2-13-2014

Functional Interactions Between the Cannabinoid System and Memory Consolidation in the Hippocampus: A Synthesis of Findings from Behavior, Morphology, Physiology, and Synaptic Signaling in the Rat

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FUNCTIONAL INTERACTIONS BETWEEN THE CANNABINOID SYSTEM AND MEMORY CONSOLIDATION IN THE HIPPOCAMPUS: A SYNTHESIS OF FINDINGS FROM BEHAVIOR, MORPHOLOGY, PHYSIOLOGY, AND SYNAPTIC SIGNALING IN THE RAT

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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Psychology

The University of New Mexico
Albuquerque, New Mexico

December, 2013

DEDICATION

To my husband, Adam,
for his never-ending patience, reassurance, love, and charming sarcasm.

To my Mom,
for first believing in me and supporting my dreams, no matter where they took me.

To the Candelaria & Cook families,
for their unwavering support and encouragement over the years.

ACKNOWLEDGEMENTS

First and foremost, I give special thanks to my advisor and dissertation chair Derek Hamilton. Your mentoring has been invaluable throughout the years. I also thank my committee members, Kevin Caldwell, Ron Yeo, and Michael Weisend, for their advice, careful document editing, and suggestions on improving the quality of these experiments.

Thanks are extended to the entire Hamilton lab, past and present, for their support, commiseration, and comical relief throughout grad school. I would like to specifically thank Clark Bird for designing and generating my IEG primers. I would also like to thank the Psychology Animal Facility Staff for taking excellent care of my subjects.

I thank my husband Adam Cook without whom I could not have finished this dissertation. Thank you for believing in me, supporting my dreams, and alleviating my stress daily. I'm grateful to have you by my side, as my partner in crime, to learn from and grow old with. Grad school was a difficult chapter in my life, but I will see you in Zihuatanejo my friend, for the Pacific has no memory.

I thank my Mom without whom none of this would be possible for she raised me to value hard work and first believed I could do anything I set my mind to. My mom and the entire Candelaria-Cook families have always provided an incredible amount of support, love, and encouragement throughout my life. I am very lucky to have such a wonderful family by my side. I also thank a special family member, my cat, Chester. He has been an irreplaceable member of my emotional support team who always lifted my spirits after a long day in the lab and while writing this dissertation.

Last, but not least, I would like to acknowledge the support and mentoring I have received from the UNM-RWJF Center for Health Policy, specifically Carolyn Richardson. Carolyn thanks for your leadership and encouraging a kindred spirit in my final stages of dissertation writing. I would also like to thank Margaret Werner-Washburn and the entire UNM-IMSD program for assisting my scientific curiosity from undergraduate throughout graduate school. I am truly grateful that so many wonderful people believed in my potential and helped me along the way.

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ABSTRACT

The hippocampus is essential for long-term memory storage and consolidation. The dentate gyrus acts as a gateway into the hippocampal formation, specifically with reference to medial and lateral perforant path projections from the entorhinal cortex. The dentate gyrus and the hippocampus contain a high density of cannabinoid receptors and may be a key location in which cannabinoids exert influence to disrupt memory. Administration of a cannabinoid agonist often leads to short-term and long-term memory deficits in a variety of tasks. Cannabinoids can impair memory acquisition, task performance, along with memory consolidation and retrieval mechanisms. Endogenous cannabinoids are retrograde messengers involved in the rapid modulation of synaptic transmission. In the following study the effects of a potent cannabinoid agonist, WIN 55,212-2, on rodent learning and memory in the dentate gyrus were examined using a

combination of morphological, behavioral, electrophysiological, and gene targeting approaches.

The current study found that the cannabinoid agonist, WIN 55,212-2, altered granule cell spine density in the dendritic targets of the associational-commissural afferents and medial perforant path projections, but not lateral perforant path. Although intraperitoneal injections of WIN 55,212-2 resulted in a 24-hour consolidation deficit mediated by CB_1 receptors, direct infusion of WIN 55,212-2 into the dentate gyrus did not influence 24-hour memory consolidation or alter immediate early gene expression in the dentate gyrus or parietal cortex. When examining *in vivo* electrophysiology, the cannabinoid agonist altered perforant path to dentate gyrus responses. WIN 55,212-2 blunted the magnitude of baseline population spike amplitude, without changing baseline fEPSP response. Furthermore, WIN 55,212-2 altered fEPSP paired pulse facilitation indicating decreased glutamate release and impaired GABAergic inhibition. Conversely, following high frequency stimulation, WIN 55,212-2 increased fEPSP fractional change. And lastly, WIN 55,212-2 elevated expression of the immediate early gene Arc in the high frequency stimulated dentate gyrus. These findings indicate cannabinoid modulation throughout the dentate gyrus and hippocampus is necessary for memory consolidation processes. When taken together, these results suggest cannabinoids alter normal learning and memory processes in the dentate gyrus by selectively altering medial perforant path projections, changing GABAergic feedforward inhibition, reducing glutamate release, and increasing expression of the immediate early gene Arc.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

Cannabis sativa has been used for centuries but despite its long history, very little is known about its repercussions on cognition. Even though cannabis is illegal in most countries, many people in the United States and Europe use it regularly for its medicinal purposes and for its psychotropic effects. Recently, legislation has passed in several states allowing cannabis to be used for pain relief and to aide in immune defense in many debilitating illnesses such as AIDS, cancer, multiple sclerosis, and rheumatoid arthritis to name a few (Ranganathan & D'Souza, 2006). Aside from a role in pain relief, cannabinoid modulation is also important in processes underlying learning and memory, appetite regulation, neuroprotection, synaptic plasticity, and retrograde signaling at synapses (Robinson & Riedel, 2004).

Consolidation

Memory is typically described in two phases: short-term and long-term (Atkinson & Shiffrin, 1971; Glickman, 1961; Hebb, 1949; McGaugh, 1966). Short-term (or recent) memory is transient in nature and lasts no longer than a few minutes. Long-term (or remote) memory is stored for weeks to years. Over time, new memories are gradually transferred and stabilized from short-term memory stores to long-term memory stores by a process of consolidation (Lechner, Squire, & Byrne, 1999; McGaugh, 2000; Muller & Pilzecker, 1900). Stabilization occurs through the expression of genes and the synthesis of new proteins which result in growth or pruning of synaptic connections (Davis & Squire, 1984; Squire, 1986).

Traditionally, consolidation has been viewed as a finite process that occurs only once, beginning shortly after initial learning and ending at the completion of

consolidation hours to days later (Dudai & Morris, 2001; McGaugh, 1966; Muller & Pilzecker, 1900). After memory consolidation has occurred, memory is relatively immune to forgetting and disruption, whereas memory not yet consolidated is susceptible to interference (Duncan, 1949; Squire, 1992). Regardless of when the exact transition between short-term and long-term memory occurs, researchers generally agree that (1) different stages involve different levels of cascade activation with only long-term storage requiring gene expression, and (2) synaptic consolidation is universal, found in all species, preparations, and memory tasks (Dudai & Morris, 2001).

Role of CB_1 receptors & endocannabinoids in learning & memory

The pharmacological analysis of the cannabinoid system started in 1964 when two chemists, Gaoni and Mechoulam, first isolated the psychoactive component of marijuana, delta-9-tetrahydrocannabinol (THC), using column chromatography and mass spectrometry to elucidate its chemical structure (Julien, 2005). An important breakthrough in understanding the target on which THC acts occurred in 1988 when Howlett and colleagues discovered the first cannabinoid receptor (Julien, 2005). Soon after its discovery and with the rise in genetic technology, the cannabinoid receptors were cloned, endocannabinoids were discovered and cannabinoid receptor knockout mice were generated. Since then abundant knowledge has flooded the scientific literature regarding the pharmacology of the cannabinoid system. The term cannabinoid, originally used to describe a 21-carbon substance found in cannabis plant extracts, is now used to define any compound that is specifically recognized by the cannabinoid receptor.

Cannabinoids, both endogenously and exogenously produced, signal through a distinct set of receptors: CB_1 and CB_2 receptors. CB_1 receptors are located in the central

nervous system and in parts of the peripheral nervous system, while CB_2 receptors are found predominately in the peripheral nervous system where they assist in immune regulation and endogenous opioid release. CB_1 receptors are coupled with inhibitory G_i proteins and are heterogeneously expressed in the central nervous system with particularly high concentrations in the cerebral cortex, hippocampus, cerebellum, basal ganglia, brainstem, thalamus, and hypothalamus (Pertwee, 1997; Pertwee & Ross, 2002). The presence of CB_1 receptors throughout the basal ganglia and cerebellum are primarily responsible for the movement and postural disturbances seen with cannabinoid administration, while their presence in the hippocampus is responsible for memory disturbances, and their presence in the cortex, especially the frontal cortex, is responsible for perceptional disturbances while taking cannabinoid agonists (Robinson & Riedel, 2004). CB_2 receptors have a low overall homology with CB_1 receptors and are found primarily in immune cells. Although recently, it was reported that CB_2 receptors exist in the CNS, as well as a potential yet undetermined and unnamed CB_3 receptor which possess a slight level of binding to cannabinoid agonists (Hashimotodani, Ohno-Shosaku, & Kano, 2007).

CB_1 receptors are predominately localized on presynaptic terminals where they modulate the release of several neurotransmitters, primarily gamma-aminobutyric acid (GABA) and glutamate, but also dopamine, epinephrine, or norepinephrine, depending on location (Pertwee & Ross, 2002; Riedel & Davies, 2005; Robinson & Riedel, 2004). In the hippocampus and neocortex, CB_1 receptors are expressed by a defined subpopulation of GABAergic interneurons (Katona et al., 1999), as well as by hippocampal glutamatergic neurons (Katona et al., 2006) where they act as glutamate release

inhibitors. They exert their modulatory influence by inhibiting Ca^{2+} currents, facilitating K^+ channel gating, inhibiting adenylyl cyclase and the subsequent activation of the MAP kinase pathway and other signaling cascades which regulate gene expression (Chevaleyre, Takahashi, & Castillo, 2006). The CB_1 receptor has been shown to have a high level of constitutive activity or ligand-independent activation (Howlett et al., 2011). The property of constitutive activity is essential for the receptor to maintain a cannabinoid tone in the central nervous system and also permits the receptor to be modulated not only by agonists, but also by inverse agonists.

Effects of cannabinoid agonists on memory

Cannabinoids impair a wide range of spatial memory tasks selectively hindering task acquisition, consolidation, and retention. In the Morris water task (MWT), a single dose of cannabinoids has been shown to impair task acquisition initially (Ferrari, Ottanim, & Giuliani, 1999), impair 24-hour consolidation (Candelaria-Cook, 2009), and impair long-term/one month consolidation (Yim, Hong, Ejaredar, McKenna, & McDonald, 2008). In other memory tasks cannabinoid administration also results in memory impairment. Working memory is impaired in the radial arm maze (Nakamura, da Silva, Concilio, Wilkinson, & Masur, 1991), T-maze (Suenaga, Kaku, & Ichitani, 2008), and delayed-match-to-sample tasks (Heyser, Hampson, & Deadwyler, 1993). Memory retrieval is impaired in the radial arm maze (Wegener, Kuhnert, Thuns, Roese, & Koch, 2008) and object recognition memory is impaired following novel object placement (Schneider & Koch, 2002). However, there is one type of learning that is enhanced, not diminished, by cannabinoid administration. In extinction learning cannabinoids facilitate the rate of extinction following fear conditioning (Pamplona,

Bitencourt, & Takahashi, 2008; Pamplona, Prediger, Pandolfo, & Takahashi, 2006) and also facilitate reversal learning in the MWT (Pamplona, et al., 2006).

Following chronic treatment cannabinoid receptors and the cannabinoid system exhibits tolerance to some of the memory impairing disruptions in tasks [MWT acquisition (Boucher et al., 2009), delayed-match-to-sample (Deadwyler, Heyser, & Hampson, 1995), object recognition (Schneider & Koch, 2003), and extinction (Lin, Mao, Chen, & Gean, 2008)] but not all tasks [MWT reversal (Boucher, et al., 2009), radial arm maze (Stiglick & Kalant, 1982), or T-maze (Nava, Carta, Colombo, & Gessa, 2001)]. It is possible that tolerance to all memory impairments would develop after a long duration of cannabinoid administration (i.e., a treatment period over 30 days) given that the studies which found significant tolerance used longer administration protocols (delayed-match-to-sample, objection recognition).

Cannabinoids have also been shown to impair both long-term potentiation (LTP) (Collins, Pertwee, & Davies, 1995; Hill, Froc, Fox, Gorzalka, & Christie, 2004; Nowicky, Teyler, & Vardaris, 1987; Terranova, Michaud, LeFur, & Soubrie, 1995) and long-term depression (LTD) (Misner & Sullivan, 1999), thereby altering long lasting synaptic plasticity in a significant manner. A modulatory role for endocannabinoids in LTD was first shown in dorsal striatal neurons, where LTD was facilitated by blocking cellular endocannabinoid uptake, while LTD was impaired in CB_1 receptor knockout mice (Gerdeman, Ronesi, & Lovinger, 2002). Since then, endocannabinoid modulated LTD has also been shown in the nucleus accumbens, basolateral amygdala, hippocampus, neocortex, and cerebellum (Hashimotodani, et al., 2007). Besides a role in modulating LTD in various regions, endocannabinoids have also been implicated in hindering LTP.

CB_1 receptor knockout mice have enhanced LTP in the Schaffer collateral-CA1 pathway when compared to wild type controls (Bohme, Laville, Ledent, Parmentier, & Imperato, 2000). It has also been shown by Lawston et al. (2000) that treatment with WIN 55,212-2 resulted in shorter, disjointed dendrites in the CA1 region which could account for the observed decrements in LTP. But, it may be the case that the reduction in LTP is due to reduced glutamate transmission (Shen, Piser, Seybold, & Thayer, 1996; Sullivan, 2000). By inhibiting glutamatergic responses, they may be indirectly impacting normal LTP, and possibly changing gene expression underlying the neural mechanisms of long term memory storage. It is possible that cannabinoids may be producing deficits by either modulating glutamate or GABA, however, cannabinoids are coupled more highly to GABA in a ratio of 20:1, and therefore are most likely modulating a deficient GABAergic response (De Oliveira Alvares, Genro, Diehl, Molina, & Quillfeldt, 2008).

CB_1 receptors are modulatory in nature and impact numerous neurotransmitters and responses throughout the brain. It is recognized that due to the fact that cannabinoids are found regulating several neurotransmitter systems throughout the brain, they will have numerous functions, not solely functions pertaining to memory. The exact mechanism behind the cannabinoid deficit in spatial learning remains unclear. Cannabinoid receptors are coupled to G-proteins that inhibit adenylyl cyclase. In general, stimulation of adenylyl cyclase activity promotes learning via activation of protein kinase A, whereas inhibition of adenylyl cyclase inhibits learning. It is important to note that this is a generalization, and that, in some instances, receptors that are negatively coupled to adenylyl cyclase promote learning.

CHAPTER 2: OBJECTIVES

Rationale

Exposure to exogenous cannabinoids can have vast negative repercussions on cognitive systems by altering underlying neurocircuitry permanently. Acute and long-term abuse of cannabinoids in rats leads to deficits in memory processing (Ferrari, et al., 1999; Robinson et al., 2008; Yim, et al., 2008), neuronal cell death (Katona, et al., 2006), detrimental neuronal cellular changes (Robinson & Riedel, 2004; Sullivan, 2000), and changes in dendritic morphology (Kolb, Gorny, Limebeer, & Parker, 2006; Rubino et al., 2009). Recent studies have shown that the cannabinoid agonist WIN 55,212-2 impairs rodent spatial memory consolidation in the Morris Water Task (MWT) (Candelaria-Cook, 2009; Yim, et al., 2008) and this impairment is mediated by CB_1 receptors (Candelaria-Cook & Hamilton, 2010). When these deficits in MWT short-term consolidation (Candelaria-Cook, 2009) are taken together with previous finding that WIN 55,212-2 impairs long-term consolidation processes in the MWT (Yim, et al., 2008) it becomes evident further research is needed to investigate the specificity and mechanisms behind the response. Thus, identifying the conditions in which disruption of consolidation processes does or does not have deleterious effects on memory represent important contributions to learning and memory research.

Hypothesis

Spatial memory consolidation deficits are seen when exogenous cannabinoid agonists are administered due to deficient signaling within the hippocampal dentate gyrus. Further, the consolidation deficits are due to CB_1 receptors directly reducing the amount of glutamate and GABA release from the presynaptic cell, thereby reducing

synaptic signaling. Cannabinoid receptors alter the release of glutamate from mossy cells and GABA from basket cells (see Figure 1). By disrupting synaptic signaling and impairing feedforward and feedback inhibition, the cannabinoid induced memory impairment should manifest itself on various physiological and behavioral levels.

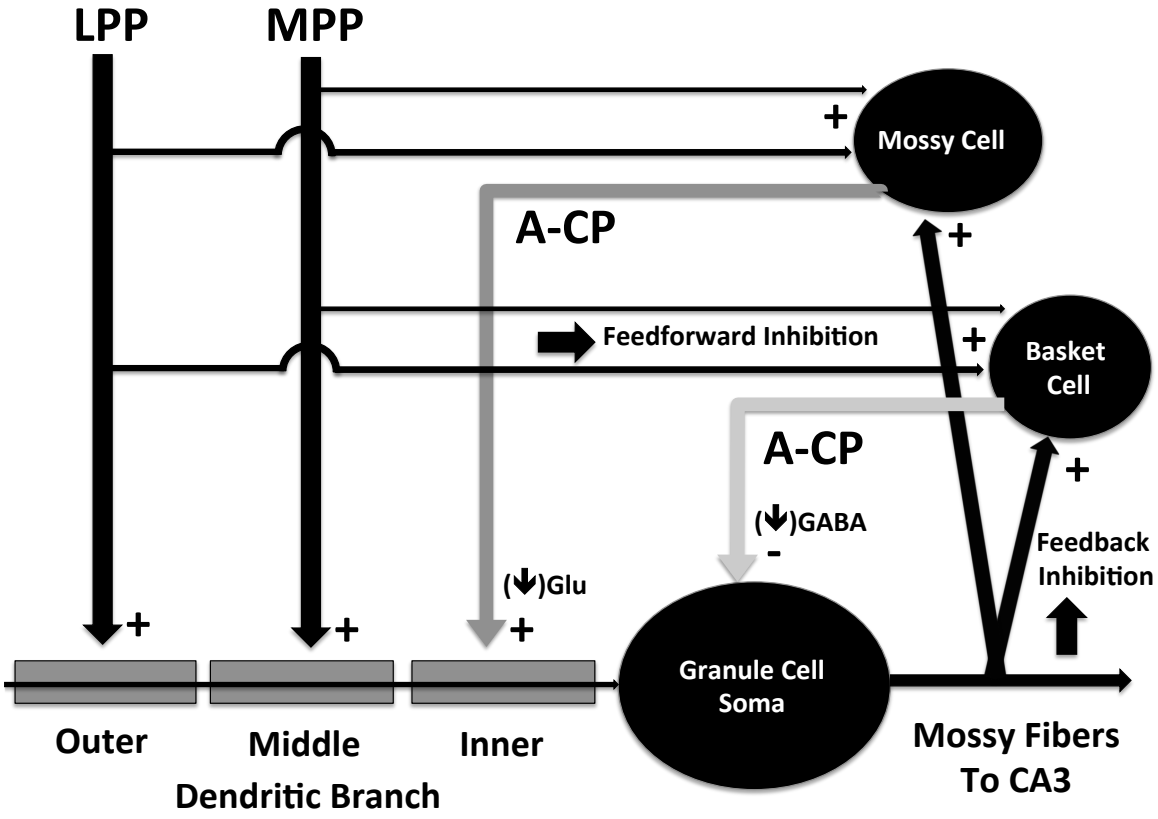

Figure 1: **Dentate gyrus signaling pathways.** Cannabinoids may negatively impact the medial perforant path and associational-commissural projection afferents onto the dentate granule cell. Cannabinoids decrease glutamate release from mossy cells and decrease GABA release from basket cells. Abbreviations: LPP, lateral perforant path; MPP, medial perforant path; A-CP, associational-commissural projection; (+), excitatory synapse; (-), inhibitory synapse.

Specific aims

In order to address the role of cannabinoids in spatial memory consolidation the following aims were developed to address the regional specificity of a specific behavioral response, underlying physiology, and functional activity:

Aim 1: To address regional specificity for a cannabinoid induced deficit in spatial memory consolidation by examining the hippocampal dentate gyrus. The dentate gyrus acts as a gateway into the hippocampal formation, specifically with regards to medial and lateral perforant path projections from the entorhinal cortex; and therefore, may be a key location cannabinoids exert influence to disrupt memory.

- Experiment 1.1: Evaluate differences in dendritic morphology of the lateral perforant path, medial perforant path, and commissural/associational path into the hippocampal dentate gyrus following chronic cannabinoid agonist WIN 55,212-2 administration.
- Experiment 1.2: Evaluate 24-hour consolidation in the MWT following direct infusion of the cannabinoid agonist WIN 55,212-2 via cannulation into the hippocampal dentate gyrus.

Aim 2: To address changes in physiology that may account for a cannabinoid induced deficit in spatial memory consolidation by examining effects of cannabinoids on synaptic plasticity. This aim will explore how cannabinoids impact a physiological marker of plasticity critically involved in long-term cellular memory formation.

- Experiment 2.1: Evaluate changes to long-term potentiation *in vivo* (pop-spike amplitude, decay) following medial and lateral perforant path to dentate gyrus stimulation with the cannabinoid agonist WIN 55,212-2 on board.

Aim 3: To address functional activity changes that may account for a cannabinoid induced deficit in spatial memory consolidation by examining neurotransmitter release and signal transduction pathways. By changing neurotransmitter release and signal transduction pathways CB_1 receptors may be critically involved in dampening neuronal signals to foster forgetting naturally in the body.

- Experiment 3.1: Evaluate paired pulse facilitation ratios following long-term potentiation *in vivo* to gather information regarding glutamate and GABA transmission (Aim 2).
- Experiment 3.2: Evaluate expression of immediate early gene products (Arc, cFos, zif268) by quantitative RT-PCR to address differences in signal transduction pathways following cannabinoid administration and MWT training (Aim 1.1) and long-term potentiation (Aim 2).

Significance

The set of experiments outlined above will investigate the effect of an exogenous cannabinoid agonist on spatial memory consolidation using a range of experimental techniques, which will capture systems levels phenomena down to cellular level phenomena. When taken together, these findings should reveal how cannabinoids are detrimental to long-term memory formation, consolidation and storage in the brain. These studies will also inform the role of CB_1 receptors in hippocampal circuitry. Although it is widely known CB_1 receptors are densely packed in the dentate gyrus, their

exact function remains mysterious. It is possible that CB_1 receptors interact with their endogenous ligands, anandamide and 2-AG, after synaptic activity to modulate consolidation and produce the natural process of forgetting. Given the scarcity of information regarding the effects cannabinoids have on long-term memory processes, the proposed study will provide important and novel information which may be used to inform drug policy and/or establish treatments for cognitive disorders.

Distribution of specific aims across chapters

The effects of cannabinoid administration on consolidation of spatial memory will be assessed in three aims that cumulatively investigate the regional specificity, physiology and functional activity changes induced by cannabinoid administration. To benefit publication purposes the five experiments will be combined into three concise dissertation chapters. Cannabinoid regional specificity will be broken down into two chapters. A portion of Aim 1 will be addressed in Chapter 3 (Experiment 1.1) which focuses on dendritic morphology of various pathway projections from the entorhinal cortex, specifically the lateral perforant path, medial perforant path, and associate-commissural path that terminate in the upper blade of the dentate gyrus. The second part of Aim 1 will be addressed in Chapter 4 (Experiment 1.2), which focuses on 24-hour consolidation in the MWT following direct infusion of WIN 55,212-2 into the dorsal dentate gyrus. Aim 2 examining the effects of cannabinoids on electrophysiology, will be addressed in Chapter 5 (Experiment 2.1), which examines the effect WIN 55,212-2 has on LTP following medial perforant path-to-dentate gyrus stimulation. Aim 3 examining the effects of cannabinoids on functional activity changes in the dentate gyrus will be found in Chapters 4 and 5 combined with their respective experimental

manipulation. The differences in immediate early gene products (Arc, cFos, and zif268) following MWT behavior can be found in Chapter 4, while the differences following induction of LTP can be found in Chapter 5. Chapter 5 also contains Experiment 3.1 of Aim 3, which evaluates differences in GABAergic and glutamatergic transmission via paired pulse ratios.

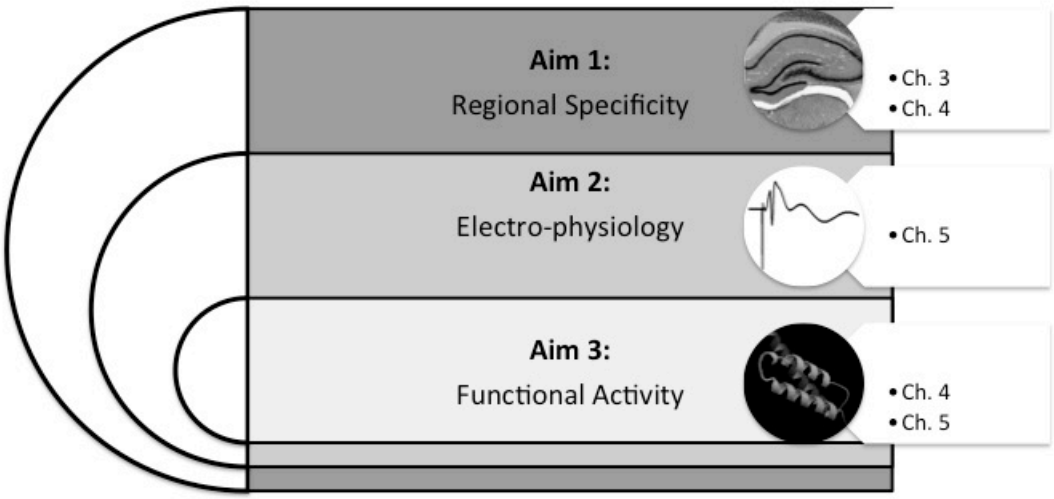

Figure 2: **Aims distributed across chapters.** The three aims are located in various sections of chapters 3-5.

CHAPTER 3: DENTATE GYRUS REGIONAL SPECIFICITY PART ONE (DENDRITIC MORPHOLOGY)

The region of interest to investigate the cannabinoid influence on memory consolidation is the hippocampus. The focus is on the hippocampus because this region contains the highest density of CB_1 receptors, has a well-defined synaptic circuitry, expresses several forms of well-studied synaptic plasticity, and is critically involved in spatial memory. The dentate gyrus acts as a gateway into the hippocampal formation, specifically with regards to medial and lateral perforant path projections from the entorhinal cortex (Figure 3); and therefore, may be a key location cannabinoids exert influence to disrupt memory. It is assumed that after the medial perforant path and lateral perforant path circulate the entire hippocampus, the transformations it undergoes are presumably essential for long-term memory storage and consolidation.

In order to assess the morphological changes in these projections produced by cannabinoid administration, a chronic administration model will be used as changes to dendritic morphology and spine density take repeated administration over time to produce. An acute dose of cannabinoids would not be sufficient to produce the type of dendritic morphological change captured by the Golgi-Cox technique. It should be noted though, that the overall goal of these Golgi-Cox studies is to assess changes in hippocampal dentate gyrus regional specificity following cannabinoid agonist exposure and changes to specific regional projections.

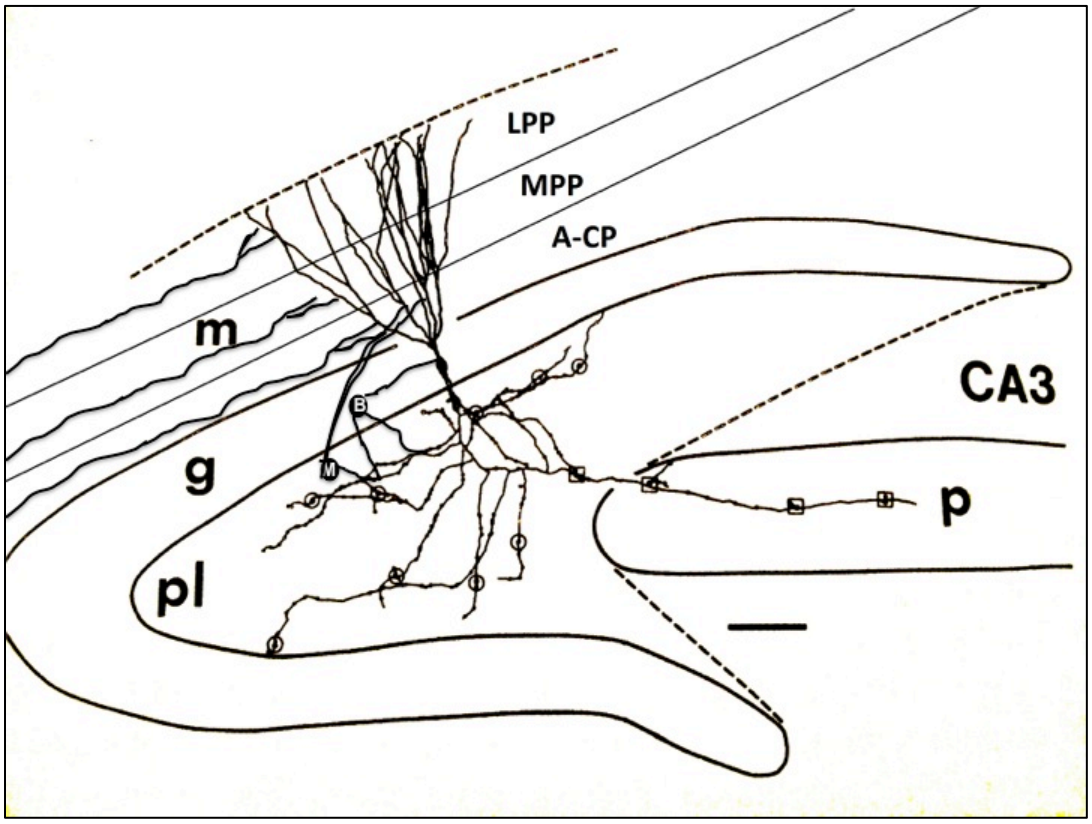

Figure 3: Line drawing of dentate gyrus connectivity. [Adapted from Johnston & Amaral (2004)]. Projections from the entorhinal cortex target specific regions of dentate dendritic branches. Granule cell axons and mossy fibers project to CA3 and the A-CP of unilateral and contralateral hemispheres. GABAergic basket cells (B) are located in the granule cell layer, while glutamatergic mossy cells (M) are located in the polymorphic layer. Abbreviations: m, molecular layer; g, granule cell layer; p, pyramidal cell layer; pl, polymorphic layer; LPP, lateral perforant path; MPP, medial perforant path; A-CP, associational-commissural projection

Chronic cannabinoid agonist (WIN 55,212-2) exposure alters hippocampal dentate gyrus spine density in adult rats

Accepted to *Brain Research* on October 20th, 2013

Abstract

Chronic abuse of drugs can result in vast negative repercussions on behavioral and biological systems by altering underlying neurocircuitry. Long-term cannabinoid administration in rats leads to detrimental cellular and dendritic morphology changes. Previous studies have found that chronic treatment with delta-9-THC selectively decreases dendritic morphology and spine density in the dentate gyrus of adolescent rats (Rubino, et al., 2009); however, whether these changes are specific to a particular developmental age is not known. The present study evaluated the effects of chronic exposure (7 or 21 days) to WIN 55,212-2 (i.p., 3.7 mg/kg), a potent cannabinoid agonist, on dendritic morphology of dentate gyrus neurons in adult rats. Upon completion of treatment brains were processed for Golgi-Cox staining. No significant effects of WIN 55,212-2 exposure were observed for dendritic branching or length. Spine density was quantified in the inner (proximal), middle, and outer (distal) thirds of the dendritic fields selected to approximate the spatial loci of afferents comprising the associational-commissural pathway, medial perforant path, and lateral perforant path, respectively. Compared to vehicle controls there was a significant reduction in spine density (~1 spine/10 μm) in the inner and middle dendritic segments. The spine density reduction was significant in inner segments following 7 days of treatment. These results suggest that chronic cannabinoid treatment specifically alters spine density in the dendritic targets of the associational-commissural afferents and medial perforant path projections, but not

lateral perforant path. The resulting loss of dendritic spine density may be an important factor underlying cannabinoid induced memory impairments.

Introduction

Long-term exposure to exogenous cannabinoids can result in persistent changes to dendritic morphology and spine density (Kolb, et al., 2006; Rubino, et al., 2009). Changes to dendritic morphology represent potential mechanisms by which cannabinoid exposure may influence behavioral and cognitive processes. Previous studies have demonstrated that chronic treatment with delta-9-tetrahydrocannabinol (THC) after 10-12 days selectively alters dendritic morphology of neurons depending on developmental age and region of interest. In adolescent rats THC administration altered dendritic morphology of dentate gyrus granule cells (Rubino, et al., 2009), while in adult rats THC administration increased dendritic morphology of medium spiny neurons of the nucleus accumbens shell and pyramidal neurons of the medial prefrontal cortex with no changes to the CA1 field of the hippocampus, striatum, orbital frontal cortex, parietal cortex, or occipital cortex (Kolb, et al., 2006). Accompanying the changes in granule cell dendritic morphology (i.e., lowered dendritic length, branching, spine density) produced by chronic exposure to cannabinoids in adolescence, Rubino et al. (2009) found deficits in spatial working memory in a radial arm maze, decreased protein expression (GFAP, VAMP2, PSD95) and NMDA receptor levels across the hippocampus. When taken together, previous studies imply chronic cannabinoid abuse in adolescence results in decreased synaptic plasticity and long-term cognitive deficits in adulthood. The impact of cannabinoids with use beginning in adulthood remains an open area of research, as little has been reported in this area. Whether the pattern of hippocampal alterations seen by

Rubino et al., (2009) is observed in adult animals that begin cannabinoid use in adulthood has yet to be determined. Also, given that previous differences in dendritic morphology were found with THC, it is pertinent to see if the effects generalize to other cannabinoid agonists with different receptor binding affinities.

Cannabinoid administration induces memory deficits in a wide assortment of behavioral paradigms (Riedel & Davies, 2005). The most commonly reported outcomes of cannabinoid use or exposure are pronounced short-term memory deficits (Robinson & Riedel, 2004) mediated by CB_1 receptors in the CA1 region of the hippocampus (Wise, Thorpe, & Lichtman, 2009). Cannabinoids also disrupt long-term spatial memory storage by interfering with memory consolidation processes mediated by CB_1 receptors in the dorsal hippocampus (Yim, et al., 2008). It is likely CB_1 receptors in other regions of the hippocampal formation modulate other forms of memory and consolidation processes.

The dentate gyrus acts as a gateway into the hippocampal formation, specifically with regard to medial and lateral perforant path projections from the entorhinal cortex that represent major neocortical afferents into the hippocampus, and therefore, may be a key location cannabinoids exert influence to disrupt memory. Mossy cells of the dentate hilus contain the highest levels of CB_1 receptors amongst excitatory hippocampal neurons (Kawamura et al., 2006; Monory et al., 2006). There is also a dense representation in the inner third of the molecular layer of the dentate gyrus (Katona, et al., 2006), which corresponds spatially to the associational-commissural projection, where mossy cells form synapses onto dendrites of the granule cells (Johnston & Amaral, 2004). Afferents to dentate gyrus granule cells also include the medial perforant path and lateral perforant path projections from the entorhinal cortex, which form synapses in the middle third and

outer third (distal) of the granule cell dendritic fields. The medial and lateral entorhinal inputs have been shown by Hargreaves et. al. (2005) to relay different types of information, with spatial information conveyed by the medial perforant path and non-spatial information conveyed by the lateral perforant path. It is important to further understand the role cannabinoids have on the dentate gyrus as this region contains the highest density of CB_1 receptors, has well-defined synaptic circuitry and synaptic plasticity, and is critically involved in spatial memory consolidation.

The present study was designed to examine changes in dendritic morphology produced by chronic treatment with a potent cannabinoid agonist WIN 55,212-2. The drug treatment began once the animals were mature adults (six months of age), which distinguishes this study from previous reports. Adult rats were given daily intraperitoneal (i.p*)* injections of WIN 55,212-2 or vehicle for 7 or 21 days after which the brains were processed for Golgi-Cox staining. Dendritic branching and length were quantified in granule cells sampled from the medial portion of the upper blade of the dentate gyrus, Zilles' area DG, Figure 4 (Zilles, 1985). By focusing on the spatial distribution of dentate gyrus afferents, it is also possible to infer how cannabinoids impact various hippocampal projections and their targets. Toward this goal, spine density was quantified in segments sampled from the inner, middle and outer thirds of the dendritic fields, relative to the soma, to estimate changes in the targets of the associational-commissural projection, medial perforant path projection, and lateral perforant path projection, respectively (Figure 5).

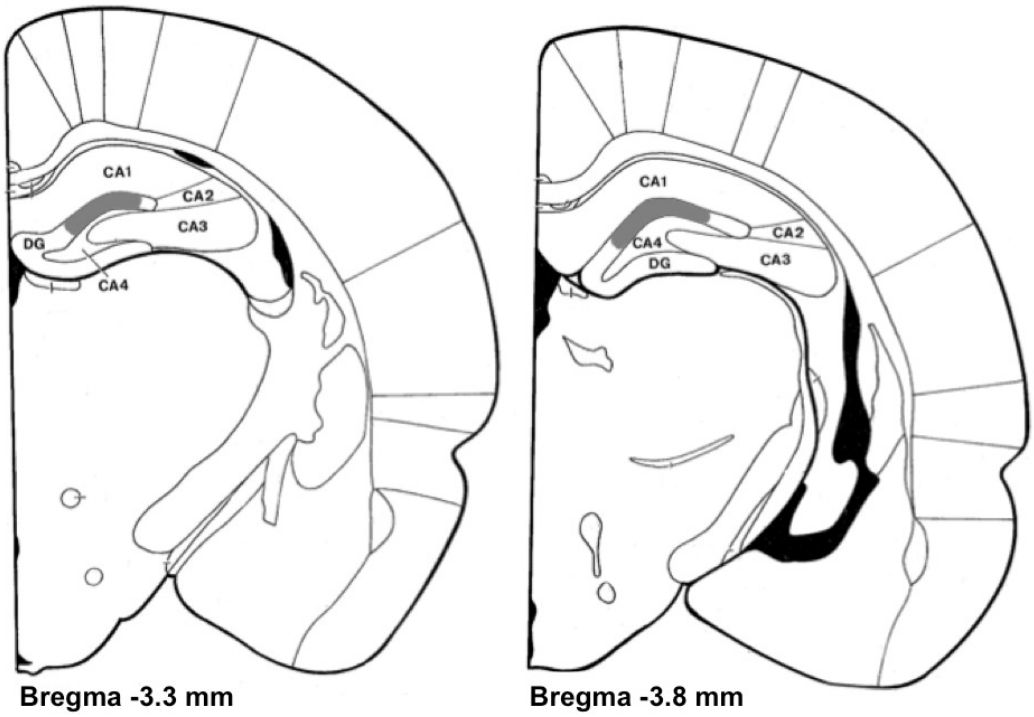

Figure 4: Dentate gyrus coronal sections. Coronal sections [adapted from Zilles (1985)] showing the region from which dentate granule cells were drawn. Sampling occurred in the upper blade of the dentate gyrus (DG), highlighted in gray, in sections ranging from 3.3 mm to 3.8 mm posterior to Bregma.

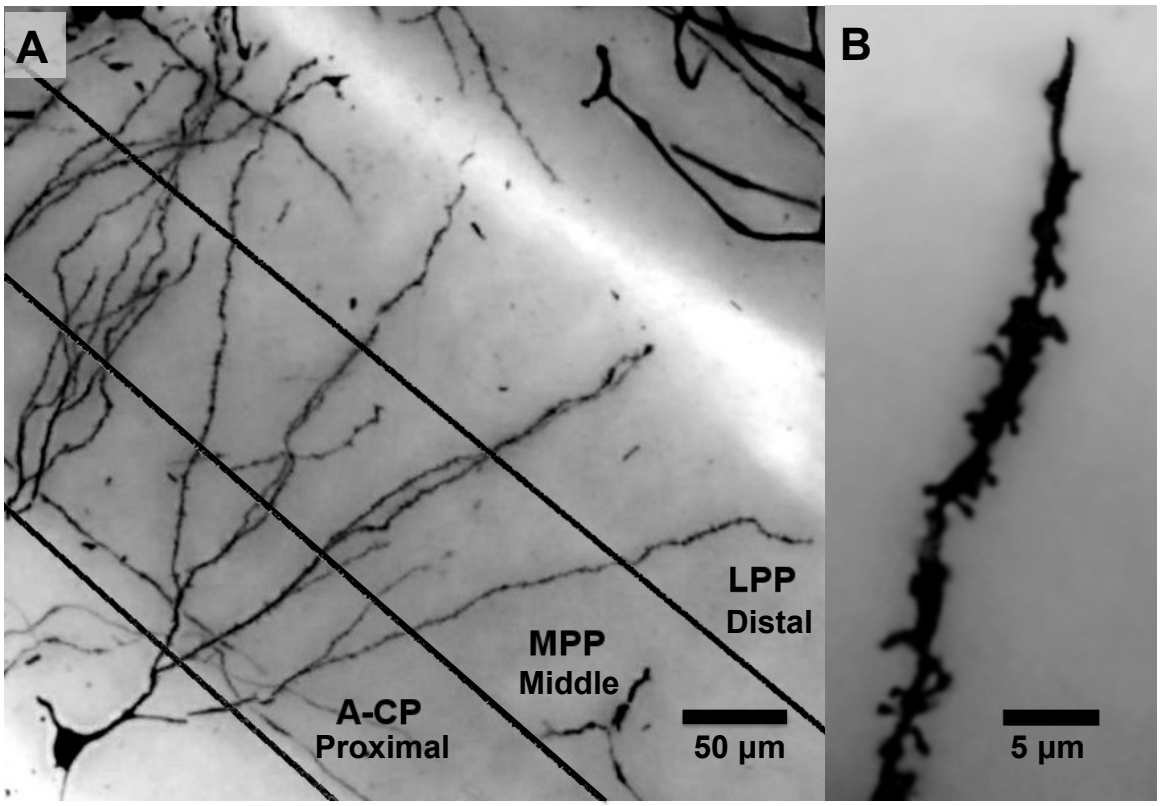

Figure 5: Granule cell divisions. (A) For spine analysis the dentate granule cell dendritic field was divided into three layers (proximal, middle, and distal) to correspond to major afferent pathways: A-CP (ssociational-commissural pathway), MPP (medial perforant path), and LPP (lateral perforant path). (B) Example illustrating the staining quality of granule cell spines (1200X magnification).

Experimental procedures

Animals

Subjects were 24 male Long-Evans rats acquired from Harlan Laboratories (Indianapolis, IN). All rats were six months of age at the beginning of experiment. Rats were pair-housed in standard clear plastic cages and maintained in a temperature and humidity controlled vivarium with food and water available *ad libitum*. The University of New Mexico Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

Drugs treatments

The cannabinoid agonist WIN 55,212-2 (Sigma-Aldrich), was dissolved in 5% DMSO (Sigma-Aldrich) with 0.1% Tween 80 (Sigma-Aldrich) and brought to volume with sterile Dulbecco's phosphate buffered saline (Sigma-Aldrich). WIN 55,212-2 was prepared at a concentration of 3.7 mg/mL. The control solution consisted of the drug vehicle. Both drug and vehicle solutions were prepared daily and injected at a volume of 1 mL/kg of body weight. All drug doses and vehicle preparations were selected according to previous literature (Pamplona, et al., 2006; Pamplona & Takahashi, 2006; Schneider, Drews, & Koch, 2005; Yim, et al., 2008). The rats were divided into four experimental groups (n = 6): 7 day vehicle, 7 day WIN, 55-212-2, 21 day vehicle, and 21 day WIN 55,212-2. Shortly after the beginning of the light cycle (1000 h), rats were weighed in the colony room and given an i.p. injection of WIN, 55, 212-2 or vehicle control, once daily, for 7 or 21 days. Rats were immediately returned to their cage following injection.

Golgi-Cox staining and analysis

At the conclusion of the experiment, one day following their last injection, rats were given an overdose of sodium pentobarbital and perfused transcardially with 0.9% (w/v) saline. The brains were extracted and immediately immersed in 30 mL of Golgi-Cox solution (Glaser & Van der Loos, 1981) for 14 days, followed by immersion in 30% (w/v) sucrose for three days. Coronal sections, 200 μm thick, were cut on a vibrating blade microtome, mounted on 2% gelatinized slides, stained, dehydrated, cleared, and cover slipped, as described by Gibb and Kolb (1998).

Granule cells of the dentate gyrus, Zilles' area DG (Zilles, 1985), were selected for analysis from the medial portion of the upper blade (Figure 4). An Olympus light microscope (Model BX51) equipped with a drawing attachment was used for analysis. Five neurons from each hemisphere (10 neurons per rat) were traced using the *camera lucida* technique (250X magnification). Selection was limited to unobscured, complete, and well-impregnated neurons with representative dendritic morphology for the region of interest. Sampling included sections ranging from 3.3 mm to 3.8 mm anterior to Bregma. An experimenter blind to drug conditions performed all morphology, spine tracing, and analysis.

Dendritic branching was measured by counting bifurcations on each dendrite (Coleman & Riesen, 1968). First-order branches were dendritic segments prior to the first bifurcation from the soma and branch order was incremented by one for each subsequent bifurcation on a given dendritic branch. The number of first through sixth-order (and higher) branches was quantified and an estimate of total branches was determined from these values. Dendritic length was measured using the Sholl analysis (Sholl, 1981). A printed transparency of a series of 20 μm concentric ring intersections (calibrated to 250X

final magnification) was centered over the cell body and the total number of intersections between each ring and dendritic branch was counted. The Sholl values were converted to estimates of dendritic length as a function of distance from the soma.

For spine density analysis, each dendritic branch was divided into three different segments at various distances from the cell body: an inner (proximal) segment, middle segment and outer (distal) segment (see Figure 5). Each segment was approximately 50 μm in length. These divisions were selected to correspond to the three major afferent pathways projecting into the dentate gyrus: associational-commissural path, medial perforant path, and lateral perforant path, correspondingly. Selection was limited to branches for which all three segments were continuous (i.e., not uninterrupted by a branch point). Spine density was measured by tracing each dendritic segment at high power (2000X magnification) followed by tracing all spines present in each segment. Total spine density per 10 μm was calculated from these values. Spine density was quantified on 10 granule cells, five cells per hemisphere. Mean spine density was calculated on 10 measurements per segment, three segments per cell. The unit of analysis for each rat was mean spine density for the entire dendritic field or for each layer.

Results

Analyses of Variance (ANOVAs) were performed using SPSS (version 20 for Macintosh). All test statistics reported here were significant at $p < 0.05$ unless otherwise noted. For all analyses Drug condition (vehicle, WIN 55,212-2) and Exposure length (7 day, 21 day) were between-subjects factors. Within-subjects factors included in separate ANOVAs for branch order, length, and spine density were distance from soma (Sholl analysis), branch order, or segment (spine density only). Effect sizes (partial eta squared;

η_p^2) are reported for all effects. One vehicle animal was identified as an outlier with respect to spine density quantification and excluded from analysis as its spine density values were greater than three standard deviations below the group mean, consistent with suboptimal staining. There were a total of 23 brains included in analyses of dendritic length, branching, and spine density (11 vehicle and 12 WIN 55,212-2).

Dendritic length and branching

Mean dendritic branching and length are presented in Figure 6A and B. Because there were no significant effects involving duration of exposure, the data were collapsed over this factor to simplify presentation of the results. There were no significant main effects or interactions for branch order [all ps > 0.355] or total branches [M_{VEH} = 22.317, M_{WIN} = 22.042, all ps > 0.439]. There were also no significant main effects or interactions with distance from soma for dendritic length [all ps > 0.226], or for total dendritic length [M_{VEH} = 32.891, M_{WIN} = 33.725, all ps > 0.275].

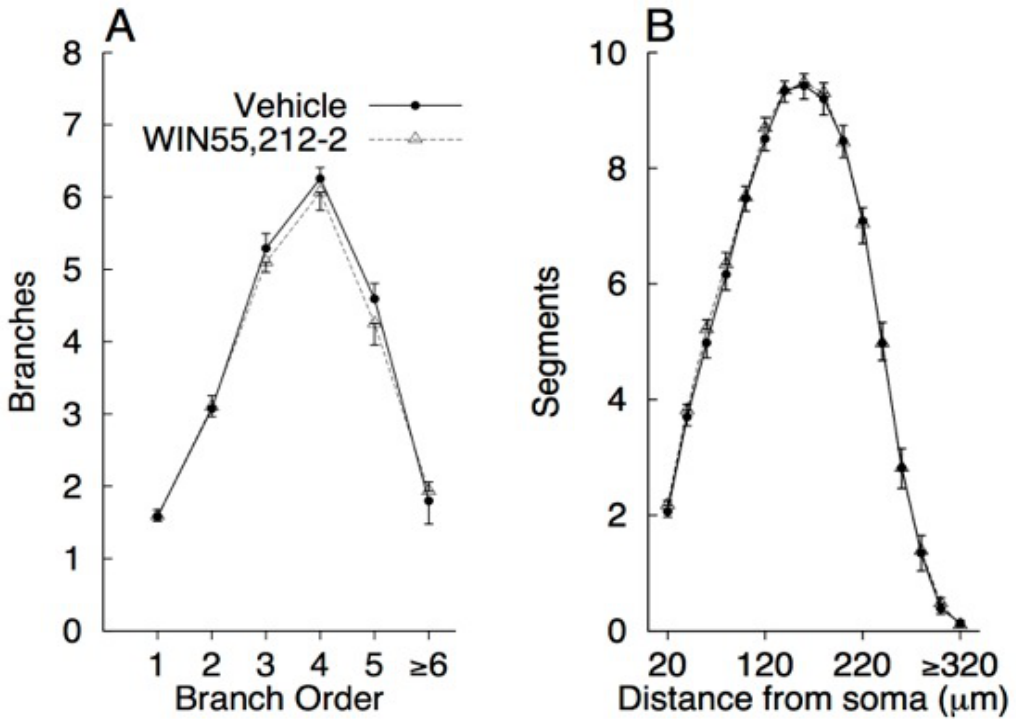

Figure 6: Dendritic branching and length group means. Mean (+SEM) of total branches for first through sixth (and greater) branch orders (A) and dendritic length as a function of distance from the soma (B) for vehicle and WIN 55,212-2 exposed rats.

Spine density

Mean spine density for the three segments of interest: inner, middle, and outer thirds (association-commissural path, medial perforant path, and lateral perforant path, respectively), in the dentate gyrus are presented in Figure 7. Because there were no interactions involving duration of exposure and drug treatment the data were collapsed over levels of exposure duration to simplify presentation of the results. The Segment X Drug interaction approached significance [$F(2, 38) = 3.126$, $p = 0.055$, $\eta_p^2 = 0.141$]. Inspection of the means suggests that WIN 55,212-2 exposure reduced spine density on the order of 1 spine per 10 microns in the inner and middle segments. Comparisons of the drug groups for each segment revealed significant differences for the inner segment [$F(1, 21) = 5.511$, $p = 0.029$, $\eta_p^2 = 0.208$] and middle segment [$F(1, 21) = 4.661$, $p = 0.043$, $\eta_p^2 = 0.182$], but not the outer segment [$p = 0.557$].

In addition, there was a main effect of Segment [$F(2, 38) = 17.106$, $\eta_p^2 = 0.474$], which was due to increased spine density in the middle segment compared to each of the other two segments [all $ps < 0.0004$]. There was also a significant interaction between Segment X Exposure duration [$F(2, 38) = 9.775$, $\eta_p^2 = 0.340$]. Rats that received injections (vehicle or drug administration) for 7 days had greater spine density in the inner segment than rats that received injections for 21 days [$M_{7d} = 15.876$, $M_{21d} = 14.750$, $F(1, 21) = 6.704$, $\eta_p^2 = 0.242$]. No other main effects or interactions were significant.

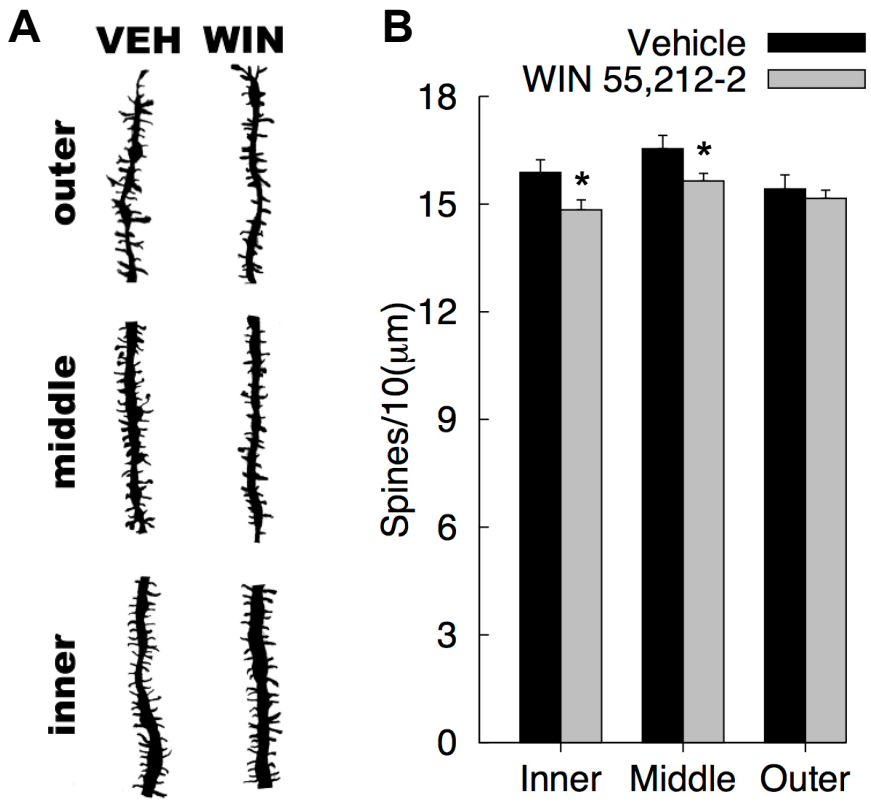

Figure 7: Dendritic spine density group means. Dentate granule cell dendritic field spine density in vehicle and WIN 55,212-2 exposed rats: (A) Representative *camera lucida* drawings of the cell layers from each group (1200X magnification), (B) Mean (+SEM) spine density from the inner, middle, and outer segments, corresponding to the Associational-Commissural Pathway, Medial Perforant Path, and Lateral Perforant Path (respectively). WIN 55,212-2 exposed rats had lower spine density in inner and middle segments compared to Vehicle controls [*$p<0.05$].

Discussion

Long-term cannabinoid administration was associated with a marked reduction in dendritic spine density within granule cells of the dentate gyrus. More specifically, the spine density loss (1 spine per 10 μm) was limited to the inner and middle dendritic segments of the granule cell, which correspond spatially to targets of the associational-commissural afferent and medial perforant path afferent, respectively. These observations do not reflect a generalized reduction in overall dendritic morphology, as no differences were found between drug and vehicle groups in measures of dendritic length and branching, but instead indicate select spine sensitivity in spatially relevant afferents from the entorhinal cortex following long-term cannabinoid exposure. When taken together, these results suggest long-term WIN 55,212-2 treatment specifically alters targets of the associational-commissural path and medial perforant path afferent from entorhinal cortex but not the lateral perforant path.

A previous study that examined dendritic morphology after long-term THC exposure in adolescence found reductions in dendritic branching, length and spine density in the dentate gyrus that persisted into adulthood (Rubino, et al., 2009). The current study extends these findings by showing that spine loss in the dentate gyrus also occurs in adult rats that began chronic treatment in adulthood after the brain was fully matured; therefore, some changes in dendritic morphology following long-term cannabinoid administration are not specific to a particular developmental age. Conversely, the fact that we did not find differences in overall dendritic branching and length in adult rats implies that adolescent granule cells may be more sensitive to cannabinoids. There is clear evidence for adolescent cannabinoid sensitivity that results in persistent, long-term

decreases in hippocampal dendritic morphology, protein expression, NMDA receptor levels, and deficits spatial working memory (Rubino, et al., 2009). Given that the negative consequences observed after adolescent exposure far outnumber the effects observed after mature adulthood exposure, there is an indication that hippocampal cannabinoid sensitivity may be partially resolved by adulthood. In fact, cannabinoid-induced structural plasticity was only found in specific circuits (associational-commissural path and medial perforant path) within the hippocampus and only in relation to spine density within those circuits. Reductions in overall dendritic branching and length in adult rats might have been expected given the reductions in spine density; however, no significant reductions in branching or length were observed. One possibility is that the lack of spine density reductions in the most distal (outer) portions of the granule cell dendritic fields helped prevent overall reductions in length and branching. Taken together with the current observations, the marked reductions in branching and length that have been reported in adolescent rats suggest that granule cells are more sensitive to cannabinoids prior to adulthood.

Another study that examined dendritic morphology following long-term THC exposure in adult rats found increased dendritic morphology on medium spiny neurons of the nucleus accumbens and pyramidal neurons of the medial prefrontal cortex and a lack of drug differences in pyramidal neurons in the CA1 field of the hippocampus (Kolb, et al., 2006). These authors did not investigate morphology differences in the dentate gyrus, but taken together the available data indicate that regionally specific changes within the hippocampus and hippocampal formation occur following long-term cannabinoid

exposure. Potentially, increases in dendritic morphology in other brain regions may compensate for reductions in dendritic spine density in the dentate gyrus.

The current study also provides evidence that a different cannabinoid agonist WIN 55,212-2, with higher binding affinities for the CB_1 receptor than THC, produces similar changes in dendritic morphology following long-term administration; therefore, the changes in dendritic morphology may be a consequence of all cannabinoid agonists. Previously it was shown that WIN 55,212-2 administration elevated spine densities in the zebra finch song regions, area X and XVC, following long-term exposure (Gilbert & Soderstrom, 2011); thus, future studies should further examine alterations in spine density in different brain regions following exposure to WIN 55,212-2.

The current study examined differences in dendritic morphology 24 hours after the last drug injection. With this design it is possible to examine the immediate changes produced by WIN 55,212-2, but it remains unknown if the effects are long lasting, and more research is needed to verify the persistence of spine density changes observed following exposure and withdrawal. Previous cannabinoid research using THC by Kolb et al. (2006) and Rubino et al. (2009) found the morphology changes produced by cannabinoids to be long lasting and persistent even after several weeks of withdrawal (from one to two months). Therefore, it is likely the WIN 55,212-2 spine density alterations are long-lasting. Although following cocaine treatment Kolb et al. (2003) found that persistence of spine alterations (in this case enhancements) varies depending on drug treatment regiment, which may also play an important role in the persistence of changes observed with cannabinoids.

Due to the fact that there is a dense band of CB_1 receptors present in the lower layer of the dentate gyrus stratum moleculare (Katona, et al., 2006) this area is likely to be impacted by long-term cannabinoid exposure. This region also corresponds to the ipsilateral associational-commissural projection originating from axons of the mossy cells of the hilus that form excitatory synapses back onto granule cells creating a feedback loop to regulate dentate gyrus responsiveness (Johnston & Amaral, 2004; Laurberg & Sorensen, 1981). Interestingly, endocannabinoids have been shown to signal at glutamatergic synapses on dendritic spines throughout the hippocampus. Katona et. al. (2006) found that diacylglycerol lipase α, the precursor to 2-arachidonoyl-glycerol, is highly concentrated on the heads of dendritic spines indicating involvement with retrograde signaling at glutamatergic synapses to presynaptic CB_1 receptors. Expanding on these results, Uchigashima et. al (2011) showed that within the dentate gyrus specifically, mossy cells contained abundant presynaptic CB_1 receptors and granule cell spines released 2-arachidonoyl-glycerol forming a bridge for retrograde signaling to adjust network activity in the dentate gyrus following excitation. If the CB_1 receptors are located at the end of a mossy cells synapses releasing glutamate onto granule cell spines, following exogenous cannabinoid agonist stimulation the $G_{i/o}$ coupled CB_1 receptor would prevent glutamate release and the postsynaptic spine may retract due to loss of excitation.

Within the associational-commissural afferents may also be where exogenous cannabinoids are most disruptive by creating feedback inhibition disruption. If cannabinoids inhibit mossy cell excitation of proximal granule cell dendrites following feedback inhibition, the end result may be reduced excitation of the granule cell, reduced

synaptic plasticity, and reduced hippocampal dependent learning and memory. Given that the medial perforant path is involved in long-term potentiation following high frequency stimulation of entorhinal inputs, the medial perforant path is a critical site of memory processing into the hippocampus. It has been shown that during high frequency stimulation of the medial perforant path *in vivo*, CB_1 activation increases glutamate release from perforant path synapses while inhibiting release of GABA from local interneurons (Sokal, Bennetti, Girlanda, & Large, 2008). Additionally it has been shown that medial perforant path stimulation preferentially recruits inhibitory basket cells in a feedforward fashion, bypassing excitatory granule cell inputs and feedback inhibition (Ewell & Jones, 2010). The cumulative effects of cannabinoids on these processes may contribute to the pattern of spine density loss observed here.

Alterations in spine density resulting from long-term cannabinoid exposure in adult rats may be an important contributing factor underlying behavioral findings of cannabinoid induced memory impairment and memory consolidation deficits. Segment specific changes in spine density may have important consequences for spatial learning and memory processes in the dentate gyrus, especially considering the pattern of reductions observed here. Hargreaves et. al. (2005) showed that the medial perforant path transferred spatial information from the medial entorhinal cortex, while the lateral perforant path transferred nonspatial information. Alterations in the synaptic targets of medial perforant path projection in the dentate gyrus may contribute to spatial learning and memory impairments, possibly through reductions in processes involved in consolidation of spatial information. WIN 55,212-2 given post-training in the Morris water task impaired long-term spatial memory consolidation by activating CB_1 receptors

in the dorsal hippocampus (Yim, et al., 2008). Prior work from our laboratory found similar Morris water task post-training administration consolidation deficits following a 24-hour consolidation time frame (Candelaria-Cook, 2009; Candelaria-Cook & Hamilton, 2008). We also found that the WIN 55,212-2 spatial memory consolidation deficit could be recovered by administration of the cannabinoid antagonist AM 251 (Candelaria-Cook & Hamilton, 2010), further supporting the role of CB_1 receptors in spatial memory consolidation. Future research should examine the relationship between cannabinoid induced reductions in spine density and memory consolidation. One prediction derived from the present data would be that cannabinoids may have a more profound effect on spatial memory consolidation than consolidation of non-spatial information processed via the lateral perforant path.

A few limitations of the current study should be taken into consideration. The present study evaluated dendritic morphology only within the dentate gyrus and its afferents. It is likely that different, yet complementary changes in morphology exist in other regions of the hippocampus and in other regions of the brain, as indicated by Kolb et al. (2006). The present study was also limited to analysis of males. Future work should address if sex differences exist in granule cell dendritic morphology. The present study was also limited to one WIN 55,212-2 drug dose. A high dose was selected based on previous literature on cannabinoid-induced deficits on spatial memory. While the results obtained here with a high dose of cannabinoids were limited to spine density, in the future different doses ranging from low to high should be investigated.

In summary, the present findings demonstrate a significant reduction in dendritic spine density of the dentate gyrus following long-term administration of WIN 55,212-2 in

adult rats. This loss in spine density on the granule cells was specific to the portions of the dendritic fields that receive associational-commissural afferents from within the hippocampal formation and medial perforant path afferents from the entorhinal cortex, and did not generalize to overall changes in dendritic length and branching. Given the involvement of granule cell plasticity in memory, these observations suggest exposure to exogenous cannabinoids can have profound and long-lasting effects on long-term spatial memory storage and consolidation.

CHAPTER 4: DENTATE GYRUS REGIONAL SPECIFICITY PART TWO (CANNULATION)

The dentate gyrus is a critical component of the hippocampal formation that bridges connections from the entorhinal cortex to the hippocampus proper. By acting as a relay board, the dentate gyrus has the potential to alter all information that enters the hippocampus; therefore, the dentate gyrus has the potential to alter the initial processes underlying memory consolidation and memory storage. Given its potential role in memory consolidation, the dentate gyrus is the primary focus of the following studies on regional specificity of cannabinoid-induced deficits in spatial memory consolidation.

In earlier experiments (Candelaria-Cook, 2009) it was found that cannabinoid administration (via intraperitoneal injections) impaired spatial memory acquisition and consolidation in a hidden platform version of the Morris water task (MWT). First, using the Repeated Acquisition Procedure (Keith & Galizio, 1997), 16 rats were given daily injections over 10 days of WIN 55,212-2 (3.7 mg/kg) or vehicle prior to beginning the MWT to address potential learning and sensorimotor deficits produced when the cannabinoid agonist is on board and the rat is performing the MWT. Rats that received WIN 55,212-2 had a significant learning/acquisition deficit across all days of training, as indicated by longer latencies and routes taken to reach the platform. These rats also had an initial performance deficit during the first three to four days of MWT training, indicating WIN 55,212-2 impacted the rat's ability to swim and perform the MWT normally; however, all sensorimotor deficits exhibited quick tolerance and by the end of the 10 days of training WIN 55,212-2 rats had equivalent performance to vehicle controls. The second experiment assessed the role of cannabinoids on memory

consolidation by giving the drug immediately post-training, thereby avoiding acquisition confounds. In this experiment, 32 rats received WIN 55,212-2 or vehicle injections immediately following fixed platform MWT training and memory was assessed 24 hours later by a non-reinforced probe and four trials of retraining. Rats that received the cannabinoid agonist showed reduced memory for the platform location when compared to vehicle controls indicating a significant 24-hour consolidation deficit during the memory probe and first trial of retraining (Figure 8). Through these sets of experiments cannabinoids were to shown to impair acquisition and consolidation, with minimal disturbance to performance in the MWT. Because these experiments were performed with i.p. injections, further research was needed to investigate receptor specificity for this cannabinoid-induced memory loss.

A follow-up study (Candelaria-Cook & Hamilton, 2010) showed that the MWT consolidation deficit was mediated by CB_1 receptors. Immediately following MWT training rats were given either: vehicle, WIN 55,212-2, AM 251, or a combined WIN 55,212-2 + AM 251 i.p. injection. When the cannabinoid antagonist (AM 251) was given 30 minutes prior to cannabinoid agonist (WIN 55,212-2), the antagonist blocked the memory impairment of the agonist. When given alone, the cannabinoid agonist (WIN 55,212-2) impaired 24-hour consolidation similar to original findings, Figure 8H. Meanwhile, the antagonist alone (AM 251) had no impact on consolidation. This brief follow-up study was able to determine receptor specificity for the cannabinoid-induced memory loss in the MWT. The focus now turns to determining regional specificity for the cannabinoid-induced consolidation deficit. Given the role of the dentate gyrus in memory formation and knowing WIN 55,212-2 can alter spine density in this region (Chapter 3),

perhaps the dentate gyrus is also a region critical for cannabinoid-induced memory consolidation deficits.

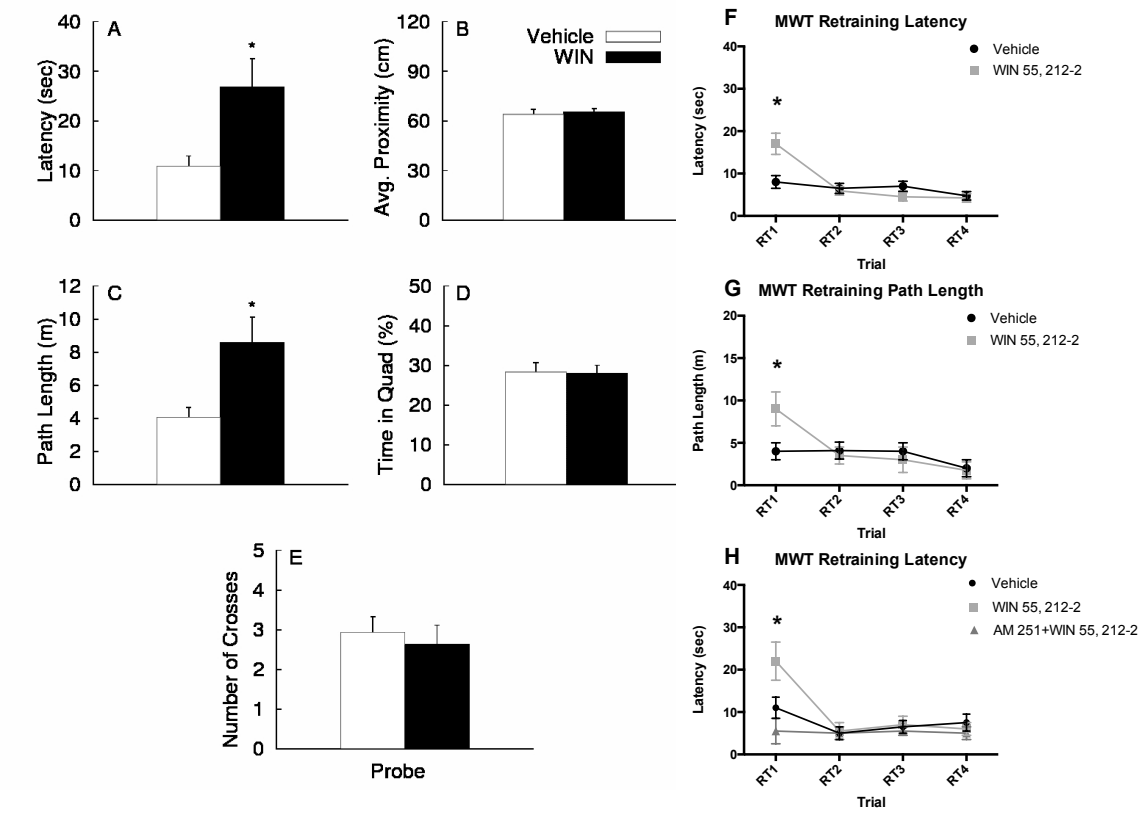

Figure 8: Post-training cannabinoid administration impairs 24-hour consolidation via CB_1 receptors. Morris water task probe and retraining measures following 24-hour consolidation showing WIN 55,212-2 impairment of retention path length and latency. WIN 55,212-2 rats had impaired memory for previous platform location (* denotes $p < 0.05$).

Post-training cannabinoid agonist intrahippocampal infusion does not influence spatial memory consolidation.

Abstract

Cannabinoids have repeatedly been shown to be detrimental to the maintenance of learning and memory processes. A previous study by Yim et al. (2008) reported that an infusion of WIN 55,212-2 into dorsal hippocampus disrupted long-term spatial memory in the Morris water task. The present study evaluated if cannabinoid infusion of WIN 55,212-2, a potent cannabinoid agonist, into the dentate gyrus produced similar results and disrupted short-term 24-hour spatial memory consolidation processes. WIN 55,212-2 (10 μg/μL), AM 251 (40 μg/μL)+WIN 55,212-2 (10 μg/μL), or vehicle was bilaterally infused into the dorsal dentate gyrus. WIN 55,212-2 or vehicle was bilaterally infused into the parietal cortex immediately following Morris water task training. Upon completion of behavior, brains were processed for immediate early gene expression and cannula placement verification. The data suggest WIN 55,212-2 delivered into the dentate gyrus or parietal cortex had no impact on 24-hour memory consolidation, as memory retention was comparable to vehicle controls during the MWT probe test. Interestingly, the combination infusion of AM 251 followed by WIN 55,212-2 30 minutes later did impair consolidation as seen by impaired probe latency, path length and 2nd trial of retraining measures. Further, WIN 55,212-2 did not alter immediate early gene expression in the dentate gyrus or parietal cortex. This behavioral data is contrary to earlier findings using systemic injections. This indicates that it is likely another region of the hippocampal formation, or the summation of all hippocampal areas working together that is responsible for proper memory consolidation. Cannabinoid

neuromodulation in the dentate gyrus is necessary but not sufficient to produce cannabinoid-induced memory impairments.

Introduction

Cannabinoids are widely known to be detrimental to processes underlying normal learning and memory. The most commonly reported cannabinoid-induced deficit is impaired working memory. It known that CB_1 receptors in the CA1 region of the hippocampus are important for short-term memory deficits (Wise, et al., 2009). Following cannabinoid agonist administration working memory is impaired in the radial arm maze (Nakamura, et al., 1991), T-maze (Suenaga, et al., 2008), and delayed-match-to-sample tasks (Heyser, et al., 1993). CB_1 agonists have been shown to impair a wide range of spatial memory tasks selectively hindering acquisition, consolidation, and retention. In the Morris water task (MWT), a single dose of cannabinoids has been shown to impair task acquisition initially (Ferrari, et al., 1999), impair 24-hour consolidation (Candelaria-Cook, 2009; Candelaria-Cook & Hamilton, 2008, 2010) and impair long-term/one month consolidation (Yim, et al., 2008). Due to cannabinoid-mediated acquisition deficits, memory consolidation must be studied with a post-training design. It is likely that CB_1 receptors in other regions of the hippocampal formation modulate other forms of memory and consolidation processes.

Using a post-training administration design, prior work from our laboratory found that a single systemic dose of WIN 55,212-2 resulted in a 24-hour short-term consolidation deficit in the MWT (Candelaria-Cook, 2009; Candelaria-Cook & Hamilton, 2008). A deficit which could be recovered by administration of the cannabinoid antagonist AM 251 (Candelaria-Cook & Hamilton, 2010), further supporting the role of

CB_1 receptors in spatial memory consolidation. Yim et al. (2008) found similar consolidation deficits in the MWT with systemic injection of WIN 55,212-2, although they found the consolidation deficit one month following training and not one week after training, concluding cannabinoids impacted long-term consolidation processes. Yim et al. (2008) further showed regional specificity for the cannabinoid consolidation deficit with post-training intracranial microinfusions of WIN 55,212-2. Inspection of the hippocampal cannula placements used by Yim et al. (2008) determined the authors targeted regions CA1 and dentate gyrus of the dorsal hippocampus. From these studies (Candelaria-Cook, 2009; Candelaria-Cook & Hamilton, 2008, 2010; Yim, et al., 2008) it is clear the cannabinoid agonist WIN 55,212-2 can impair short and long-term consolidation processes. Further, it has been shown that the memory consolidation disruption is mediated by CB_1 receptors, however the specific brain regions or systems responsible remain unidentified. It remains unknown if other regions of the hippocampus are involved in the cannabinoid-induced consolidation deficit, or if the deficit is solely driven by cannabinoid modulation within region CA1 of the hippocampus.

It is important to further understand the role cannabinoids have on the dentate gyrus because mossy cells of the dentate hilus contain the highest levels of CB_1 receptors amongst excitatory hippocampal neurons (Kawamura, et al., 2006; Monory, et al., 2006). The dentate gyrus is also is critically involved in spatial memory consolidation. Medial entorhinal inputs and the medial perforant path terminate in the middle portion of the upper blade of the dentate gyrus and contain spatial information (Hargreaves, et al., 2005) which is sent to the other regions of the hippocampus. Lesions to the medial perforant path input into the dentate gyrus result in impaired place learning in the MWT suggesting

medial perforant path terminals have a higher efficiency in activating hippocampal neurons (Ferbinteanu, Holsinger, & McDonald, 1999). Lesions to the dentate gyrus result in impaired MWT acquisition and delayed matching-to-place tasks when compared to lesions of other hippocampal subregions (Okada & Okaichi, 2009). Lee & Kesner (2002) suggest the different hippocampal subregions are important for different parts of spatial memory, with CA1 and dentate gyrus important for memory acquisition and long-term retrieval and CA3 important for reorganization of spatial representation. However, it may be that all subregions of the hippocampus act as a functional unit for spatial memory and all regions are necessary for proper memory consolidation (Okada & Okaichi, 2009). Whether one part of the hippocampus is more important for memory consolidation remains to be seen.

Immediate early genes (IEGs) are named for their rapid response to cellular stimuli. There are several involved in various components of cellular signaling pathways, but three IEGs in particular are of interest to memory consolidation researchers: Arc (Activity-dependent cytoskeleton associated protein), cFos, and zif268. Arc has been shown to play an important role in behavioral plasticity, when Arc expression is blocked there is interference with consolidation of spatial memory within granule cells (Guzowski et al., 2000). Guzowski et al. (2001) found correlations between the IEGs Arc, cFos, and zif268 and memory consolidation with the MWT. Specifically, they found that Arc is the most responsive IEG to differences in behavioral task demands and Arc mRNA levels were correlated with learning in hippocampal-dependent versions of the MWT. In other tasks of spatial exploration, following environment specific spatial exploration Arc expression within the upper blade of the dentate gyrus exhibits a four-fold increase over

control animals (Chawla et al., 2005). Aside from Arc, other IEGs are also important for memory consolidation. Zif268 is known to be essential for memory consolidation and is involved in the expression of long-term memories (Jones et al., 2001). In zif268 knockout mice early phases of LTP are normal, but later phases of LTP underlying consolidation are not present; therefore, 24-hour consolidation issues arise (Bozon, Davis, & Laroche, 2002).

The present study was designed to examine the impact of a potent cannabinoid agonist (WIN 55,212-2) on spatial memory consolidation and resulting immediate early gene expression (Arc, cFos, and zif268) within the hippocampal dentate gyrus. Immediately following MWT training, a high-dose intrahippocampal infusion of vehicle, WIN 55,212-2, AM 251+WIN 55,212-2, targeting the upper blade of the dentate gyrus, was given to adult rats. Memory retention was tested 24-hours later with a no-platform probe and four trials of retraining. In the first subset of rats, IEG mRNA levels were measured in the dentate gyrus four hours after MWT behavioral training. In the second subset of rats, cannula locations within the dentate gyrus were verified with fluorescent muscimol imaging. Together, these manipulations should reveal if the dentate gyrus is important for cannabinoid-induced memory consolidation deficits and if certain IEGs within the dentate gyrus are changed following cannabinoid exposure.

Experimental procedures

Animals

Subjects were 36 male Long-Evans rats acquired from Harlan Laboratories (Indianapolis, IN). All rats were three months of age at the beginning of the experiments. Rats were pair-housed in standard clear plastic cages and maintained in a temperature and

humidity controlled vivarium with food and water available *ad libitum*. The University of New Mexico Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

Drug treatments

All drug doses and vehicle preparations were selected according to previous literature (Lichtman, Dimen, & Martin, 1995; Yim, et al., 2008). WIN 55,212-2 (Sigma-Aldrich) was prepared for microinfusion at a high dose concentration of 10 μg/μL in vehicle solution. AM 251 (Tocris) was prepared for microinfusion at a high dose concentration of 40 μg/μL in vehicle solution (Sink et al., 2009). The vehicle solution consisted of a 1:1:18 ratio ethanol:alkamuls:saline, respectively. WIN 55,212-2 was first dissolved and vortex in a 1:1 mixture of ethanol and alkamuls EL-620 (kindly provided by Rhodia Pharma Solutions). The mixture brought to volume with 18 parts sterile Dulbecco's phosphate buffered saline (Sigma-Aldrich), vortex and sonicated. AM 251 was first dissolved and vortex in a 1:1 mixture of ethanol and alkamuls. The mixture brought to volume with 18 parts sterile Dulbecco's phosphate buffered saline, vortex, and sonicated. All drug and vehicle solutions were prepared fresh daily.

Microinfusions

Rats were divided into five experimental groups: 10-Veh/DG, 11-WIN/DG, 5-AM 251+WIN/DG, 5-Veh/PC, and 5-WIN/PC. Immediately following the last MWT training trial on day one, within three to five minutes, all rats received a direct cannula infusions over two unilateral infusions, altering hemisphere order. Rats in the antagonist condition received the antagonist initially following training followed 30 minutes later by the agonist. For infusions, animals were gently held in a towel by the experimenter while

the infusion cannula (Plastics One) was attached to the implant via a connector assembly (Plastics One). The infusion cannula was attached to polyethylene tubing (PE20) and connected to a 10 mL Hamilton syringe (Hamilton Company) controlled by a Stoelting microinfusion pump. The Stoelting microinfusion pump was set at a rate of 0.5 μL/min for one minute. Each infusion cannula extended 1.0-2.5 mm below the implanted cannula tip, depending on region, parietal cortex and dentate gyrus, respectively. Infusion cannula was left in place for an additional two minutes to allow for drug diffusion. The extent of diffusion was estimated, based on fluorescent imaging, to be one mm radially from the cannula tip. Prior to the start of each experiment, all rats were handled and wrapped in a towel several times to habituate to the infusion procedure.

Cannulation

Animals were anesthetized with isoflurane (4%) oxygen mixture in an induction chamber for surgical preparation and placed into a stereotaxic frame (David Kopf Instruments) with anesthetic administered via facemask. Once a surgical plane of anesthesia was achieved, the isoflurane concentration was reduced to 2%. After cleaning the scalp, a midline incision was made to expose the skull. Holes were drilled using a dental burr at the following coordinates relative to Bregma for bilateral implants into dorsal hippocampus: AP -4.0, ML ±3.5, and for parietal cortex: AP -3.5, ML±3.5 (Paxinos & Watson, 2005). Sterile guide cannulas (Plastics one; 26-gauge) were implanted by stereotaxic guidance so that the guide tip was located for dentate gyrus at: AP -4.0, ML ±3.5, DV -1.9, and for parietal cortex at: AP -3.5, ML ±3.0, DV -1.0 (Paxinos & Watson, 2005). The cannulas were permanently fixed in dental cement with five small jewelers' screws as anchors for stability. All animals were given

buprenorphrine subcutaneously every 12 hours after surgery for analgesia 48 hours and individually housed for the duration for the experiment. Animals were allowed to recover for two weeks before behavior testing.

Morris water task

The procedure for the Morris water task was modified from the original guidelines outlined by R. G. M. Morris (Morris, 1981, 1982, 1984). The apparatus was a circular swimming pool (1.5 m in diameter) located in a room with various distal visual cues. The pool was filled with water, maintained at 25°C, and made opaque by the addition of a small amount (~2 oz.) of nontoxic white tempera paint. Inside the pool, a removable rectangular Plexiglas platform (12 cm x 12 cm) was submerged to a depth of 1 cm below the surface of the water. The platform was located in a defined quadrant of the maze (NE, NW, SE, and SW). Four release points were used and the sequence of release points was determined by a pseudorandom sequence. Each trial consisted of releasing the rat into the water facing the outer edge of the pool and letting the rat escape to the platform before 60 seconds elapsed. The rat's swimming behavior was videotaped by an overhead camera and transferred to a computer workstation for tracking and analysis.

All rats received MWT pre-training a few days prior to MWT training and testing. The pre-training was done in a separate room from the actual testing room to provide a distinct learning environment and consisted of 12 trials during one day. The MWT pre-training was necessary to ensure rats learned the non-specific aspects of the task (e.g., that escape cannot be achieved at the pool wall, and that there is an escape platform). MWT pre-training also guaranteed all rats could effectively solve the task before further experimental manipulations. All were at asymptotic performance after pre-training.

MWT training occurred over the course of a single day with 12 fixed platform trials. The trials were massed with one-minute inter-trial-intervals. Each rat was run individually to minimize training duration and control initiation of consolidation. Immediately following the last training trial, rats were wrapped in a towel and infused with either vehicle, WIN 55,212-2, or AM 251+WIN 55,212-2 then returned to their home-cage. Twenty-four hours later, the rat's memory of the platform location was tested with a 60 second probe trial followed by four trials of retraining.

Twenty-three rats underwent a second round of MWT testing one week later (see experimental timeline in Figure 9B). A new room and pool position were used to create a novel environment. To provide various IEG controls rats were divided into three experimental groups to account for swimming behavior only, drug only, and combination swimming behavior and drug conditions (MWT/NoDrug, NoMWT/Veh^{1hemi}WIN^{1hemi}, MWT/Veh^{1hemi} WIN^{1hemi}). Rats were unilaterally infused with vehicle into one hemisphere and drug into the second hemisphere to provide a within subjects measure for IEG analysis. Rats in the swimming behavior only condition received 12 massed trials of MWT and were returned to their home-cage. Rats in the drug only condition were taken to the novel MWT environment but did not receive MWT testing. Instead, drug only rats were wrapped in a towel and received infusions of vehicle into one hemisphere and WIN 55,212-2 into the other hemisphere and returned to their home-cage. Rats in the combination swimming and drug condition were given 12 massed trials and immediately infused with vehicle into one hemisphere and WIN 55,212-2 into the other hemisphere in a pseudorandom order. No retention tests were performed for any of the animals; four hours later rats were euthanized for IEG expression.

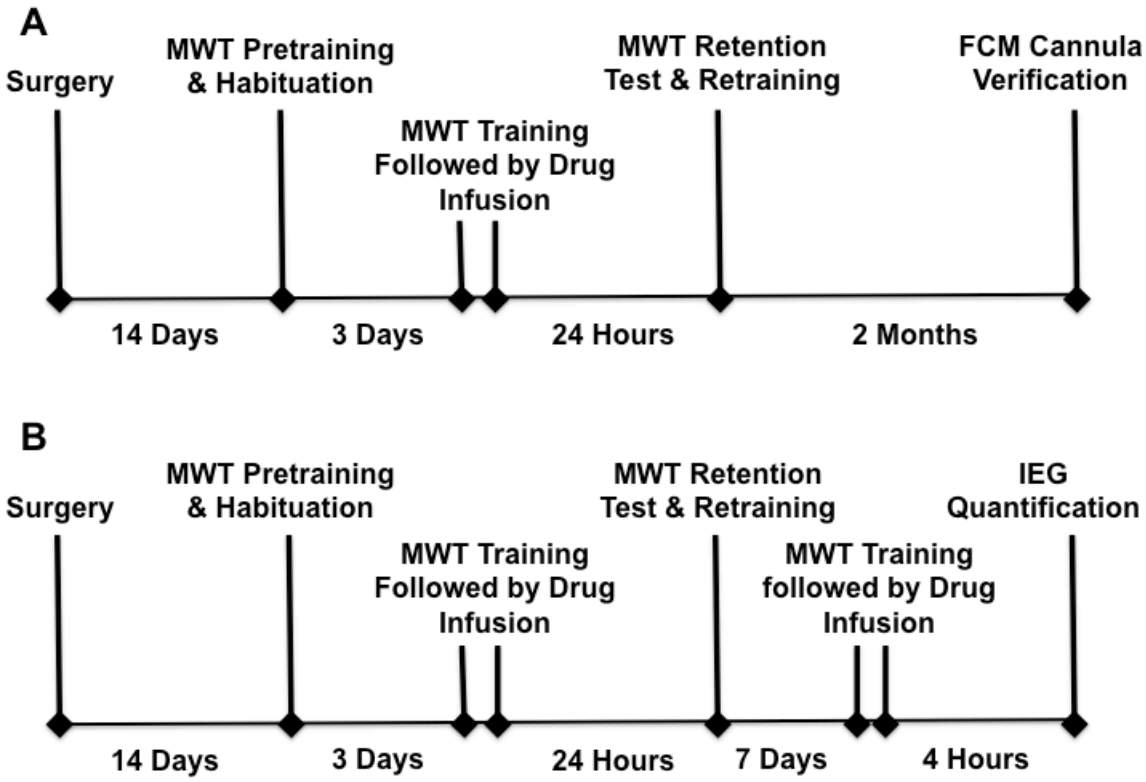

Figure 9: Experimental timeline for cannulation. Rats were divided into two cohorts following MWT retention tests. FCM cannula verification was performed on 13 rats (A), while a second round of behavioral training and IEG quantification was performed on 23 rats (B).

Immediate early gene expression

IEG quantification was performed on 23 rats not involved in FCM cannula verification (Figure 9B). Four hours following experimental manipulation (swimming only, drug only, combination swimming and drug condition) rats were rapidly anesthetized in an isoflurane chamber and euthanized. The brain was quickly extracted, and placed in ice-cold phosphate buffered saline. The brains were visually inspected for proper cannula placements. The dentate gyrus and parietal cortex were micro-dissected, placed in RNA free tubes, and stored at -80 °C. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to a previous protocol (Caldwell et al., 2008) and Arc, cFos, zif268 mRNA levels were measured using primers designed by Dr. Clark Bird. Primers were designed from national database sequences of rat reference mRNA and validated with NCBI primer BLAST software. First, mRNA was extracted from the stored tissue using a RNeasy mini kit (Qiagen) and mRNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies). cDNAs were created using 1 μg total mRNA and Superscript II Reverse Transcriptase (Invitrogen Corporation) following manufacturer's protocol and stored at -20 °C until used. qRT-PCR reactions were performed on an Applied Biosystems 7100 Real Time PCR System using 96-well plates (Applied Biosystems). Gene expression levels were analyzed using SYBR Green (Life Technologies) detection in triplicate reactions against GAPDH values. Based on dissociation curves, abnormal samples were discarded. Relative levels of IEG (Arc, cFos, and zif268) expression compared to control GAPDH expression were calculated using the $2^{-\Delta\Delta Ct}$ method described by (Livak & Schmittgen, 2001).

Cannula placement verification with fluorophore-conjugated muscimol

Cannula verifications were performed on the 13 remaining rats not involved in IEG quantification (Figure 9A). Following previous protocols (Allen et al., 2008; Stackman, Lora, & Williams, 2012) 1 mg of fluorophore-conjugated muscimol (FCM) (BODIPY TMR-X; Invitrogen) was dissolved in 1 mL of Dulbecco's phosphate buffered saline (Sigma Aldrich), sonicated and aliquotted for storage. Rats were anesthetized with isoflurane (4%) oxygen mixture in an induction chamber and placed in a stereotaxic frame (David Kopf Instruments) with anesthetic administered via facemask. Infusion cannula (Plastics One) targeting hippocampal dentate gyrus (2.5 mm extension below implant) and parietal cortex (1.0 mm extension below implant) were attached to polyethylene tubing (PE20), connected to a 10 mL Hamilton syringe (Hamilton Company) controlled by a Stoelting microinfusion pump. Rats received bilateral intracranial microinfusions of FCM at a rate of 0.5 μL/min for two minutes. Infusions were confirmed by monitoring fluid flow in the tubing via a small bubble. Infusion cannula was left in place for an additional two minutes to allow for drug diffusion. One hour following FCM infusion, rats were euthanized with an overdose of isoflurane. Brains were removed, frozen in isopentane chilled in a dry-ice/methanol bath and stored at -80 °C. Brains were sliced into 40 μm thick coronal sections with a freezing microtome and mounted onto 2% gelatinized slides. Sections were briefly air-dried then fixed in 4% buffered paraformaldehyde for 20 minutes. Slides were cover-slipped using fluorescent glue and stored at -4 °C until image processing.

To visualize the locations of infusion cannula tips and quantify the spread of FCM, fluorescent images were acquired on an Olympus microscope (Model BX51)

equipped with a fluorescent lamp and digital camera (Olympus Model DP70) attached to a personal computer. Images were acquired at 20X magnification with FITC filter sets (Olympus). Exposure times were manually adjusted to maximize image quality and visualize area of fluorescence. Proper placement of cannulas within the dentate gyrus was confirmed. All brains sectioned from rats with hippocampal cannulation showed cannula placement in the upper blade of the dentate gyrus, Figure 10. Example FCM images of distribution within the dentate gyrus are shown in Figure 11. Proper placement of cannulas within the parietal cortex was confirmed. All brains sectioned from rats with parietal cortex cannulation showed cannula placement in the dorsal portion of the medial parietal association cortex and lateral parietal association cortex, Figure 12. Example FCM images of distribution within the parietal cortex are shown in Figure 13. No animals were excluded from behavioral analysis due to improper cannula placement.

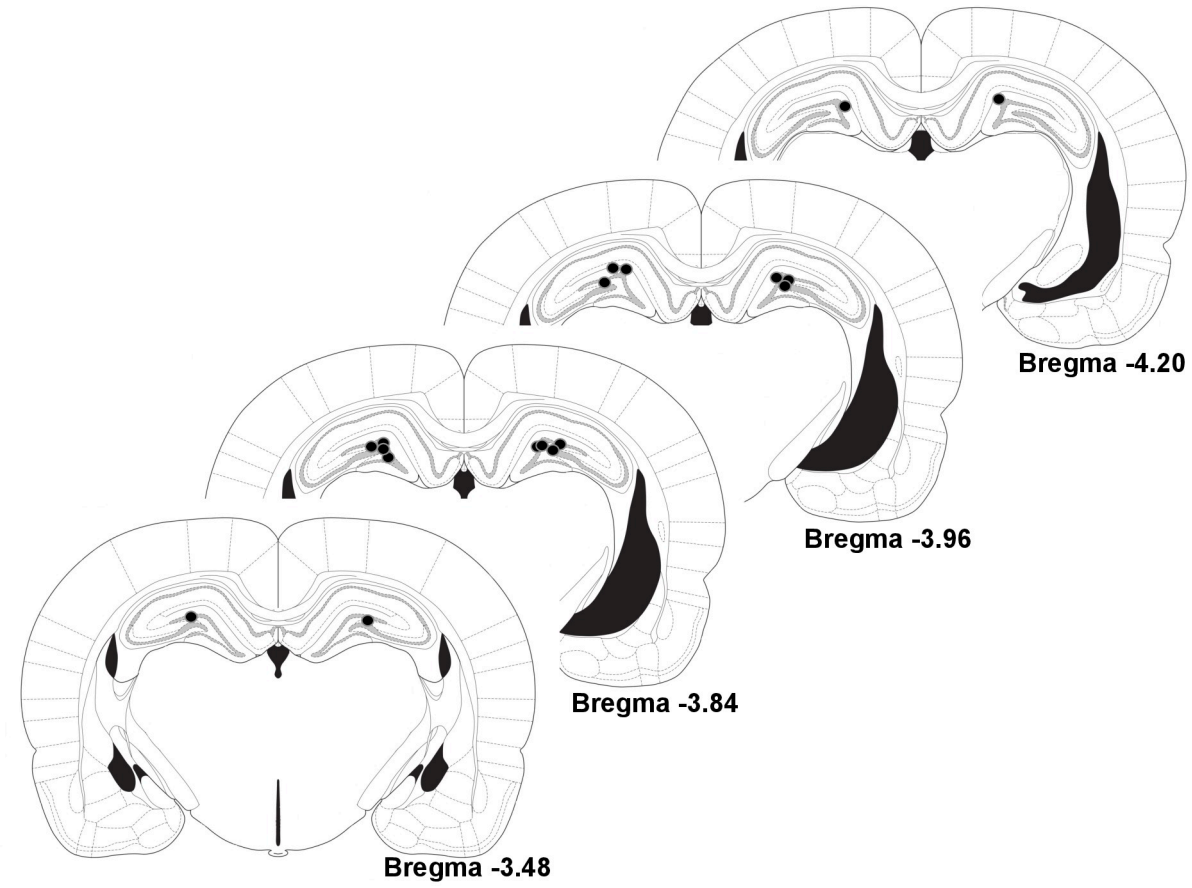

Figure 10: Placement of cannulas within the dentate gyrus. Coronal sections [adapted form Paxinos and Watson (2005)] showing proper placement of cannulas within the dentate gyrus. All brains sectioned showed infusion sites and infusion cannula tip placement in the upper blade of the dentate gyrus.

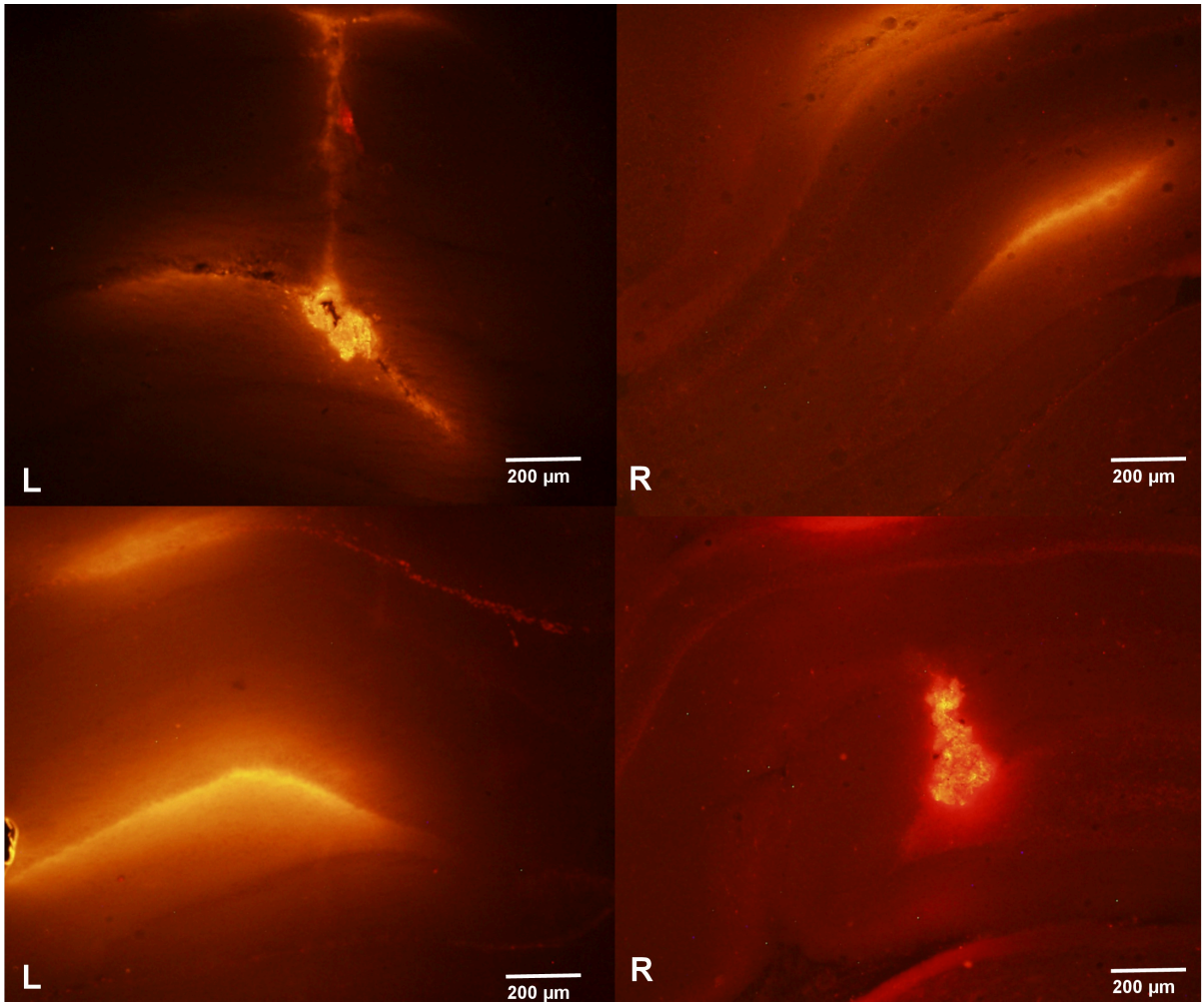

Figure 11: Muscimol verifications of dentate gyrus cannula placement. Fluorescent images of hippocampal coronal sections at 40X magnification showing FCM infusion site distribution within the left and right hemispheres, respectively. Images confirm FCM was infused and distributed into the upper blade of the dentate gyrus.

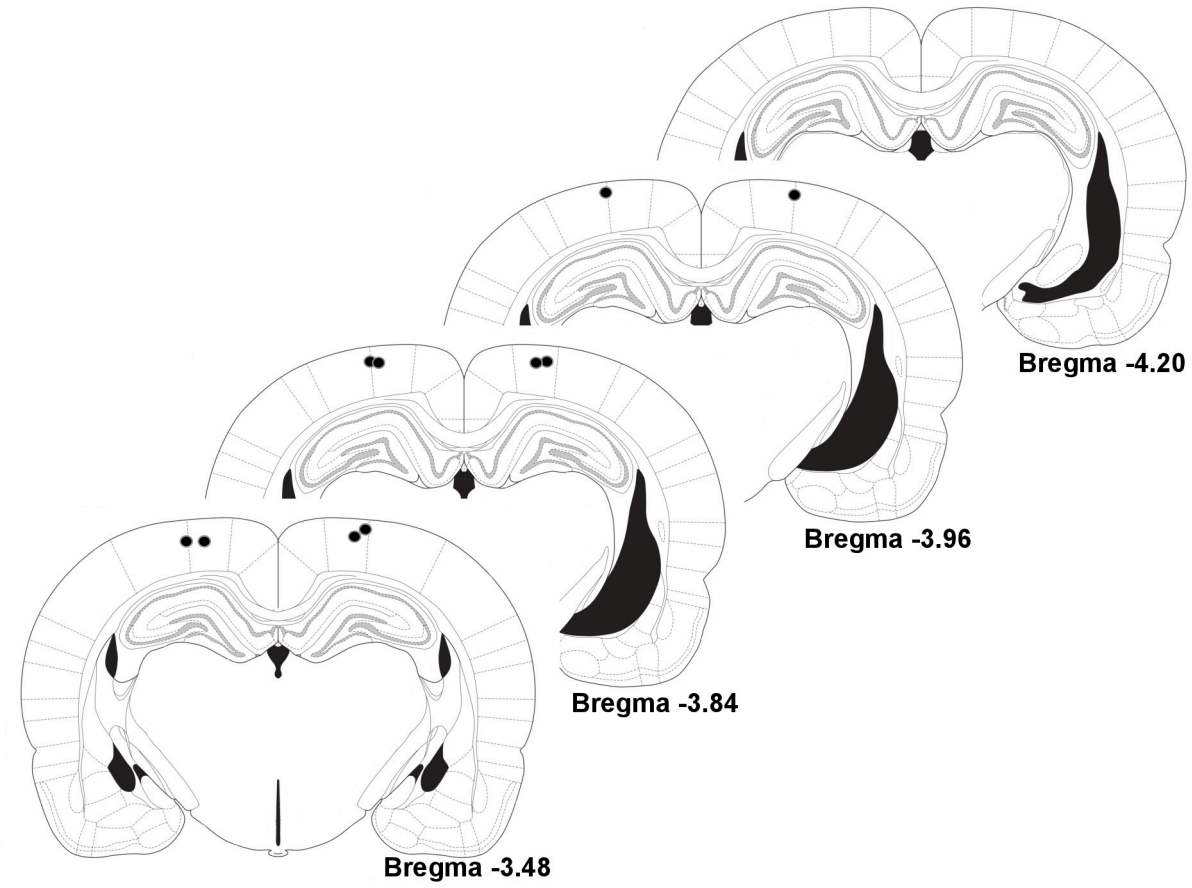

Figure 12: Placement of cannulas within the parietal cortex. Coronal sections [adapted form Paxinos and Watson (2005)] showing proper placement of cannulas within the parietal cortex. All brains sectioned showed infusion sites and infusion cannula tip placement in the dorsal portion of the medial parietal association cortex and lateral parietal association cortex.

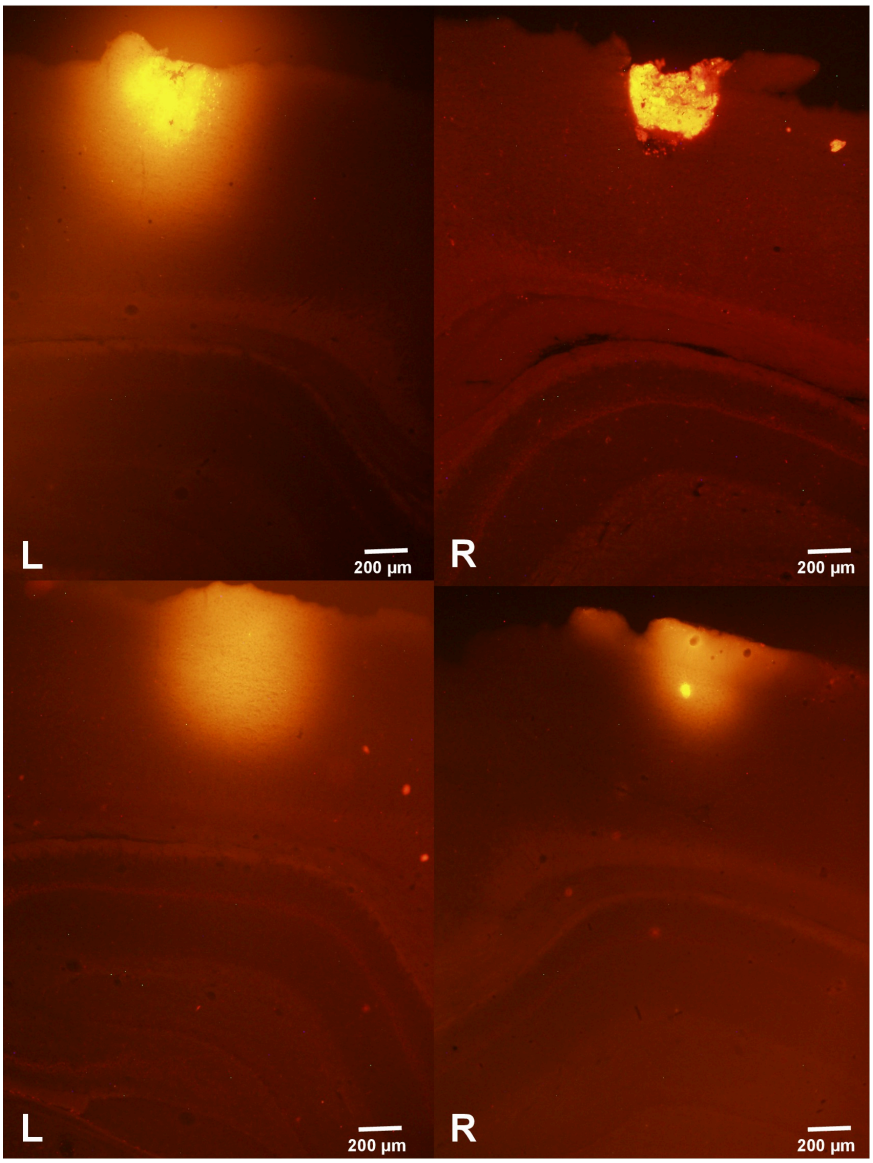

Figure 13: Muscimol verifications of parietal cortex cannula placement. Fluorescent images of parietal cortex coronal sections at 40X magnification showing FCM infusion site distribution within the left and right hemispheres, respectively. Images confirm FCM was infused and distributed into the dorsal portion of the medial parietal association cortex and lateral parietal association cortex.

Results

Analyses of Variance (ANOVAs) were performed using SPSS (version 22 for Macintosh). All test statistics reported here were significant at $p < 0.05$ unless otherwise noted. A total of 36 rats were analyzed for MWT behavior (10 Veh-DG, 11 WIN-DG, 5 AM-DG, 5 Veh-PC, 5 Veh-PC). For IEG analysis 23 brains were used (7 MWT only, 5 Drug only, 8 DG infusion, 3 PC infusion). For the Drug only, DG infusion, and PC infusion conditions, vehicle was infused into one hemisphere and WIN 55,212-2 into the second hemisphere. Separate ANOVAs were conducted for various experimental components: MWT training, MWT retention, IEG. Effect sizes (partial eta squared; η_p^2) are reported for all effects.

MWT- dentate gyrus microinfusion

Training. To evaluate group differences in the training data prior to experimental manipulation, swim data was analyzed in a two-way mixed model ANOVA with drug condition (vehicle, WIN 55,212-2, AM 251+WIN 55,212-2) as the between-subjects factor and trial block as within subject factor. There was a significant main effect of Trial on latency [$F(11,253) = 5.614$, $\eta_p^2 = 0.196$] and path length [$F(11,253) = 6.652$, $\eta_p^2 = 0.224$]. Follow-up contrasts revealed significant linear trends for latency [$F(1,23) = 16.276$, $\eta_p^2 = 0.414$] and path length [$F(1,23) = 18.802$, $\eta_p^2 = 0.450$] indicating that latency and path length to reach the platform decreased across training trials. No other main effects or interactions were significant ps> 0.823. All drug groups were equivalent in performance at the end of training, Figure 14.

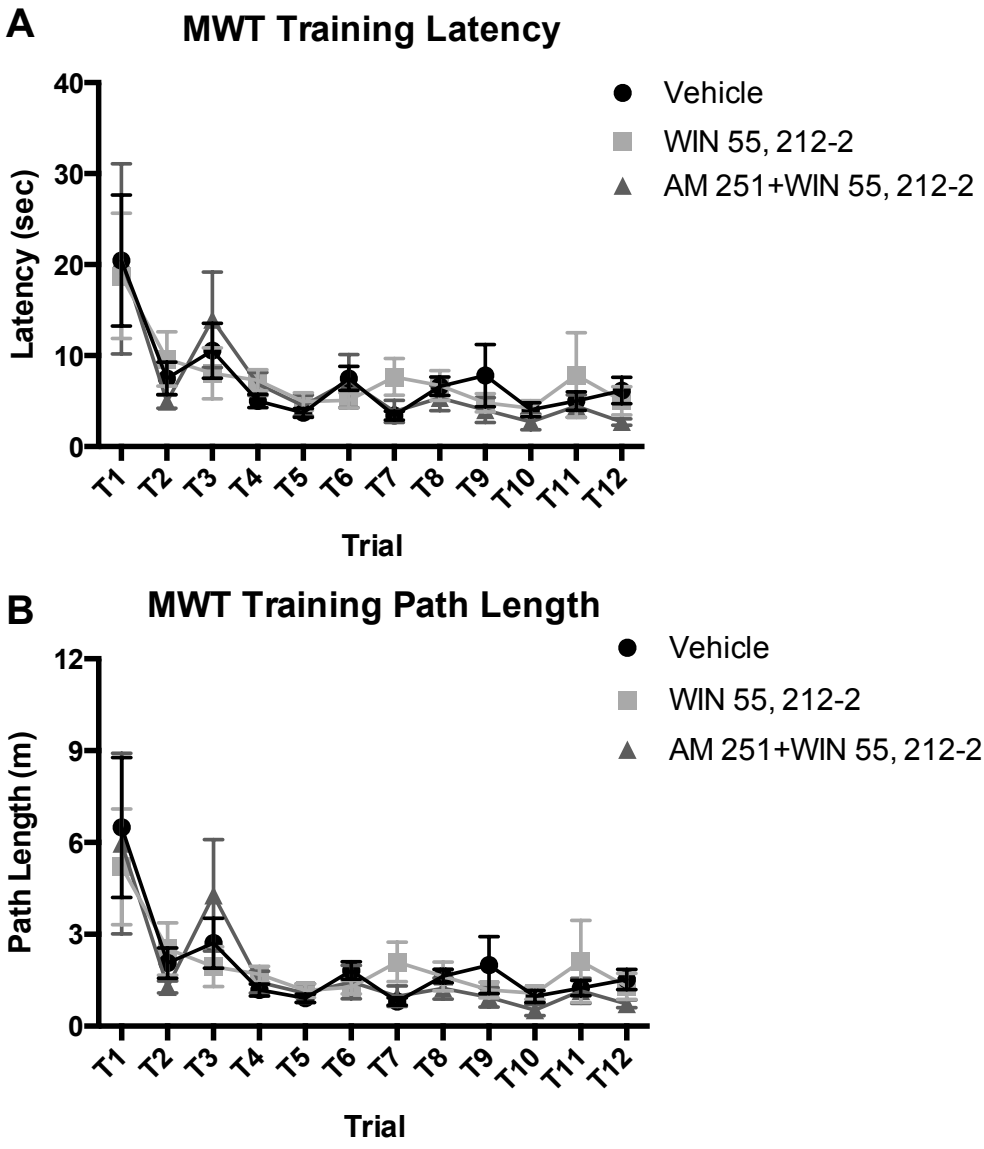

Figure 14: MWT training latency and path length- dentate gyrus. MWT behavior prior to dentate gyrus infusions: (A) mean latency to platform (±SEM), (B) mean path length to platform (±SEM). There were no significant differences between groups during training.

Probe. To evaluate 24-hour consolidation following post-training dentate gyrus microinfusion, 30 second no-platform probe swim data was analyzed by univariate ANOVAs with latency to reach target location, average proximity of entire swim path to target location, path length to reach target location, percent time in target quadrant, and number of target location crosses as dependent measures, Figure 15. There was a significant main effect of Drug in latency to target location [$F(2,23) = 4.963$, $\eta_p^2 = 0.301$] and path length to target location [$F(2,23) = 6.121$, $\eta_p^2 = 0.347$]. Follow-up pairwise comparisons revealed AM 251+WIN 55,212-2 rats had significantly higher latency to target location and significantly longer path lengths to target location compared to vehicle and WIN 55,212-2 rats representing impaired retention. This suggests the cannabinoid antagonist AM 251 in combination with WIN 55,212-2 microinfused into the dentate gyrus impaired 24-hour consolidation and memory retention. The main effect of Drug in average distance to target location approached significant [$F(2,23) = 3.303$, $p = 0.055$, $\eta_p^2 = 0.223$]. Follow-up pairwise comparisons revealed WIN 55,212-2 rats had significantly shorter average distances to target location compared to vehicle and AM 251+WIN 55,212-2 rats. This indicates rats that received the cannabinoid agonist WIN 55,212-2 had more precise searching behavior than vehicle controls and the cannabinoid antagonist AM 251+WIN 55,212-2. There were no further differences between drug groups, $ps > 0.146$. When taken together, the data indicates microinfusion of the cannabinoid agonist WIN 55,212-2 into the dentate gyrus did not impair memory consolidation, and in fact search precision was improved in one measure of probe performance, while the cannabinoid antagonist AM 251+WIN 55,212-2 impaired memory retention.

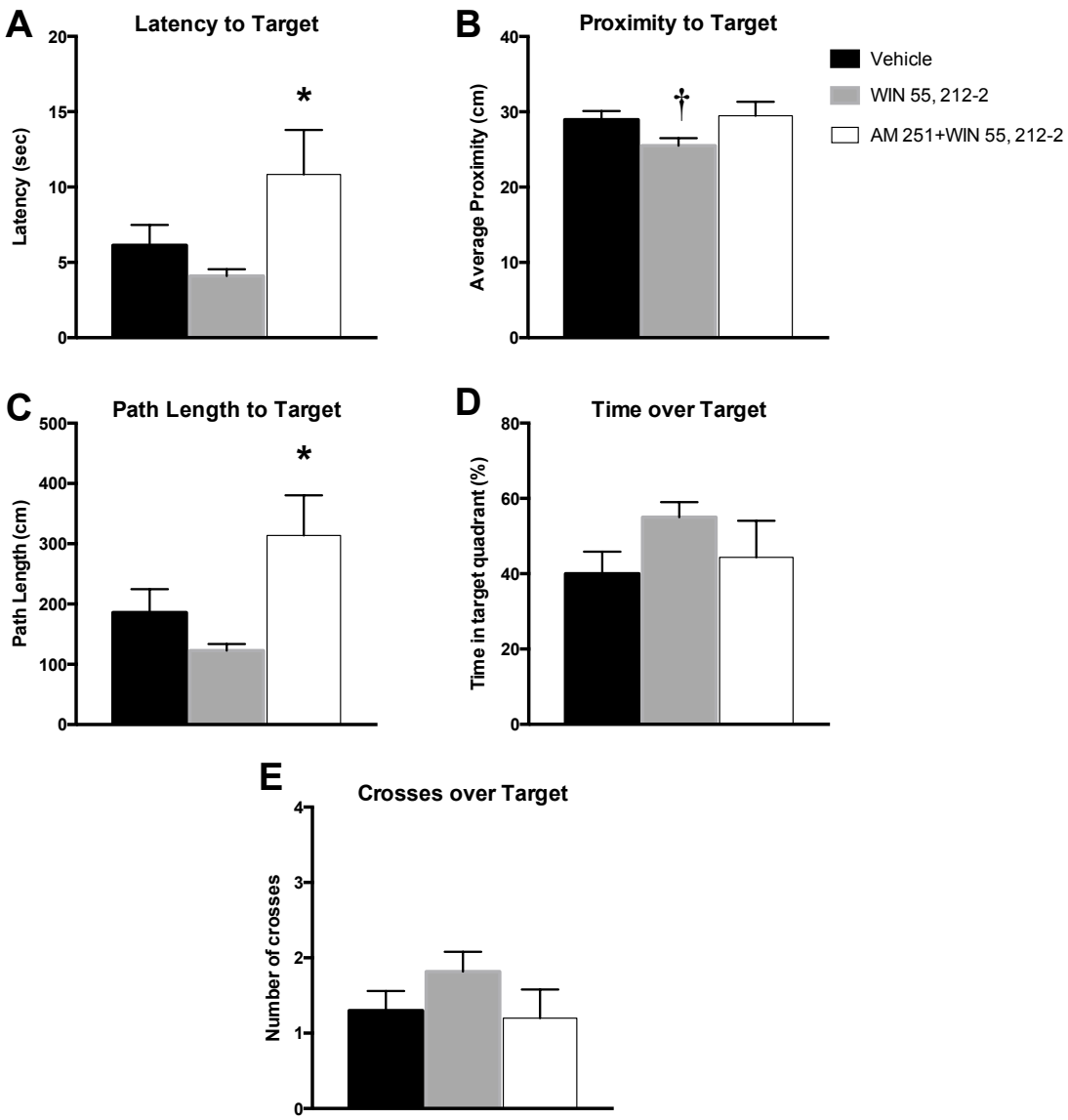

Figure 15: MWT no-platform probe test- dentate gyrus. Probe dependent measures evaluating memory for former platform location 24 hours following drug infusion into dentate gyrus (+SEM): (A) latency to reach target location, (B) average proximity of entire swim path to target location, (C) path length to reach target location, (D) percent time in target quadrant, (E) number of target location crosses. AM 251+WIN 55,212-2 rats had impaired latency and path length (* denotes $p < 0.05$). WIN 55,212-2 rats had more precise search behavior demonstrated by shorter average distance from target († denotes $p = 0.055$).

Retraining. To further evaluate 24-hour consolidation following post-training dentate gyrus microinfusion, four trials of retraining were analyzed in a two-way mixed model ANOVA with drug condition (vehicle, WIN 55,212-2, AM 251+WIN 55,212-2) as the between-subjects factor and trial block as within subject factor. Overall, there were no significant main effects or interactions. The primary trial of interest was the first trial of retraining, immediately after the MWT probe; however, no differences between drug groups existed during retraining Trial 1. There was a Trial X Drug interaction that was approaching significance for latency [$F(6,69) = 2.170$, $p = 0.056$, $\eta_p^2 = 0.159$] and path length [$F(6,69) = 1.919$, $p = 0.090$, $\eta_p^2 = 0.143$]. Follow-up pairwise comparisons revealed that AM 251+WIN 55,212-2 rats had significantly higher latencies than vehicle and WIN 55,212-2 rats during the second retraining trial, Figures 16 C&D. Meanwhile, the difference between WIN 55,212-2 rats and vehicle rats during the first retraining trial approached significance ($p = 0.095$), Figures 16 A&B. All drug groups were equivalent in performance by the end of retraining.

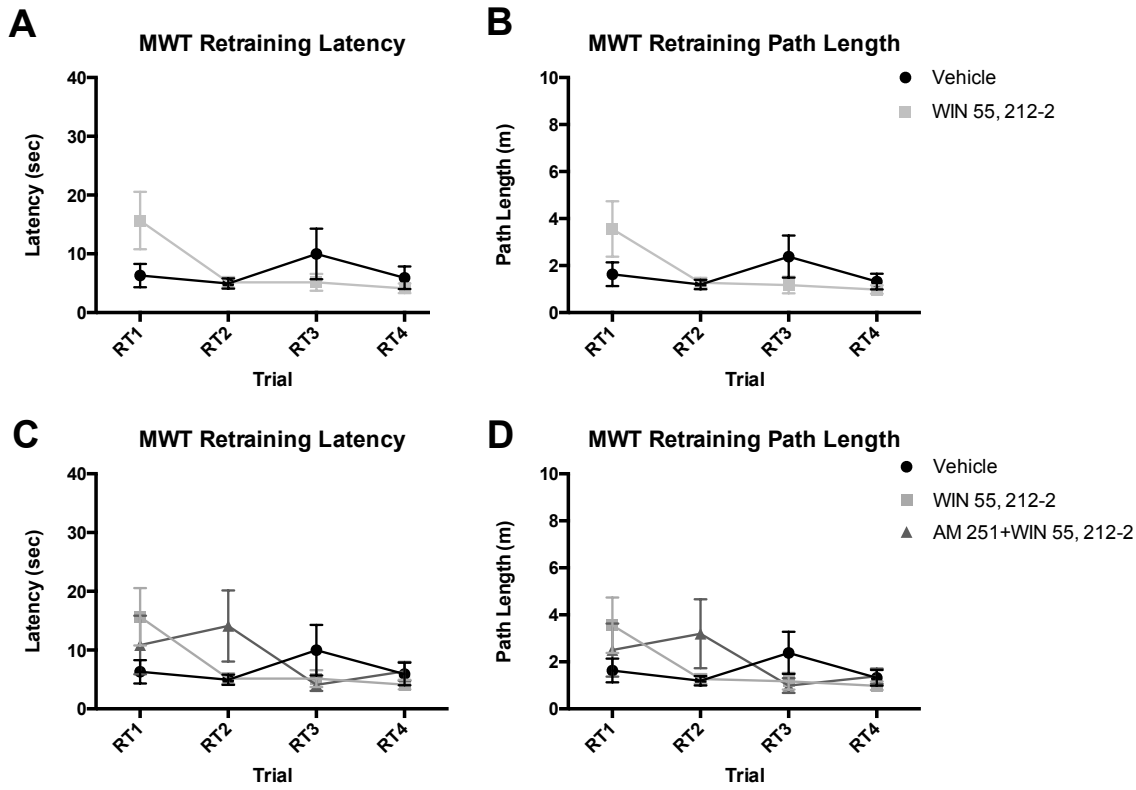

Figure 16: MWT retraining latency and path length- dentate gyrus. MWT behavior 24 hours after dentate gyrus infusions evaluating memory for former platform location (±SEM): (A) mean latency to platform (±SEM), (B) mean path length to platform (±SEM). There were no significant differences between groups during retraining.

MWT- parietal cortex microinfusion

Training. To evaluate if group differences existed in the training data prior to experimental manipulation, swim data was analyzed in a two-way mixed model ANOVA with drug condition (vehicle, WIN 55,212-2) as the between-subjects factor and trial block as within subject factor. There was a significant main effect of Trial on latency [$F(11,88) = 4.356$, $\eta_p^2 = 0.353$] and path length [$F(11,88) = 4.470$, $\eta_p^2 = 0.358$]. Follow-up contrasts revealed significant linear trends for latency [$F(1,8) = 37.912$, $\eta_p^2 = 0.826$] and path length [$F(1,8) = 61.511$, $\eta_p^2 = 0.885$] indicating that latency and path length to reach the platform decreased across training trials. There was a significant Trial X Drug interaction on latency [$F(11,88) = 2.099$, $\eta_p^2 = 0.208$] and path length [$F(11,88) = 2.173$, $\eta_p^2 = 0.214$]. Follow-up Helmert contrasts revealed Trial 1 and Trial 3 were significantly different than remaining trials in terms of latency [$F(1,8) = 5.358$, $\eta_p^2 = 0.401$, $F(1,8) = 7.342$, $\eta_p^2 = 0.479$, Trial 1 & 3, respectively] and path length [$F(1,8) = 5.897$, $\eta_p^2 = 0.424$, $F(1,8) = 5.271$, $\eta_p^2 = 0.397$, Trial 1 & 3, respectively]. Although rats were assigned to drug conditions based on MWT pre-training performance to equate the drug groups, WIN 55,212-2 rats showed impaired latency and path length initially. However, by the end of training there was no difference between groups. No other main effects or interactions were significant $ps > 0.146$.

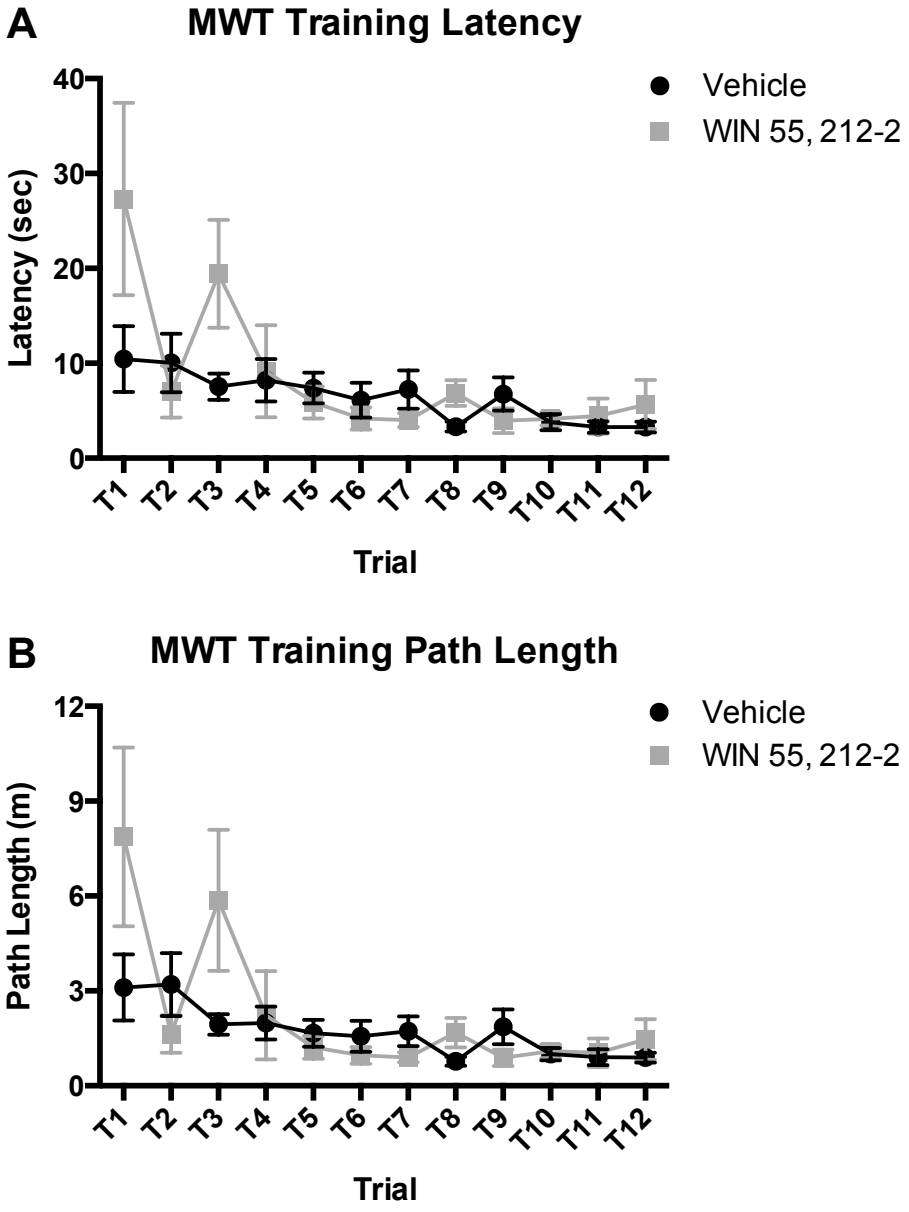

Figure 17: MWT training latency and path length- parietal cortex. MWT behavior prior to parietal cortex infusions: (A) mean latency to platform (±SEM), (B) mean path length to platform (±SEM). There were no significant differences between groups by the end of training.

Probe. To evaluate 24-hour consolidation following post-training parietal cortex microinfusion, 30 second no-platform probe swim data was analyzed by univariate ANOVAs with latency to reach target location, average proximity of entire swim path to target location, path length to reach target location, percent time in target quadrant, and number of target location crosses as dependent measures. There were no significant differences between vehicle and WIN 55,212-2 rats in any of the probe measures, ps > 0.105. Microinfusion of WIN 55,212-2 into the parietal cortex did not impact memory consolidation or influence memory retention.

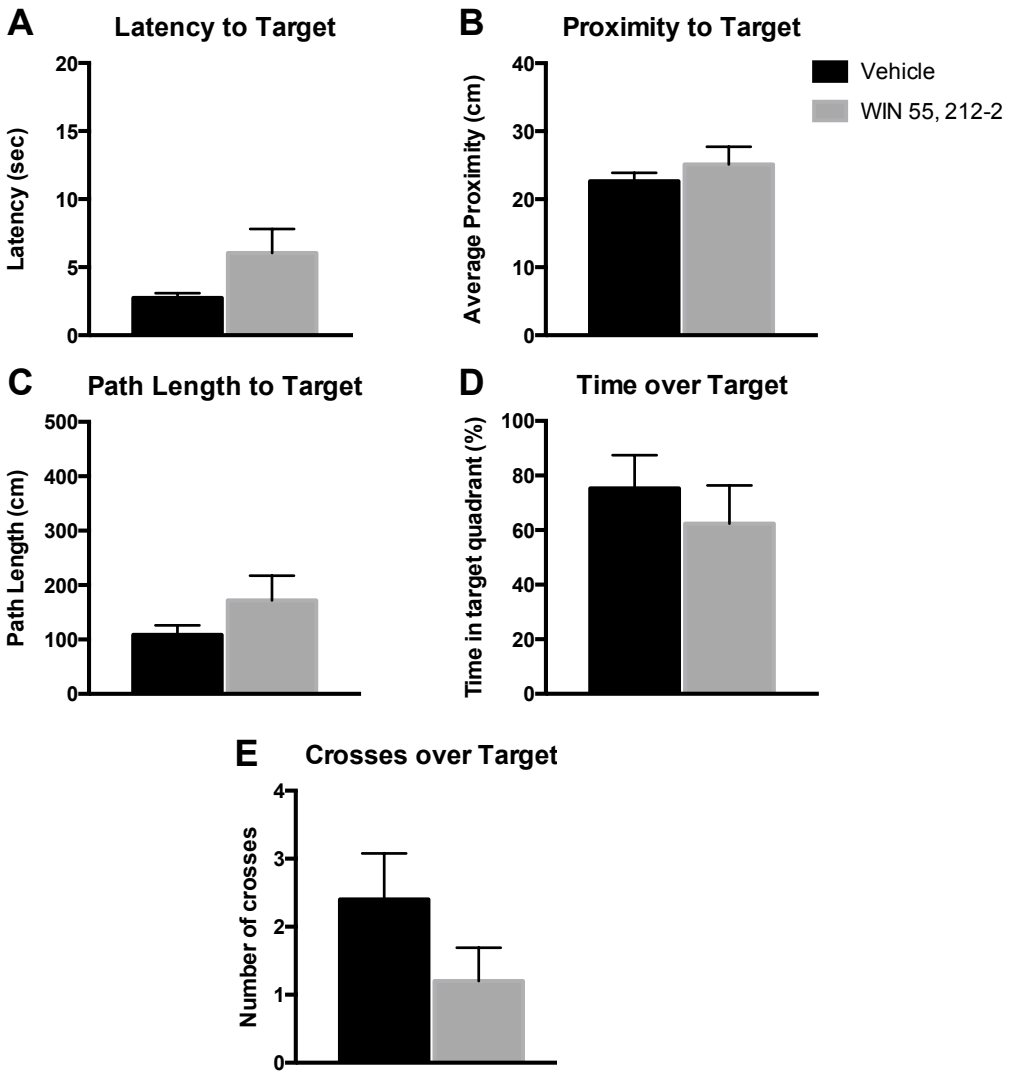

Figure 18: MWT no-platform probe test- parietal cortex. Probe dependent measures evaluating memory for former platform location 24 hours following drug infusion into the parietal cortex (+SEM): (A) latency to reach target location, (B) average proximity of entire swim path to target location, (C) path length to reach target location, (D) percent time in target quadrant, (E) number of target location crosses. There was no significant difference between groups in platform memory retention. Microinfusion of WIN 55,212-2 into the parietal cortex did not impact memory consolidation or influence memory retention.

Retraining. To further evaluate 24-hour consolidation following post-training parietal cortex microinfusion, four trials of retraining were analyzed in a two-way mixed model ANOVA with drug condition (vehicle, WIN 55,212-2) as the between-subjects factor and trial block as within subject factor. No main effects or interactions were significant, ps > 0.167. There were no significant differences between groups at any trial of retraining. Both drug groups were equivalent in performance during retraining. Microinfusion of WIN 55,212-2 into the parietal cortex did not impact memory consolidation or influence memory retention.

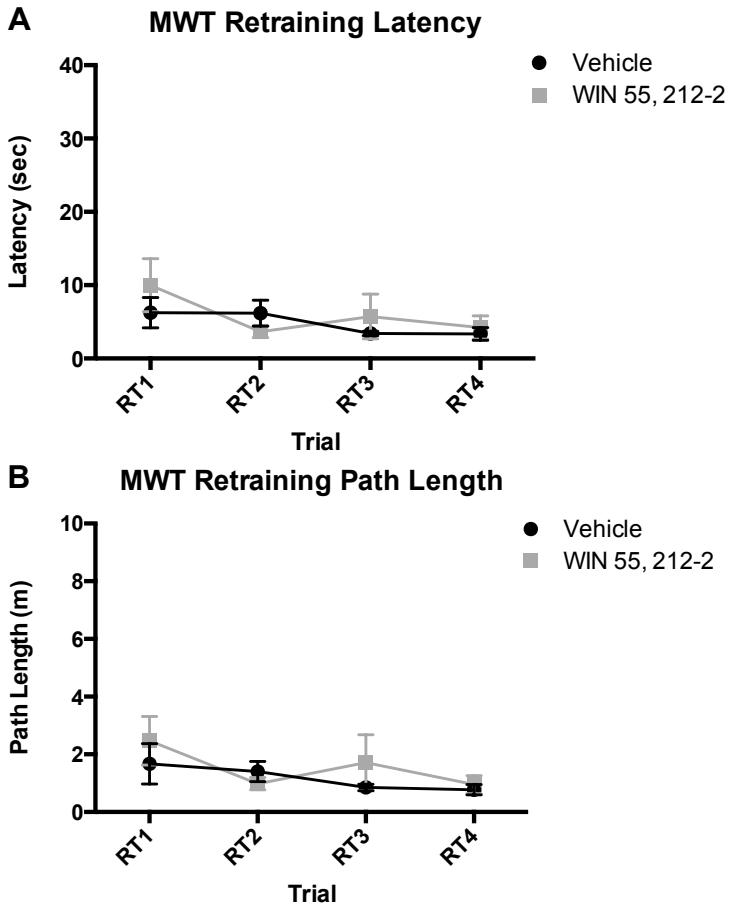

Figure 19: MWT retraining latency and path length- parietal cortex. MWT behavior 24 hours after parietal cortex infusions evaluating memory for former platform location (±SEM): (A) mean latency to platform (±SEM), (B) mean path length to platform (±SEM). There were no significant differences between groups during retraining. Microinfusion of WIN 55,212-2 into the parietal cortex did not impact memory consolidation or influence memory retention.

IEG mRNA levels- dentate gyrus microinfusion

The effects of WIN 55,212-2 on IEG (Arc, cFos, zif268) activity following MWT training are shown in Figure 20. Repeated measures ANOVAs were conducted on IEG mRNA levels with IEG (Arc, cFos, zif268), Region (dentate gyrus, parietal cortex) and Drug (vehicle, WIN 55,212-2) as within-subjects factors. Control conditions (Swim only, Drug only) were compared to combination conditions (Drug + MWT training). Within the dentate gyrus, there were no significant main effects of Drug for any of the IEGs of interest (Arc, cFos, zif268), ps > 0.238. RT-PCR analysis revealed vehicle and WIN 55,212-2 mRNA levels were not significantly different on average. However, there were other significant interactions and main effects reported below which are divided by gene of interest.

For the IEG Arc there was a significant main effect of Region [$F(1,17) = 8.371$, $\eta_p^2 = 0.330$] indicating IEG Arc mRNA average values were higher in the dentate gyrus than in the parietal cortex [$M_{DG}= 5.768$, $M_{PC}= 1.277$], Figure 20 A&D. There was a significant main effect of Condition [$F(2,17) = 3.934$, $\eta_p^2 = 0.316$] indicating the average Arc mRNA value were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that MWT+Drug was significantly higher than MWT only [$M_{MWT} = 0.494$, $M_{MWT+DRUG} = 5.416$]. Within the dentate gyrus, the significant main effect of Condition approached significance [$F(2,17) = 3.373$, $p = 0.058$, $\eta_p^2 = 0.284$] indicating the average Arc mRNA values were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that Drug Only and MWT+Drug were both significantly higher than MWT only. Within the dentate gyrus MWT+Drug condition, the Drug difference showed a trend towards significance

[F(1,7) = 3.776, p = 0.093, η_p^2 = 0.350; M_{VEH} = 7.47, M_{WIN} = 10.7983], WIN 55,212-2 rats showed slightly elevated Arc mRNA expression. No other interactions or main effects were significant, ps > 0.092.

For the IEG cFos there was a significant main effect of Region [F(1,17) = 8.063, η_p^2 = 0.322] indicating IEG cFos mRNA average values were higher in the parietal cortex compared to the dentate gyrus [M_{DG} = 1.584, M_{PC} = 2.691]. The main effect of Condition approached significance [F(2,17) = 3.009, p = 0.076, η_p^2 = 0.261] indicating the average cFos values were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that MWT+Drug was significantly higher than MWT only [M_{MWT} = 1.047, $M_{MWT+DRUG}$ = 2.662]. Within the dentate gyrus, the main effect of Condition was significant [F(2,17) = 6.624, η_p^2 = 0.438] indicating the average cFos mRNA value were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that Drug Only and MWT+Drug were both significantly higher than MWT only. Within the dentate gyrus MWT+Drug condition, the Drug difference showed a trend towards signficance [F(1,7) = 3.721, p = 0.095, η_p^2 = 0.347, M_{VEH} = 1.713, M_{WIN} = 2.6818], WIN 55,212-2 rats showed slightly elevated cFos mRNA expression. No other interactions or main effects were significant, ps > 0.237.

For the IEG zif268 there was a significant interaction between Region X Condition [F(2,17) = 4.526, η_p^2 = 0.347]. Simple contrasts revealed that IEG zif268 mRNA values were higher in the parietal cortex for MWT only, Drug Only, and MWT& Drug Conditions compared to the dentate gyrus. There was also a significant main effect of Region [F(1,17) = 97.613, η_p^2 = 0.852] indicating IEG zif268 mRNA average values in the parietal cortex were higher than the dentate gyrus [M_{DG} = 1.613, M_{PC} = 3.375]. The

main effect of Condition approached significance [$F(2,17) = 3.038$, $p = 0.074$, $\eta_p^2 = 0.263$] indicating the average zif268 value was different between MWT only, Drug only, and MWT+Drug conditions. No other interactions or main effects were significant, ps > 0.249.

IEG mRNA levels- parietal cortex microinfusion

The effects of WIN 55,212-2 on IEG (cFos, Arc, zif268) activity following MWT training are shown in Figure 20. Repeated measures ANOVAs were conducted on IEG mRNA levels with IEG (cFos, Arc, zif268), Region (dentate gyrus, parietal cortex) and Drug (vehicle, WIN 55,212-2) as within-subjects factors. Control conditions (Swim only, Drug only) were compared to combination conditions (Drug + MWT training). Within the parietal cortex, there were no significant differences between drug conditions for any of the IEGs of interest (Arc, cFos, zif268), ps > 0.177.

For the IEG Arc there was a significant interaction between Region X Condition [$F(2,12) = 6.638$, $\eta_p^2 = 0.525$] indicating IEG Arc mRNA average values were higher in the parietal cortex over the dentate gyrus for MWT only and MWT + Drug conditions, but not Drug only. There was a significant main effect of Condition [$F(2,12) = 13.519$, $\eta_p^2 = 0.693$] indicating the average Arc mRNA values were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that MWT+Drug was significantly higher than MWT only. Within the dentate gyrus, the main effect of Condition approached significance [$F(2,12) = 3.182$, $p = 0.078$, $\eta_p^2 = 0.347$] indicating the average Arc mRNA values were different between MWT only, Drug only, and MWT+Drug conditions. Pairwise comparisons revealed that Drug Only was significantly higher than MWT only.

For the IEG cFos the main effect of Condition was significant [$F(2,12) = 3.852$, $\eta_p^2 = 0.391$] indicating the average cFos values, were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that Drug only was significantly higher than MWT only [$M_{MWT} = 1.047$, $M_{DRUG} = 2.703$]. No other interactions or main effects were significant, $ps > 0.214$.

For the IEG zif268 there was a significant main effect of Region [$F(1,12) = 15.010$, $\eta_p^2 = 0.556$] indicating IEG zif268 mRNA average values in the parietal cortex were higher than the dentate gyrus [$M_{DG} = 1.810$, $M_{PC} = 3.259$]. The main effect of Condition was significant [$F(2,12) = 3.852$, $\eta_p^2 = 0.410$] indicating the average zif268 values were different between MWT only, Drug only, and MWT+Drug conditions. Simple contrasts revealed that MWT Only was significantly elevated compared to Drug only. No other interactions or main effects were significant, $ps > 0.221$.

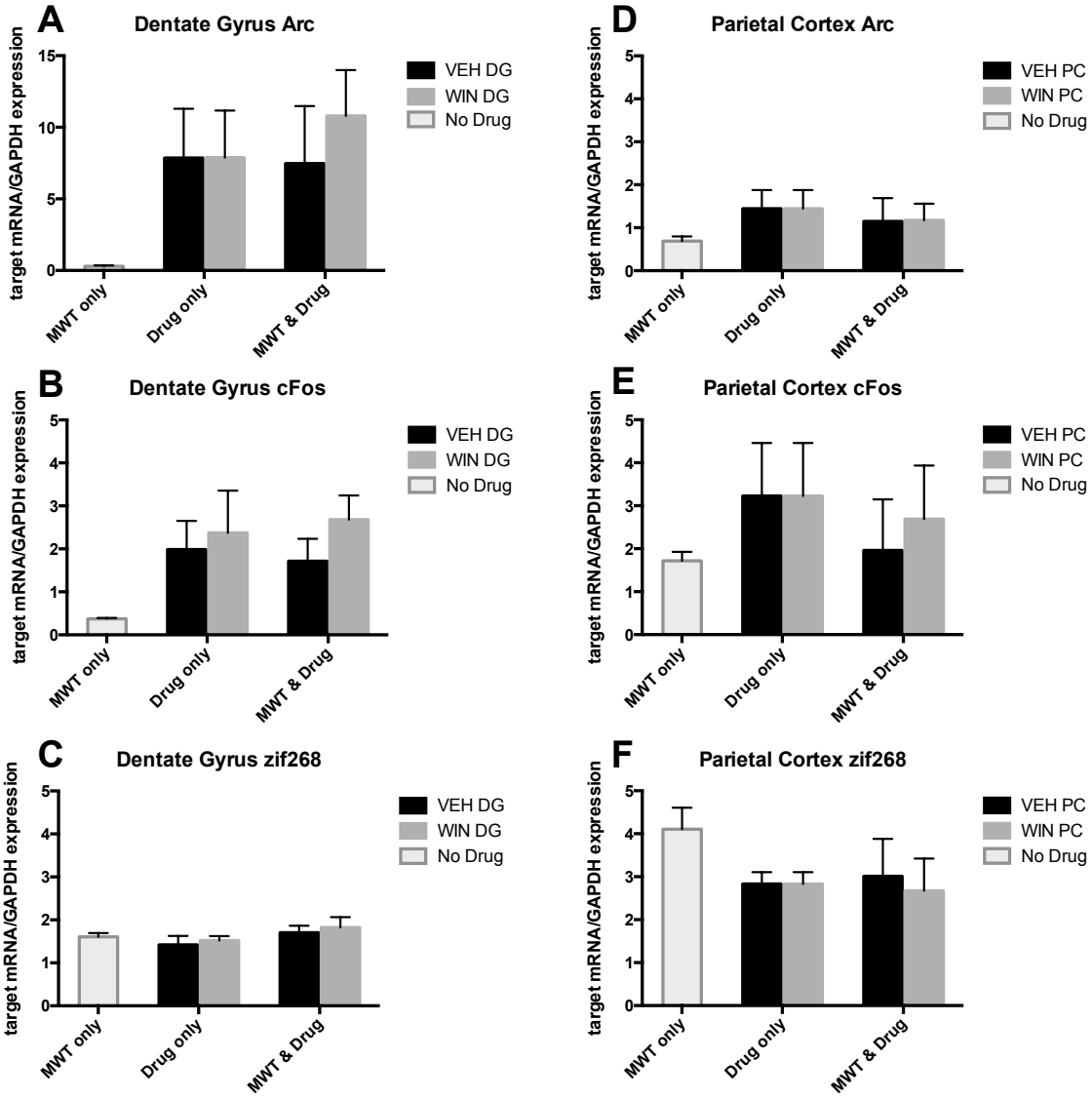

Figure 20: Immediate early gene mRNA expression following MWT training. Effect of WIN 55,212-2 on IEG (Arc, cFos, zif268) mRNA levels in the dentate gyrus (A-C) and parietal cortex (D-F). Data represent group mean (+SEM). There were no significant differences in Arc (A, D), cFos (B, E), or zif268 (C, F) expression between drug groups.

Discussion

It was hypothesized that the cannabinoid agonist WIN 55,212-2 infused into the dentate gyrus would result in a consolidation deficit in the MWT, however, this did not occur. The present study found that a single dose of vehicle and WIN 55,212-2 were indistinguishable on resulting MWT behavioral retention tests. Moreover, search performance during the probe for WIN 55,212-2 rats was more precise in average search distance from previous platform location. Conversely, during the first trial of retraining, the WIN 55,212-2 rats were slightly impaired compared to vehicle controls. These findings together may indicate search persistence is impacted in WIN 55,212-2 rats, and that their precise search strategy during the no-platform probe resulted in diminished search during the first trial of retraining. This may be partially explained by WIN 55,212-2 facilitating reversal learning (Pamplona, et al., 2006). Interestingly, the present study found that the cannabinoid antagonist + cannabinoid agonist condition did significantly impair search performance during the memory probe. The AM 251+WIN 55,212-2 rats had longer latencies and path length to the previous platform location. However, no significant drug differences were evident during the first trial of retraining. When vehicle or WIN 55,212-2 was infused into the parietal cortex, there was no difference between groups in memory retention; therefore, the parietal cortex is not critically important to memory consolidation in the MWT. Together, these results did not parallel previous findings found with systemic injections and are unable to provide dentate gyrus regional specificity for cannabinoid induce MWT consolidation deficits, although FCM verification confirmed proper cannula placement in microinfusion site. It is likely that other regions of the hippocampal formation in combination, or CA1 in combination with

dentate gyrus, contribute to cannabinoid induced memory impairment. Further, it may be that spatial memory consolidation requires multiple brain regions simultaneously, i.e. integration between the frontal cortex, and hippocampus to be stored properly.

In terms of the impact the cannabinoid agonist had on immediate early gene expression in the dentate gyrus and parietal cortex, it was expected that WIN 55,212-2 would reduce overall IEG expression compared to controls, however, this did not occur. The present study found no differences in Arc, cFos, and zif268 IEG expression between vehicle and WIN 55,212-2 conditions. There were significant differences between swim only, drug only, and combination swim+drug conditions, with swim only resulting in the lowest Arc, cFos, zif268 IEG expression. Low immediate early gene expression resulting from MWT swimming behavior is consistent with drug only controls showing similar expression to swim+drug conditions (i.e. swimming provided little elevation in expression). This also indicates the vehicle solution can result in significant IEG expression by itself. Trends in the date indicate WIN 55,212-2 elevated Arc and cFos expression in the dentate gyrus following dentate gyrus microinfusion, but would need more data to be conclusive. Although the results are not in line with expectations, there is no surprise. The signaling pathways evoked by cannabinoid administration are complex and poorly understood. Although cFos, zif268, and Arc expression appear to be important for LTP, their specific role in memory consolidation and behavior remains unclear.

In term of addressing regional specificity, the present findings show that the dentate gyrus alone is not the region responsible cannabinoid-induced memory consolidation deficits. An alternative explanation may be the dentate gyrus is not critically involved in short-term memory consolidation. Yim et al. (2008) showed that

WIN 55,212-2 infused into the dorsal hippocampus impaired long-term/one-month consolidation processes, with no impact on short-term consolidation processes. Although the MWT training procedures were different between Yim et al. (2008) and the present study, perhaps a memory retention evaluation at 24 hours is too soon to detect a consolidation deficit. It is unknown if a consolidation deficit in the present study would occur if memory retention was performed at the two-week or one-month time frame. Conversely, we were previously able to reliably detect a consolidation difference at 24 hours using systemic i.p. injections several times (Candelaria-Cook, 2009; Candelaria-Cook & Hamilton, 2008, 2010); therefore, the lack of consolidation deficits in the present study is likely more related to insufficient regional inactivation.

The role of the dentate gyrus in spatial memory consolidation can be debated. Previous research by Okada & Okaichi (2009) has shown that the hippocampus is a functional unit for spatial memory and a certain degree of cooperation between subregions is necessary for proper memory storage. Further, lesions to the dentate gyrus, CA3, and CA1 cause varying degrees of impairment in MWT acquisition depending on if the hippocampal commissure was also severed. This indicates that the associational-commissural pathway is critical to maintaining proper spatial memory consolidation as it relays spatial information between subregions on unilateral and contralateral sides. The complexity and redundancy present in hippocampal networks is resilient enough to endure partial subregion deactivations and lesions without impaired spatial memory.

It is also necessary to consider what memory phase was being studied. Post-training drug infusion and consolidation require different hippocampal subregions than acquisition. For MWT acquisition, rats can still learn the task with as little as a quarter of

the dorsal hippocampus intact (Moser, Moser, Forrest, Andersen, & Morris, 1995). However, two-thirds of the dorsal hippocampus is necessary for memory retrieval, when tested with lesions and muscimol temporary inactivation (Moser & Moser, 1998a, 1998b).

Not detecting a significant difference in immediate early gene expression between drug groups following MWT training and drug infusion may not be too surprising. Previous research has shown that the expression of the IEGs zif268 and cFos in hippocampal subregions following training in the MWT is not a direct predictor of explicit spatial location learning (Guzowski, et al., 2001). Also, it is important to have proper control conditions due because immediate early genes are activated by an array of behavior, learning, and experiences. Previously, Shires and Aggleton (2008) looked at IEG correlates of hippocampal-dependent learning in the MWT and concluded that problems in interpretation may arise due to the nature of the control conditions. They found a significant difference between cage controls, which were the baseline minimum in IEG expression, compared to free-swimming controls.

A few limitations of the present study should be taken into consideration. The present study was limited to the analysis of males. Future work should address if sex differences exist in spatial behavior following cannabinoid administration. The present study was also limited to one WIN 55,212-2 and AM 251 drug dose. A high dose was selected based on previous literature on cannabinoid-induced deficits on spatial memory. The results obtained here with a high dose of cannabinoids were subtle, but future doses ranging from low to high should be investigated, in case low doses produce a different result. Considering the unexpected nature of the AM 251 finding, the drug dosing may

need to be modified or the timing between infusions changed. Future research should also use an antagonist only condition in addition to the antagonist + agonist condition used here. This would help in determining if the AM 251 dose was too high in the current study. Also, given the uncertainty of drug distribution within the dentate gyrus, it may be useful to use a fluorescent cannabinoid agonist in the future to map the degree of diffusion. It may be that the amount of cannabinoid agonist infused was too little to completely inactivate the entire dorsal dentate gyrus, and the amount of dentate spared was enough to successfully aide in memory consolidation.

Additional limitations of the present study's IEG expression should also be taken into consideration. Here, IEG expression was compared in two areas (dentate gyrus and parietal cortex), given that immediate early genes expression is complex and varies between regions; there may be complementary changes elsewhere in the brain. Even though the present study included a modest set of control measures for IEG expression, more controls should be investigated in the future (i.e. cage controls with no behavior or drug administration). Although, swim only behavior was correlated with slight changes in gene expression, it may be important to control the amount time of spent swimming during MWT training prior to drug infusion with yoked swim controls. Furthermore, given the design of the present study, the rats of this study had abundant MWT experience; in the future it may be useful to use rats with novel experience in the MWT task prior to analyzing IEG expression. Perhaps changes in IEG expression are higher with task novelty and new learning experiences. Lastly, the current study microdissected the entire dentate gyrus with dorsal and ventral aspects, but the drug was only infused into the dorsal dentate. Perhaps using only the dorsal dentate, with tissue punch

techniques, would reveal differences in IEG expression more precisely. Alternatively, fluorescent in situ hybridization would reveal IEG expression within granule cells. These techniques were not further explored in the current study given the lack of group differences found.

In summary, the present findings demonstrated that the dentate gyrus and its activity related immediate early gene expression were not altered by a cannabinoid agonist microinfusion following MWT training. Conversely, the combined cannabinoid antagonist + agonist microinfusion following MWT training did impair 24-hour consolidation. These findings imply that cannabinoid activity within the dentate gyrus is essential but not required for proper memory consolidation. It is likely that other regions of the hippocampal formation in combination, or CA1 individually, contribute to cannabinoid induced memory impairment. Further, it may be that spatial memory consolidation requires multiple brain regions simultaneously, i.e. integration between the frontal cortex and hippocampus to be stored properly.

CHAPTER 5: DENTATE GYRUS ELECTROPHYSIOLOGY

In order to further investigate the influence cannabinoids have on memory consolidation in the dentate gyrus, we now turn to cellular forms of memory and synaptic plasticity present in the well-defined dentate gyrus circuitry, specifically two projections from the entorhinal cortex which terminate in the dentate gyrus, the medial perforant path and lateral perforant path. The following chapter discusses the effects the cannabinoid agonist WIN 55,212-2 has on long-term potentiation (LTP), paired-pulse facilitation (PPF), and specific aspects of functional activity such as neurotransmitter release and immediate early gene (IEG) signaling pathways, which may underlie memory consolidation processes in the dentate gyrus. A brief introduction to each technique can be found below.

Long-term potentiation is usually discussed as the analog to cellular memory and refers to a persistent increase in synaptic strength that is produced by brief high-frequency stimulation at excitatory afferents (Andersen, Eccles, & Loyning, 1964; Lomo, 1971). Within in the dentate gyrus, when the perforant path is stimulated a positive fEPSP is recorded which is reflective of the depolarization of a granule cell population (Lomo, 1971). Another response to stimulation of the perforant path is the population spike, which reflects the number and synchrony of the granule cell population (Andersen, Bliss, & Skrede, 1971). In dentate gyrus medial perforant path and mossy fiber projections, LTP is considered NMDA-dependent (Harris & Cotman, 1986). CB_1 receptor activation can acutely block the induction of LTP via the inhibition of presynaptic glutamate release (Shen, et al., 1996; Sullivan, 2000), which would prevent sufficient postsynaptic depolarization required to elevate calcium levels.

Paired-pulse facilitation is a form of short-term plasticity that is indicative of changes in neurotransmitter release probability and can be used in various stimulation protocols to reveal differences in GABA and glutamate release from dentate granule cells (Andersen, et al., 1971; Andersen, et al., 1964; Lomo, 1971; Sloviter, 1991). PPF is studied by giving a pair of pulses to the synaptic pathway and comparing the amplitude of the second response with that of the first response. PPF is related to a nonlinear calcium dependence of vesicular release and the ability of residual calcium to markedly augment the effectiveness of successfully timed stimuli. It should be noted that the amount of PPF depends on the interval between the two pulses; PPF decreases as the interval between stimuli increases. The formula to calculate the PPF is: (PPR= PSC #2/ PSC #1). It is generally accepted that a reduction in PPR ratio is equivalent to facilitation of neurotransmitter release. When the second response is enhanced it represents reduced neurotransmitter release with the first pulse. The phenomenon of PPR reduction being equivalent to paired pulse facilitation is explained by the fact that sufficient neurotransmitter release occurred during the first stimulus, which thereby rendered the second stimulus smaller.

The PPR can also be used as a measure of feedback and feedforward inhibition in the dentate gyrus. At inter-pulse intervals of 40 ms or less, a decrease in the second population spike is due to recurrent/feedforward inhibition, a process by which granule cell stimulation triggers basket cell inhibition of other granule cells via the perforant path (Ribak & Seress, 1983; Sloviter, 1991), Figure 21. However, at inter-pulse intervals greater than 40 ms an increase in the second pop-spike occurs due to recurrent excitatory synapses (Johnston & Amaral, 2004).

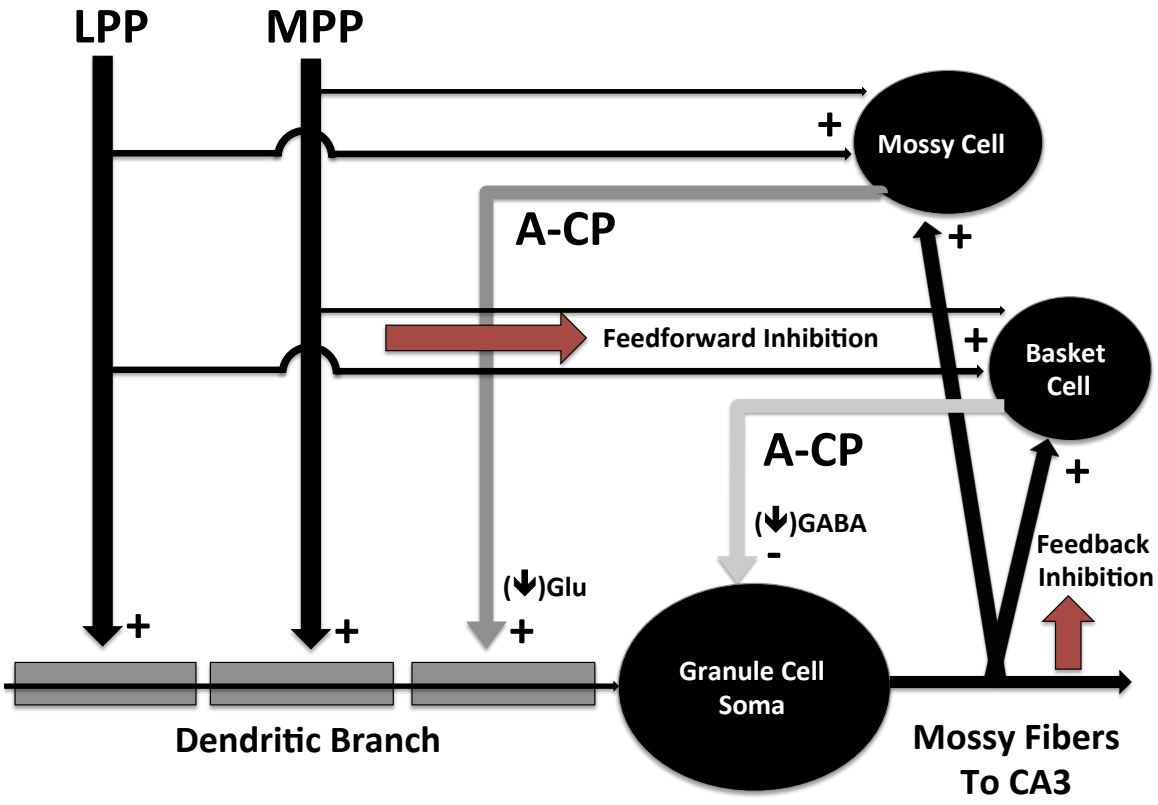

Figure 21: **Dentate gyrus feedforward and feedback inhibition.** Feedforward inhibition is a process by which granule cell stimulation triggers basket cell inhibition of other granule cells via the perforant path. Feedback inhibition is a process by which granule cell stimulation triggers basket cell inhibition of other granule cells and mossy cell excitation via mossy fibers. Abbreviations: LPP, lateral perforant path; MPP, medial perforant path; A-CP, associational-commissural projection

Cannabinoid agonist WIN 55,212-2 alters dentate gyrus perforant path paired-pulse facilitation and long-term potentiation *in vivo*.

Abstract

Endogenous cannabinoids are fundamental retrograde messengers involved in the rapid modulation of synaptic transmission. The present study evaluated the effects of WIN 55,212-2, a potent cannabinoid agonist, on dentate gyrus paired pulse facilitation and long-term potentiation *in vivo*. WIN 55,212-2 (10 μg/μL) or vehicle was unilaterally infused into the right dorsal hippocampus prior to commencing perforant path to dentate gyrus electrophysiology recordings. Upon completion of physiology brains were processed for immediate early gene expression. The data suggest WIN 55,212-2 blunted the magnitude of baseline population spike amplitude at current intensities between 500-600 **μ**A, without changing baseline fEPSP response. The cannabinoid agonist also altered fEPSP PPRs indicating WIN 55,212-2 decreased the probability of glutamate release and changed GABA mediated inhibition. WIN 55,212-2 also significantly increased fEPSP amplitude following perforant path to dentate gyrus high frequency stimulation. And lastly, WIN 55,212-2 rats had significantly higher Arc expression in the stimulated dentate gyrus compared to vehicle rats. WIN 55,212-2 may modulate LTP through a combined reduction of glutamate, and GABA via feedforward and feedback processes. WIN 55,212-2 may disturb baseline and activity-dependent changes via different mechanisms in different inhibitory circuits. When taken together, these results suggest cannabinoids selectively alter medial perforant path projection. However, the reduced baseline granule cell population spike amplitude and altered GABA mediated feedback

inhibition may be an important factors underlying cannabinoid induced memory impairments.

Introduction

Endocannabinoids are involved in the rapid modulation of synaptic transmission in the CNS by retrograde signaling causing local inhibitory effects on both excitatory and inhibitory neurotransmitter release that persists for minutes to hours. CB_1 receptors are found on both GABAergic and glutamatergic nerve terminals and may inhibit either of those two neurotransmitters. In the hippocampus and neocortex, CB_1 receptors are expressed by a defined subpopulation of GABAergic interneurons (Katona, et al., 1999), as well as by hippocampal glutamatergic neurons (Katona, et al., 2006) where they act as glutamate release inhibitors. Mossy cells of the dentate hilus contain the highest levels of CB_1 receptors amongst excitatory hippocampal neurons (Kawamura, et al., 2006; Monory, et al., 2006). Within the dentate gyrus, CB_1 receptors alter the release of glutamate from mossy cells and GABA from basket cells. The critical location of cannabinoid receptors have the potential to disrupting synaptic signaling and impair feedforward and feedback inhibition, creating long-term memory impairments.

Paired pulse facilitation (PPF) is a form of short-term plasticity that is indicative of changes in neurotransmitter release probability and can be used to assess differences in GABA and glutamate release (Ribak & Seress, 1983; Sloviter, 1991). Previous studies examining cannabinoids and PPF ratios suggest a presynaptic or retrograde mechanism is indicated if an increased PPF ratio exists. This is based on prior research showing that postsynaptic generated endocannabinoids and CB_1 receptor activation are correlated with increased PPF ratios (Gerdeman, et al., 2002). PPF ratios can also be used through two

difference stimulation protocols to indicate probability of glutamate release in the dentate gyrus and GABAergic inhibition of dentate granule cells. It may be that in the dentate gyrus cannabinoids selectively alter the release of glutamate inhibiting maintenance of Long-term potentiation (LTP) and memory consolidation.

LTP is a more persistent form of long-term synaptic plasticity than PPF. Cannabinoids have been shown to impair LTP (Collins, et al., 1995; Hill, et al., 2004; Nowicky, et al., 1987; Terranova, et al., 1995) thereby impacting long lasting synaptic plasticity in a significant manner. It may be the case that the reduction in LTP is due to reduced glutamate transmission following CB_1 receptor activation (Shen, et al., 1996; Sullivan, 2000). By inhibiting glutamatergic responses, cannabinoids may be indirectly impacting normal LTP, and possibly changing gene expression underlying the neural mechanisms of long term memory storage.

Within the ipsilateral associational-commissural projection, a feedback loop is created to regulate dentate gyrus responsiveness between excitatory mossy cells axons onto granule cells (Johnston & Amaral, 2004; Laurberg & Sorensen, 1981). Endocannabinoids have been known to adjust network activity in the dentate gyrus following excitation by retrograde signaling from granule cell spines onto CB_1 receptor containing mossy cells (Uchigashima, et al., 2011). Within the associational-commissural afferents may also be where exogenous cannabinoids are most disruptive by creating feedback inhibition disruption. If cannabinoids inhibit mossy cell excitation of proximal granule cell dendrites following feedback inhibition, the end result may be reduced excitation of the granule cell, reduced synaptic plasticity, and reduced hippocampal-dependent learning and memory.

The medial perforant path is involved in long-term potentiation following high frequency stimulation of entorhinal inputs. It has been shown that during high frequency stimulation of the medial perforant path *in vivo*, CB_1 activation increases glutamate release from perforant path synapses while inhibiting release of GABA from local interneurons (Sokal, Bennetti, et al., 2008). Additionally it has been shown that medial perforant path stimulation preferentially recruits inhibitory basket cells in a feedforward fashion, bypassing excitatory granule cell inputs and feedback inhibition (Ewell & Jones, 2010).

Following induction of LTP in the dentate gyrus, immediate early genes (IEGs) rapidly respond to the cellular stimulus. Three IEGs in particular are of interest to memory consolidation researchers: Arc (Activity-dependent cytoskeleton associated protein), cFos, and zif268 which all show elevation following perforant path LTP. Arc plays a critical role in regulating translational machinery during LTP consolidation (Bramham, Worley, Moore, & Guzowski, 2008). Induction of LTP in the dentate gyrus is associated with a rapid increase in Arc up to two hours following high-frequency stimulation (Abraham et al., 1993). The Arc protein exists in high quantities on hippocampal dendrites (Lyford et al., 1995), and Arc mRNA accumulates rapidly following recent dendritic synaptic activity (Steward, Wallace, Lyford, & Worley, 1998). Disruption of Arc protein expression impairs LTP maintenance and consolidation, without impacting LTP induction (Guzowski, et al., 2000). The IEG zif268 also rapidly increases following high frequency stimulation for up to two hours (Abraham, et al., 1993; Richardson et al., 1992). In fact, in zif268 knockouts late LTP is absent suggesting

zif268 is essential for transitioning from short-term to long-term synaptic plasticity (Jones, et al., 2001).

The present study was designed to examine the impact of a potent cannabinoid agonist (WIN 55,212-2) on synaptic plasticity and resulting immediate early gene expression within the hippocampal dentate gyrus. In adult rats, a high-dose of WIN 55,212-2 or vehicle was delivered directly into the upper blade of the dentate gyrus preceding unilateral stimulation of the perforant path to dentate gyrus circuit. To observe differences in glutamate and GABA transmission paired pulse ratios were examined along with LTP to evaluate changes in synaptic plasticity. Immediate early gene (Arc, cFos, and zif268) mRNA levels were measured in the dentate gyrus after physiological recordings were completed. Together these manipulations should reveal if a cannabinoid agonist produces changes in synaptic plasticity in the dentate gyrus, changes in neurotransmission and changes to immediate early gene levels. By changing neurotransmitter release and signal transduction pathways CB_1 receptors may be critically involved in dampening neuronal signals to foster forgetting naturally in the body.

Experimental procedures

Animals

Subjects were 16 male Long-Evans rats acquired from Harlan Laboratories (Indianapolis, IN). All rats were three months of age at the beginning of the experiments. Rats were pair-housed in standard clear plastic cages and maintained in a temperature and humidity controlled vivarium with food and water available *ad libitum*. The University of New Mexico Institutional Animal Care and Use Committee (IACUC) approved all experimental procedures.

Drug treatments

All drug doses and vehicle preparations were selected according to previous literature (Lichtman, et al., 1995; Yim, et al., 2008). WIN 55,212-2 (Sigma-Aldrich) was prepared for microinfusion at a high dose concentration of 10 μg/μL in vehicle solution. The vehicle solution consisted of a 1:1:18 ratio ethanol:alkamuls:saline, respectively. WIN 55,212-2 was first dissolved and vortex in a 1:1 mixture of ethanol and alkamuls EL-620 (kindly provided by Rhodia Pharma Solutions). The mixture brought to volume with 18 parts sterile Dulbecco's phosphate buffered saline (Sigma-Aldrich), vortex and sonicated. Both drug and vehicle solutions were prepared fresh daily.

Microinfusions

Rats were divided into two experimental groups (n = 8): vehicle control and WIN 55,212-2. Drug microinfusions occurred after rats were deeply anesthetized with urethane and positioned in the stereotaxic frame in the initial stages of surgery before electrodes were lowered into recording position. WIN 55,212-2 or vehicle was unilaterally infused into the right dorsal hippocampus with a Hamilton Neuros 1.0 μL 32 gauge syringe (Hamilton Company) attached to a stereotaxic arm on the stereotaxic frame (David Kopf Instruments). The syringe tip coordinates were -0.02 mm above the perforant path recording site (AP: -3.5; ML: -2.2; DV: -3.3 in millimeters relative to the skull surface at Bregma). The rate of infusion was 0.5 μL/min for one minute. The syringe was left in place for an additional two minutes to allow for drug diffusion. The extent of diffusion was estimated to be one mm in diameter from the injector tips (see Chapter 4 fluorescent microscopy).

***In-vivo* electrophysiology**

Rats were deeply anesthetized with urethane at a dose (1.35 g/kg) dissolved in a concentration of 250 mg/mL (two injections of 0.75 g/kg, 30 min apart). Rectal temperature was monitored and maintained at 37 °C. Once animals were non-responsive to a hind-limb pinch they were placed into a stereotaxic frame (David Kopf Instruments). The stereotaxic procedure was conducted as previously described by Sutherland et al., (1997). After cleaning the scalp, a midline incision was made to expose the skull. Five holes were drilled using a dental burr for positioning circuit components. Five stainless steel self-tapping screws (Small Parts Inc.) attached to gold Amphenol pins (Allied Electronics) were inserted into the skull; two served as reference and ground components of the differential recording circuit, one served as a return component for the stimulating circuit, and two provided additional structural support. Bregma was carefully measured with the drill bit and two additional holes were drilled for electrode placement. Rats were surgically implanted with one recording electrode and one stimulating electrode composed of Teflon- coated stainless steel wires (114 μm outer diameter; A-M Systems). The recording electrode was placed unilaterally in the right hemisphere into the hilar region of the dentate gyrus at the following coordinates relative to Bregma: AP -3.5 mm, ML -1.8 mm, DV -3.5 mm (Paxinos & Watson, 2005). The stimulating electrode was placed unilaterally in the right hemisphere into the medial perforant path at the following coordinates relative to Bregma: AP -8.1 mm, ML -4.3, DV -4.0 mm (Paxinos & Watson, 2005). Electrodes were connected to an isolated pulse stimulator (Model 2100; A-M Systems) and differential AC amplifier (Model 1800; A-M Systems). Recording signals were amplified (1000X) and converted to digital (Models PCI 6221 and BNC- 2090; National Instruments) then transferred to a personal computer for monitoring and

recording of the dentate gyrus evoked potential. Final electrode depths were optimized, varying recording and stimulating depth, until ideal placement for stimulation of the medial perforant path occurred under electrophysiological guidance. Optimal electrode placement was evident by a positive excitatory postsynaptic potential (EPSP) with a superimposed maximal negative population spike evoked by test pulses at amplitude of 400 μA (100 μs pulse duration, 10 s inter-pulse interval). Rats failing to exhibit a population spike (PS) were discarded from the study.

Baseline input/output curve

Following optimal placement of electrodes an input/output curve was generated using 100 μA, 300 μA, 500 μA, and 600 μA pulse amplitude intensities. Each amplitude was repeated five times with a 30 s inter-pulse interval. The I/O curve data was transferred to a Macbook where a Matlab program calculated the average evoked response at each pulse amplitude intensity, 40% fEPSP, 40% PS, and 1% PS values. The amplitude of the fEPSP was calculated at the initial slope midpoint at a time point after the stimulus artifact. The amplitude of the PS was calculated using the tangent method, defined as the voltage difference between a tangent to the two positive peaks and the trough of the negative peak. The PS latency was the time between the stimulation and the population spike.

Paired pulse facilitation

Following I/O curve generation, paired pulse facilitation was measured with two protocols. First, paired pulse submaximal ratios were obtained. Following a previous protocol (Varaschin, 2012) the fEPSP was measured without a population spike present to measure the probability of glutamate release. The stimulating pulse amplitude

intensity used was 90% of baseline current needed to evoke a population spike (90% of Matlab 1% PS calculation). The paired pulse submaximal ratios were obtained at inter-pulse intervals of 30, 40, and 80 milliseconds (five pulses, 100 μs duration, 30 s inter-pulse interval). The PPR was calculated by dividing the fEPSP slope from the second pulse by that of the first pulse.

Next, paired pulse near-maximal ratios were obtained. Following a previous protocol (Varaschin, 2012) the population spike was maximized to assess GABAergic inhibition of dentate granule cells. The stimulating current used for all animals was 600 μA. The paired pulse near-maximal ratios were obtained at inter-pulse intervals of 10, 20, 30, 40, 60, 80, 100, 200, 400, 600, and 1000 milliseconds (five pulses, 100 μs duration, 30 s inter-pulse interval). The PPR was calculated by dividing the fEPSP slope or population spike magnitude from the second pulse by that of the first pulse.

Long-term potentiation

Immediately following the PPF protocols, the LTP protocol began. A pulse amplitude stimulus intensity of 40% maximum population spike (ES_{40}), roughly 300-400 μA, was used for baseline, high frequency stimulation (HFS), and post-HFS recordings. Baseline (pretetanus) responses were recorded to single pulses with a 30 s inter-pulse interval over 10 minutes. LTP was induced by three trains of 10 pulses of HFS at 400 Hz with 30 s inter-train intervals. After tetanus, post-HFS evoked responses were measured to single pulses with a 30 s inter-pulse interval over 60 minutes. Baseline (pretetanus) responses were compared to the post-tetanus data. The fractional change in fEPSP slope, PS amplitude, and PS latency were compared between vehicle and WIN 55,212-2 conditions. Fractional change was calculated by subtracting the second response

amplitude from the first response amplitude then dividing by the first response amplitude. The data was further sampled into bin sizes of one minute in length to minimize random fluctuations. The post-tetanic potentiation phase (immediately four to five minutes post HFS) was also measured separately from LTP.

Immediate early gene expression

After post-HFS recordings were completed, the rat was euthanized. The brain was quickly extracted, and placed in ice-cold PBS. The brain was quickly extracted, and placed in ice-cold phosphate buffered saline. The brains were visually inspected for proper cannula placements and the dentate gyrus and parietal cortex were microdissected, placed in RNA free tubes, and stored at -80 °C. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed according to a previous protocol (Caldwell, et al., 2008) and Arc, cFos, zif268 mRNA levels were measured using primers designed by Dr. Clark Bird. Primers were designed from national database sequences of rat reference mRNA and validated with NCBI primer BLAST software. First, mRNA was extracted from the stored tissue using a RNeasy mini kit (Qiagen) and mRNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Technologies). cDNAs were created using 1 μg total mRNA and Superscript II Reverse Transcriptase (Invitrogen Corporation) following manufacturer's protocol and stored at -20 °C until used. qRT-PCR reactions were performed on an Applied Biosystems 7100 Real Time PCR System using 96-well plates (Applied Biosystems). Gene expression levels were analyzed using SYBR Green (Life Technologies) detection in triplicate reactions against GAPDH values. Based on dissociation curves, abnormal samples were discarded. Relative levels of IEG

(Arc, cFos, and zif268) expression compared to control GAPDH expression were calculated using the $2^{-\Delta\Delta Ct}$ method described by (Livak & Schmittgen, 2001).

Results

Analyses of Variance (ANOVAs) were performed using SPSS (version 22 for Macintosh). All test statistics reported here were significant at $p < 0.05$ unless otherwise noted. For all analyses Drug condition (vehicle, WIN 55,212-2) was a between-subjects factor. I/O analysis was generated on the complete data set of 16 brains (7 vehicle, 9 WIN 55,212-2). Due to recording difficulties (i.e. PPF software coding issues) and incomplete data sets (i.e. premature urethane overdose), animal numbers varied for paired pulse recordings and LTP recordings. All rats included in the analyses had a measured population spike greater than 2 mv at 600 μA current intensity. For paired pulse analyses a total of 14 brains were included (5 vehicle, 9 WIN 55,212-2) due to difficulties with the PPF recording software with two vehicle rats. For LTP analysis a total of 14 brains were included in analysis (6 vehicle, 8 WIN 55,212-2) due to one vehicle premature death and one WIN 55,212-2 recording difficulty. Separate ANOVAs were conducted for various experimental components: I/O curve, PPR, LTP, IEG. Effect sizes (partial eta squared; η_p^2) are reported for all effects.

Input/output

The effects of WIN 55,212-2 on baseline perforant path stimulation and dentate gyrus evoked potential at 100 μA, 300 μA, 500 μA, and 600 μA current intensities are shown in Figure 22. Repeated measures ANOVAs were conducted on fEPSP and PS amplitude with the four current intensities as within-subjects factors. There was a significant main effect of Current in both fEPSP response [$F(3,42) = 44.375$, $\eta_p^2 = 0.756$]

and PS amplitude [$F(3,42) = 30.739$, $\eta_p^2 = 0.687$]. Follow-up contrasts revealed significant linear trends in fEPSP response [$F(1,14) = 47.837$, $\eta_p^2 = 0.774$] and PS amplitude [$F(1,14) = 35.296$, $\eta_p^2 = 0.716$] indicating higher current intensities resulted in higher evoked responses. The fEPSP slope and PS amplitude increased proportionally to larger current intensities (Figure 22C).

There was a significant Current X Drug interaction in PS amplitude [$F(3,42) = 3.892$, $\eta_p^2 = 0.218$]. Based on visual inspection of Figure 22B showing large mean differences in PS amplitude at 500 μA [$M_{Veh} = 5.464$, $M_{WIN} = 2.166$] and 600 μA [$M_{Veh} = 6.929$, $M_{WIN} = 3.620$] follow-up pairwise comparisons were conducted. WIN 55,212-2 rats had a significantly reduced PS amplitude compared to vehicle rats at the 500 μA current intensity [$F(1,14) = 4.985$, $\eta_p^2 = 0.263$], and a difference approaching significant at the 600 μA current intensity [$F(1,14) = 3.645$, $p = 0.077$, $\eta_p^2 = 0.207$]. The main effect of Drug approached significance in PS amplitude [$F(1,14) = 3.953$, $p = 0.067$, $\eta_p^2 = 0.220$] indicating WIN 55,212-2 rats had reduced overall PS amplitude compared to vehicle rats [$M_{Veh} = 3.326$, $M_{WIN} = 1.563$]. No other effects or interactions were significant for fEPSP [all $ps > 0.450$].

It was determined there were no overall differences between vehicle and WIN 55,212-2 I/O evoked fEPSP responses. However, it was clear that WIN 55,212-2 administration impacted I/O evoked PS amplitude at higher current intensities primarily 500 μA, but also possibly at 600 μA. Given these I/O findings it is possible WIN 55,212-2 administration changed granule cell responsiveness.

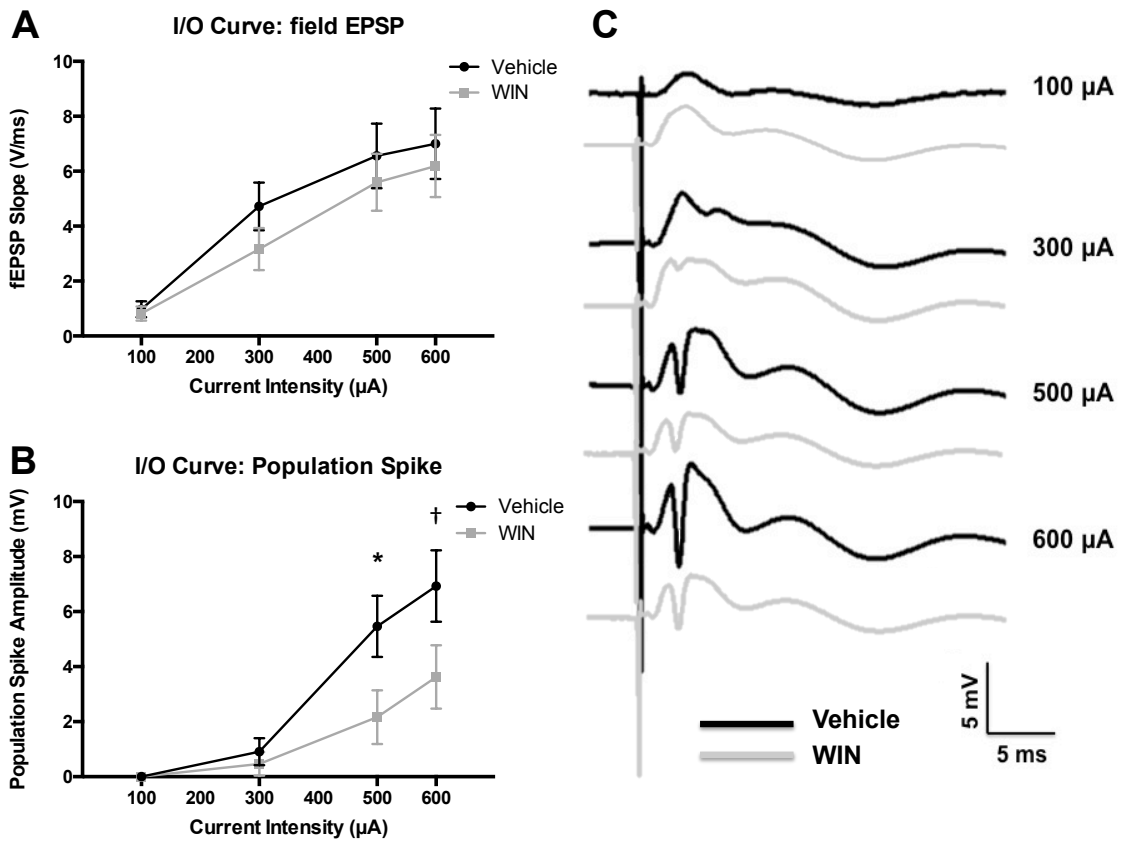

Figure 22: Baseline granule cell responsiveness. Effect of WIN 55,212-2 on fEPSP (A) and PS amplitude (B) in the dentate gyrus at four perforant path stimulus intensities, with representative traces for each group (C). Data represent group mean ± SEM. WIN 55,212-2 rats had significantly lower PS amplitude at 500 μA current intensity (* denotes $p < 0.05$), and a difference approaching significance at 600 μA († denotes $p = 0.077$). No statistically significant fEPSP differences were found between groups.

Paired pulse ratios at sub-maximal stimulation levels

The effects of WIN 55,212-2 on dentate gyrus paired pulse ratios at sub-maximal stimulation levels at 30 ms, 40 ms, 80 ms inter-pulse intervals are shown in Figure 23. Repeated measures ANOVAs were conducted on fEPSP PPRs with the three inter-pulse intervals as within-subjects factors. There was a significant main effect of Inter-pulse Interval on fEPSP PPR [$F(2,22) = 6.235$, $\eta_p^2 = 0.362$]. Follow-up contrasts revealed a significant linear trend in fEPSP PPR [$F(1,11) = 8.972$, $\eta_p^2 = 0.449$] indicating paired pulse ratios decreased linearly as inter-pulse interval increased. There was a significant main effect of Drug [$F(1,11) = 11.232$, $\eta_p^2 = 0.505$] indicating WIN 55,212-2 rats had higher PPRs than vehicle rats across all submaximal stimulation inter-pulse intervals [$M_{Veh} = 0.875$, $M_{WIN} = 1.064$]. Based on visual inspection of Figure 23A showing large mean differences in fEPSP ratios at 40 ms [$M_{Veh} = 0.879$, $M_{WIN} = 1.169$] and 80 ms [$M_{Veh} = 0.813$, $M_{WIN} = 0.927$] follow-up pairwise comparisons were conducted. WIN 55,212-2 rats had higher PPRs than vehicle rats specifically at inter-pulse intervals of 40 ms [$F(1,11) = 15.146$, $\eta_p^2 = 0.579$] and 80 ms [$F(1,11) = 6.313$, $\eta_p^2 = 0.365$]. No other effects or interactions were significant for fEPSP PPR [all $ps > 0.206$]. Based on these results it is clear WIN 55,212-2 administration impacted dentate gyrus paired pulse ratios at submaximal stimulation levels, primarily at 40 ms and 80 ms inter-pulse intervals, indicating WIN 55,212-2 administration reduced glutamate release.

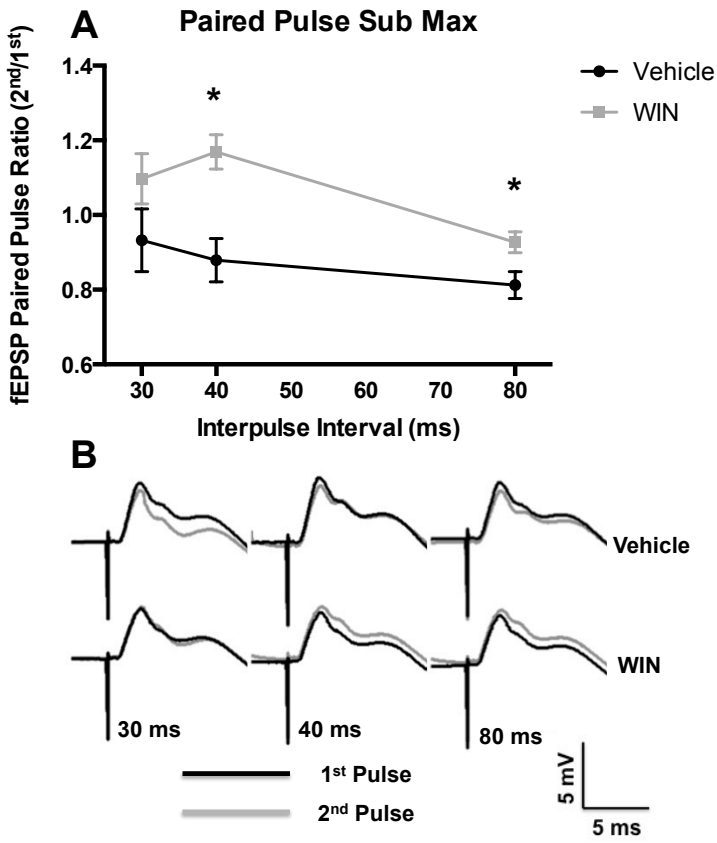

Figure 23: Paired pulse sub-maximal responsiveness. Effect of WIN 55,212-2 on fEPSP paired pulse ratios (A) in the dentate gyrus at three different inter-pulse intervals, with representative traces for each group (B). Data represent group mean ± SEM. WIN 55,212-2 rats had significantly higher PPRs at 40 ms and 80 ms inter-pulse intervals (* denotes $p < 0.05$).

Paired pulse ratios at near-maximal stimulation levels

The effects of WIN 55,212-2 on dentate gyrus paired pulse ratios at near-maximal stimulation levels at 10 ms, 20 ms, 30 ms, 40 ms, 60 ms, 80 ms, 100 ms, 200 ms, 400 ms, 600 ms, 1000 ms inter-pulse intervals are shown in Figure 24. Repeated measures ANOVAs were conducted on PPRs with the 11 inter-pulse intervals as within-subjects factors and fEPSP and PS measures as between-subjects factors. There was a significant main effect of Inter-pulse Interval in both fEPSP PPR [$F(10,120) = 25.679$, $\eta_p^2 = 0.682$] and PS PPR [$F(10,120) = 20.133$, $\eta_p^2 = 0.627$] indicating different inter-pulse intervals evoked different paired pulse ratios. As expected, responses showed paired pulse inhibition at shorter inter-pulse intervals, paired pulse facilitation at middle inter-pulse intervals, and paired pulse inhibition at higher inter-pulse intervals (Figure 24A and 24B). There was a significant interaction between Inter-pulse Interval X Drug [$F(10,120) = 2.862$, $\eta_p^2 = 0.193$]. Based on visual inspection of Figure 24A showing large mean differences in fEPSP ratios at 10 ms [$M_{Veh} = 0.668$, $M_{WIN} = 0.785$] and 30 ms [$M_{Veh} = 0.911$, $M_{WIN} = 0.986$] follow-up pairwise comparisons were conducted. WIN 55,212-2 rats had higher fEPSP PPRs than vehicle rats specifically at the inter-pulse interval of 10 ms [$F(1,12) = 5.919$, $\eta_p^2 = 0.330$]. The fEPSP PPR difference was approaching significant at 30 ms [$F(1,12) = 4.014$, $p = 0.068$, $\eta_p^2 = 0.251$]. The PS PPR difference was approaching significant at 10 ms [$F(1,12) = 3.431$, $p = 0.080$, $\eta_p^2 = 0.222$]. No other effects or interactions were significant for fEPSP PPR [all $ps > 0.173$] and PS PPR [all $ps > 0.512$]. Based on these results it is clear WIN 55,212-2 administration impacted dentate gyrus fEPSP paired pulse ratios at near-maximal stimulation levels, primarily at 10 ms inter-pulse intervals and possibly at 30 ms inter-pulse intervals. There is also some

indication WIN 55,212-2 administration may impact PS paired pulse ratios at 10 ms inter-pulse interval. Although these results are not as striking as sub-maximal stimulation PPRs, there is some indication WIN 55,212-2 administration can alter GABAergic inhibition feedback circuits within the dentate gyrus and GABA release.

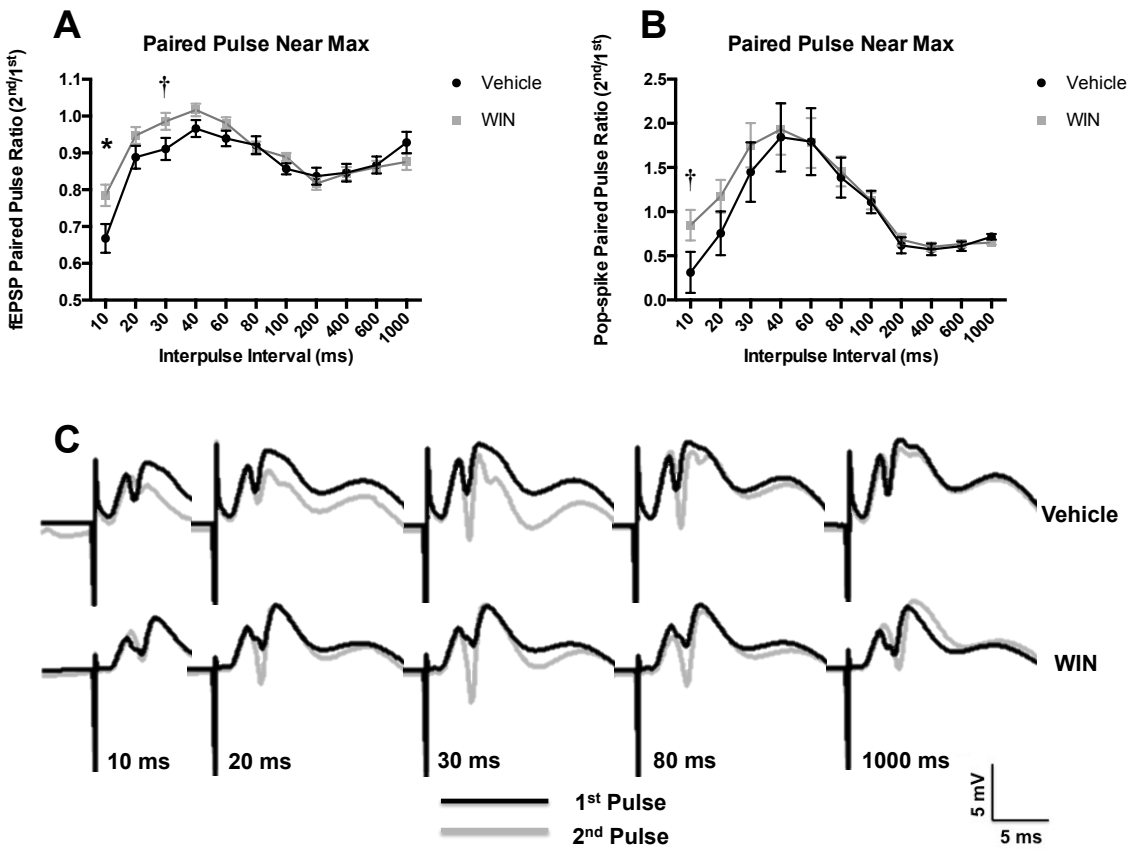

Figure 24: Paired pulse near-maximal responsiveness. Effect of WIN 55,212-2 on fEPSP paired pulse ratios (A) and PS paired pulse ratios (B) in the dentate gyrus at eleven different inter-pulse intervals, with representative traces for each group (C). Data represent group mean ± SEM. WIN 55,212-2 rats had significantly higher fEPSP and PS PPRs at 10 ms (* denotes $p < 0.05$). The difference was approaching significance on fEPSP PPRs at 30 ms († denotes $p = 0.068$) and PS PPR at 10 ms († denotes $p = 0.08$).

Long-term potentiation

The effect of WIN 55,212-2 on dentate gyrus perforant path LTP *in vivo* is shown in Figure 25. Repeated measures ANOVAs were conducted on 60 minutes of post-HFS recordings with change over time as within-subjects factors and fEPSP and PS measures as between-subjects factors. All data was binned into one-minute intervals. Baseline recording showed no difference between groups for fEPSP measures or PS measures (ps > 0.753). Following HFS, there was no difference between groups in PS latency (M_{Veh} = -0.119, M_{WIN} = -0.103, p = 0.342), data not shown.

During the first 5 minutes of post-HFS recordings there was a significant effect of Drug on fEPSP [$F(1,12) = 10.220$, $\eta_p^2 = 0.460$] indicating WIN 55,212-2 rats had higher fEPSP average fractional change compared to vehicle rats [M_{Veh} = 0.118, M_{WIN} = 0.263]. WIN 55,212-2 rats had a higher increase in fEPSP from baseline following high frequency stimulation than vehicle controls. There was also a main effect of Time on fEPSP [$F(4,48) = 22.013$, $\eta_p^2 = 0.647$] showing significant linear trends [$F(1,12) = 32.720$, $\eta_p^2 = 0.732$] indicating the post-HFS response reduced over time. No other fEPSP effects were significant (p > 0.943). During the same 5 minutes of post-HFS recordings there were no significant differences in PS amplitude between groups (ps > 0.857).

During the entire 60 minutes of post-HFS recordings there was a significant effect of Drug on fEPSP [$F(1,12) = 5.380$, $\eta_p^2 = 0.310$] indicating WIN 55,212-2 rats had higher fEPSP average fractional change compared to vehicle rats [M_{Veh} = 0.035, M_{WIN} = 0.158]. The difference between groups in fEPSP responses was specific to the first 24 minutes. By the end of the 60 minutes the two groups were not statistically different p =

0.102. There was also a main effect of Time on fEPSP [$F(59,708) = 4.561$, $\eta_p^2 = 0.275$] showing significant quadratic trends [$F(1,12) = 78.751$, $\eta_p^2 = 0.868$] indicating the post-HFS response reduced over time. No other fEPSP effects were significant ($p > 0.99$).

During the same 60 minutes of post-HFS recordings there was a significant main effect of Time in PS response [$F(59,708) = 6.250$, $\eta_p^2 = 0.342$] both showing significant linear trends [$F(1,12) = 18.727$, $\eta_p^2 = 0.609$] indicating the change over time in post-HFS response returned to baseline. No other effects or interactions were significant (ps> 0.695). The average PS fractional change between WIN 55,212-2 and vehicle rats was similar [$M_{Veh} = 2.085$, $M_{WIN} = 2.410$].

Based on these results it is clear WIN 55,212-2 administration impacted fEPSP amplitude following dentate gyrus LTP *in vivo* without impacting PS measures. This is indication WIN 55,212-2 administration can alter glutamatergic responses following LTP induction but the transient increase may decay quickly.

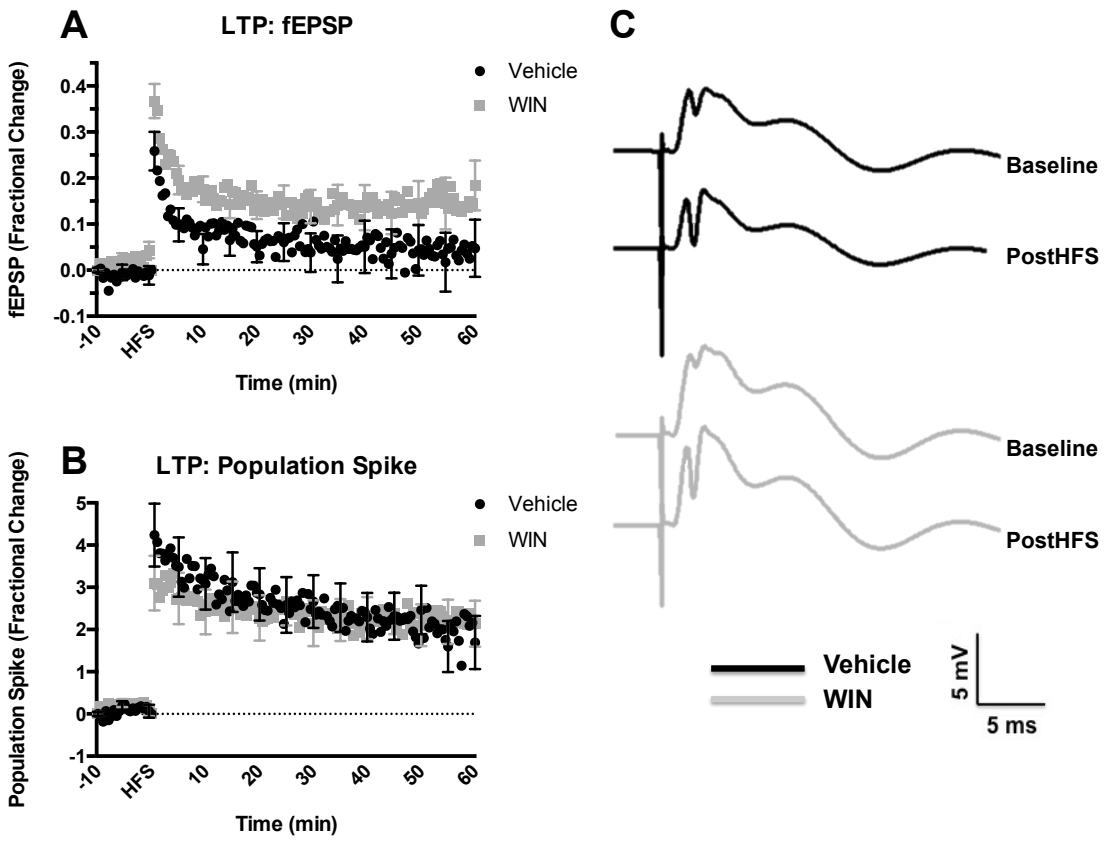

Figure 25: Stimulation of the dentate gyrus perforant path *in vivo*. Effect of WIN 55,212-2 on fEPSP LTP response (A) and PS LTP response (B) in the dentate gyrus following perforant path stimulation, with representative traces for each group (C). Data represent group mean ± SEM, error bars are shown for every 10th stimulation. WIN 55,212-2 rats had significantly higher fEPSP responses following HFS compared to vehicle rats ($p < 0.05$). No significant difference between groups was seen in population spike fractional change.

Immediate early gene expression

The effects of WIN 55,212-2 on IEG (cFos, Arc, zif268) activity following paired pulse facilitation and long-term potentiation recordings in the dentate gyrus and parietal cortex are shown in Figure 26. Repeated measures ANOVAs were conducted on IEG mRNA levels with IEG (cFos, Arc, zif268) and Region (right dentate gyrus, left dentate gyrus, parietal cortex) as within-subjects factors and Drug as between subjects factor.

The IEG Arc had a significant interaction between Region X Drug [$F(2,28) = 37.118$, $\eta_p^2 = 0.726$]. Simple contrasts revealed that WIN 55,212-2 produced higher IEG Arc levels in the stimulated dentate gyrus region ($M_{Veh} = 13.084$, $M_{WIN} = 52.489$) compared to un-stimulated left dentate gyrus [$M_{Veh} = 0.401$, $M_{WIN} = 0.450$, $F(1,14) = 39.020$, $\eta_p^2 = 0.736$] and parietal cortex [$M_{Veh} = 1.346$, $M_{WIN} = 1.714$, $F(1,14) = 35.704$, $\eta_p^2 = 0.718$]. There was a significant main effect of Region [$F(2,28) = 97.865$, $\eta_p^2 = 0.875$]. Simple contrasts revealed the stimulated right dentate gyrus was significantly different from the un-stimulated left dentate gyrus [$F(1,14) = 105.531$, $\eta_p^2 = 0.883$] and the un-stimulated parietal cortex [$F(1,14) = 91.562$, $\eta_p^2 = 0.867$]. There was a significant main effect of Drug [$F(1,14) = 40.486$, $\eta_p^2 = 0.743$]. Pairwise comparisons revealed WIN 55,212-2 rats had higher levels of Arc mRNA expression compared to vehicle rats [$M_{Veh} = 4.944$, $M_{WIN} = 18.217$].

The IEG cFos had a significant main effect of Region [$F(2,28) = 16.894$, $\eta_p^2 = 0.547$]. Simple contrasts revealed the stimulated right dentate gyrus was significantly different from the un-stimulated left dentate gyrus [$F(1,14) = 35.668$, $\eta_p^2 = 0.718$] but not statically different from the parietal cortex ($p = 0.126$). No other effects of interactions were significant ($ps > 0.584$).

For the IEG zif268, simple contrasts revealed the stimulated right dentate gyrus was significantly different from the un-stimulated left dentate gyrus [$F(1,14) = 7.113$, $\eta_p^2 = 0.337$] but not statically different from the parietal cortex ($p = 0.984$). No other effects or interactions were significant ($ps > 0.115$).

When comparing LTP stimulated and un-stimulated regions of the dentate gyrus, LTP stimulation increased all IEG mRNA expression levels, Arc [$F(1,14) = 105.531$, $\eta_p^2 = 0.883$], cFos [$F(1,14) = 35.668$, $\eta_p^2 = 0.718$], zif268 [$F(1,14) = 7.113$, $\eta_p^2 = 0.337$]. However, a Stimulation X Drug interaction only occurred in Arc mRNA expression levels [$F(1,14) = 39.020$, $\eta_p^2 = 0.736$] indicating LTP stimulation in combination with WIN 55,212-2 administration elevated Arc mRNA levels above Arc mRNA levels seen with LTP stimulation alone. Based on these results it is clear WIN 55,212-2 administration elevated Arc expression in the dentate gyrus following LTP stimulation.

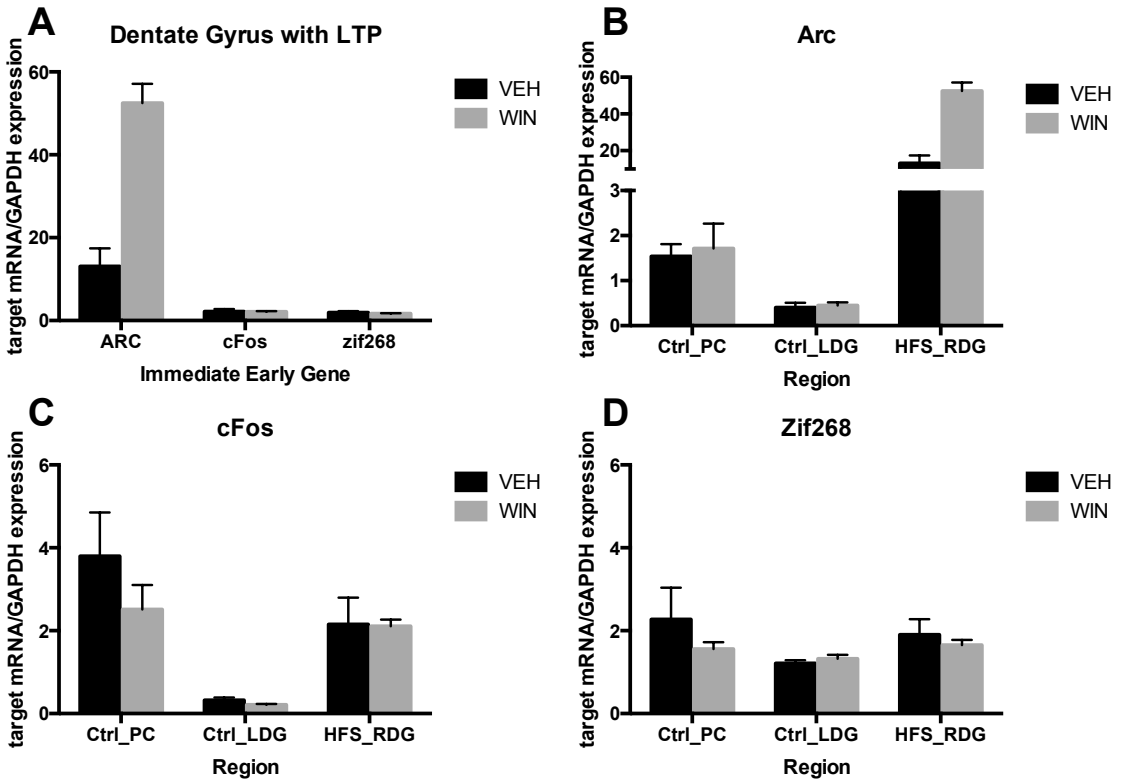

Figure 26: Immediate early gene mRNA expression following dentate gyrus perforant path stimulation *in vivo*. Effect of WIN 55,212-2 on IEG (Arc, cFos, zif268) mRNA expresssion levels in the stimulated dentate gyrus (A). The remaining graphs are divided by IEG of interest: Arc (B), cFos (C), zif268 (D) comparing un-stimulated regions (parietal cortex control, left dentate gyrus control) to the high-frequency stimulated right dentate gyrus. Data represent group mean (+ SEM). WIN 55,212-2 rats had significantly higher Arc expression in the stimulated dentate gyrus compared to vehicle rats ($p < 0.05$). No significant differences between drug groups were seen in cFos or zif268 expression. The stimulated dentate gyrus region had higher IEG expression levels (Arc, cFos, zif268) compared to the non-stimulated dentate gyrus region ($p < 0.05$).

Discussion

The cannabinoid agonist, WIN 55,212-2, altered perforant path electrophysiology responses in the dentate gyrus and elevated immediate early gene Arc expression within the dentate gyrus. When taken together, these results suggest cannabinoids selectively alter perforant path projections. The dampened population spike may be an important factor underlying cannabinoid induced memory impairments. Based on the I/O curve data, there is indication WIN 55,212-2 significantly blunted baseline granule cell responsiveness due to the fact that population spike amplitude at higher current intensities (500-600 μA) was decreased in WIN 55,212-2 rats. However, WIN 55,212-2, did not impact baseline fEPSP responses. It has been shown that feedforward inhibition can alter the amplitude of the population spike (Sloviter, 1991) which may partially explain the decreased population spike seen as differences in feedforward inhibition were detected by the cannabinoid agonist.

During paired pulse sub-maximal evaluation, by removing the population spike, there is removal of potential GABAergic inhibitory circuitry; therefore, those PPRs are an index of glutamate release from perforant path terminals (Varaschin, 2012). Even at sub-maximal stimulation levels, increased PPRs reflect a reduction in the probability of release (Zucker & Regehr, 2002). In the present study the cannabinoid agonist WIN 55,212-2 increased PPRs suggesting WIN 55,212-2 reduced the probability of glutamate release and granule cell responsiveness. At sub-maximal stimulation levels, WIN 55,212-2 significantly increased fEPSP paired pulse ratios at 40 ms and 80 ms inter-pulse intervals indicating reduced glutamate within the dentate gyrus.

At paired pulse near-maximum levels there is evaluation of GABA release and GABAergic feedback inhibition (Jedlicka et al., 2011; Sloviter, 1991; Varaschin, 2012). The findings of the present study also indicate GABA mediated inhibition of dentate granule cells is impacted by WIN 55,212-2, as higher PPRs were present. At near-maximal stimulation levels, WIN 55,212-2 significantly increased fEPSP paired pulse ratios at 10 ms and 30 ms, with possible indication that it may increase PS paired pulse ratios at 10 ms as well. This indicates that WIN 55,212-2 can impair GABAergic feedback inhibition and reduce GABA release. Together, the paired pulse measures indicate fEPSP PPRs were impacted by WIN 55,212-2 more than PS PPRs. However, it is a possibility that the blunted baseline population spike amplitude initially made it difficult to detect any difference in PS PPRs. WIN 55,212-2 may influence baseline and activity-dependent changes via different mechanisms in different inhibitory circuits.

Paired pulse protocols are often used to evaluate local circuit inhibition within the dentate gyrus. Following granule cell excitation mossy fibers engage a feedback loop to basket cells which leads to GABAergic inhibition of granule cells (Sloviter, 1991). The PPR is generally greater when the initial probability of release in response to an action potential is low. With paired pulse near-maximal stimulation levels, at short intervals (< 40 ms) the second population spike is inhibited relative to the first and this is indicative of the degree of feedforward/recurrent inhibition occurring (Seress & Ribak, 1983; Sloviter, 1991). At intervals between 20 and 100 ms, it has been suggested that fEPSP PPRs reflect probability of neurotransmitter release. Given that PPR is inversely related to the probability of neurotransmitter release, the increase in PPR at short inter-pulse intervals by WIN 55,212-2 found here indicates a reduction in paired pulse inhibition and

is suggestive of a reduction in GABA release during the first pulse. This is supported by previous research showing that WIN 55,212-2 administration can decrease dialysate GABA levels measured using microdialysis and cortical GABA levels *in vivo* (Ferraro et al., 2001). In slice physiology models, cannabinoid agonists have been shown to enhance paired pulse facilitation (Sullivan, 2000). Furthermore, CB_1 knockout mice display decreased paired pulse facilitation suggesting altered inhibitory feedback on granule cells (Jacob, Marsch, Marsicano, Lutz, & Wotjak, 2012). Together, these findings imply the CB_1 receptor is important for regulating GABAergic feedback on granule cells. It is a possibility that reducing feedforward inhibitory synapses would lead to increased overall network excitation. The inhibitory impact might be disproportionate given there are fewer interneurons than granule cells.

After high frequency stimulation of the perforant path, WIN 55,212-2 administration increased fEPSP amplitude without changing population spike amplitude. However, the elevated fEPSP amplitude showed quick decay and was equivalent to vehicle controls by 24 minutes post high frequency stimulation. These data were surprising given the inhibitory nature of cannabinoid agonists. It was expected that the cannabinoid agonist would inhibit glutamate transmission in the synapse, impairing LTP on the post-synaptic cell. Instead, this data indicates the possibility that glutamatergic release may be elevated temporary following high frequency stimulation.

The increase in fEPSP following LTP induction produced by WIN 55,212-2 may be partially explained by the complexity of the current protocol, specifically, paired pulse measures at near-maximum stimulation levels given prior to high frequency stimulation. This high stimulation protocol may have changed the responsiveness of the granule cell.

The current findings are unusual, because it has typically been proposed that cannabinoid modulation within the hippocampus acts to dampen signaling and neurotransmitter release following excitation (Sullivan, 1999, 2000). Cannabinoid receptor activation does not directly inhibit mechanisms underlying LTP but impairs LTP by reducing presynaptic neurotransmitter release which prevents significant depolarization to relieve the Mg^{2+} blockade on NMDA receptors (Misner & Sullivan, 1999). In slice physiology models cannabinoid agonists have been shown to impair LTP induction and enhance paired pulse facilitation (Sullivan, 2000). Additionally, cannabinoids and CB_1 receptor signaling have been shown to be critically involved in depolarization-induced suppression of inhibition (DSI) within CA1 pyramidal neurons (Wilson, Kunos, & Nicoll, 2001). DSI protocols involve brief trains of action potentials which transiently suppress GABAergic neurotransmission (Pitler & Alger, 1992). Interesting, in some cases LTP induction can be enhanced by a cannabinoid agonist if a depolarizing pulse (DSI) is given immediately preceding high frequency stimulation (Carlson, Wang, & Alger, 2002) because the DSI inhibits GABAergic responding allowing previously ineffective excitatory inputs to induce LTP. Given that changes to GABAergic inhibition can facilitate LTP, perhaps in the present study the paired pulse facilitation protocol altered GABAergic signaling short-term which then impacted the level of excitatory, glutamatergic neurotransmission in LTP through a non-CB_1 mechanism.

A previous study (Sokal, Benetti, Girlanda, & Large, 2008) which assessed the impact of a cannabinoid antagonist on granule cell physiology used a complex protocol similar to the one in this study and found similar differences in fEPSP only following high frequency stimulation. In dentate gyrus perforant path stimulation *in vivo*, the

cannabinoid antagonist SR141716A impaired fEPSP magnitude (Sokal, Benetti, et al., 2008) without impacting the population spike amplitude compared to controls. Studies have indicated SR141716A acts as an antagonist and inverse agonist (Pertwee, 2005). AM 251 has also been shown to hinder fEPSP responses following LTP in CA1 pyramidal cells (Alvares et al., 2006). Sokal et al., (2008) suggest that CB_1 receptors selectively impair fEPSP responses following high frequency stimulation. They further suggest CB_1 activation in the medial perforant path increases glutamate release from perforant terminals but inhibit GABA release from interneurons. Interestingly, Sokal and colleagues and the present study used similar electrophysiology protocols, in that paired pulse facilitation was measured along with LTP. Both studies found unique differences in fEPSP magnitude following cannabinoid administration and the direction of results is similar if you take into consideration the fact that the present study found post-HFS elevations with a cannabinoid agonist, while Sokal and collegues found post-HFS decreases with a cannabinoid antagonist. Also similar are the results in paired pulse facilitation, the cannabinoid agonist used here, resulted in decreased paired pulse inhibition, whereas, the cannabinoid antagonist resulted in increased paired pulse inhibition. If HFS can reduce the degree of local circuit inhibition (Sokal, Benetti, et al., 2008), perhaps PPF can also change neurotransmitter release levels prior to LTP.

The effect of cannabinoids on LTP may also vary due to the number of stimulation trains used for LTP induction. Delta-9-tetrahydrocannabinol (THC) altered LTP in CA1 hippocampal slices in a dose dependent manner. Nowicky et al. (1987) found that three doses of THC resulted in a biphasic change to population spike amplitude; a low THC dose resulted in increased baseline population spike amplitude

while high doses of THC resulted in decreased baseline population spike amplitude prior to LTP induction. Following high frequency stimulation, there were no differences in LTP induction between groups, but there was a difference in LTP decay, with high doses showing the quickest decay. These authors concluded that cannabinoids did not impact LTP levels, or elevate LTP; instead, cannabinoids modulated the decay constant of LTP, which could impact short-term memory processes.

Aside from altered granule cell responsiveness, WIN 55,212-2 administration resulted in significantly higher Arc mRNA expression within the stimulated dentate gyrus compared to vehicle controls. No differences were seen between vehicle and WIN 55,212-2 rats in cFos and zif268 expression. Unfortunately, a drug difference in zif268 in the stimulated dentate gyrus was not detected, even though zif268 is the IEG linked to memory consolidation and late LTP processes. Aside from drug effects, LTP stimulation alone elevated immediate early gene expression. The dentate gyrus hemisphere that was stimulated had increased IEG (Arc, cFos, zif268) expression levels compared to the un-stimulated dentate gyrus hemisphere.

We know LTP will increase IEGs in the hippocampus. Usually increases in Arc expression are known to facilitate learning (Guzowski, 2002; Guzowski, et al., 2000; Guzowski, et al., 2001). Therefore, this suggest the drastic increase in Arc expression produced by WIN 55,212-2 administration is significantly elevating plasticity within the synapse. In one scenario this may elevate learning, but if the level of elevation is too high, learning may be impaired. In this present study the magnitude of increase is substantial, WIN 55,212-2 rats showed a 300% increase in Arc expression in the stimulated dentate gyrus over controls; therefore, the synapse may be too destabilized to

facilitate proper memory consolidation, though this is just speculation. Nonetheless, the direction of Arc elevation in WIN 55,212-2 rats is consistent with the elevation in LTP observed because Arc is known to be elevated following LTP protocols. While cFos, zif268, and Arc expression appear to be important for LTP, their specific role in memory storage is still unclear. It was expected that LTP would result in a rise in zif268 expression. It has been shown that zif268 is important for processes underlying late LTP in the dentate gyrus (Jones, et al., 2001). Increasing the number of trains in the LTP protocol had been correlated with increased zif268 and Fos-related proteins (Abraham, et al., 1993). Perhaps increasing the number of trains for LTP induction from three to ten would have resulted in a larger zif268 increase and a drug difference between groups.

A few limitations of the present study should be taken into consideration. The present study was limited to a single, high dose of WIN 55,212-2. Future work should explore if different doses produce similar results. Or, as observed by Nowicky et al., (1987) low doses may produce different results on population spike amplitude and LTP decay than high doses of cannabinoids. Due to the fact that baseline granule cell population spike responses in WIN 55,212-2 rats were impaired compared to controls, there is difficulty interpreting the overall electrophysiology findings. In a potential follow-up it would be important to artificially equalize drug rats to controls through LTP clamping.

The current design looked at paired pulse facilitation prior to LTP induction. In pilot work with a small animal number, alternating paired pulse protocols before and after LTP did not impact LTP responses, but did impact the level of paired pulse inhibition. Perhaps the paired pulse protocol before LTP also impacted LTP. Future studies should

look at these two phenomena in a separate cohort of animals to avoid potential confounds. Further, given the design of the current study it is difficult to infer what triggered the rise in Arc expression in the stimulated dentate gyrus. Here, we collected paired pulse responses for approximately an hour prior to the high frequency stimulation hour-long protocol. It is a possibility that either or both of the paired pulse intervals or high frequency stimulation triggered the rise the Arc in the WIN 55,212-2. Another issue with the current design is the length of time it took to run all experimental procedures. On average, the drug was infused into the right dentate gyrus one hour prior to paired pulse protocols and two hours prior to the LTP protocol. It is unknown how quickly WIN 55,212-2 diffused away from its target location; therefore, it is unknown if the drug was still present in the dentate gyrus at the end of recordings. Perhaps the rapid decay in LTP may be explained by lack of drug at the synapse.

Additional limitations, which pertain to IEG expression, should also be taken in consideration. This study lacked a couple additional controls for IEG analysis. Previously, Shires and Aggleton (2008) looked at IEG correlates of hippocampal-dependent learning in the MWT and concluded that problems in interpretation may arise due to the nature of the control conditions. Here, we used the un-stimulated dentate gyrus and parietal cortex as controls, but are lacking cage controls and a condition with stimulation but no vehicle or drug. In Chapter 4, it was evident that the vehicle solution by itself triggered an elevation in IEG expression; therefore, it is possible similar elevations are occurring here. Future studies should closely examine what cell type is responsible for the substantial increase in Arc expression. It is believed the current rise in Arc expression is occurring within granule cells, but given that IEG mRNA levels were

performed on homogenized dentate gyrus tissue, it is a possibility nearby cell types may be contributing to the response. In the future, fluorescent in situ hybridization would be able to address signaling location.

Follow-up studies should examine if there are differences in LTP following medial versus lateral perforant path stimulation. There have been indications the medial perforant path projection is more sensitive to the cannabinoid agonist than lateral perforant path projections based on Golgi morphology findings (Chapter 3). Also, future studies should pursue in-vitro slice physiology recordings in the dentate gyrus to better control neuronal inputs and rule out elevations in endocannabinoid responding.

In summary, the present findings demonstrate that the dentate gyrus electrophysiology and its activity related immediate early gene expression is altered by a cannabinoid agonist microinfusion. These finding imply that cannabinoid signaling within the dentate gyrus is very complex and the alteration in the signaling found here may be responsible for some cannabinoid-induced memory consolidation deficits. CB_1 receptors can alter GABAergic and glutamatergic signaling via feedforward and feedback inhibition with different consequences for memory processing and memory consolidation. WIN 55,212-2 may modulate LTP through a combined reduction of glutamate, and GABA via feedforward and feedback processes. More research is needed to elucidate these findings, specifically to clarify why an increase in fEPSP following LTP was detected. By changing neurotransmitter release and signal transduction pathways CB_1 receptors may be critically involved in dampening neuronal signals to foster forgetting naturally in the body.

CHAPTER 6: GENERAL DISCUSSION

Summary and significance of results

The effect of a potent cannabinoid agonist, WIN 55,212-2, on rodent learning and memory in the dentate gyrus was examined using a combination of morphological, behavioral, electrophysiological, and gene targeting approaches. The dentate gyrus was selected as the region of interest for its role in memory formation and consolidation combined with its high density of CB_1 receptors. The goal was to capture systems levels phenomena (decreases in spatial memory in the MWT) down to cellular level phenomena (difference in immediate early gene expression and neurotransmitter release). For a more complete discussion of the findings in relation to relevant literature refer to the individual discussions at the end of Chapters 3-5. A brief overview of the findings is presented below.

In Chapter 3, which addressed changes to granule cell morphology following chronic WIN 55, 212-3 administration, there was suggestion that the cannabinoid agonist specifically alters associational-commissural projections from within the dentate gyrus and medial perforant path projections from the entorhinal cortex. Long-term cannabinoid administration for seven and 21 days resulted in a marked reduction in granule cell dendritic spine density. The magnitude of spine density loss was on the order of ~1 spine per 10 μm in dendritic segments proximal and middle to the cell body. These observations do not reflect a generalized reduction in overall dendritic morphology, as no differences were found between drug and vehicle groups in measures of dendritic length and branching, but instead indicate select spine sensitivity in spatially relevant afferents from the entorhinal cortex following long-term cannabinoid exposure. Segment specific

changes in spine density may have important consequences for spatial learning and memory processes in the dentate gyrus. Hargreaves et. al. (2005) showed that the medial perforant path transferred spatial information from the medial entorhinal cortex, while the lateral perforant path transferred nonspatial information. The current study extends previous adolescent findings by showing that spine loss in the dentate gyrus also occurs in adult rats that began chronic treatment in adulthood after the brain was fully matured; therefore, some changes in dendritic morphology following long-term cannabinoid administration are not specific to a particular developmental age. Conversely, the fact that we did not find differences in overall dendritic branching and length in adult rats implies that adolescent granule cells may be more sensitive to cannabinoids. Given that the negative consequences observed after adolescent exposure far outnumber the effects observed after mature adulthood exposure, there is an indication that hippocampal cannabinoid sensitivity may partially resolve by adulthood. Considering the involvement of granule cell plasticity in memory, these observations suggest exposure to exogenous cannabinoids can have profound and long-lasting consequences on long-term spatial memory storage and consolidation.

In Chapter 4, which focused on 24-hour MWT consolidation deficits following microinfusion of WIN 55,212-2 in the dentate gyrus, there was suggestion that CB_1 receptor activation in the dentate gyrus alone is insufficient to create a memory deficit. Prior work from our laboratory found WIN 55,212-2 given post-training resulted in retention deficits following a 24-hour consolidation time frame (Candelaria-Cook, 2009; Candelaria-Cook & Hamilton, 2008). This memory consolidation deficit could be recovered by administration of the cannabinoid antagonist AM 251 (Candelaria-Cook &

Hamilton, 2010), further supporting the role of CB_1 receptors in spatial memory consolidation. In contrast, the present study found that direct infusion of WIN 55,212-2 into the dentate gyrus did not influence 24-hour memory consolidation or alter immediate early gene expression in the dentate gyrus or parietal cortex. This indicates that another region of the hippocampal formation, or the summation of all hippocampal areas working together is responsible for proper memory consolidation following cannabinoid administration. The present study also found that the cannabinoid antagonist AM 251 followed 30 minutes later by the cannabinoid agonist WIN 55,212-2 resulted in impaired MWT probe retention 24 hours following training. Considering the unexpected nature of this drug effect, the drug dosing used in this protocol may need to be modified. The role of the dentate gyrus in spatial memory consolidation can be debated. Previous research by Okada & Okaichi (2009) has shown that the hippocampus is a functional unit for spatial memory and a certain degree of cooperation between subregions is necessary for proper memory storage. Further, lesions to the dentate gyrus, CA3, and CA1 cause varying degrees of impairment in MWT acquisition depending on if the hippocampal commissure was also severed. This indicates that the associational-commissural pathway is critical to maintaining proper spatial memory consolidation as it relays spatial information between subregions on unilateral and contralateral sides. It is also necessary to consider what memory phase was being studied. Post-training drug infusion and consolidation require different hippocampal subregions than acquisition. For MWT acquisition, rats can still learn the task with as little as a quarter of the dorsal hippocampus intact (Moser, et al., 1995). However, two-thirds of the dorsal hippocampus is necessary for memory retrieval, when tested with lesions and muscimol temporary

inactivation (Moser & Moser, 1998a, 1998b). The complexity and redundancy present in hippocampal networks is resilient enough to endure partial subregion deactivations and lesions without impaired spatial memory.

In Chapter 5, which focused on changes in perforant path to dentate gyrus electrophysiology following microinfusion of WIN 55,212-2, there was suggestion that cannabinoid modulation via GABAergic feedforward inhibition results in reduced glutamate release and corresponding elevation in the mRNA levels of the immediate early gene Arc. WIN 55,212-2 blunted the magnitude of baseline population spike amplitude at current intensities between 500-600 μA, without impacting baseline fEPSP response. The cannabinoid agonist also altered fEPSP paired pulse ratios indicating decreased glutamate release and altered GABAergic inhibition via feedforward projections. Together, the paired pulse measures indicate fEPSP PPRs were impacted by WIN 55,212-2 more than PS PPRs. However, it is a possibility that the blunted baseline population spike amplitude initially made it difficult to detect any difference in PS PPRs. WIN 55,212-2 may alter baseline and activity-dependent changes via different mechanisms in different inhibitory circuits. Following high frequency stimulation WIN 55,212-2 also increased fEPSP amplitude transiently. This indicates the possibility that glutamatergic release may be elevated temporary following high frequency stimulation, but given the lack of support in the literature for this finding, more research is needed to clarify this result, as it may not replicate with a different protocol design. And lastly, WIN 55,212-2 rats resulted in elevated Arc immediate early gene expression in the stimulated dentate gyrus. WIN 55,212-2 may modulate LTP through a combined reduction of glutamate and GABA via feedforward and feedback processes. When taken

together, these results suggest cannabinoids selectively alter the perforant path projection. However, the reduced baseline granule cell population spike amplitude and altered GABA mediated feedback inhibition may be important factors underlying cannabinoid induced memory impairments.

Critique and future studies

Although these experiments were carefully designed, there are several limitations of each which need to be considered when interpreting the results found. First, all studies used a high drug dose of WIN 55,212-2. It is known that WIN 55,212-2 is a potent cannabinoid agonist but its exact half-life and metabolism are largely unknown. Moving forward a proper dose-response curve and half-life metabolism should be investigated. These would aide greatly in the analysis of results produced by the WIN 55,212-2 cannabinoid agonist. Second, throughout most of these experiments, better control conditions could be added. Most of the needed controls would address changes in immediate early gene expression evoked by changes in various behaviors and activity. There was indication that the vehicle alone elevated immediate early genes above baseline, therefore, having additional baseline controls would be useful. There were other limitations of each design but for complete description refer to the end of each chapter discussion.

Final remarks

When taken together, these findings partially clarify ways in which cannabinoids are detrimental to long-term memory formation, consolidation and storage in the brain. Cannabinoids alter normal learning and memory processes in the dentate gyrus by selectively altering medial perforant path projections, influencing GABAergic

feedforward inhibition, reducing glutamate release, and increasing expression of the immediate early gene Arc. Cannabinoid modulation throughout the dentate gyrus and other hippocampal subregions are necessary for memory consolidation processes.

LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol268)
A-CP	Associational-commissural projection
AM 251	1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide
ANOVA	Analysis of Variance
CA1	*Cornu Ammonis* area 1
CA3	*Cornu Ammonis* area 3
CB_1	Cannabinoid Receptor subtype 1
CB_2	Cannabinoid Receptor subtype 2
DAG	Diacylglycerol
DG	*Dentate gyrus*
DMSO	Dimethyl sulfoxide
ES40	Effective stimulus intensity to elicit 40% of maximal response
fEPSP	Field excitatory postsynaptic potential
GABA	Gamma-Aminobutyric acid
HFS	High frequency stimulation
IEG	Immediate early gene
i.p.	Intraperitoneal
I/O	Input/Output
LPP	Lateral perforant path
LTD	Long-term depression

LTP	Long-term potentiation
MPP	Medial perforant path
mGluR	Metabotropic glutamate receptor
mRNA	Messenger ribonucleic acid
NMDA	N-methyl-D-aspartate
MWT	Morris water task
PPR	Paired-pulse ratio
PPF	Paired-pulse facilitation
PS	Population spike
RT-PCR	Real time polymerase chain reaction
THC	delta-9-tetrahydrocannabinol
WIN 55,212-2	[R-(+)-(2,3-dihydro-5-methyl-3-[{4-morpholinyl} methyl] pyrol[1,2,3-de-]-1,4-benzoxazin-6-yl)(1-naphthalenyl)-methanone mesylate]

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