre et al., 2006). In other studies such as the one performed by Kim and Thayer, it was shown that cannabinoids inhibited the recruitment and formation of new synapses in the hippocampus – thus illustrating a possible mechanism by which cannabinoids affect synaptic plasticity independent of neurotransmitter release (Kim and Thayer, 2001).

Expression of cannabinoid-evoked LTD can also be associated with presynaptic neurotransmitter release. This is controlled by Ca^{2+} levels and allows strength modifications of the synapse to be made based on decreasing the amount of neurotransmitter release (Dobrunz, 2002). Cannabinoids are thought to work this way by

activating CB_1Rs on the presynaptic terminal (induction) and thus causing inhibition of transmitter release (expression).

1.5.2 Acute versus chronic treatment

A great deal of the research being done with the cannabinoid system involves a single treatment of one of the various forms of cannabinoids. Depending on the brain location, the type of recordings/experiments being performed, and whether it is *in vivo* or *in vitro* can play a role in the affects seen by an acute treatment of cannabinoids. Our study, viewing the affects of CA3-CA1 synaptic plasticity in association with spatial working memory (SWM) impairment showed affects lasting more than 24 hours from a single cannabinoid treatment (Han et al., 2012). Some future research of the lab will involve chronic treatment and recordings performed on freely moving rats with implanted electrodes, to understand the affects that occur in terms of synaptic transmission and SWM.

Some studies exist, whereby researchers have treated animals chronically with various cannabinoids and viewed the resulting consequences on synaptic plasticity. In 2004, Christie's group at the University of British Columbia did a study whereby they treated rats for 15 days with HU210. They utilized electrical methods to induce LTP including the weak and strong theta-patterned stimulus (TPS) protocol. The HU210 treated groups showed a significant impairment of LTP compared with the control groups (Hill et al., 2004).

In a very recent study, done by Abush and Akirav, the chronic treatment of the CB_1R agonist WIN-55,212-2 was performed on adolescent rats for two weeks and the

resulting lasting affects were examined at various time intervals post-treatment including 24 hours, 10 days and 30 days (Abush and Akirav, 2012). Recordings were made at the nucleus accumbens and dentate gyrus. WIN55,212-2 did not have an affect on LTP induction in the PP-DG pathway, while it greatly impaired electrically induced LTP in the NAc pathway for the 24 hour and 10 day post treatment tests (Abush and Akirav, 2012). These studies provide *in vivo* evidence of the alteration of synaptic plasticity via chronic cannabinoid treatment.

1.5.3 Transient verses long-term effects

One concept as to whether or not a process is long term or transient in terms of receptor-ligand relationships is whether or not the produced affect is easily reversible by means of antagonism – i.e. is a continuous activation of CB_1Rs by cannabinoid agonists required. In our paper, we show that a single treatment of HU210 is blocked by pretreatment of a CB_1R antagonist (AM 281) but not post-treatment (see Figure 3-2). However, in many *in vitro* studies, this has not been the case. In Freund's 2001 paper, the depressing effects of WIN55,212-22 was reversed with an antagonist treated 10 minutes after the cannabinoid (Hajos et al., 2001). Similar observations were made by Kano's group and such results as suggested by Castillo display the likelihood that due to the fact of reversibility by antagonism, these observations are of a form of depression that is transient and not long-lasting (Kawamura et al., 2006; Chevaleyre et al., 2006).

Making observations for time-passing phenomena is a difficult thing to do as it is subject to the limitations of current electrophysiological experimentation. The first issue one faces when attempting to determine if treatments (both acute and chronic) will have

long-lasting effects is the necessity for *in vivo* recording. It is very difficult to preserve a brain slice for a period of time necessary to make these recordings. Another issue that arises is that even within the bounds of *in vivo* recording, an anesthetized rat or mouse can only be held under for a certain period of time before fatigue, damage or other considerations will occur and alter the recording – thus for recordings where the desire is to observe long-term affects, although anesthetized rats can be used, freely moving rat electrophysiology can be as well (procedures such as optogenetics may provide even more insight if used in these types of studies in the future).

Although numerous studies have shown the immediate effects of cannabinoids on synaptic plasticity, very few have looked at animal recordings to see the long-lasting effects of chronic or acute treatments. Two 2012 studies have shown evidence of electrophysiological effects that are long-lasting. In the Akirav study, using anesthetized rats, but waiting various periods after the 2 week-long treatment ended (i.e. 24 hours, 10 days and 30 days), they would record. What they discovered was that a 2 week long cannabinoid treatment, (14 i.p. injections – one per day) caused impairment of electrically-induced LTP 24 hours later and 10 days later but not 30 days later, giving a time frame whereby more chronic treatment lasts (Abush and Akirav, 2012). Our 2012 study showed, by utilizing freely moving rats in order to establish a common baseline and observe the relationship from the starting point of a single treatment of HU210 to later time points, that the observed LTD lasted over 24 hours after a single treatment (Han et al., 2012).

1.6 Working Memory

1.6.1 Overview

Working memory is understood to be the holding of information in the brain to be used later on for processes such as reasoning and comprehension (Baddeley, 2003). This type of memory is linked heavily to the hippocampus and ultimately the output from this leads to the cortex and the performing of tasks of higher function. The current understanding of the model of working memory relates to the Baddeley and Hitch proposed three component system. These include the two storage sub-systems: the phonological loop and the visuospatial sketchpad, both linked to the control system known as the central executive (Baddeley, 2003) (see Fig. 1-3).

The phonological loop is the element of working memory that deals with language and sound. In this system of memory, serial recall tests are heavily implemented where strings of letters or words are recalled based on similarity or dissimilarity of sound. The notion comes into clarity that for short-term memory, the actual acoustics are key for memorization, where for long-term memory, the meaning of the sound/language becomes far more significant (Baddeley, 2003). Other interpretations imply that the cue of the previous letter/word is what is significant for the memorization to occur, known as the "chaining interpretation" (Brown et al., 2000). This association of cues may involve the amygdala or another brain region in this form of working memory.

The visuospatial sketchpad, which is the relevant system for this study, focuses, as its name suggests, on memory associated with form, colour and location (Luck and Vogel, 1997). The portmanteau of the name of this loop refers to the what (visual) and where (spatial) of working memory. Numerous tasks are used to study this loop including

the Morris water maze (see Fig. 1-3) and the Brooks' matrix task (Baddeley, 2003). It is understood that this loop is related to developing an understanding of objects, their function, how to utilize them and their architecture as well as perhaps being linked with kinesthetic learning (Baddeley, 2000).

The central executive is responsible for the regulation of the two sub-systems as well as having functions of its own. These potentially include, intervention when normal control by the loops has gone awry, fusion of multiple sources of input, implementation of habitual patterns, etc. (Repovs and Baddeley, 2006). However, the function of the central executive continues to be misunderstood and much more research has to be done in this area to determine a clearer function and comprehension.

1.6.2 Memory and the hippocampus

A great deal of research has gone into the associations between the role of the hippocampus and the spatial element of working memory. An array of experimental techniques have been utilized in the cognitive dissection of hippocampal sub-regions and how they relate to the normal and impairing function of SWM.

The hippocampus is a part of the limbic system and thus, beyond its role in learning and memory has been associated with other functions including fear and emotion (Zigmond et al., 1999). The hippocampus has many subfields or regions that are extremely well organized and allow for fairly simple studying. The main input path that exists within the hippocampus is known as the perforant path. This path links input from the entorhinal cortex to granule cells of the dentate gyrus. From here, mossy fibers connect to the CA3 region, where finally, connections arise via ipsilateral Schaeffer

collaterals as well as contralateral commisural fibers to the CA1 region (which ultimately provides most of the output of the hippocampus) (Lavenex and Amaral, 2000).

In the 1950s, it became understood that the hippocampus was associated with memory when a surgeon had removed the hippocampus from a patient and discovered that they began to suffer from a form of amnesia (Scoville and Milner, 1957). Since then, a great deal of work has been done in human and animal models in the hippocampus, a significant amount of which has been the relation of memory to place cells, brain lesions and synaptic plasticity in this area.

1.6.3 Place cells and memory

Place cells in the hippocampus were named that way due to the fact that their firing is dependent on the location of the animal in the environment. They were discovered in the 1970s and extensive research has been done in rodent models (little evidence has been shown in primates) (Eichenbaum et al., 1999). The existence of place cells has allowed researchers to develop a theory that one of the roles of the hippocampus is to create a cognitive map of space, defined by the firing of these cells in response to environmental cues (Martin and Clark, 2007). They believe this heavily plays into the role of the hippocampus in spatial working memory (see Fig. 1-3).

1.6.4 Brain lesions and memory

The use of lesions made in specific areas of the hippocampus gave more insight into how the hippocampus relates to memory. This technique allowed researchers to implicate

different structures with different forms of memory. Although, as with all brain research, it is understood that various brain regions work as networks, often in tandem with one another, thus, lesion experiments did not conclude results in which scientists claimed that certain areas of the hippocampus were solely responsible for a type of memory, but merely that a certain form of learning and memory was dependent on a certain region. Lesions in hippocampal regions have been associated with impairment of spatial working memory, pattern separation and disrupted novelty detection (Gasbarri et al., 1996; Gilbert et al., 1998; Honey et al., 1998).

1.6.5 Synaptic plasticity and memory

Finally, a great deal of work has been done in the field, relating synaptic plasticity in the hippocampus to various forms of memory. Years of studies involving the use of NMDAR and mGluR antagonists, inducing or blocking LTP and LTD, modulating AMPAR subunits, and many other experiments have provided a window into the relationship between synaptic plasticity in the hippocampus and memory (Moser et al., 1998; Nakao et al., 2002; Escobar and Derrick, 2007; Han et al., 2012). Countless research has shown the association of synaptic plasticity to SWM with the ultimate conclusion, thus far, that bidirectional synaptic plasticity is vital in regulating SWM (Martin and Clark, 2007) (see Fig. 1-3).

1.6.6 Association with cannabinoids

From general patient observation to the relationship of cannabinoids to alterations in synaptic plasticity of the hippocampus, a number of associations have been made that correlate both chronic and acute treatment of cannabinoids to behavioural tasks associated with one or more types of working memory. The 2008 paper from Cammarota's group studied the effects that CB_1R activation (utilizing WIN-55,212-2 as their agonist and VDM-11 to test the endocannabinoid system) after training on novel object recognition tasks. They determined that both of these substances, when given post-training had no affect on short-term memory, yet completely blocked long-term object recognition memory (Clarke et al., 2008).

Beyond this was the Akirav study which showed results for both object recognition tasks and water maze ones and although both showed impairment, among the two, the more pronounced impairment was in the object recognition task (they suggest that the difference in the robustness of these results may be due to the varying aversive levels of the tasks) (Abush and Akirav, 2012).

Using differing cannabinoid treatments and various experimental protocols (T-maze, water maze paradigms, knockout models, etc.) groups including those of Lichtman, Gessa, Martin and Christie among others observed and concluded that cannabinoids cause an impairment of SWM performance (Lichtman and Martin, 1996; Nava et al., 2001; Varvel and Lichtman, 2002; Hill et al., 2004; Wise et al., 2009). The Riedel 2007 work produced seemingly contrasting results to the Christie paper and they suggest in their discussion that the spatial memory deficit that they do see comes from a spatial processing deficit – which in itself arises from alterations in hippocampal neuron firing

(Robinson et al., 2007). Partially due to contrasting opinions in this field, the HU210-induced SWM implication was further evaluated in the paper presented in this thesis (see Discussion).

1.7 Aims

The primary aim of this study is to determine the relation of astroglial and neuronal CB_1Rs with the induction of cannabinoid-evoked LTD at excitatory CA3-CA1 synapses. Based on the work of Araque, Akirav and others and utilizing previous knowledge obtained with hippocampal signalling pathways, as well as various knockout models, I hypothesize that astroglial, and not neuronal, CB_1Rs induce cannabinoid-evoked LTD in the hippocampal CA3-CA1 synapses.

The secondary objective of this thesis is to show the pre-treatment versus post-treatment affects of the CB_1R antagonist AM 281. Castillo suggests that when synaptic alteration can be easily reversed by antagonism, the effect is normally transient. I hypothesize that utilizing the *in vivo* protocol that we do, we will not see a reversal of the synaptic depression by post-treatment with AM 281.

Thirdly, is to determine the relation of protein synthesis with the expression of cannabinoid-evoked LTD at excitatory CA3-CA1 synapses. In this case, based on the work by Kandel, Frey and others, showing a protein synthesis-dependent component of hippocampal long-term depression, I hypothesize that cannabinoid-evoked LTD expression in the hippocampus is maintained by protein synthesis (from pre-existing and newly synthesized mRNA).

The fourth element is to determine the relationship of NMDAR activation with cannabinoid-evoked LTD at excitatory CA3-CA1 synapses. In this aim, our lab has already observed the involvement of these receptors in the induction phase (see Han et al., 2012); I hypothesize that the activation of extrasynaptic NR2B-specific NMDARs may play a role in the early, transient stages post-induction during cannabinoid-evoked LTD in the hippocampus. This NMDAR dependency may persist until further downstream mechanisms take over (i.e. protein synthesis). Furthermore, I hypothesize that other forms of LTD, including DHPG-LTD will be long-lasting as the cannabinoid-evoked form is (i.e. not reversed by antagonism) but will not be dependent on CB_1Rs.

The final aim of this study is two-fold. To first determine whether direct intra-CA1 treatments will match the i.p. injections of HU210 and secondly to observe whether the viewed LTD occurs in the PP-DG pathway (due to the potential involvement of this pathway in spatial working memory impairment). I predict that HU210 treatment will cause a similar LTD when given intra-CA1, related to the involvement of astrocytic CB_1Rs within the CA1 area and that this will not occur when recording from the DG (perhaps due to possible implications that SWM impairments in this area may be related to paradigms beyond synaptic plasticity (Martin and Clark, 2007)).

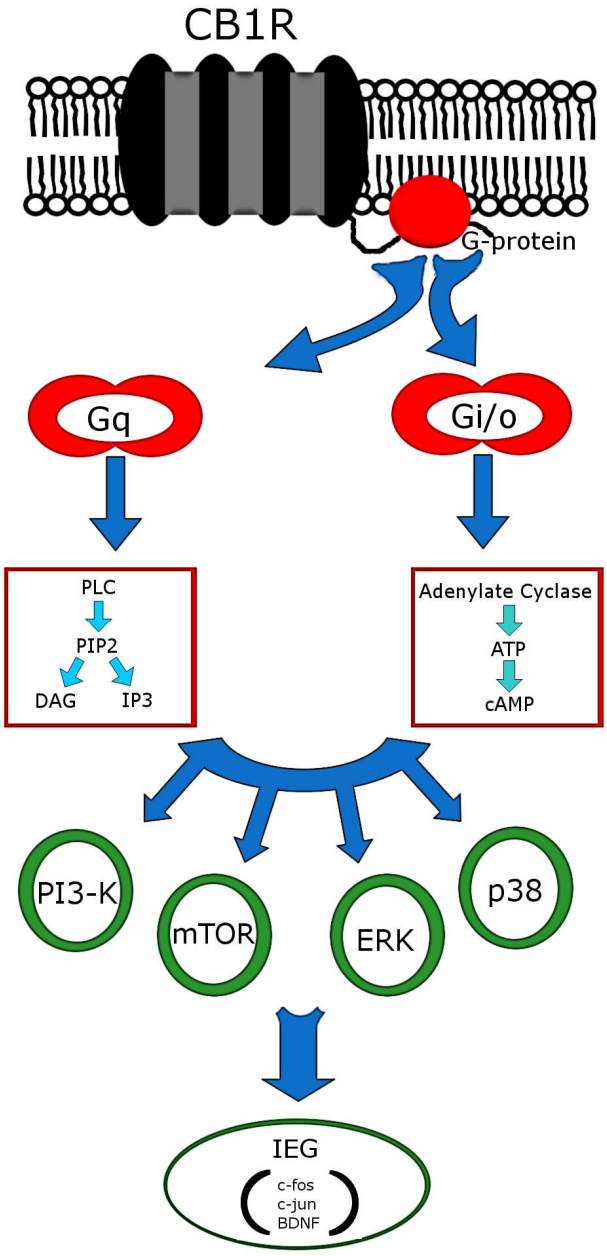

Figure 1-1 Downstream pathways involved in CB_1R activation by cannabinoids. This figure illustrates the numerous downstream pathways that are potentially involved in the activation of CB_1Rs. The top of the figure shows the CB_1R, coupled to a G-protein. The majority of the research shows that, most commonly, the G-protein type is of the $G_{i/o}$ nature (the right column), but it is also believed that in some cases $G_{q/11}$ type proteins are involved (left column) (Navarrete and Araque, 2008). The $G_{i/o}$ type proteins are involved in decreasing the amount of adenylate cyclase, which in turn inhibits the conversion of cAMP from ATP. The $G_{q/11}$ type proteins cause the activation of phospholipase C, which in turn metabolizes PIP2 producing DAG and IP3. These second messengers and/or signal amplifiers (including PKA and others beyond what have been mentioned) have then been seen to activate various pathways including the PI3-K, mTOR, MAPK/ERK, and the p38, ultimately leading to alterations in nuclear transcription or translation, in particular immediate early genes (IEG) including c-fos, c-jun and BDNF.

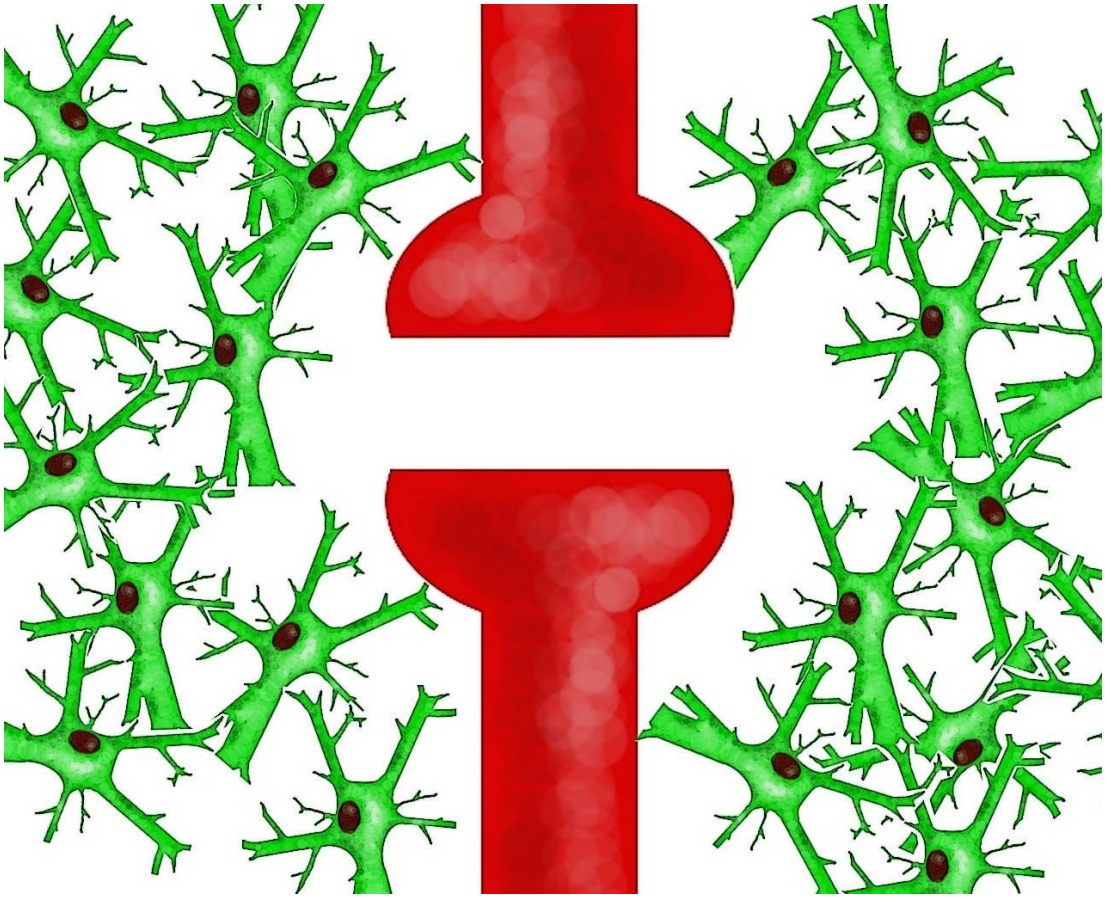

Figure 1-2 Astrocyte network surrounding a neuronal synapse. Glia outnumber neurons 9 to 1 in the brain (Zigmond et al., 1999). Neuronal synapses, including those found in the hippocampus, are surrounded by a vast array of astrocytes, existing in a network. This network is heavily due to the existence of gap junctions on the astrocytes, connecting the individual glial with one another. This intricate connection aids with normal functioning of the brain as well as neuronal modulation. In a thus far unpublished result, our lab discovered that a half knockout of connexin 43 (a glial gap junction protein) using a short hairpin RNA vector (shRNA Cx43) prevented the induction of cannabinoid-evoked CA3-CA1 LTD.

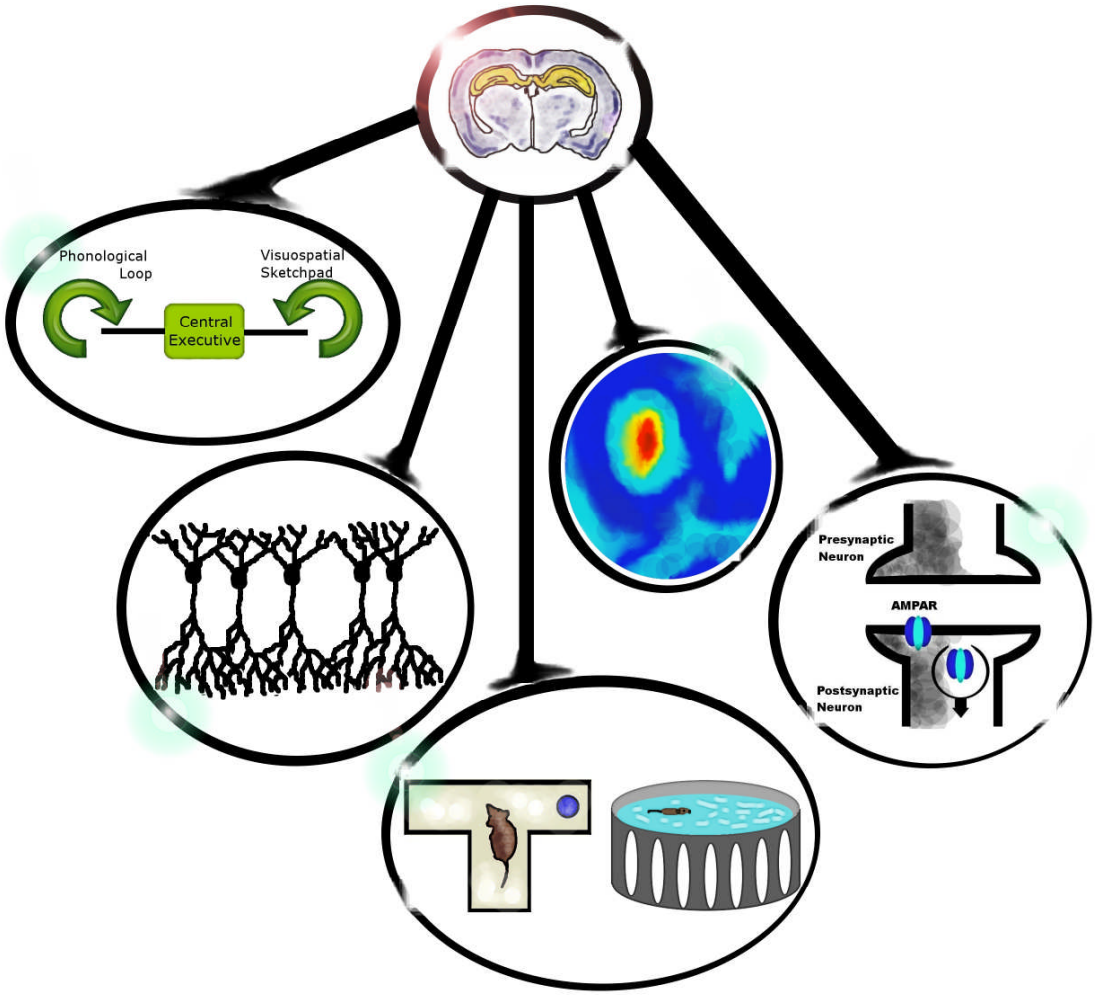

Figure 1-3 Schematic of various components of the working memory paradigm. This schematic shows some of the theories and protocols utilized to show the relationship between the hippocampus and working memory. At the top is a coronal view of the brain with the hippocampus highlighted in yellow. The connections are made (from left to right) first to the Baddeley and Hitch concept of the three elements of WM: the phonological loop, visuospatial sketchpad and central executive. The second circle shows highly branched neurons very organized and in close proximity. This refers to the extremely high organization of the hippocampus into various regions, which has allowed researchers to easily perform electrophysiological experimentation to help decipher the involvement of neuronal firing and transmission in working memory. Thirdly is the illustration of two behavioural mechanisms whereby scientists study WM. These are the T-maze and the Morris water maze. After that, we see a place field, data obtained from studying place cells in the hippocampus and determining the relationship of their firing with the location of objects in space. Finally, we see a synapse whereby an AMPAR is being endocytosed. This is meant to illustrate the involvement of synaptic plasticity in the hippocampus with WM.

Chapter 2: Materials and Methods

2.1 Animal Protocol

Adult male Sprague-Dawley rats (Charles River, Saint-Constant, QC, Canada) weighing 250-350 g were used. Animals were housed 1 or 2 per cage at standard experimental conditions (12 hour 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*. All procedures were performed in accordance with the guidelines established by the Canadian Council on Animal Care as approved by the Animal Care Committee (ACC) of the University of Ottawa Institute Mental Health Research.

Conditional Glu-*CB_1R*-KO and GABA-*CB_1R*-KO mice and inducible GFAP-CB_1R KO mice were provided by Dr. Giovanni Marsicano's lab at University Bordeaux (see Sections 2.4.2 and 2.4.3 as well as Fig. 2-3 for knockout construct details). Mice were housed 2-8 per cage at standard experimental conditions as above.

2.2 Drugs and Vehicles

HU210 (Sigma, St. Louis, USA) was dissolved in DMSO: Tween 80: 0.9 % NaCl (2:1:37). THC (Sigma, St. Louis, USA) had the vehicle Ethanol: Tween 80: 0.9% NaCl (1:1:10). Anisomycin (Sigma, St. Louis, USA) was dissolved in saline. Actinomycin-D (Sigma, St. Louis, USA) was dissolved in DMSO: Tween 80: 0.9 % NaCl (2:1:37). The vehicle for AM 281 (Tocris Bioscience, Ellisville, USA) was DMSO: Tween 80: 0.9 % NaCl (2:1:37). The vehicle for Ro25-6981 (Sigma, St. Louis, USA) was also be 0.9 %

saline. NVP-AAM077 (Novartis Pharma, AG) was dissolved in ddH_2O. Ifenprodil (Sigma, St. Louis, USA) was dissolved in ddH_2O. E4CPG and S-DHPG (Tocris Bioscience, Ellisville, USA) had 0.9% NaCl as their vehicles.

2.3 Electrophysiology

2.3.1 *In vivo* setup

The hippocampal field excitatory postsynaptic potentials (fEPSPs) were recorded using the following protocol. Rats were anesthetised using 40 % urethane (3 ml/kg of body weight, i.p.) complemented with 4 % chloral hydrate (3 ml/kg, i.p.). The rat's temperature was regulated by the use of a temperature therapy mat and T/PUMP at 37 °C (Gaymar, Orchard Park, NY) throughout the entirety of the experiment. The rat's skull was then fixed to a stereotaxic frame (Model 1730, David Kopf Instruments) followed by drilling a hole in the skull and inserting two electrodes into the CA1 area or the angular bundle and dentate gyrus with the following coordinates: (I) CA1 area, B/P -4.00 mm, M/L -2.80 mm, D/V 2.40 mm for the recording electrode (glass micropipette, tip diameter – approx. 0.20 mm, Stoelting, Illinois, USA), and B/P +3.50 mm, M/L +3.84 mm, D/V -2.40 mm for the stimulating electrode (SNEX-200X 70mm, Rhodes Medical Instruments Inc., Summerland, CA) (see Fig. 2-2); (II) dentate gyrus, B/P -2.80 mm, M/L -1.05 mm, D/V 4.10 mm for the recording electrode, and angular bundle, B/P +8.00 mm, M/L +5.42 mm, D/V -2.98 mm for the stimulating electrode (where B/P is posterior to the bregma, M/L is mediolateral, D/V is dorsoventral and the atlas used was Paxinos and Watson, 1998). The stimulating electrode was placed into the correct region manually while the glass

microelectrode containing 2 M NaCl (resistance of 2-4 MΩ) was moved first manually to the brain surface and then by a Hydraulic Micropositioner (Model 2650, David Kopf Instruments) to ensure accuracy. The two electrodes were adjusted to find the best wave (negative wave – to ensure that it is extracellular; apx. 6-18 ms from stimulation point to trough; baseline amplitude of 1-2 mV – gain = 100x).

The recordings were performed with a Model MDA-3 AC Differential Amplifier (BAK Electronics, Inc.). Using a concentric bipolar electrode, fEPSPs were evoked by applying single pulses of stimulation at 0.067 Hz. Stimulus pulse intensities were typically 20-60 nA with a duration of 500 μs. A stimulus intensity that yielded approximately 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). To conclude the experiment, a high frequency stimulation (HFS, 100 Hz train for one sec x 3 times - 20 sec apart) was performed (see Fig. 2-1). If the HFS induced a potentiation the neurons were assumed to be in good condition and the observed LTD was not due to damage or fatigue (see Fig. 2-2). If the HFS did not evoke a response, the result could not be considered a consequence of the pharmacological treatment alone and thus was not accepted as a legitimate observation.

2.3.2 Intraperitoneal treatments

In all cases, once the ideal placement of the electrodes was established, a recording was made of baseline conditions for at least 20 minutes. The initial study involved HU210 (50 μg/kg, i.p.) treatment and AM 281 (3 mg/kg, i.p.) (Cui et al., 2001) a CB_1R antagonist or its vehicle was administered 10 minutes before, 10 minutes after (Fig. 3-2) and 30

minutes after (Fig. 3-5). The fEPSPs were recorded for 120 minutes and then HFS was performed on the rat. Using the 50 μg/kg, i.p. dose for HU210 was chosen for several reasons: (I) by testing different doses, it was determined that both 50 and 100 μg/kg, i.p. gave a functional effect; (II) this dose fits well within the confines of the dose response of this agonist of 3-100 μg/kg to various tests including rectal temperature, catalepsy, locomotor behaviour and tail-flick (Little et al., 1989); (III) previous literature reveals the use of this dose and higher doses in numerous plasticity and memory-related experimentation (Ferrari et al., 1999; Hill et al., 2004; Robinson et al., 2007).

Anisomycin (18 mg/kg, i.p.) (Puighermanal et al., 2009), a protein translation inhibitor, or vehicle was given to the rat. The baseline conditions were established in the recording and 2 hours after the anisomycin treatment, HU210 (50 μg/kg, i.p.) was administered to the rat. The fEPSPs were recorded for 120 minutes and then the HFS control was done (Fig. 3-3 A).

Ro25-6981 (6 mg/kg, i.p.) (Fox et al., 2006) and ifenprodil (5 mg/kg, i.p.) (Higgins et al., 2005), NR2B subunit antagonists, NVP-AAM077 (1.2 mg/kg, i.p.) (Fox et al., 2006), an NR2A antagonist, or their vehicles were given 30 min. after HU210 (50 μg/kg, i.p.) treatment (Fig. 3-4, 3-5). The experiment was conducted for 120 minutes. Following this, the experiment in which the same conditions existed, however, the rat was treated with Ro25-6981 (6 mg/kg, i.p.) 1 hour after HU210 treatment was performed (Fig. 3-4 C). Recording went for 120 minutes followed by HFS. Ro25-6981 (6 mg/kg, i.p.) was also tested alone, without any cannabinoid treatment (Fig. 3-4 C).

2.3.3 Intracerebroventricular treatments

Actinomycin-D (72 µg/12 µl, i.c.v.) (Manahan-Vaughan et al., 2000), a transcription inhibitor, or vehicle was given to the rat. The intracerebroventricular microinjections were performed by implanting a cannula guide (22G, 20 mm, HRS Scientific, Montreal, QC) just above the lateral ventricle (LV). The needle (30G, 20 mm, PlasticsOne, Roanoke, VA) threaded through the cannula releases the drug directly in the LV where it is understood that it will spread throughout the brain. The coordinates for the cannula guide from the bregma were: B/P: -0.92 mm, M/L: -1.40 mm, D/V: -2.60 mm (Peterfi et al., 2010). The needle was 0.90 mm deeper than the guide cannula. The placement coordinates were verified by colleagues, who injected fast green through the cannula guide then immediately performed a decapitation, removing and freezing the brain to prevent spreading of the fast green. Finally brain slices were made to determine that it was in the LV. Movement of the cannula was prevented by the use of screws and cement mix. The microinjection was given at a pace of approximately 1 µl/min. The baseline conditions were established in the recording and 2 hours after the actinomycin-D treatment, HU210 (50 µg/kg, i.p.) was administered to the rat. The fEPSPs were recorded for 120 minutes and then the HFS control was performed (Fig. 3-3 A).

As controls, experiments were done to evaluate the affects of AM 281 on DHPG-induced LTD. E4CPG (35 nM/3.5 µl, i.c.v) was given 5 min. prior or 5 min. after an injection of S-DHPG (100 nM/5 µl, i.c.v) (Fig. 3-6 A). Comparatively, AM 281 (3 mg/kg i.p.) was given 5 min. prior to an injection of S-DHPG (100 nM/5 µl, i.c.v) (Fig. 3-6 C). HFS was performed at the end of the 120 min. recordings.

2.3.4 Iontophoresis treatments

Iontophoresis was used for experiments with anesthetized rats, where the procedure paralleled the above electrophysiology protocol, except for the treatment. In these experiments, a multi-barreled micropipette was utilized to both record from the CA1 region and dispense the drug. The experimentation consisted of HU210 administration at 50 µg/ml, iontophoretic ejection at -40 nA for 30 sec. The same iontophoretic ejection was performed using the HU210 vehicle: DMSO: Tween 80: 0.9 % NaCl, as a control (Fig. 3-7 A).

Initially, the literature was searched for general iontophoretic experimentation with various drugs, including antidepressants and glutamate (Haddjeri and Blier, 1995; Kiyatkin and Rebec, 1999). The current and duration were chosen primarily because of a functional effect. The HU210 had a slightly negative charge and a pH of 8.5, and thus the chosen negative current would repel it out of the barrel into the CA1 region.

2.4 Knockout Mice Models

2.4.1 Electrophysiology

The CA3-CA1 field excitatory postsynaptic potentials were recorded using the following protocol. Mice were initially anesthetized in a box with 4 % halothane. The mouse's temperature was regulated at approx. 37 ±1 °C by the use of a temperature therapy mat and T/PUMP and verified with a rectal thermometer (Harvard Apparatus, S.A.R.L., France) throughout the entirety of the experiment. The mouse's skull was then fixed to a stereotaxic frame (Unimecanique, France) and nose placed in an anesthetic mask, whereby the mouse received 1.5% halothane anesthesia for the duration of the

experiment. The locations for the two electrodes (stimulating and recording) were found, using the bregma as a guide. The coordinates for the recording electrode (glass micropipette, tip diameter – approx. 0.10-0.20 mm, Harvard Apparatus Ltd., France) from the bregma were: B/P: -1.94 mm, M/L: -1.20 mm, D/V: 1.33 mm. The coordinates for the stimulating electrode (concentric biopolar, 250 μm diameter, Phymep, Paris, France) from the bregma were: B/P: +1.82 mm, M/L: +2.13 mm, D/V: -1.70 mm. The stimulating electrode was implanted into the correct region manually while the recording electrode was moved first manually to the brain surface and then by Micropositioner (Unimecanique, France) to ensure accuracy. The two electrodes were then adjusted to find the best wave.

The recordings were performed with a Model 1700 Differential AC Amplifier (A-M Systems). The fEPSPs were evoked at 0.067 Hz using a concentric bipolar electrode every 15 seconds. Stimulus pulse intensities were typically 1-3 mA with a duration of 100 μs (selected due to equipment available in Bordeaux – less than minimal degree of stimulation but still physiologically relevant). A stimulus intensity that yielded approximately 60% of the maximal response was selected for baseline measurements – using a Model DS3 Isolated Constant Current Stimulator (Digitimer Ltd.). A glass microelectrode full of 0.5M sodium acetate and 2% pontamine sky blue was used as the recording electrode. Spike2 software was utilized to record data. KO or wild-type mice were treated with either THC (5 mg/kg, i.p.) or vehicle and recorded for 120 min.

2.4.2 $CB_1^{f/f}$ mice

$CB_1^{f/f}$ mice were generated and genotyped (PCR analysis of genomic material obtained through tail clippings) by Dr. Giovanni Marsicano's lab using the following protocol. The $CB_1^{f/f}$ were generated by crossing two mouse lines. The first carried the *CB1*-floxed-neo allele. A genomic library was screened to isolate fragments of DNA that contained the *CB1* locus and two loxP sites were used to flank the *CB1* gene. In order to create the final construct, two homology arms and a FRT-flanked PGK-neo cassette (encoding the neomycin resistance gene with a eukaryotic promoter) were also used (Marsicano et al., 2002). The *CB1*-floxed-neo allele was thus created by electroporating this construct into E14 mouse embryonic stem cells (Marsicano et al., 2002). These mice were then crossed with flipase-deleter mice. Flipase-deleter mice involved utilizing a vector containing Flp recombinase target sites (pFRTZ). This vector was developed by inserting the *Hind*III/*Sal*I fragment from pSLh3APr-lacZ-pA into the *Hind*III and *Sal*I sites of $pFRT_2$neo.lacZ (Dymecki, 1996). From these plasmid sequences, the transgenes were purified and injected into fertilized eggs, allowing the development of a mouse line. This cross is performed for it allows for the deletion of the FRT-PGK-Neo selection cassette (Marsicano et al., 2003) (see Fig 2-3).

2.4.3 Glu-*CB1R*-KO mice

Conditional Glu-*CB1R*-KO mice were generated and genotyped by Dr. Giovanni Marsicano's lab using the following protocol. The Glu-*CB1R*-KO mice were generated by crossing $CB_1^{f/f}$ (floxed *CB1R* gene carrying mice) and *NEX*-Cre mice (Monory et al.,

2006). The mutant mice had variable genetic backgrounds but the majority contribution came from the C57BL6/6NCrlBR (Monory et al., 2006).

This $CB_1^{f/f}$ transgenic line (generation previously described in section 2.4.2) was crossed with *NEX*-Cre mice. The Cre-recombinase sequence in these mice is associated with the *NEX* locus (prominent in the hippocampal neurons) (Kleppisch et al., 2003) (see Fig 2-3).

2.4.4 GABA-CB_1R-KO mice

Conditional GABA-CB_1R-KO mice were generated and genotyped by Dr. Giovanni Marsicano's lab using the following protocol. The GABA-CB_1R-KO mice were generated by crossing $CB_1^{f/f}$ (floxed CB_1R gene carrying mice) and *Dlx5/6*-Cre mice (Monory et al., 2006). The generation of $CB_1^{f/f}$ is described previously in section 2.4.2.

The *Dlx5/6*-Cre mice are generated using the following protocol. I56i and I56ii intergenic enhancer sequences were used to control the *Cre* gene. A plasmid with a *Cre* coding sequence was created and a fragment from the zebrafish *dlx5a/dlx6a* locus was added (Monory et al., 2006). The transgenic mice were produced by excising the transgene from the plasmid and injecting it into eggs (Zerucha et al., 2000) (see Fig 2-3).

2.4.5 GFAP-CB_1R-KO mice

GFAP-CB_1R-KO mice were generated at the Marsicano lab in Bordeaux, France utilizing the following procedure. Two genetically altered mouse lines were crossed using a three-

step backcrossing procedure; these were $CB_1^{f/f}$ (floxed CB_1R gene carrying mice) and GFAP-CreERT2 mice (Hirrlinger et al., 2006). The mutant mice had variable genetic backgrounds but the majority contribution came from the C57BL6-N strain (Han et al., 2012). The generation of $CB_1^{f/f}$ is described previously in section 2.4.2.

The GFAP-CreERT2 mice were generated following the protocol developed by Kirchhoff's group. A fusion protein was developed combining an estrogen receptor's (ER) mutated ligand-binding domain and DNA recombinase Cre. This fusion protein was controlled by the human GFAP promoter (Hirrlinger et al., 2006). The overall vector used was generated using the previously mentioned promoter along with the open reading frame of CreERT2, tamoxifen-inducible Cre-recombinase, an immunoglobulin G splice acceptor site and an adenovirus splice donor (together make an intron used to express the transgene in a broad range of tissues), as well as the human growth hormone polyA site (for structural stability) (Hirrlinger et al., 2006; Choi et al., 1991; Fig. 2-3). The GFAP-CreERT2 DNA fragment was eventually injected into fertilized oocytes. The backcrossing procedure thus gave $CB_1R^{f/f;GFAP\text{-}CreERT2}$ (GFAP-CB_1R-KO**)** and $CB_1R^{f/f}$ (GFAP-CB_1R-WT) littermates (see Fig 2-3).

Tamoxifen (1 mg, i.p. – vehicle of 90% sunflower oil and 10% ethanol – given at a concentration of 10 mg/ml – Hirrlinger et al., 2006) – an ER agonist, was used to activate the CreERT2 protein (causes the release of CreERT2 and thus allows it to move into the nucleus where gene alteration can occur – Hirrlinger et al., 2006). The treatment was eight injections/day for eight weeks in order to delete the astroglial CB_1R gene with a recovery period of four weeks. The lack of protein was verified by a double immunohistochemistry analysis (Han et al., 2012).

2.5 Statistical Analysis

SPSS 9.0 for Windows was used for analysis of statistics. Results are reported as means ± SE. Statistical analysis will be performed utilizing one-way ANOVAs, followed with the Tukey HSD *post hoc* test. Statistical significance is noted for $p < 0.05$.

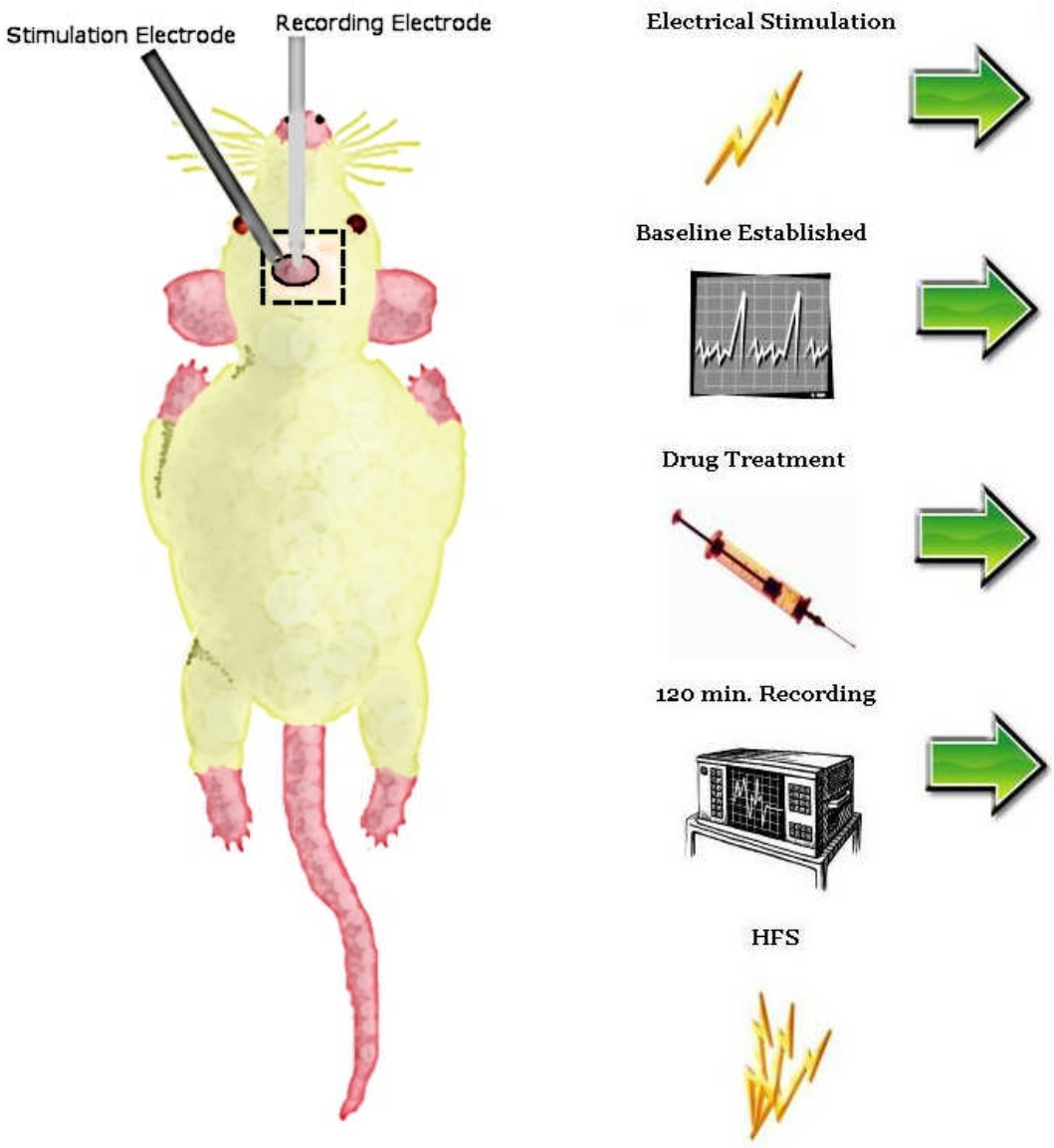

Figure 2-1 Illustration of *in vivo* electrophysiology protocol. The illustration shows a rat in an anesthetised state as would exist when it would be placed in the stereotaxic frame. The two electrodes are inserted into the brain at the proper coordinates, through a small hole drilled into the skull. In the right column is the general protocol for many of the experiments performed in this study. This follows the general outline: electrical stimulation, given in a constant, unchanging fashion to implement basal transmission, this is recorded as the baseline for a minimum of twenty minutes. Following this, whatever drug treatment that we were interested in was given, followed by a recording for a minimum of two hours. Finally, high frequency stimulation was given at the end of the experiment to ensure that the neurons were still responsive and the synaptic transmission was not altered due to damage.

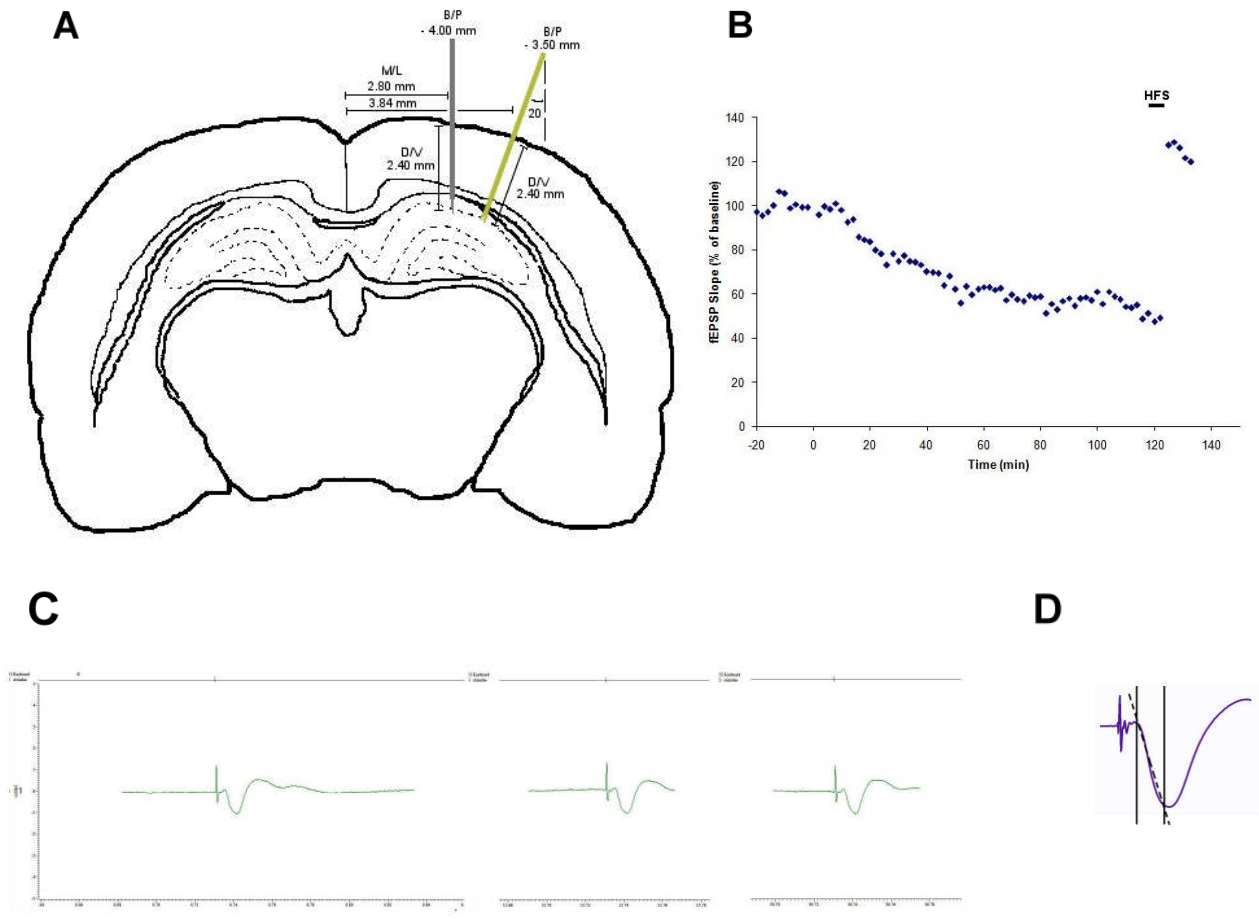

Figure 2-2 Schematic showing various protocols involved in the study. A. Brain cross section showing placement of the two electrodes: stimulating (gold – B/P +3.50 mm, M/L +3.84 mm, D/V -2.40 mm) and recording (grey – B/P -4.00 mm, M/L -2.80 mm, D/V 2.40 mm) for the CA1 region. **B.** Sample graph showing the HFS induced response at approx. 120 min. (black bar above the graph) and the resulting potentiation. **C.** Sample of data recorded – 3 fEPSP responses 15 seconds apart. Ticks on the top bar represent when the stimulation was given. Voltage on the y-axis and Time on the x-axis. **D.** The paramaters for a measured slope of a typical fEPSP.

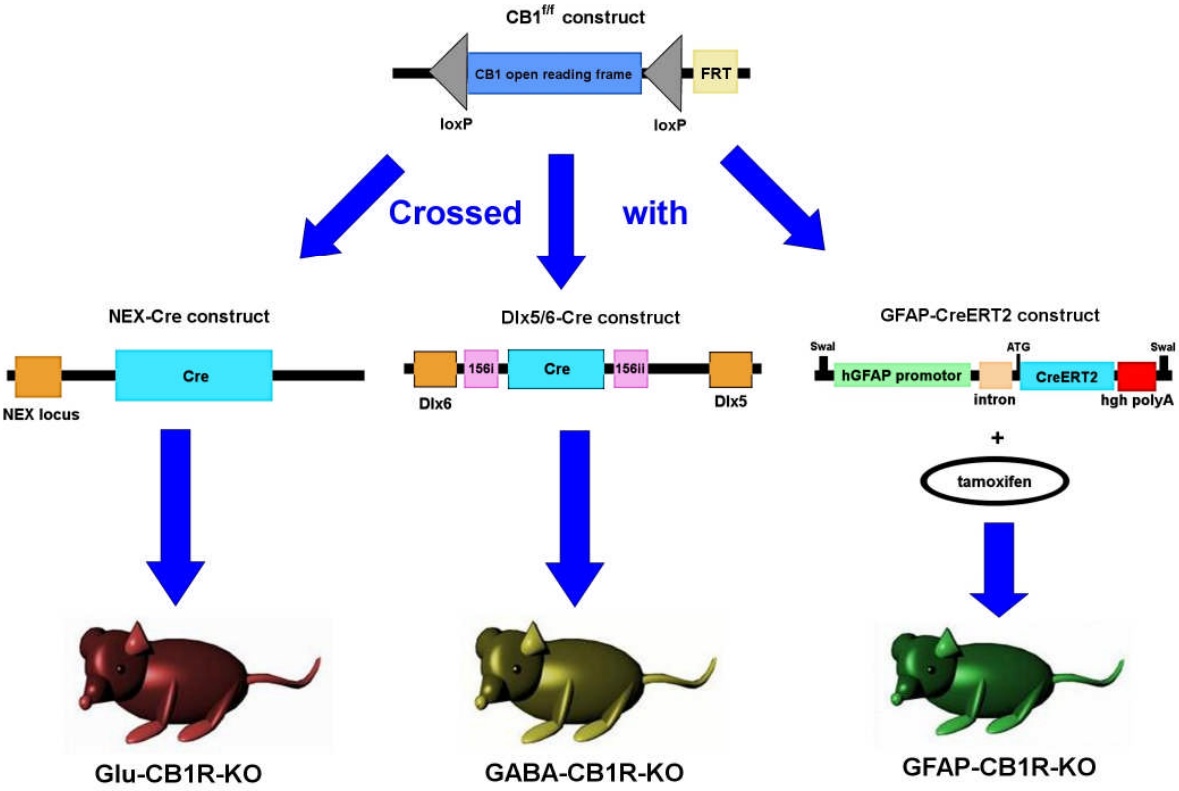

Figure 2-3 Illustration of various knockout mice models and the schematics of their constructs. The figure shows the three lines of knockout mice used in this study. All were developed by crossing $CB_1^{f/f}$ with different other transgenic models. The $CB_1^{f/f}$ construct has the *loxP* sites (grey triangles) flanking the CB_1 open reading frame (blue box), with FRT standing for the Flp recombinase recognition target site (beige box). In the left column, the $CB_1^{f/f}$ mice are crossed with *NEX*-Cre mice (containing the Cre-recombinase sequence (light blue box) associated with the *NEX* locus (orange box)) to generate the Glu-CB_1*R*-KO mouse line. The middle column shows the $CB_1^{f/f}$ mice crossed with *Dlx5/6*-Cre mice (containing the *Cre* coding sequence (light blue box) contained by 156i and 156ii intergenic enhancer sequences (pink boxes) at the *Dlx5* and *Dlx6* sites (orange boxes)) to generate the GABA-CB_1*R*-KO mouse line. The right column shows the $CB_1^{f/f}$ mice crossed with GFAP-CreERT2 mice (containing the human GFAP promoter (green box), the tripartite intron (beige box), the CreERT2 open reading frame (light blue box) and the human growth hormone polyA site (red box), as well as SwaI restriction sites and ATG – the transcription initiation site), then treated with tamoxifen to generate the GFAP-CB_1*R*-KO mouse line.

Chapter 3: Results

3.1 GFAP-*CB1R*-KO mice do not experience cannabinoid-evoked hippocampal LTD while Glutamate and GABA-*CB1R*-KO mice still do

GFAP-CB_1R-KO mice as well as their GFAP-CB_1R-WT line (containing the floxed sites and tamoxifen treatment) received an injection of THC (5 mg/kg, i.p.). The GFAP-CB_1R-KO mice did not experience any change in synaptic transmission, while the GFAP-CB_1R-WT experienced the equivalent LTD as seen in the rats (to around 40% of baseline conditions). The wild-type mice also received vehicle treatment as a control, which resulted in no affect to synaptic transmission (see Fig. 3-1 A).

Glu-CB_1R-KO and GABA-CB_1R-KO mice as well as their WT line all received an injection of THC (5 mg/kg, i.p.). Both KO mice and their WT counterparts all experienced the equivalent LTD as seen in the rats (to around 40% of baseline conditions) (see Fig. 3-1 C).

THC (5 mg/kg, i.p.) was used for the mice while HU210 (50 μg/kg, i.p.) was utilized for the rats due to the lack of availability of HU210 at the Marsicano lab in Bordeaux. Experimentation was performed by myself and colleagues to verify that a very similar LTD effect was seen when treating both rats and mice with these doses of these two cannabinoids.

3.2 AM 281 pretreatment but not post-treatment, prevents HU210 -evoked hippocampal LTD

The rats received a treatment of AM 281 (3 mg/kg, i.p.) 10 min prior to an HU210 injection. In these conditions, the HU210-evoked LTD was completely blocked. This was not the case for the vehicle treatment. When the AM 281 treatment was given 10 min after the HU210 injection, the normally viewed LTD to approximately 40% of baseline conditions occurred (see Fig. 3-2).

3.3 Protein synthesis is a component of cannabinoid-evoked expression of later-phase LTD at excitatory CA3-CA1 synapses

It was shown by my colleagues, that AMPAR endocytosis plays a key role in the expression of cannabinoid-evoked hippocampal CA3-CA1 LTD, thus my desire was to look forward to discover what else is involved in the expression stage, in particular the late phase. When the rats received a two hour pre-treatment with anisomycin (18 mg/kg, i.p.), which acts on the 60S ribosomal subunit and prevents the formation of the 80S complex therefore preventing elongation and thus protein synthesis (van Venrooij et al., 1977), followed by HU210 treatment, L-LTD was blocked (approximately +40 min after HU210 treatment) without altering E-LTD (initial approximately 30 min). Baseline conditions were gradually re-established after dropping to around 70% during the initial 40 minutes of LTD (see Fig. 3-3).

Another experiment was designed where rats received a two hour pre-treatment with actinomycin-D (72 μg/12 μl, i.c.v.), which acts on the transcription initiation complex and therefore inhibits RNA-chain elongation (Sobell, 1985), and then HU210 treatment, L-LTD was blocked (approximately +30 min after HU210 treatment) without altering E-LTD (initial approximately 30 min). Baseline conditions were gradually re-established after dropping to around 70% during the initial 30 minutes of LTD (see Fig. 3-3).

3.4 NR2B-containing NMDAR antagonists return depressed transmission to baseline conditions in HU210-evoked hippocampal LTD

It had previously been discovered (in Han et al., 2012) that pre-treatment with ifenprodil, and Ro25-6981 but not NVP-AAM077 prevented the induction of cannabinoid-evoked LTD, so we continued on this thought process to see if we could determine a time-line for the involvement of NMDARs (see Fig. 3-4 A, 3-5 A). No change in LTD (i.e. the fEPSP slopes decreased to approximately 40 % of the baseline) was seen when the rats were treated with the NR2A-specific antagonist NVP-AAM077 (1.2 mg/kg, i.p.) or the CB_1R antagonist AM 281 (3 mg/kg, i.p.) as well as their vehicles, 30 minutes after HU210 treatment (see Fig. 3-5 A). This indicates that beyond the knowledge we previously obtained of the lack of involvement of these types of receptors in induction, the NR2A subunit of NMDARs and the CB_1Rs are not involved in the transient post-induction depression of cannabinoid-evoked LTD in the CA3-CA1 synapses.

When treated with the NR2B-specific antagonist Ro25-6981 (6 mg/kg, i.p.) or vehicle, 1 hour after HU210 (50 μg/kg, i.p.) treatment the following was observed. The slope of the fEPSPs decreased to approximately 40 % of baseline conditions in the first hour after HU210 treatment. After Ro25-6981 treatment, the slopes of fEPSPs stayed at 40 % of baseline (see Fig. 3-4 C). When the treatment of Ro25-6981 or another structurally different NR2B-specific antagonist ifenprodil was given 30 minutes after HU210 administration, LTD was blocked and the fEPSPs returned to baseline conditions (see Fig. 3-4 A). This was not seen with the vehicle treatments. This begins to imply that the activation of NR2B-containing NMDARs is involved in the transient post-induction depression of cannabinoid-evoked LTD in the CA3-CA1 synapses.

3.5 E4CPG blocks S-DHPG induced LTD through pretreatment but not post-treatment while AM 281 does not block S-DHPG induced LTD

To understand this process better, numerous controls were performed to compare the NMDAR-dependent cannabinoid-LTD that we viewed with an mGluR-dependent form of LTD. The rats received a treatment of the mGluR antagonist E4CPG (35 nM/3.5 μl, i.c.v) 5 min prior to an injection of the mGluR agonist S-DHPG (100 nM/5 μl, i.c.v). In these conditions, the S-DHPG-evoked LTD was completely blocked by E4CPG. When the E4CPG treatment was given 5 min after the S-DHPG injection, the normally viewed LTD occurred. This was the same in the case for the vehicle treatment (see Fig. 3-6 A).

In another experiment, rats were given AM 281 (3 mg/kg, i.p.) 5 min before S-DHPG treatment and no change in the LTD occurred (see Fig. 3-6 C). These experiments acted as controls, showing that AM 281 is specific for cannabinoid-evoked LTD and not other forms of LTD.

3.6 Equivalent HU210-evoked LTD occurs in excitatory CA3-CA1 synapses when given intra-CA1 as in systemic injections

The rats received a treatment of HU210 (50 μg/ml, iontophoretic ejection at -40 nA for 30 sec). In this experiment, the HU210-evoked LTD was equivalent to the intraperitoneal treatments shown by our lab previously (i.e. a reduction of fEPSP slope to approximately 40% of baseline conditions) (see Fig. 3-7 A). Due to the notion that the drug in these experiments was given directly into the brain opposed to i.p. may lead one to believe that the onset of LTD would have been quicker. However, the observation made showed a similar latency to the intraperitoneal treatments (see Fig. 3-1 to 3-5). The speculation that can thus be made is that the onset of action of the cannabinoid occurs within the first 5 min. even when given i.p. From here, the physiological processes proceed slowly and reach a plateau at approximately 90 minutes. The same iontophoretic ejections (-40 nA for 30 sec.) was performed using the vehicle for HU210, where no fEPSP reduction occurred (see Fig. 3-7 A).

3.7 HU210 has very little affect in synaptic transmission in the perforant path-dentate gyrus pathway

The rats received a treatment of HU210 (50 μg/mg, i.p.) while recording in the dentate gyrus region of the hippocampus. The HU210 did not evoke significant depression of synaptic transmission in this region. The reduction of fEPSP slopes only went to approximately 82 % of baseline conditions (see Fig. 3-7 C).

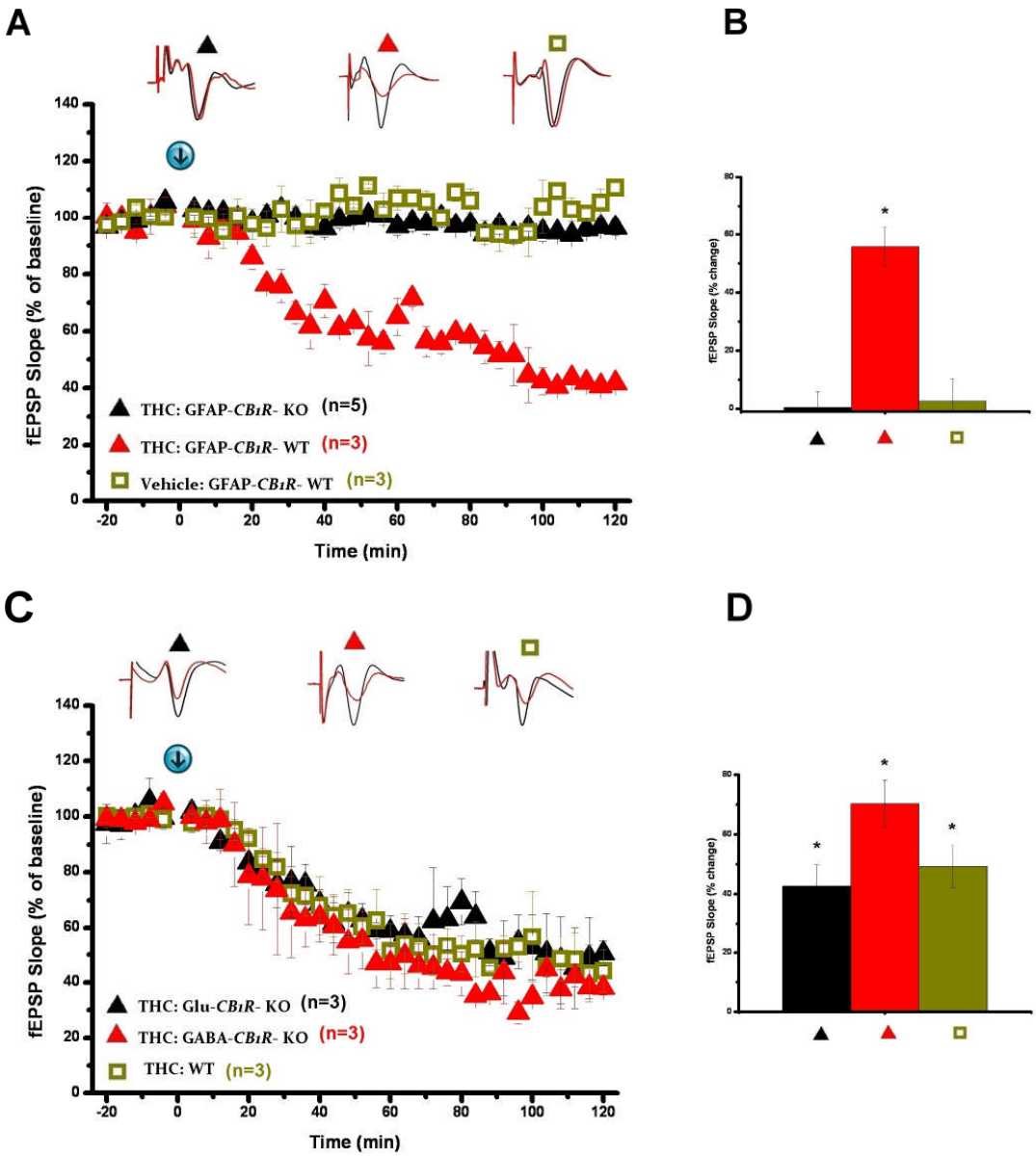

Figure 3-1 GFAP-*CB$_1$R*-KO mice do not experience cannabinoid-evoked hippocampal LTD while Glutamate and GABA-*CB$_1$R*-KO mice do. A. THC (5 mg/kg, i.p.) was injected into GFAP astrocytic *CB$_1$R* KO (black triangles) or WT mice (red triangles). Ethanol: Tween 80: 0.9 % NaCl vehicle was injected into GFAP WT mice (gold squares). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for THC or vehicle treatment from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test). **C.** THC (5 mg/kg, i.p.) was injected into Glu-*CB$_1$R*-KO (black triangles), GABA-*CB$_1$R*-KO (red triangles) or WT mice (gold squares). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **D.** Percent change of fEPSPs for THC or vehicle treatment from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

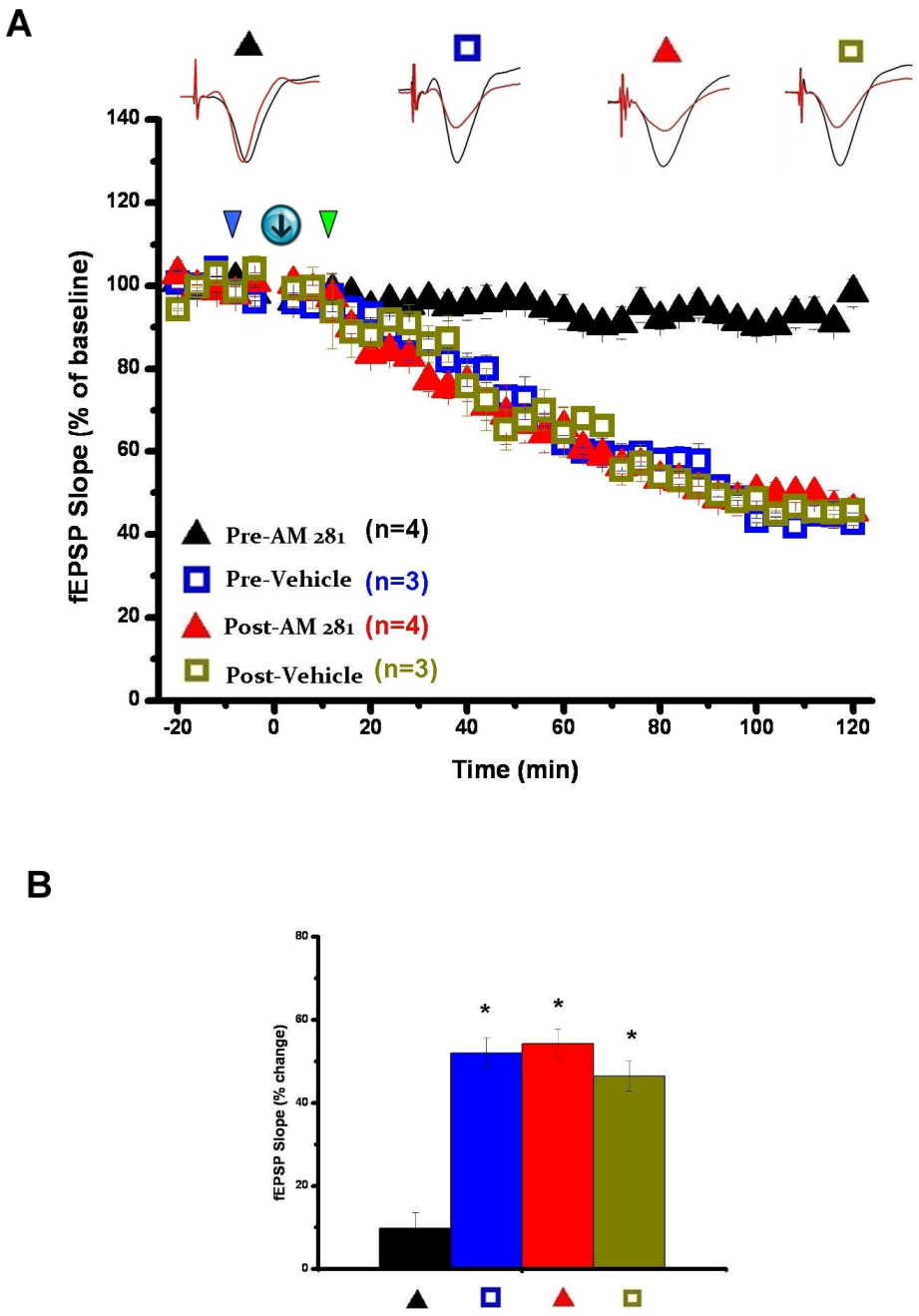

Figure 3-2 AM 281 pretreatment but not post-treatment, prevents HU210-evoked hippocampal LTD. **A.** AM 281 (3 mg/kg, i.p.) injected 10 minutes before (blue arrowhead) HU210 (50 µg/kg, i.p.) treatment (black triangles) or 10 min. after (red triangles) (green arrowhead). DMSO: Tween 80: 0.9 % NaCl vehicle injected 10 minutes before (blue arrowhead) HU210 (50 µg/kg, i.p.) treatment (blue squares) or 10 min. after (gold squares) (green arrowhead). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for AM 281 or vehicle treatment from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

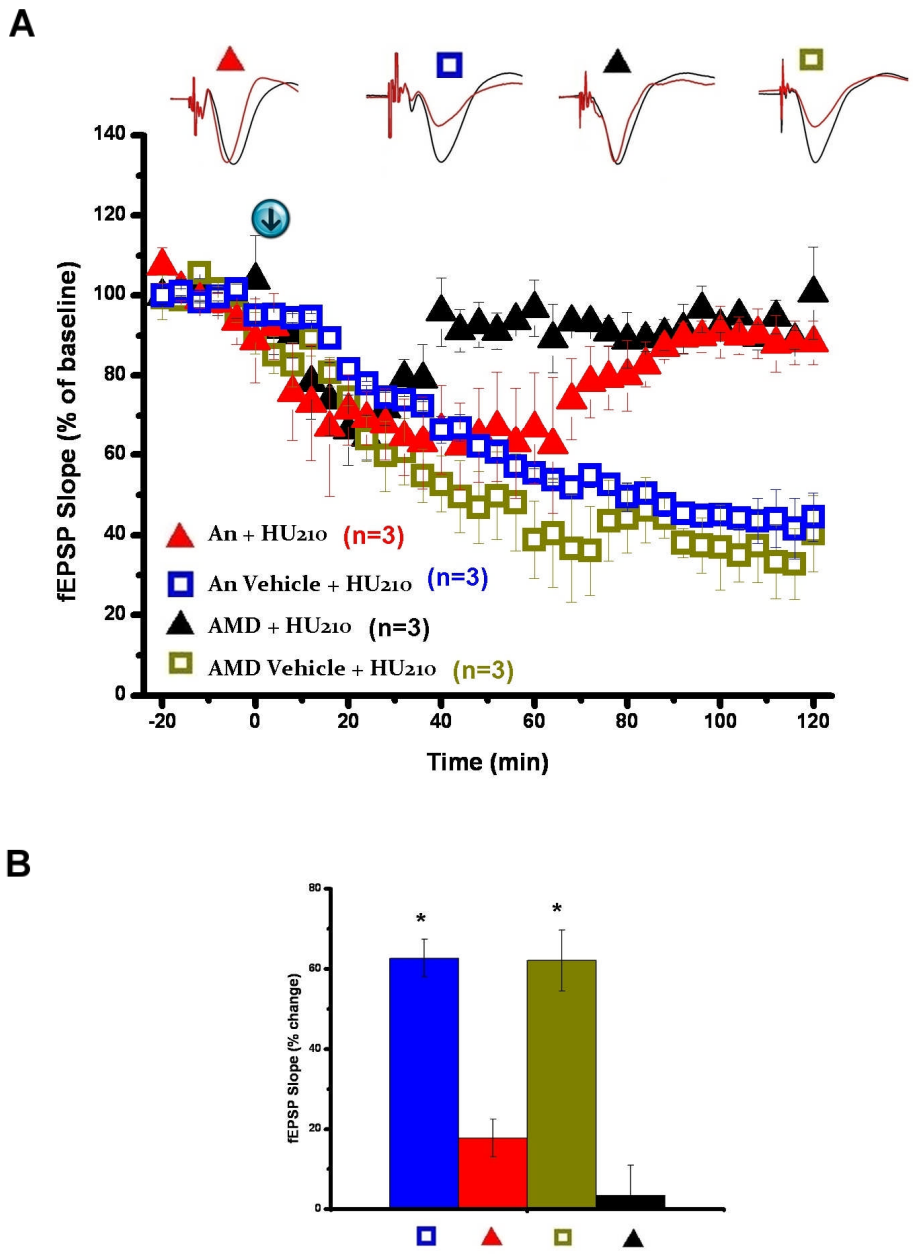

Figure 3-3 Anisomycin and actinomycin-D return depressed transmission to baseline conditions in HU210-evoked hippocampal LTD. A. 2 hour pretreatment of anisomycin (18 mg/kg, i.p.), followed by HU210 (50 µg/kg, i.p.) treatment (red triangles) or 2 hour pretreatment of actinomycin-D (black triangles). 0.9 % saline vehicle for anisomycin vehicle injected 2 hours prior to HU210 (50 µg/kg, i.p.) treatment (blue squares) or 0.9 % saline vehicle for actinomycin-D vehicle injected 2 hours prior (gold squares). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for anisomycin, actinomycin-D or vehicle treatments from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

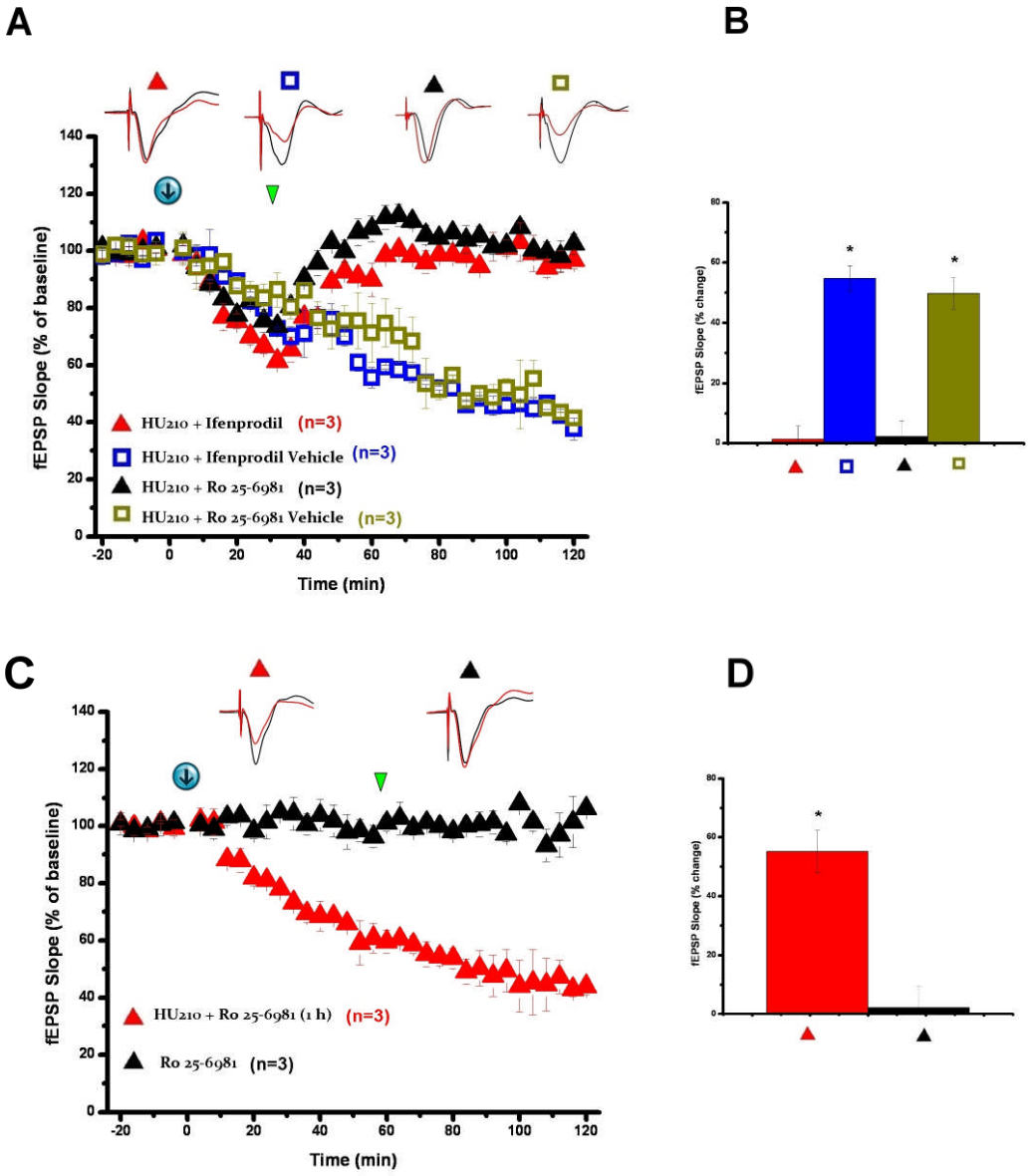

Figure 3-4 NR2B-containing NMDAR antagonists return depressed transmission to baseline conditions in HU210-evoked hippocampal LTD. A. Ro25-6981 (6 mg/kg, i.p.) injected 30 minutes after HU210 (50 µg/kg, i.p.) treatment (black triangles) or ifenprodil (5 mg/kg, i.p.) injected 30 minutes after HU210 (50 µg/kg, i.p.) treatment (red triangles). 0.9 % saline vehicle injected 30 minutes after HU210 (50 µg/kg, i.p.) treatment (gold squares) or ddH_2O vehicle injected 30 minutes after HU210 (50 µg/kg, i.p.) treatment (blue squares). 30 minute post HU210 treatment denoted by a green arrowhead. Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for Ro25-6981, ifenprodil or vehicle treatments from baseline to 110 min. into recording. **C.** Ro25-6981 (6 mg/kg, i.p.) injected 1 hour after HU210 (50 µg/kg, i.p.) treatment (red triangles) (treatment time denoted by green arrowhead). Ro25-6981 (6 mg/kg, i.p.) injected alone (black triangles). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **D.** Percent change of fEPSPs for Ro25-6981 treatments from baseline to 110 min. into recording. All Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

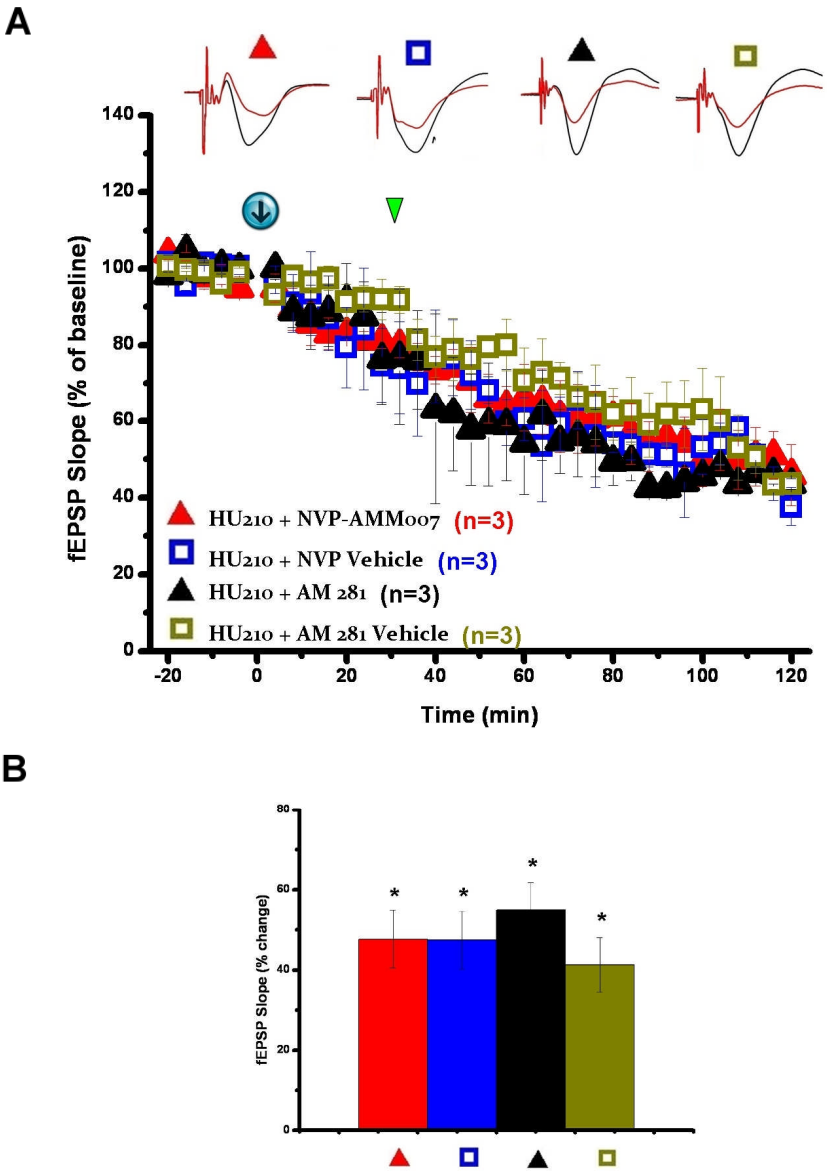

Figure 3-5 NR2A-containing NMDAR antagonists and AM 281 do not return depressed transmission to baseline conditions in HU210-evoked hippocampal LTD. A. NVP-AAM077 (1.2 mg/kg, i.p.) injected 30 minutes after HU210 (50 µg/kg, i.p.) treatment (red triangles) or AM 281 injected 30 min. after (black triangles). ddH_20 vehicle injected 30 minutes after HU210 (50 µg/kg, i.p.) treatment (blue squares) or DMSO: Tween 80: 0.9 % NaCl vehicle injected 30 min. after (gold squares) (treatment time denoted by green arrowhead). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for NVP-AAM077, AM 281 or vehicle treatments from baseline to 110 min. into recording. All Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

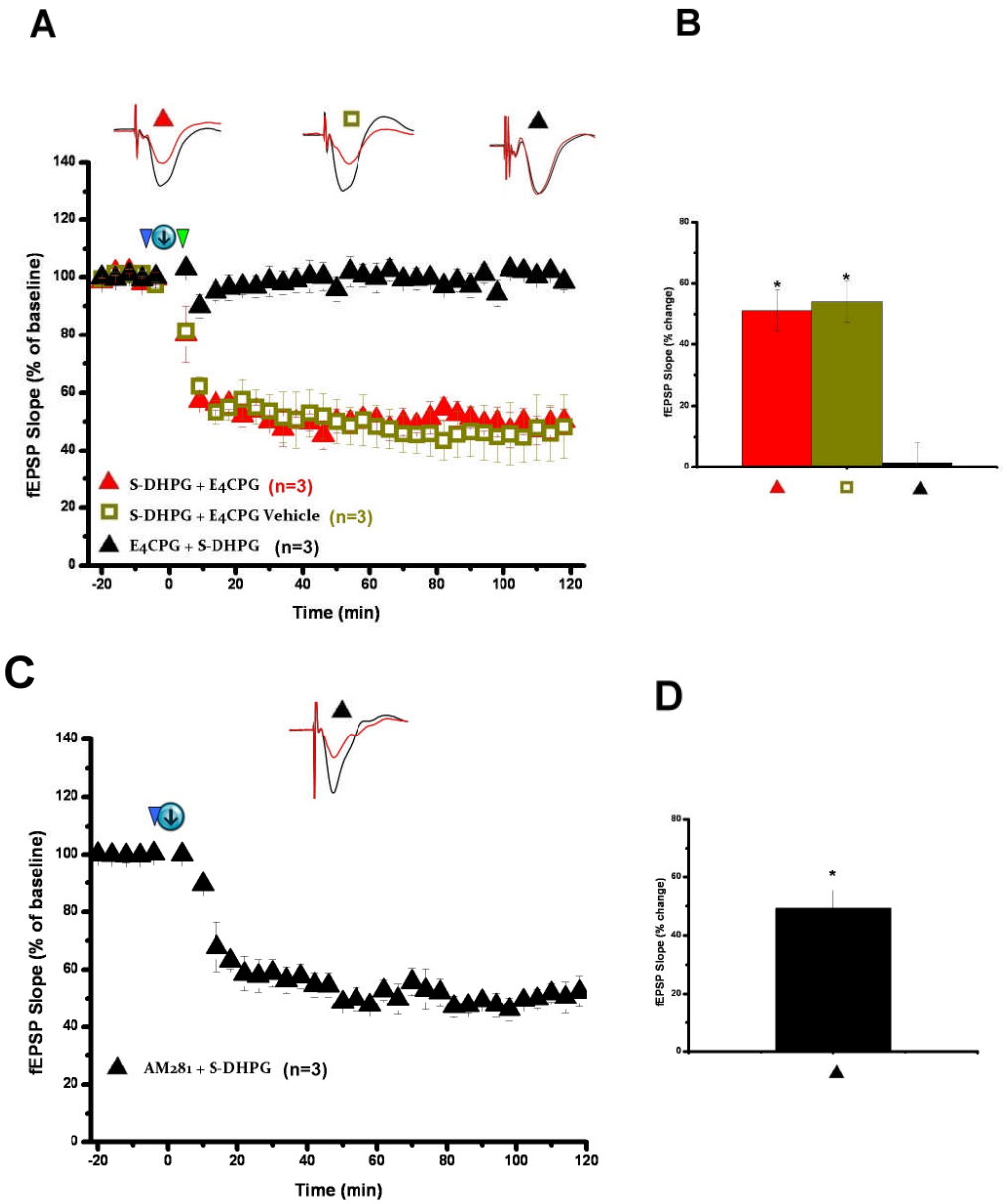

Figure 3-6 E4CPG blocks S-DHPG induced LTD through pretreatment but not post-treatment while AM 281 does not block S-DHPG induced LTD. A. E4CPG (35 nM/3.5 µl, i.c.v.) injected 5 minutes before S-DHPG (100 nM/5 µl, i.c.v.) treatment (black triangles) or 5 min. after (red triangles). 0.9 % saline vehicle injected 5 minutes after S-DHPG (100 nM/5 µl, i.c.v.) treatment (gold squares). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for E4CPG and S-DHPG or vehicle and S-DHPG treatment from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test). **C.** AM 281 (3 mg/kg, i.p.) injected 5 minutes before S-DHPG (100 nM/5 µl, i.c.v.) treatment (black triangles). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **D.** Percent change of fEPSPs for AM 281 treatment from baseline to 110 min. into recording. Blue arrowheads denote 5 min. before S-DHPG treatments and green arrowheads denote 5 min. after S-DHPG treatments. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

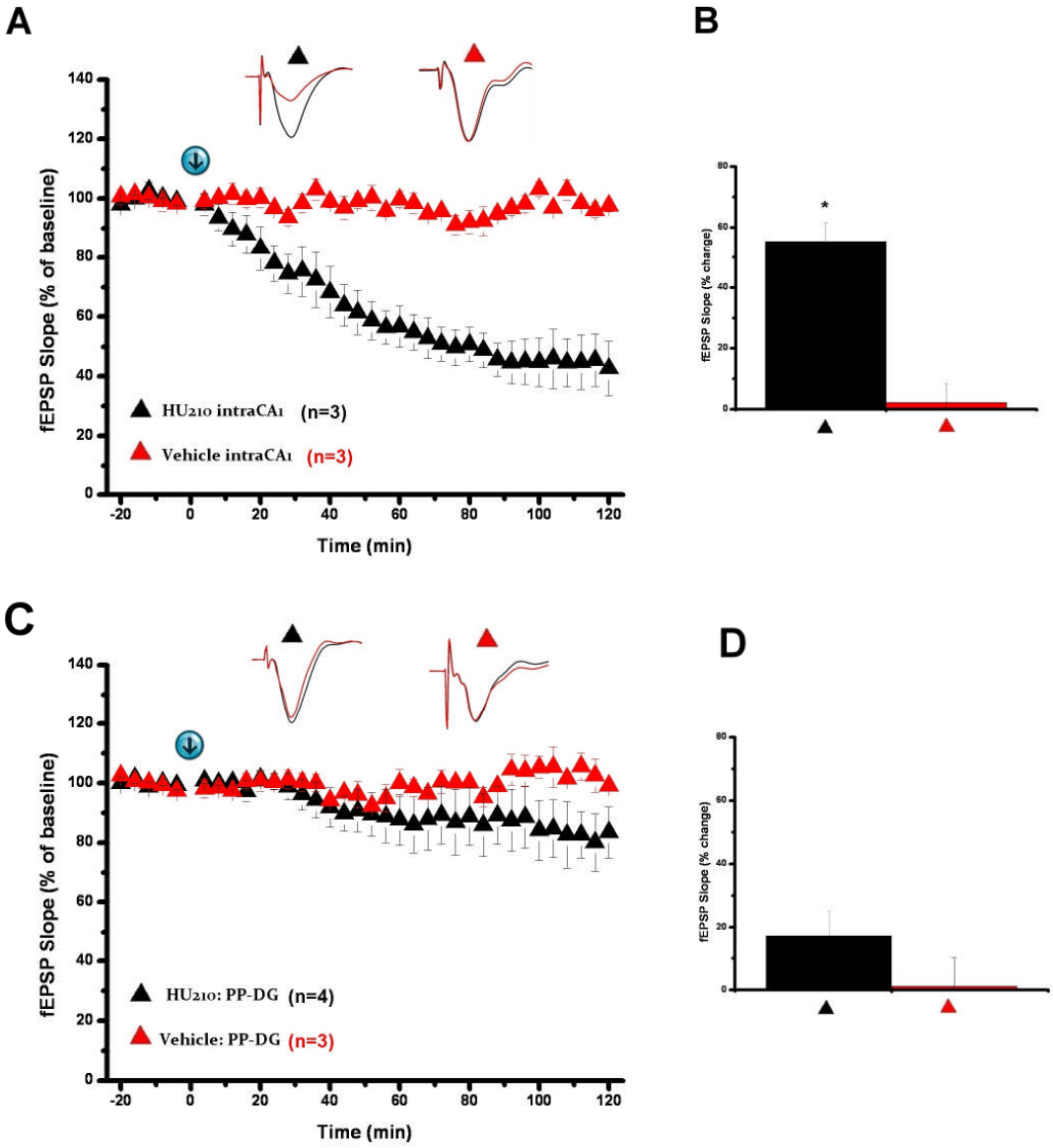

Figure 3-7 HU210 evoked LTD occurs in excitatory CA3-CA1 synapses when given intra-CA1 but has little affect with systemic treatment in the PP-DG pathway. A. HU210 (50 µg/ml, iontophoretic ejection at -40 nA for 30 sec), treatment (black triangles) or DMSO: Tween 80: 0.9 % NaCl vehicle (red triangles). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **B.** Percent change of fEPSPs for HU210 or vehcile treatments from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test). **C.** HU210 (50 µg/kg, i.p.) treatment (black triangles) or DMSO: Tween 80: 0.9 % NaCl vehicle injection (red triangles). Slope of field excitatory postsynaptic potentials are presented as a percentage of the baseline EPSPs. Average individual fEPSP traces: black – average baseline fEPSP, red – average fEPSP for 100-110 min. time frame. **D.** Percent change of fEPSPs for HU210 or vehicle treatments from baseline to 110 min. into recording. Error bars established for ±SE. * $p < 0.05$ (Tukey HSD *post hoc* test).

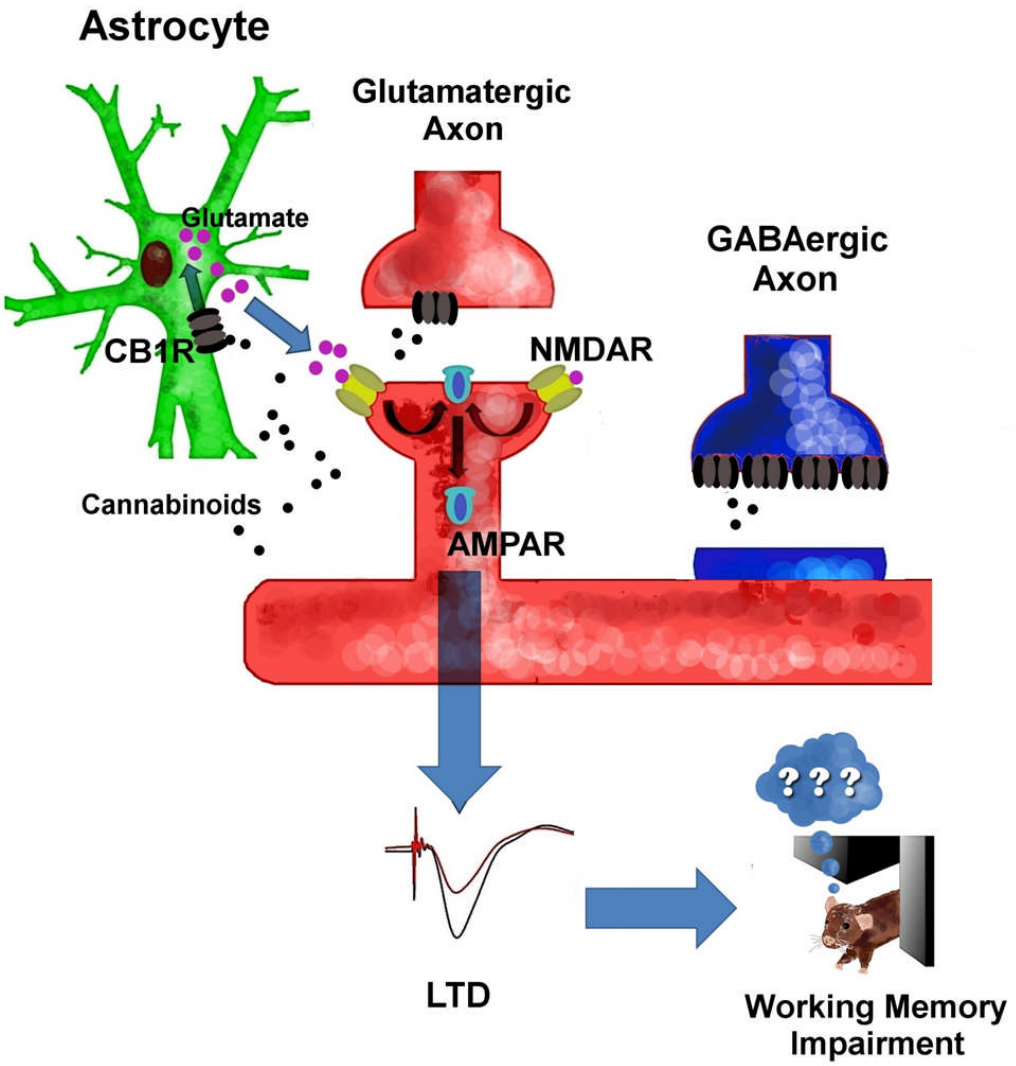

Figure 3-8 Proposed mechanism for cannabinoid-evoked hippocampal LTD. Exogenous cannabinoids enter the CA1 region of the hippocampus and bind to astroglial CB1 receptors which causes glutamate build up in the extrasynaptic area, these bind to NR2B-containing NMDARs which in turn cause AMPAR endocytosis that leads to LTD and is ultimately correlated with spatial working memory impairment.

Chapter 4: Discussion

4.1 Involvement of astroglial CB1Rs over neuronal ones

These results clearly show the importance of astroglial CB_1Rs in cannabinoid-evoked LTD at the CA3-CA1 synapses. The neuronal CB_1Rs, on the other hand, do not seem to play a large role in this mechanism. The use of conditional knockouts allows one to eliminate a gene from a target area opposed to the entire organism. This allows for more precision, accuracy and the greater chance that survival or developmental issues will not play a role in the development of the organism (in this case – mice). Because the pharmacological treatment with cannabinoids, induced the LTD we observed in both the Glu-*CB_1R*-KO and the GABA-*CB_1R*-KO, it can be speculated that these CB_1Rs are not necessary in the mechanism both acutely or developmentally. In order to understand when the gene/protein is necessary, an inducible knockout can be utilized. This type of knockout allows for temporal control – i.e. in terms of the CreER/lox system, excision of the desired gene does not occur until the ligand binding domain of the ER (which is fused to the Cre recombinase) is activated by tamoxifen; this allows the CreER to move into the nucleus and excise the DNA that is flanked by *loxP* sites (Feil et al., 1996). Therefore, by utilizing this form of inducible knockout with the GFAP-*CB_1R*-KO, it allowed for more control in terms of avoiding developmental issues.

The reasoning for why astroglial CB_1Rs are necessary for this LTD and neuronal ones seem to not be, could be the result of several circumstances. One, in the CA3-CA1 synapses; it has previously been shown that the density levels of the presynaptic CB_1Rs

are just above background levels (Kawamura et al., 2006). The sheer number of astrocytes and thus density of the receptors in comparison with the low presynaptic CB_1R densities, could account for the resultant preference to astroglial CB_1Rs. Beyond this concept, in the early 2000s, based on their own results conflicting with the prevailing theories on cannabinoid-sensitive receptors, Freund's group discussed the possibility of a novel receptor that is pharmacologically unique (Hajos et al., 2001; Hajos and Freund, 2002). Although purely speculative in nature, it is not totally implausible to conceive that perhaps the reason why cannabinoids target astroglial CB_1Rs in the CA3-CA1 synapses is due to some pharmacologically unique development that allows for their affinity for cannabinoids to increase well beyond that of the neuronal CB_1Rs. It has already been shown numerous times that the affinity of cannabinoid receptors for different cannabinoids (endogenous and exogenous) varies greatly (Pertwee, 2010), therefore, one could also speculate that this phenomena occurs by utilizing the cannabinoids used in these experiments, whereby using endogenous cannabinoids, or even different exogenous ones, could produce a different mechanism and even outcome.

Another unknown issue is whether or not this astroglial CB_1R-dependent LTD occurs throughout the brain. There exists the notion that although there are far more CB_1Rs on GABAergic terminals than glutamatergic (Kawamura et al., 2006; Marsicano et al., 2003), in some areas of the brain, cannabinoids still seem to preferentially target glutamatergic ones (as discussed in Monory et al., 2007). Due to the results my colleagues and I obtained, it is fair to speculate that beyond preferential targeting of various neuronal CB_1Rs in certain areas of the brain, that astroglial CB_1Rs could also be preferentially activated. My colleagues have found that a similar form of cannabinoid-

evoked synaptic long-term depression does occur in the VTA (Liu et al., 2010). However, based on other preliminary data, this cannabinoid-evoked LTD does not arise universally throughout the brain, as can be seen in Figure 3-7 C, where the phenomenon did not significantly occur in the PP-DG pathway. This can be related to studies whereby cannabinoids did not have the ability to alter synaptic plasticity in the DG region (Abush and Akirav, 2012). Due to these findings, it is likely that although the preferential targeting of astroglial CB_1Rs by cannabinoids may occur in other areas of the brain beyond the CA3-CA1 region, it is unlikely that it arises everywhere, the reason for this phenomenon and the extent of the areas of the brain where it occurs remains to be unknown.

Finally, as discussed earlier, the potential significance of slicing the brain and the prospective consequence this may have on changing cannabinoid targeting from astroglial CB_1Rs to neuronal ones, remains an area to be researched further.

4.2 Gliosomal fractionation and glutamate release experiments

One element of the proposed mechanism that we have yet to show is what occurs from astroglial CB_1R activation to glutamate build-up. One strong possibility, as theorized based on the work of Araque, Bonanno, Maccarrone and others, is that glutamate is released from the astrocyte upon activation of the CB_1Rs (Navarrete and Araque, 2010; Milanese et al., 2009; Bari et al., 2011).

In order to test this possibility, we can use a gliosome protocol, prepared via a Percoll gradient fractionation. As of this point, our lab has achieved this step (separated

out synaptosomes and gliosomes from hippocampal samples). From here, both fraction 2 and 4 are separated on 10% SDS-PAGE gels, and then transferred onto Nitrocellulose membranes. Following this, membranes are probed with antibodies to GFAP and MAP-2. Utilizing parallel superfusion chambers, filtration and radioactivity protocols, a liquid scintillation counting method can be used. By comparing control experiments to the drug-treated samples, one can determine a ratio by which the concentration of glutamate outside the gliosomes can be determined and by consequence, discover whether the amount of glutamate released increases with cannabinoid treatment (Milanese et al., 2009; Bari et al., 2011).

4.3 *In vitro* versus *in vivo* cannabinoid studies

In the cannabinoid-antagonist experiments (Fig. 3-2), AM 281 (3 mg/kg, i.p.) treatment 10 min. after HU210 administration does not block LTD. In certain *in vitro* studies from literature, investigators have noted that this time frame will block LTD. My colleagues and I feel that the reason for the difference is that the slice preparation for *in vitro* studies would damage the network of astrocytes that we feel is a necessity for cannabinoid-evoked LTD (discussed later).

This gives the first indication that what is being viewed is in fact long-term depression and not a transient form. Only the pretreatment was affective, implying that a single activation of CB_1Rs is sufficient to induce a long-term effect. This is different than previous findings shown in papers such as Chevaleyre et al., 2006 and Kawamura et al., 2006 among others, where they showed in *in vitro* studies that a cannabinoid-induced

synaptic depression could be reversed by post-treatment with AM 281. So the second consequence of this discovery is that there is a significant difference when doing experimentation of this sort *in vivo* opposed to *in vitro*.

A potential reason as to why there are differences in terms of how the phenomenon is studied is currently unknown but one can speculate on numerous possibilities. One such possibility could be that of CB_1R endocytosis. Vesicular trafficking of CB_1R in both neurons and astrocytes has been described (Daigle et al., 2008; Osborne et al., 2009). A potential implication as to why *in vivo* differs from *in vitro* in terms of CB_1R agonism/antagonism could be that *in vivo*, CB_1Rs are internalized upon activation, while *in vitro*, they are not. If the receptor would be internalized, AM 281 or any CB_1R antagonist would not be able to produce any affect on the receptor. However, if they are not endocytosed (potentially as in the *in vitro* state), the CB_1R antagonist may be able to outcompete the agonist and in turn prevent the effect (in this case LTD) that would normally be seen. This is speculative and the reason for this difference may be difficult to prove.

Beyond the differences seen with CB_1R antagonism, there are also more general *in vivo* variations as seen in the involvement of protein translation and transcription in synaptic plasticity. In his 2000 paper, Kandel showed, using electrophysiological methods and pharmacological agents, that there exists a protein-dependent element of long-term synaptic plasticity when observed in an *in vivo*-type environment (Kauderer and Kandel, 2000). Other labs, including Frey's, mirrored this observation in freely moving rats (Manahan-Vaughan et al., 2000). Work of this nature provided controversial comparisons between *in vivo* and *in vitro* protocols. Kandel discusses the fact that

initially, utilizing acute brain slices, the damage to the brain may prevent one from observing a protein-dependent late phase to hippocampal LTD (as their own and Huber et al., 2000 results of NMDAR-dependent LTD showed) but ultimately upon creating an *in vivo*-type environment and subsequently doing *in vivo* electrophysiological studies, the late phase expression was seen to be dependent on protein synthesis (Kauderer and Kandel, 2000). Beyond hippocampal LTD, this phenomenon has been seen in other areas of the brain including the cerebellum (Linden, 1996), implying a fairly ubiquitous nature to the protein synthesis involvement in *in vivo* experimentation.

One such reason as to the *in vivo*/*in vitro* differences (including as mentioned earlier: CB_1R antagonism, protein synthesis, as well as LTP versus LTD effects, and role of NMDARs in the MAPK/ERK pathway (Derkinderen et al., 2003)) could be the anatomical and physiological damage done by the act of slicing the brain. In the hippocampus, as well as most areas throughout the brain, neurons are surrounded by astrocytic networks (see Fig. 1-2). These networks, connected by gap junctions, provide important roles in neuronal nodulation and numerous other brain functions (Giaume et al., 2010). As further evidence of the importance of these networks, our lab has collected data showing that a half KO of connexin 43 (a glial gap junction protein) had such a significant affect as to prevent the LTD that we normally obsevered at the CA3-CA1 synapses after cannabinoid treatment (unpublished results). This leads us to hypothesize that perhaps one of the reasons why the effects differ, often very significantly (see Han et al., 2012 showing *in vivo* astrocytic CB_1R-dependent exogenous cannabinoid-induced LTD, opposed to Navarette and Araque, 2010 showing *in vitro* astrocytic CB_1R-

dependent endocannabinoid induced LTP at remote synapses) may be due to the damage inflicted on this network when the brain is sliced.

This study our lab published may also provide insight into the use of *in vivo* techniques while observing the effects of other drugs of abuse. In many studies examining drugs of abuse and their ability to alter synaptic plasticity, often *in vitro* brain slice techniques are utilized. It would be interesting to examine these same protocols, but to instead use *in vivo* methods, and beyond this, to potentially examine the role of astrocytes in these outcomes.

Ultimately, this study is an observation of the effects of a pharmacologically induced LTD, opposed to that induced by means of electrical stimulation. Due to this, we can stress the importance of physiological conditions. Many protocols involving the induction of LTD *in vitro* use non-realistic firing patterns, it has also been speculated that pathological events (including epilepsy) occur when inducing LTP due to the firing frequencies utilized, as opposed to it being a physiological condition. This is due to the fact that these frequencies are often far beyond the limits of physiological conditions (Sjostrom et al., 2008). It is known that low firing rates naturally occur in the brain, thus we attempted to keep our electrophysiology paradigm to these standards. Beyond this, utilizing exogenous cannabinoids opposed to the endogenous variety allows for another control in the separation of pharmacology and electrophysiology. If we utilized endogenous cannabinoid paradigms to view this phenomenon (i.e. JZL 184 – which inhibits the degradation of 2-AG by inhibiting the enzyme monoacylglycerol lipase (MAGL) (Long et al., 2009)), we run the risk of having our basal firing affect the release/removal of endocannabinoids as their own production/release can be induced by

electrical means (Heifets and Castillo, 2009). However, using an exogenous substance allows us to separate the effects.

Another possibility as to potentially explain the differences between *in vivo* and *in vitro* outcomes could lie in the temperature variability that may exist in *in vitro* experimentation. In numerous papers, it has been shown that slices incubated at different temperatures can produce significantly variable results in relation to the induction of synaptic plasticity. Some examples of this occurring include the following. In a 1999 study, investigators showed that by increasing the temperature of their hippocampal slices, they could inhibit excitatory transmission. They further reported that they believed this to occur due to regulation via an adenosine mechanism (Masino and Dunwiddie, 1999). Other studies showing varying synaptic transmission results due to alterations in temperature include the Verkhratsky study that showed by using an interleukin-1 receptor antagonist, they were able to block LTP at 34-36 °C, but not when the incubation temperature was set to 21-24 °C (Ross et al., 2003). Finally, while looking at short-term plasticity in hippocampal slices, Klyachko and Stevens showed that by increasing the temperature from 23 to 38 °C, the synaptic strength went from a depression in response to high frequency stimulation, to an augmentation (Klyachko and Stevens, 2006). These among other studies show the significance and potential alteration that can occur to synapses upon variation in temperature. Therefore, the results I obtained in these studies may differ from *in vitro* studies due to the idea that the temperature in those studies may have been variable and caused a different outcome to occur.

4.4 Implications of the involvement of protein synthesis

The results in this study imply the necessity for protein translation and mRNA transcription in the later-phase expression of LTD. Based on similar but not exact time courses, the implication can be made that protein synthesis may stem from pre-existing mRNA as well as newly transcribed mRNA. This fits in with the data produced by investigators including Kandel and Frey who showed that later phase LTD expression involves the synthesis of new proteins (Kauderer and Kandel, 2000; Manahan-Vaughan et al., 2000). The proteins that may be involved in this process are as of yet unknown but may involve IEGs that are transported to the dendrites (where local protein synthesis may occur) as well as nuclear mRNA (different time frames) in a similar sense that the Arc protein (involved in AMPAR endocytosis and LTD) is produced on demand (Waung et al., 2008).

4.5 Experimentation to define what proteins may be involved in cannabinoid-evoked expression of later-phase LTD

Due to the fact that anisomycin and actinomycin-D blocked L-LTD, it can only be assumed that certain genes will be up-regulated after HU210 treatment. In order to gain further knowledge as to the specific genes and proteins that are up regulated, a microarray analysis could be performed of the CA1 region and perhaps even the CA3 region (as the proteins could potentially be presynaptically translated). This knowledge can then be used to target certain proteins that would be translated from these up-

regulated genes by creating specific viral vectors or knockouts to silence their effects. This will hopefully lead to an occlusion of L-LTD and thus knowledge of the proteins involved in this overall mechanism. Greenberg and others have looked at similar experiments and noted as many as 65 altered genes after cannabinoid treatment in mice (Parmentier-Batteur, 2002).

4.6 Glutamate receptors in cannabinoid evoked LTD

Due to the fact that NMDAR activation was still important 30 min. after HU210 treatment, there is an implication that a continuous or delayed release of glutamate may be occurring. Because AM 281 had no effect when administered 30 min. into the LTD, it could be hypothesized that there is a fairly quick removal of HU210 from the synapse, thus one explanation is that a single dose of HU210 causes a continuous or prolonged release of glutamate from astrocytes, which can then in turn act upon the postsynaptic NMDARs over an extended period of time. Glutamate reuptake may be prevented in astrocytes during this form of LTD, as a similar LTD has been seen in the CA3-CA1 synapses when the glutamate-uptake inhibitor DL-threo-β-benzyloxyaspartate (TBOA) treatment is utilized (Han et al., 2012). Another possibility is that there is a delay in the release of glutamate from neighbouring astrocytes due to the fact that long-range signalling has been seen to be performed by astrocytes (Navarrete and Araque, 2010).

Beyond this, the notion of NR2B-specific NMDA receptors playing a key role over NR2A-specific ones, may be related to a developmental paradigm. NR2B-containing NMDARs are the only type of NMDARs present in the early stages of

development while the NR2A variety begin to develop and become up regulated several days postnatal. This development pattern also correlates with the development of synapses (occurring basically simultaneously with the NR2A-containing NMDAR development). This may create a correlation with localization of these two types of NMDARs (NR2B-type localizing extrasynaptically while the NR2A-type localized in the synapse) (Tovar and Westbrook, 1999; Barria and Malinow, 2002). Due to this possibility, the NR2B-containing NMDARs may be more readily available to interact with the increased levels of extracellular glutamate introduced after CB_1Rs on astrocytes are activated.

4.7 NR2B-subunit knockout mice models

A potential future experiment that could be implemented to further understand the significance of the various NMDAR subunits would be to develop a line of knockout mice models that lacked the NR2B or NR2A subunit. This would eliminate any question of the specificity of various antagonists and therefore strongly corroborate the results we observed using Ro25-6981, NVP-AAM077 and ifenprodil.

Numerous investigators have produced viable NR2B-KO or NR2A-KO mice using various protocols. In 1995, Mishina's group created a specific knockout of the NR2A subunit gene by using the targeting vector pTVGRε1 (a blunted fragment with neomycin phosphotransferase gene into NR2B genomic DNA, under phosphoglycerate kinase promoter), followed by removal of the key multi-cloning sequence (Sakimura et al., 1995). They used these mice to perform various electrophysiological and behavioural

studies. Other groups including that of Holmes' as well as Becker's, have utilized floxed-NR2B mice (which they refer to as GluN2B-floxed) crossed with CaMKII promoter driven Cre recombinase transgenic mice, to create viable NR2B subunit specific knockouts, which they then used to test various hypotheses involving synaptic plasticity, learning, metabolism, etc. (Brigman et al., 2010; Badanich et al., 2011). By utilizing one of the previously mentioned protocols, a viable KO model could be designed and then treated with various cannabinoids and the resulting observations noted. I hypothesize that the results will match those seen when utilizing the receptor antagonists (Fig. 3-4, 3-5).

4.8 AMPAR endocytosis in L-LTD

Biochemical work done for the AMPAR endocytosis experiments in our lab, involving Western blotting with antibodies specific to AMPAR subunits (i.e. GluR1, GluR2, and GluR3) will allow a comparison of band intensity between vehicle and HU210 treatments. A change in surface expression of AMPAR would be a significant indication of endocytosis during L-LTD. This is what is being seen (Han et al., 2012).

When treated with the Tat-GluR2$_{3Y}$ peptide 30 min. after HU210 treatment, I hypothesize LTD expression will be blocked and baseline conditions will be restored. This will not be the case for the scrambled peptide, where LTD will continue at around 40 % of the baseline conditions. This phenomenon has been seen to occur in cannabinoid-evoked LTD in VTA dopamine neurons (Liu et al., 2010). The GluR2$_{3Y}$ peptide functions by interfering with the GluR2 subunit tyrosine phosphorylation dependent endocytic motif, and thus does not allow the binding of proteins such as the

clathrin adaptor protein (Wang, 2008). The Tat protein (YGRKKRRQRRR) allows the peptide to be delivered into neurons (Wang, 2008).

4.9 Correlation with spatial working memory impairment

As can be seen in the paper Han et al., 2012, the data observed from electrophysiological studies was correlated with behavioural ones. Utilizing the delayed non-match to sample T-maze and the delayed-matching to place version of the Morris Water Maze, members of the lab as well as collaborators from other labs showed a correlation of the observed LTD with impairment of spatial working memory. Using the three part mechanism discovered (astroglial CB_1Rs, dependence on NMDARs and involvement of AMPAR endocytosis) the researchers studied the affects on SWM.

The knockout models along with their WTs (tamoxifen-treated GFAP-*CB_1R*-KO and GFAP-*CB_1R*-WT; as well as Glu-*CB_1R*-KO, GABA-*CB_1R*-KO and their WT) were tested in terms of SWM performance/impairment using a delayed-matching-to place (DMTP) version of the Morris water maze paradigm. In all experiments, mice were put through three trials of habituation that occurred 24 hours before the training sessions began. This was done in order for the mice to learn the rules/goal without spatial cues. The trials then began with varying organization of the platform and times (see Han et al., 2012 for details). In all trials, recordings for the latency to reach the platform were made. During the spatial learning training sessions, the submerged platform's location was altered daily (the mice were also treated with saline to become habituated to treatment – trials 1-5). These sessions had four trials where the mice had three varying starting points

each time. Vehicle treatments were given for trials 6-12 (30 min. before session) – this developed a baseline for learning. The thirteenth session involved THC treatment. Path saving ratio and latency saving ratio formulas can be seen in Han et al., 2012. The THC caused an impairment in SWM performance (both path saving ratio and latency saving ratio) in all WT groups compared to vehicle treatments. The THC did not cause an effect on the GFAP-CB_1R-KO mice. The Glu-CB_1R-KO and GABA-CB_1R-KO mice showed an impairment when treated with THC as well (see Han et al., 2012 for details).

To determine the effects of NMDAR antagonists: NVP-AAM077, ifenprodil and Ro25-6981, as well as the AMPAR endocytosis blocking peptide Tat-$GluR2_{3Y}$, a delayed nonmatching-to-sample T-maze protocol (DNMTST) was used. These experiments consisted of first, pretest training sessions (3 days – habituation: exploring of the maze, eating treats in a goal arm, blocking one arm occasionally, etc.). Following this was acquisition training which consisted of 6 days – 10 trials/day. There was a sample and choice run for each trial. For the sample run, the rat had access to one goal box with treats in the initial trial. In the choice run, the rat had access to both goal boxes, but was only rewarded with treats if they entered the one opposite to the sample run. The final step was the performance test which consisted of two days. The rats were treated with the pharmacological agent(s) of interest and SWM performance was determined by the average value of the two days (for further details, see Han et al., 2012). HU210 treatment caused an impairment of the SWM performance. This however, was blocked by pretreatment with ifenprodil, Ro25-6981, the Tat-$GluR2_{3Y}$ peptide, but not NVP-AAM077 (Han et al., 2012). These agents were also tested on the DMTP version of the Morris water maze paradigm with the same conclusions obtained. For all behavioural

experimentation, numerous control studies were undertaken including swim speed, locomotor activity, elevated-plus-maze and motor balance tests (see Han et al., 2012 for details).

These results mirror the conclusions made by the electrophysiology. Namely that in the cannabinoid-induced impairment of SWM, the underlying mechanism involves: astroglial CB_1Rs (shown in the KO experiments), NR2B-containing NMDARs (shown in the antagonist experiments) and AMPAR endocytosis (shown in the peptide experiments). This allows one to put forward the notion that in this particular form of SWM impairment, the involvement of synaptic plasticity alterations (i.e. LTD) may play a large role and by preventing these transformations, one can prevent the impairment.

4.10 Conclusion

These experiments show electrophysiological evidence of the mechanism of cannabinoid-evoked induction and expression of early- and late-phase LTD in excitatory CA3-CA1 synapses. This novel study strongly suggests the importance of astroglial CB_1Rs, protein synthesis and NR2B-containing NMDARs, along with the discoveries made by other members of our lab; we have proposed a mechanism whereby astroglial CB_1R activation by exogenous cannabinoids causes glutamate build-up in the extrasynaptic area, which activates NR2B-containing NMDARs that cause AMPAR endocytosis, which leads to LTD (see Fig. 3-8).

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