Spring 2014

Role of central cannabinoid receptors in cerebellar dependent learning

Adam Benjamin Steinmetz
University of Iowa

ROLE OF CENTRAL CANNABINOID RECEPTORS IN CEREBELLAR DEPENDENT LEARNING

by

Adam Benjamin Steinmetz

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Psychology
in the Graduate College of
The University of Iowa

May 2014

Thesis Supervisor: Professor John Freeman

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Adam Benjamin Steinmetz

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Psychology at the May 2014 graduation.

Thesis Committee: John Freeman, Thesis Supervisor

Mark Blumberg

Amy Poremba

Ryan LaLumiere

Jason Radley

Dedicated to my loving wife, son, parents, and family.

ACKNOWLEDGMENTS

I would like to thank my mentor John Freeman for giving me the opportunity to work, learn, and grow as a scientist over the past years. The environment provided allowed me to develop the critical thinking that will be necessary to be successful in the future. I would like to thank the various members of the Freeman lab who have provided important feedback and advice for the projects and experiments throughout my time in the lab.

I would also like to thank my committee members Dr. Mark Blumberg, Dr. Amy Poremba, Dr. Ryan LaLumiere and Dr. Jason Radley for their suggestions and guidance of my thesis work. They have each provided important contributions and advice that extends into my development as a scientist.

I also want to thank my parents for their love, support, and continuous encouragement to pursue my passion. To my wife I owe a debt of gratitude for being there throughout this process.

ABSTRACT

Cannabinoid receptors (CBR) are the most abundant G-protein coupled receptors in the mammalian brain with the highest densities within the cerebellum (Herkenham et al., 1990). Cannabinoid manipulations have been reported to cause deficits in cerebellar-dependent learning (Kishimoto and Kano, 2006; Skosnik et al., 2007; Steinmetz and Freeman, 2010; 2013). Cannabinoid receptors-1 (CB1R) have been hypothesized to be important in the establishment of long-term depression within the cerebellar cortex (Levenes et al., 1998; Safo and Regehr, 2005). However, all investigations *in vivo* have used global manipulations and have not attempted to localize or characterize these receptors during cerebellar-dependent learning. Chapter 2 systematically examined localization within the cerebellar cortex of cannabinoid effects on eyeblink conditioning, a type of cerebellum-dependent learning. Local infusions into a specific portion of the cerebellar cortex, the eyeblink conditioning microzone, resulted in deficits in learning similar to systemic injections. Additionally, infusions of cannabinoids into the eyeblink conditioning microzone, and no other parts of the cerebellar cortex or deep nuclei, were responsible for the deficits. Finally, tetrode recordings were made in Purkinje cells while receiving either CBR agonist or vehicle injections prior to training. Fewer Purkinje cells exhibited learning-related decreases in activity when the rat was administered a CBR agonist as compared to when it was injected with the vehicle. The CBR administered Purkinje cells also showed earlier onsets and smaller amplitudes in their learning-related activity. Purkinje cells that show a learning-related increase in activity were not affected by cannabinoid administration. The impairment in Purkinje cell plasticity was not observed after the rats reached asymptotic levels of learning. These results indicate that

CBR agonist administration disrupts the induction of plasticity within the cerebellar cortex and this may account for the behavioral deficit in eyeblink conditioning. Chapter 3 examined whether infusions of the CBR agonist into the cerebellar cortex impaired forebrain-dependent learning as well as forebrain-independent associative learning. Similar to subcutaneous injections, forebrain-dependent trace eyeblink conditioning was unimpaired, whereas forebrain independent delay eyeblink conditioning was impaired. These findings provide evidence that plasticity mechanisms that are modulated by cannabinoids do not play a significant role in trace eyeblink conditioning. Finally, in Chapter 4 the role of CBRs and endocannabinoids during memory consolidation were examined. CBR and endocannabinoid manipulations prior to training resulted in impaired eyeblink conditioning. However, a CBR agonist or a drug increasing endocannabinoid levels resulted in enhanced consolidation when administered 1 hour post-training. In contrast, a CBR antagonist or an endocannabinoid decreasing drug resulted in impairments 1 hour post-training. Thus, CBRs and endocannabinoids appear to be important in learning and consolidation of cerebellar-dependent learning.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

LIST OF ABBREVIATIONS

AIN:	Anterior interpositus nucleus
CB1R:	Cannabinoid-1 receptor
CB2R:	Cannabinoid-2 receptor
CN:	Cochlear nucleus
CR:	Conditioned response
CS:	Conditioned Stimulus
EBC:	Eyeblink Conditioning
FN:	Facial Nucleus
IC:	Inferior colliculus
LTD:	Long-term depression
LTP:	Long-term potentiation
MAT:	Medial auditory thalamus
PC:	Purkinje cell
PN:	Pontine nucleus
RN:	Red nucleus
UR:	Unconditioned response
US:	Unconditioned stimulus

CHAPTER 1. INTRODUCTION

Cannabis Sativa has been used medically and recreationally by humans for numerous years. The first use of cannabis dates back to 8000 B.C. to Central/Western Asia, specifically within China, in which the plant was used to treat a variety of medical conditions as well as for recreational consumption (Li, 1973). The use of cannabis spread throughout as a medicinal treatment to India around 1200 to 800 B.C and then to Europe around 430 B.C. (Russo, 2006; Nagy et al., 2008). Initially cannabis was legalized within Europe in 1901, until the Geneva Convention listed it as a dangerous and illicit drug in 1925. The consumption of cannabis within the United States was also initially legal until the Mexican Revolution in 1910 symbolized the use of the drug as a terrible sin. Federal Law was introduced in 1937 making cannabis illegal in the United States. Presidents John F. Kennedy and Lyndon Johnson suggested the legalization of the cannabis and it still remains a controversial topic. Presently, 18 states have passed bills in which medicinal Cannabis can be sold and two states, Colorado and Washington, recently have legalized possession.

Today, Cannabis remains one of the most widely used psychoactive substances in the world and consumption rates have increased substantially during the past 25 years. In the United States alone, over 94 million individuals have used cannabis at least once in their lifetime; while over 17 million use it regularly (The Substance Abuse and Mental Health Services Administration [SAMHSA], 2009; 2011). Furthermore, roughly 5 million individuals within the United States reported using cannabis over 300 times in a

year (SAMHSA, 2011). Among new cannabis users, 2.4 million in the past year are aged 18 and younger, a time when the brain is still developing (SAMSHA, 2011).

Cannabinoid Receptors

Numerous attempts were made to isolate the active constituent(s) and structures of Cannabis but were unsuccessful for over a century (Mechoulam & Hanus, 2000). In 1964, Gaoni and Mechoulam isolated the principal psychoactive constituent in cannabis, Δ9-tetrahydrocannabinol (THC). It was later determined that cannabis contains over 60 constituents, many with closely related structures and properties. THC was determined to affect the brain via the action of central cannabinoid receptors (CBR; Devane et al., 1988). To date two cannabinoid receptors have been discovered and cloned, cannabinoid-1 and cannabinoid-2 receptors (CB1R and CB2R, respectfully; Pertwee, 1997). However, there is evidence that additional CBRs could exist (Wiley and Martin, 2002; Ryberg et al., 2009). CB1Rs are one of the most abundant G-protein coupled receptors in the central nervous system, with high densities in areas such as the cerebral cortex, basal ganglia, hippocampus, and cerebellum (Herkenham et al., 1990; 1991; Glass et al., 1997; Pertwee, 1997; 1999; Tsou et al., 1998; Egertova and Elphick, 2000; Eggan and Lewis, 2007). CB1Rs are 7-transmembrane G protein coupled receptors that when activated inhibit adenylyl cyclase and Ca2+ channels and activate mitogen-activated protein kinase (MAPK) and K+ channels (Iversen, 2003; Howlett, 2002; 2005). CB1Rs are located primarily on presynaptic terminals and their activation leads to the inhibition of neurotransmitters, notably γ-Aminobutyric acid (GABA), glutamate, and dopamine (Iversen, 2003). Similar to CB1Rs, CB2Rs are also seven transmembrane receptors. In

contrast, CB2Rs are located primarily in the peripheral tissues of the immune system. Within the brain, CB2Rs are located primarily on microglia and but can also be located on neurons.

The presence of receptors within the central and peripheral nervous systems led researchers in search of an endogenous molecule (endocannabinoid) that is synthesized within the brain. Two endocannabinoids were isolated in the 1990s, one that is primarily found within the brain, 2-arachidonoylglycerol (2-AG), and another that is found mostly in peripheral tissue, anandamide (Devane et al., 1992; Mechoulam et al., 1995). Endocannabinoids have several properties that make them unique from traditional neurotransmitters such as dopamine and serotonin. First, endocannabinoids are not stored in vesicles but are synthesized when and where they are needed. Second, endocannabinoids serve as fast retrograde synaptic messengers that act presynaptically and not postsynaptically (Howlett et al., 2002). Thus, endocannabinoids are synthesized at a postsynaptic cell and released to affect the presynaptic cell. Inhibiting presynaptic activity is the primary activity of the cannabinoid system which will be discussed in detail below. Anandamide and 2-AG are both hydrolyzed to arachidonic acid and ethanoamine by fatty acid amide hydrolase (FAAH). 2-AG can also be hydrolyzed by monoacylglycerol lipase (MAGL). Suppression of these enzymes has been shown to prolong the activity of 2-AG and anandamide (Gaetani et al., 2009).

Following the discovery and isolation of THC and the endocannabinoids, biochemists sought to create synthetic agonists and antagonists for CB1Rs and CB2Rs and also classify additional constituents of cannabis. These have been subdivided into groups based on the molecular structures. The naming of the newly synthesized

cannabinoid receptor drugs follows these rules: if the compound is from Raphael Mechoulam's laboratory at Hebrew University the drug contains the initials HU, if the compound is from Alexandros Makriyannis' laboratory than AM proceeds the number, and if the drug was created by Pfizer then CP is attached. The classical cannabinoids include delta-9 THC and delta-8 THC, both of which are plant-derived cannabinoids and non-selective for CB1R or CB2R. Additionally in the classical cannabinoids is the synthetic agonist HU-210 that is also non-selective but has an enhanced affinity and efficacy compared to the plant derived cannabinoids. Non-classical compounds, which include the popular non-selective agonist CP 55,940, have similar structures to classical cannabinoids. Aminoalkylindole cannabinoids include the most widely used agonist in cannabinoid research WIN55,212-2, which has a high affinity and efficacy but is non-selective for CB1Rs or CB2Rs. More recently, selective cannabinoid agonists have been developed, most notably arachidonyl-2'-chloroethylamide (ACEA) which has a 2000 times greater affinity for CB1Rs than CB2Rs. Selective CB2R agonists have also been developed including AM 1241 and HU 308. Selective CB1R antagonists have also been developed and include SR141716A, which is widely used for research. SR141716A was marketed and prescribed as a weight loss supplement under the name Rimonabant in 2006. In 2008, doctors discouraged the prescription of Rimonabant due to side effects that included an increased risk of the developmental psychiatric problems such as depression and suicidal thoughts. A summary of popular cannabinoid receptor agonists and antagonists are given in Table 1.

As mentioned above, the activation of CB1Rs cause an inhibition of the presynaptic cell. This activation of CB1Rs, either by endocannabinoids or synthetic

agonists, primarily causes an inhibition or activation of ion channels. The most characterized and studied occurrence is inhibition of neurotransmitter release at the axon terminals. However, it is important to note that CB1Rs can have an effect on dendrites by interfering with conduction towards the soma or on the soma by interfering with the generation of an action potential. These conditions are difficult to study, thus most cannabinoid physiology has been studied using axon terminals. In axon terminals, CB1Rs interfere with Ca2+ and K+ channels. Initial studies using cultured cells indicated that CB1R activation inhibits N-type voltage-dependent Ca2+ channels via a G protein containing a Galphaai/o subunit (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie, Devane, and Hille, 1993). This was later replicated using rat hippocampal and cerebellar cells (Twitchell et al., 1997; Nogueron et al., 2008). Additionally Q-type calcium channels have also shown to be inhibited by CB1R activation (Mackie et al., 1995). Vesicles containing neurotransmitter do not fuse to the membrane and release the containing neurotransmitter without Ca2+ entering the cell. The activation of CB1Rs also has been demonstrated to cause an activation of K+ channels. This activation causes an inward rectification of K+ into the neuron (Mackie et al., 1995; Henry and Chavkin, 1995). The activation of K+ and inhibition of Ca2+ channels together results in decreased neurotransmitter release. The resulting activity is summarized in Figure 1.

The Cerebellum

The cerebellum contains more than half of all neurons within the mammalian brain (~50-85 billion) although the volume is relatively small (Lange, 1975). This dense structure can be divided into three lobes, the anterior lobe, posterior lobe, and the

flocculonodular lobe. Inputs into the cerebellum come into the cerebellar cortex from a variety of sources including the spinal cord, brainstem, pontine nuclei, and inferior olivary nucleus. Output from the cerebellum occurs via the deep cerebellar nuclei. The afferent and efferent connections come from the cerebellar peduncles.

The cerebellar cortex (Figure 2) contains three layers and five cell types that are repeated in a similar circuit throughout the entire cerebellum. Mossy fibers, originating from the pontine nuclei, make two synapses, onto neurons in the deep cerebellar nuclei and granule cells. Granule cells are small and one of the most numerous neurons in the adult brain. The cell bodies are located in the granule cell layer of the cortex and project axons to the most outer layer, the molecular layer. The axons in the molecular layer, termed parallel fibers, can extend for up to 6 mm in mammals in which they synapse with Purkinje cells, stellate cells, basket cells, and Golgi cells. Each parallel fiber comes in contact with 2000-3000 Purkinje cells and each Purkinje cell receives inputs from 200,000 parallel fibers. Parallel fiber activity controls the occurrence of simple spike activity in the Purkinje cells. The second input into the cerebellar cortex is via climbing fibers that originate in the inferior olive. Each climbing fiber, roughly 15 million in humans, comes in contact with 10 Purkinje cells; each Purkinje cell receives input from one climbing fiber. Each discharge from the climbing fiber results in a complex spike. The sole output of the cerebellar cortex is the Purkinje cells. These cells make synapses with the deep cerebellar nuclei in which they release GABA. The cerebellar cortex also contains three GABAergic inhibitory interneurons: basket cells, stellate cells, and Golgi cells. Basket and stellate cells are located within the molecular layer and receive input

from parallel fibers and make synapses onto Purkinje cells. Finally, Golgi cells receive input from parallel fibers and synapse with granule cells and other Golgi cells.

The cerebellum has been thought to be a contributor primarily to motor function, specifically in fine motor control, posture and gait. However, this traditional conceptualization of the cerebellum as a motor control structure has been updated and it has been implicated in a wide range of non-motor higher cognitive functions including attention, language, working memory, and learning (Strick et al., 2009). Dysfunction of the cerebellum has been associated with disorders such as Schizophrenia, Bipolar Disorder, and Specific Language Impairment (Bolbecker et al., 2009; 2011; Steinmetz and Rice, 2010).

Studies localizing CB1Rs using autoradiography found that the heaviest density of the receptors was within the cerebellum. Suarez et al. (2008) examined the specific locations of CB1Rs, CB2Rs, cannabinoid biosynthesis enzymes (DAGL-alpha, DAGL-beta, and NAPE-PLD), and endocannabinoid degrading enzymes (FAAH and MAGL) within the cerebellum. The authors reported heavy CB1R labeling within the cerebellar cortex, specifically proximal to, but not located directly on, Purkinje cells. The CB1Rs are densest in the molecular layer on parallel fibers of granule cells and moderately on basket cells and climbing fibers. In contrast, the deep nuclei (interpositus nucleus) have very low levels of CB1Rs and relatively high levels of CB2Rs. Both the cortex and nuclei contain high levels of FAAH and low levels of MAGL.

Cannabinoids and Synaptic Plasticity

Cannabinoid receptors have been linked to the induction of plasticity throughout the brain. It was first reported in 1991 that brief depolarization of Purkinje cells in the cerebellum triggers a suppression of GABAergic synapses (Llano et al., 1991). The authors reported this suppression was due to the presynaptic neuron (parallel fibers or climbing fibers), most likely from a retrograde signal. The suppression caused by the presynaptic neuron (parallel fibers or climbing fibers) was later observed in hippocampal neurons and was termed depolarization-induced suppression of inhibition (DSI; Pitler and Alger, 1992). DSI contains several key features that are similar throughout the brain. First, DSI requires a large increase in postsynaptic Ca2+. Second, DSI is associated with a decrease in the frequency but not amplitude of spontaneous IPSCs. DSI also has no effect on the response of iontophoretically applied GABA, indicating a presynaptic localization (Llano et al., 1991; Pitler & Alger, 1992). The retrograde messenger involved in DSI was later reported to be endocannabinoids (Wilson and Nicoll, 2001). Kreitzer and Regehr (2001b) reported another phenomenon in cerebellar slices called depolarization-induced suppression of excitation (DSE), which is also mediated by endocannabinoids. Most of the initial work was conducted in hippocampal and cerebellar neurons; however DSI and DSE mediated by endocannabinoids have been reported in numerous other regions. DSI has been reported in neocortex, substantia nigra, and dentate gyrus and DSE has been reported in the ventral tegmental area and hypothalamus.

In the hippocampus, depolarization of pyramidal neurons induces a suppression of GABAergic IPSCs. This DSI is blocked by CB1R antagonists and is absent in CB1R knockout mice (Ohno-Shosaku, Maejima, and Kano, 2001; Wilson and Nicoll, 2001). DSI expressed in the hippocampus is transient, and suppression of the resulting inhibition

could not account for long-term plasticity of synapses. However, it may be important in facilitating depolarization, and therefore induction of LTP. This has been shown experimentally by using a subthreshold stimulation during DSI to induce long-lasting LTP (Carlson et al., 2002). Addition of AM251 prevented this facilitation, resulting in the conclusion that the release of endocannabinoids causes a local facilitation of the induction of LTP. It has also been shown that high-frequency stimulation induces release of endocannabinoids, specifically 2-AG (Stella, Schweitzer, and Piomelli, 1997). If the synthesis and release of endocannabinoids is rapid, then the local release of endocannabinoids caused by the inducing train would tend to facilitate the induction of LTP at that particular site and therefore increase the signal to noise ratio of any potentiating synapses in that area. The role of endocannabinoids in the CA1 region of hippocampus may therefore be to cause a local facilitation of LTP. The physiological role of endocannabinoids would be to enhance hippocampal dependent learning and memory. Thus, smoking cannabis may impair learning and memory due to inappropriate global facilitation of LTP at synapses throughout the brain, rather than at discrete local sites, leading the elevation of the background noise and a reduction in the signal to noise ratio of potentiated synapses (Riedel and Davies, 2005).

Ca2+ has been shown to trigger the release and production of endocannabinoids. Synaptically evoked endocannabinoid release requires an elevation of calcium along with activation of a G-coupled protein receptor (Brenowitz and Regehr, 2005; Maejima et al., 2005). This in turn activates an intracellular cascade that results in the endocannabinoid production and release. Elevation in intracellular Ca2+ has been reported to be necessary for endocannabinoid synthesis (Freund et al., 2003). The release of endocannabinoids has

been studied mostly using in vitro Purkinje cell LTD. Calcium-dependent mechanisms can evoke the release of endocannabinoids from Purkinje cells (Kreitzer and Regehr, 2001a). Adding the calcium chelator BAPTA prevented retrograde inhibition of Purkinje cells indicating that postsynaptic calcium is essential. The authors suggested that the calcium from dendrites is what mediates climbing fiber effects of plasticity at parallel fiber-Purkinje cell synapses (Brenowitz and Regehr, 2005). Another trigger to the synthesis and release of endocannabinoids may be the activation of group I metabotropic glutamate (mGluR) receptors (Varma et al., 2001; Ohno-Shosaku et al., 2002) and muscarinic acetylcholine receptors (Kim et al., 2002). Activation of mGluR results in the production of endocannabinoids (Maejima et al., 2001). This can be dependent or independent of the Ca2+ increases (Maejima et al., 2001; Robbe et al., 2002). Furthermore, downstream the enzymes phospholipase C and DAG lipase may be required for production of endocannabinoids (Melis et al., 2004). These endocannabinoid production pathways have been reported to occur in the same cell type within the hippocampus and cerebellum (Figure 3; Stella and Piomelli, 2001; Galante and Diana, 2004). Blocking the production and release of endocannabinoids results in the loss of LTD in the cerebellum and LTP in hippocampus (Chevaleyre et al., 2007; Safo and Regehr, 2005).

The administration of cannabinoids also impairs LTD within the hippocampus. The first investigation was by Nowicky, Teyler, and Vardaris (1987) which showed the preincubation with THC could inhibit or potentiate high frequency stimulation LTP in the hippocampus depending on the concentration. A low concentration increased the duration of LTP but also increased the population spike amplitude. In contrast, two higher

concentrations reduced the duration of the LTP but also decreased the population amplitude. CB1R agonists HU-210 and WIN55,212-2 were shown to impair LTP formation and were both blocked by SR141716A (Collins, Pertwee, and Davies , 1995; Terranova et al., 1995; Paton, Pertwee, and Davies, 1998; Misner and Sullivan, 1999).

The cerebellar cortex, particularly the molecular layer, contains a high density of CB1Rs as measured by autoradiography (Herkenham et al., 1990; 1991; Ong and Mackie, 1999). CB1Rs are located on the glutamatergic terminals of parallel fibers and the GABAergic terminals of stellate cells and basket cells, and thereby modulate inhibitory and excitatory synaptic transmission to Purkinje cells (Suarez et al., 2008; Iversen, 2003). Activation of CB1Rs is necessary for Purkinje cell LTD (Safo and Regehr, 2005). Moreover, Purkinje cell LTD is dependent upon release of the endocannabinoid 2-arachidonyl glycerol (2-AG) from Purkinje cells (Safo and Regehr, 2005), which results in decreased glutamate release from parallel fibers (Kreitzer and Regehr, 2001a; b).

Cannabinoids and Cognition

Initial studies examining the effects of cannabinoids on cognition were conducted using human subjects. It was found that acute intoxication produced more reliable data than long-term use (Gonzalez, 2007). Additionally,it has been reported that the more difficult the task, the greater degree of impairment (Jager et al., 2006; Hunault et al., 2009; Tarald, 1977). Acute cannabis use also has been demonstrated to affect the perception of smell, taste, hearing, vision, and time (Adams et al., 1976; Tart, 1971). The memory that appears to be most affected by cannabis use is recognition memory

(D'Souza et al., 2009). In one version of the task, participants are presented with a list of words to remember. Following a delay, participants are shown a new list of words containing some from the previous list and some new words. Cannabis users typically are able to recognize the former words but also falsely recognize some of the new words (Dornbush, 1974). When the task is made more difficult by asking the subjects to write the words that they previously saw (recall task), the subjects perform even worse by inserting words that were never presented (Dornbush et al., 1971; Miller and Cornett, 1978). Several critiques have been made of the studies examining human subjects. First, it could be argued that differences between subjects are present prior to testing between subjects. Additionally, humans who use marijuana also are more likely to use a greater amount of alcohol and other drugs. In order to avoid the critiques mentioned, researchers turned to animal models of cognition in order to study the effects of acute and chronic administration of cannabis.

Animal models of cognition have used a variety of paradigms in order to examine the effects of cannabis on learning and memory. Spatial learning has been examined using water mazes, radial arm mazes, and T/Y mazes. The Morris Water maze has been used in order to test the effects of cannabinoid agonists on spatial learning. Ferrari et al. (1999) reported that animals given the cannabinoid agonist HU 210 were unable to learn the position of the hidden platform. Later studies showed that cannabinoids impair acquisition of spatial memory but not consolidation or recall (Riedel and Davies, 2005). Cannabinoids also impaired the acquisition when the position of the platform was changed, making this a working memory task (D'Hooge and De Deyn, 2001). Interestingly, CB1R knockout mice were able to acquire a spatial reference memory task.

The mice were impaired in reversal learning of the maze, however, suggesting a deficit in task difficulty (Varvel and Lichtman, 2002). Radial arm mazes have been extensively used and reliably have shown that manipulations in cannabinoid functioning, through either a CB1R knockout mouse or CB1R agonist, impair acquisition (Lichtman, Dimen, and Martin, 1995; Lichtman and Martin, 1996). However, the results from these maze studies are hard to interpret since cannabinoid manipulations also impair motor ability and motivation (Sañudo-Peña et al., 1999; 2000).

Pavlovian fear conditioning has also been employed to study learning in animals. CB1R knockout mice and wild-type mice treated with the antagonist SR141716A acquire auditory fear conditioning similar to wild-type littermates (Marsicano et al., 2002; Pamplona et al., 2006). However, during extinction training, both knockout mice and the SR141716A treated mice maintain high levels of freezing, whereas controls significantly reduce their freezing levels. It was also reported that the levels of anandamide and 2-AG increase during the presentation of the tone (Marsicano et al., 2002). Similar results have been reported during contextual fear conditioning with acquisition being unimpaired but extinction being impaired (Pamplona and Takahash, 2006).

Eyeblink Conditioning

Eyeblink conditioning (EBC) is a Pavlovian associative learning paradigm in which a conditioned stimulus (CS; e.g. tone) is repeatedly paired with an unconditioned stimulus (US; e.g. shock). Before training the CS does not elicit a blink response, whereas the US elicits a reliable blink response known as the unconditioned response (UR). Over repeated CS-US pairings a conditioned blink response (CR) occurs prior to

US presentation. EBC was initially developed for human participants in the 1920s to study learning without the need for verbal reports (Cason, 1922). The paradigm was then used as an animal model to study the properties and eventually the neurobiology of learning. Gormezano and colleagues were the first to transition this procedure to rabbits by studying the movement of the nictitating membrane (Gormezano et al., 1962; Scheiderman and Gormezano, 1962, Gormezano et al., 1983). Rabbits were an ideal species for EBC due to their ability to tolerate restraint and their low levels of spontaneous blinks (Gormezano et al., 1983). Most of the initial neurobiology of EBC was conducted in rabbits, but EBC has been applied to a wide array of species including rats, mice, frogs, turtles, sheep, dogs, monkeys, and cats.

Several classical conditioning paradigms were first described by Pavlov in the early 1900s continue to be employed today in EBC (Pavlov, 1927). The two most widely used to study the neurobiology and mechanisms of learning are termed delay and trace EBC. Delay EBC, the most widely used EBC paradigm, involves the US presentation terminating with the offset of the CS. Trace EBC, in contrast, has a stimulus free "trace" interval that occurs between the offset of the CS and the onset of the US. These two paradigms both rely on the cerebellum for learning, but have several noted differences.

Neural Substrates of Eyeblink Conditioning

The first indication that the engram for eyeblink conditioning was located within the cerebellum was reported by Thompson and colleagues (McCormick et al., 1981). The initial studies found that large aspirations of the cerebellar hemisphere ipsilateral to the trained eye abolish learning but spare CRs to the contralateral eye. Lesions that included

the anterior interpositus nucleus were found to be the most effective (McCormick and Thompson, 1984). Smaller, more precise lesions completely abolish learning following extensive training and after memory consolidation (McCormick and Thompson, 1984; Lavond et al., 1984). Inactivation of the anterior interpositus nucleus reversibly blocks learning (Krupa and Thompson, 1997). Additionally, anterior interpositus nucleus lesions impaired learning following extensive training, even following 150-200 sessions or 18,000-24,000 trials (Steinmetz, Louge, and Steinmetz, 1992). The studies mentioned above were all completed with delay EBC, though lesions of the anterior interpositus nucleus also impair the acquisition and retention of trace EBC (Woodruff-Pak et al., 1985).

The cerebellar cortex contributes substantially to acquisition of delay EBC (Thompson and Steinmetz, 2009; Freeman and Steinmetz, 2011). The mechanism underlying the cerebellar cortical contribution to motor learning is thought to be long-term depression (LTD) of parallel fiber synapses with Purkinje cells (Albus and Branch, 1971; Ito and Kano, 1982; Ekerot and Kano, 1985; Linden and Conner, 1991; Schreurs and Alkon, 1993; Freeman et al., 1998; Medina and Mauk, 2000; Thompson and Steinmetz, 2009). Damaging this LTD mechanism by ablation of the cerebellar cortex ipsilateral to the conditioned eye causes a severe impairment in acquisition of delay EBC (Lavond and Steinmetz, 1989; Garcia, Steele, and Mauk, 1999). Elimination of Purkinje cells, the hypothesized site of plasticity within the cerebellar cortex, in pcd mice (Chen et al., 1996; 1999), or with OX7-saporin in rats (Nolan and Freeman, 2006) produces deficits in acquisition as well. A more direct link between cerebellar cortical plasticity and delay EBC comes from studies that used genetic manipulations to impair cerebellar

LTD and acquisition of delay EBC (Aiba et al., 1994; Shibuki et al., 1996; Miyata et al., 2001; Kishimoto and Kano, 2006). Cerebellar cortical LTD releases the deep nuclei from inhibition, which increases cerebellar output to motor nuclei that produce eyelid closure and may be necessary to induce plasticity in the anterior interpositus nucleus (Mauk and Donegan, 1997; Ohyama et al., 2006; Thompson and Steinmetz, 2009).

The role of the cerebellar cortex during acquisition of trace EBC is less understood. Most results indicate that the cerebellar cortex is not necessary for acquisition of trace EBC. Mice with genetic alterations targeted towards cerebellar cortex functioning have repeatedly shown intact trace EBC but impaired delay EBC (Kishimoto et al., 2001a, b, c; Brown, Agelan, and Woodruff-Pak, 2010). Patients with cerebellar cortex lesions also demonstrate intact hippocampus-dependent trace conditioning but impaired delay and short (non-hippocampus dependent) trace eyeblink conditioning (Gerwig, Kolb, and Timmann, 2007). More recently, we have suggested that the cerebellar cortex is involved in trace conditioning when the CS duration is longer than the trace duration (Steinmetz and Freeman, 2013).

Recently, the neural substrates underlying the CS pathway have been examined for auditory and visual modalities (Halverson and Freeman, 2010; Steinmetz, Buss, and Freeman, 2013). Auditory information enters the ear and projects to the cochlear nucleus. The cochlear nucleus project to the medial auditory thalamic nuclei through direct projections and also through the superior olive, lateral lemniscus and inferior colliculus. The MATN and CN project to the lateral pontine nucleus which projects to the anterior interpositus nucleus and cerebellar cortex (Figure 4; for review see Freeman and Steinmetz, 2011). Visual information enters the retina and projects to the nucleus of the

optic tract and the ventral lateral geniculate. The NOT and LGNv project to the medial pontine nucleus. The medial pontine nucleus projects to the anterior interpositus nucleus and cerebellar cortex (for review see Freeman and Steinmetz, 2011).

The US pathway originates within the trigeminal nuclei which send information to the inferior olive (Harvey et al., 1984; Schreurs, 1988; van Ham and Yeo, 1996). The inferior olive synapses onto neurons within the interpositus nucleus and Purkinje cells (Van der Want et al., 2004; Shinoda et al., 2000; Sugihara, Wu, and Shinoda, 2001). Stimulation of neurons within the dorsal accessory division of the inferior olive (DAO) can replace a peripheral US in conditioning (Mauk, Steinmetz, and Thompson, 1986; Steinmetz, Lavond, and Thompson, 1989; Jirenhed, Bengtsson, and Hesslow, 2007). Disruption of the inferior olive signaling impairs the acquisition and retention of eyeblink conditioning (McCormick et al., 1985; Yeo, Hardiman, and Glickstein, 1986).

The interpositus nucleus sends projections via the superior cerebellar peduncle to the magnocellular division of the red nucleus and then to the brainstem motor nuclei that innervate eyelid, ocular, and facial muscles (McCormick et al., 1982; Rosenfield and Moore, 1983; Chapman et al., 1990; Desmond and Moore, 1991; Krupa et al., 1993; Krupa and Thompson, 1995). Inactivation of red nucleus abolishes CRs while leaving learning related activity in the interpositus nucleus intact (Chapman et al., 1990).

Cannabinoid Receptors and Cerebellar Learning

The role of CB1Rs during delay and trace EBC has been previously examined in several human and animal studies. Animal work examining the role of cannabinoids in cerebellar function have shown impairments during delay EBC providing evidence for

the role of CB1Rs during the acquisition, retention, and extinction of cerebellum-dependent learning. For example, CB1R knockout mice exhibit impaired acquisition of delay EBC but not trace EBC (Kishimoto and Kano, 2006). Similarly, mice administered SR141716A (CB1R antagonist) subcutaneously exhibit impaired learning during delay EBC but learn trace EBC to control-like levels. The authors conclude from these experiments that cerebellar-dependent learning (delay EBC) requires CB1Rs. We have administered the CB1R agonist WIN55,212-2 and antagonist SR141716A subcutaneously in varying doses to rats during delay EBC. The rats exhibited a dose-dependent impairment in acquisition with both WIN55,212-2 and SR141716A (Steinmetz and Freeman, 2010). The deficits resulting from WIN55,212-2 administration were greater than the highest concentration of SR141716A. This was attributed to differing actions of the drugs since the middle and highest concentrations of SR141716A did not differ from each other. Importantly, this study showed that blink parameters were not altered by the drugs (i.e., sensitivity to the US, spontaneous blink rates, or blinks to CS presentation). Administration of SR141716A 30 mins prior to WIN55,212-2 administration blocked the impairment that was previously observed, suggesting that the deficit was CB1R dependent. We also reported that rats administered WIN55,212-2, but not SR141716A, following asymptotic learning and during extinction show impairments as compared to vehicle injections (Steinmetz and Freeman, 2011). More recently, we administered WIN55,212-2 during trace conditioning and reported no significant differences compared to vehicle treated animals (Steinmetz and Freeman, 2013). However, animals that were trained with long delay EBC (750 ms CS) were more impaired than animals to a shorter delay (250 ms CS). These studies suggest that the

CB1Rs play roles in the acquisition, retention, and extinction of delay EBC, but not trace EBC.

In humans, current and former cannabis use has been associated with robust decreases in percentage of CRs and altered CR timing in delay EBC (Skosnik et al., 2008; Steinmetz et al., 2011). It was also demonstrated that current users showed intact URs and CS processing, indicating that the deficits in delay EBC were due to learning and not to altered CS processing or habituation to the US. Thus, the impairment was hypothesized to be related to the downregulation of CB1Rs in the cerebellar cortex in chronic cannabis users. This hypothesis is supported by a follow-up study showing that active cannabis users can accurately acquire CRs during trace conditioning, which is thought to be less cerebellar-cortical dependent, and is mediated by more forebrain cortical structures (Edwards et al., 2008).

The current hypothesis from both the animal and human studies is that the deficits in EBC with systemic administration of CB1R agonists are caused by effects localized to the cerebellar cortex. CB1Rs are the most abundant G-coupled protein receptors in the brain and areas outside the cerebellar cortex could be contributing to the EBC deficits. The current proposal attempts to localize the EBC impairments with CB1R administration within the cerebellar cortex and characterize the responses of Purkinje cells following CB1R agonist administration during acquisition, retention, and extinction.

Prior to the start of the current experiments the critical area within the cerebellar cortex was examined. Initial studies examining the role of the cerebellar cortex indicated that large aspirations and lesions would disrupt the rate of learning (Chen at al., 1996; Lavond and Steinmetz, 1989). Studies from the Mauk and Yeo laboratories localized the

critical area within the cortex in rabbits to the anterior lobe and lobule HVI, respectively (Garcia et al., 1999; Yeo et al., 1984; Yeo and Hardiman, 1992). Examining data published from these laboratories led to a hypothesized common area at the border of anterior lobe and lobule HVI. Unpublished data from our lab show that electrolytic lesions of this area severely impaired the rate of learning in rats. Lesions that included only one of the areas (i.e., lobule HVI or anterior lobe) were not as effective as lesions of both. Additionally, large lesions that created a lot of damage did not differ from smaller lesions that affected both lobule HVI and anterior lobe. Thus, we determined the border between the areas to be the critical area and targeted it in the experiments described below.

Overview of Experiments

As mentioned above, previous work has hypothesized that global manipulations to CB1Rs specifically impair LTD formation within the cerebellar cortex. The goals of the current experiments are to examine the localization and function of CB1Rs during motor learning within the cerebellum. This will directly test whether CB1R dysfunction within the cerebellar cortex results in impaired cerebellar learning.

The first set of experiments in Chapter 2 set out to localize the effects of CB1Rs during delay EBC. The first experiment used intra-cerebellar cortex infusions of differing concentrations of a CB1R/CB2R agonist during delay EBC to test if dose-dependent impairments are witnessed similar to previous reports using subcutaneous administration. The next experiment examined areas within the cerebellum in order to test whether a specific region within the cerebellar cortex is the critical area within the cerebellum.

Infusions were made into the anterior lobe/lobule HVI area (EBC microzone), vermis, or the anterior interpositus nucleus. Experiment 3 within Chapter 2 tested if blocking the receptors only in the cerebellar cortex with a selective CB1R antagonist while administering a peripheral dose of a CB1R/CB2R agonist would block the impairment. The final experiment of the chapter examined if CB1Rs or CB2Rs within the cerebellar cortex were responsible for impairments in acquisition of delay EBC. To test this a CB1R selective antagonist was administered before both a non-selective CB1R/CB2R agonist and a CB1R preferring agonist. These experiments localize the effects of CB1Rs during delay EBC to the cerebellar cortex.

During delay EBC Purkinje cells develop pauses in simple spike firing within the CS period during acquisition that then return to baseline during extinction training (Green and Steinmetz, 2005; Jirenhed and Hesslow, 2007; 2011). The pauses in simple spike activity reflect LTD at parallel fiber synapses. Research examining *in vitro* manipulations of CB1Rs during Purkinje cell LTD procedures demonstrates impaired LTD formation as compared to control conditions (Safo and Regehr, 2005). Chapter 2 examines *in vivo* recordings of Purkinje cells during acquisition and retention of delay EBC following subcutaneous injections of CB1R/CB2R agonist or vehicle administration.

CB1R manipulations have been shown to impair cerebellar-dependent delay but not forebrain-dependent trace EBC. Chapter 3 examined infusions of WIN55,212-2 into the cerebellar cortex during delay, long delay, and trace EBC. Subcutaneous injections of WIN55,212-2 impair delay and long delay but not trace EBC. The impairment observed during long delay EBC was greater than delay EBC indicating a differential role of the cerebellar cortex and CB1Rs during long delay compared to delay. The role of the

cerebellar cortex during trace conditioning has remained controversial. Mice with impaired LTD formation within the cerebellar cortex have intact trace EBC (Kishimoto et al., 2001a, b, c; Brown et al., 2010). Chapter 4 will examine if infusions of CB1R agonist made into the cerebellar cortex results in deficits during delay and long delay, but not trace conditioning.

Chapter 4 examined the role of CB1Rs and endocannabinoids during acquisition and consolidation of delay EBC. Consolidation of cerebellar learning has not been examined with CB1Rs and endocannabinoids. CB1Rs may be important for the consolidation of memory. Endocannabinoids have been reported to be necessary for the induction of LTD within Purkinje cells (Safo and Regehr, 2005). Endocannabinoid manipulations, increasing or decreasing levels, were also examined during acquisition and consolidation. Rats received 5 sessions of paired CS-US training with infusions either before of following each session. The infusions following the session occurred at intervals between immediately following the session to 6 hours.

Compound	CB1Ki (nM)	CB2Ki (nM)	Affinity ratio CB1/CB2
Antagonists			
SR141716A	11.8	13200	1118.64
Agonists			
ACEA	1.4	>2000	>1428
AM 1241	0.75	2.4	3.2
CP 55,940	1.37	1.37	1
Δ-8 THC	47.6	39.3	0.83
Δ-9 THC	53.3	75.3	1.41
HU-210	0.1	0.17	1.7
HU-308	10000	22.7	0.0022
WIN55,212-2	4.4	1.2	0.27
Endocannabinoids			
Anandamide	71.7	279	3.89
2-AG	472	1400	2.97

Table 1. Summary table of cannabinoid agonists and antagonists used commonly in research. Binding properties for CB1Rs and CB2Rs and corresponding affinity ratios are given.

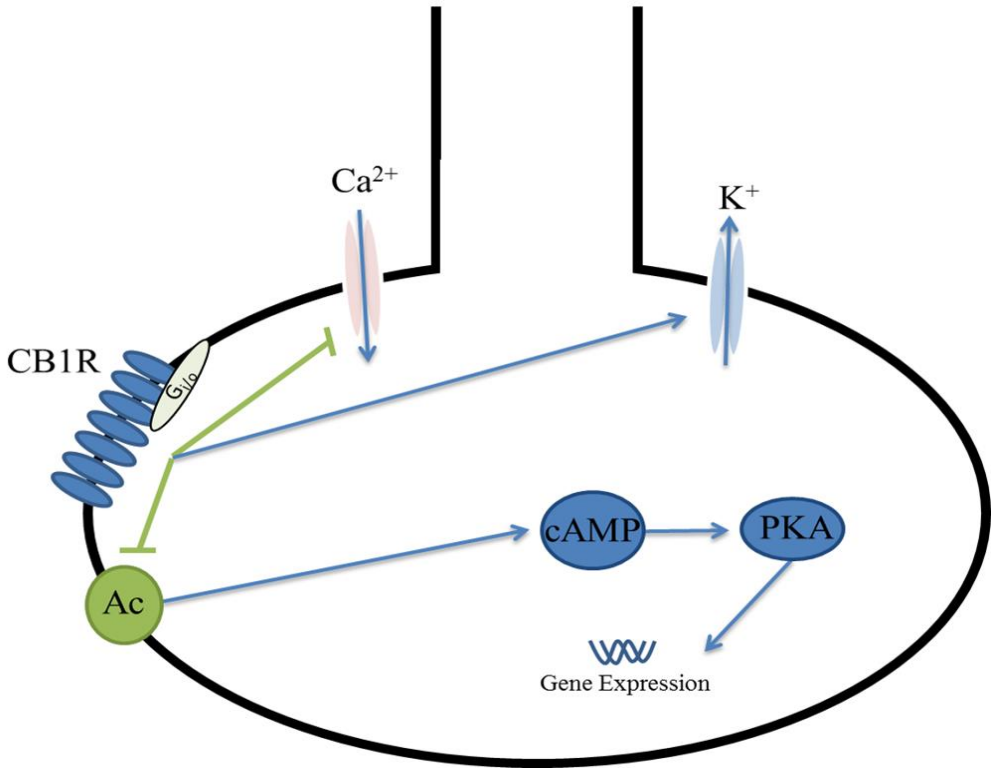

Figure 1. Result of Cannabinoid-1 receptors (CB1R) activation. Inward rectifying Calcium receptors are inhibited whereas outward rectifying Potassium receptors are activated. Additionally, adenylyl cyclase (Ac) activity is inhibited, resulting in a decrease in cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and gene expression.

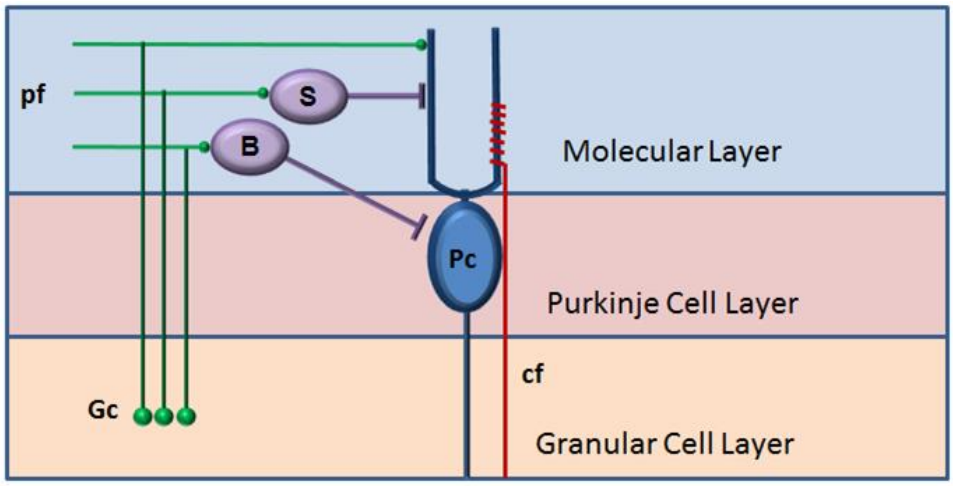

Figure 2. Organization of the cerebellar cortex. Purkinje cells (Pc) receive projections from climbing fibers (cf) and granule cells (Gc). Granule cells project to Purkinje cells, basket cells (B), and stellate cells (S) via parallel fibers (pf). Purkinje cells project to the deep nuclei of the cerebellum.

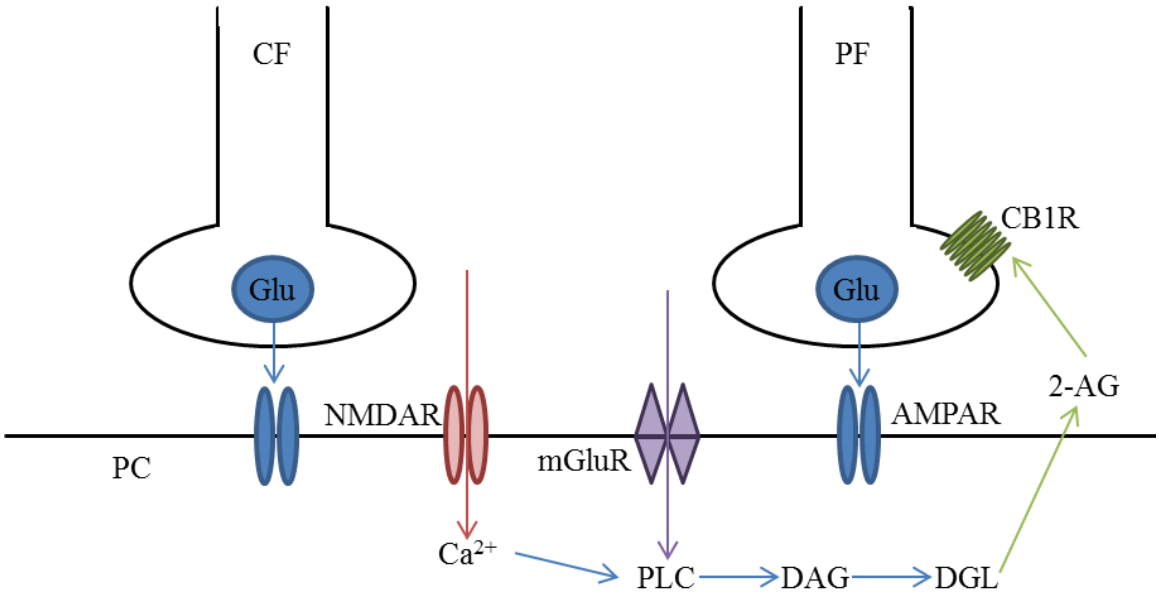

Figure 3. Calcium influx into Purkinje cells (PC) via activation of climbing fibers (CF) initiates endocannabinoid production. Calcium or metabotropic glutamate receptor (mGluR) activates the cascade of phospholipase C (PLC), diacylglycerol (DAG), and diacylglycerol lipase (DGL). DGL activation results in the production and release of endocannabinoids.

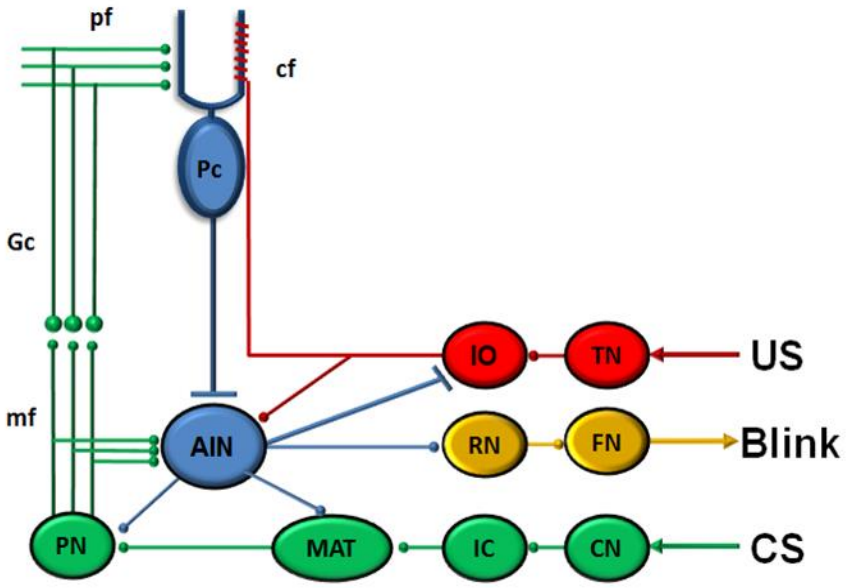

Figure 4. The neurocircuitry involved in delay eyeblink conditioning. Conditioned stimulus (CS) is projected to the medial auditory thalamus (MAT) from the inferior colliculus (IC) and cochlear nucleus (CN). Pontine nucleus sends projections to the cerebellum, Purkinje cells (Pc) and anterior interpositus nucleus (AIN). Unconditioned stimulus (US) information projects to the Pc and AIN via inferior olive. Conditioned response (CR) information is sent from the AIN to the red nucleus (RN) to the facial nucleus (FN). The AIN also projects to the PN, MAT, and IO.

CHAPTER 2. CANNABINOID ADMINISTRATION WITHIN THE CEREBELLAR CORTEX IMPAIRS EYEBLINK CONDITIONING AND PURKINJE CELL PLASTICITY

Cannabinoid-1 receptors (CB1R) are the most abundant G-coupled protein receptors in the mammalian brain (Herkenham et al., 1990; 1991). Their physiology and function have been tied to a variety of processes including learning and memory. The largest density of these receptors lies within the cerebellum, specifically in the cerebellar cortex (Herkenham et al., 1990; 1991; Suarez et al., 2008). This has led researchers to examine CB1R function in a cerebellar-dependent learning paradigm, delay eyeblink conditioning (EBC). In delay EBC, a conditioned stimulus (CS; e.g. tone) is paired with an unconditioned stimulus (US; e.g. perioribital stmulation). Before training the CS does not elicit eyelid closure, whereas the US elicits a reliable eyelid closure known as the unconditioned response (UR). Over repeated CS-US pairings an eyelid closure conditioned response (CR) occurs prior to US presentation. The neural substrates necessary for EBC include the anterior interpositus nucleus and the cerebellar cortex (for review see Freeman & Steinmetz, 2011).

Learning of eyeblink conditioning involves at least two distinct plasticity events to occur: long-term depression (LTD) of parallel fiber to Purkinje cell synapses and long-term potentiation (LTP) of mossy fiber synapses within the anterior interpositus nucleus (Freeman & Steinmetz, 2011). During delay EBC Purkinje cells develop pauses in simple spike firing within the CS period that then return to baseline during extinction training which has been termed as an LTD-like response (Jirenhed et al., 2007). The pauses in simple spike activity (LTD) are dependent upon the timing of the US, indicating that they

play a role in CR timing (Green and Steinmetz, 2005; Jirenhed et al., 2007; 2011). Purkinje cells tonically inhibit the anterior interpositus nucleus. These pauses in simple spike activity then release the anterior interpositus nucleus from inhibition and allow for the induction of LTP (Mauk and Donegan, 1997; Pugh and Raman, 2008). Research examining *in vitro* manipulations of CB1Rs during Purkinje cell LTD procedures demonstrates impaired LTD formation as compared to control conditions. Levenes et al. (1998) found that the CB1R agonists WIN55, 212-2 and CP55,940 impaired the induction of LTD, an effect which was blocked by the antagonist SR141716A. Furthermore, CB1R knockout mice or mice treated with high concentrations of the CB1R antagonist AM251 were unable to form LTD in cultured slices (Safo and Regehr 2005). The authors hypothesized that CB1R activation regulates the release of an anterograde messenger that acts on Purkinje cells to induce the LTD. This anterograde messenger was later speculated to be nitric oxide (Safo et al., 2006).

Manipulations of CB1R function in humans and non-human mammals produce decrements in the rate of learning of cerebellar-dependent delay EBC (Kishimoto and Kano, 2006; Skosnik et al., 2007; Edwards et al., 2008; Steinmetz and Freeman, 2010; 2011). The manipulations in animals include a CB1R gene knockout and subcutaneous administration of the CB1R agonist WIN55,212-2. The hypothesis that has been drawn from these studies is that the decrement in acquisition of delay EBC is the result of impaired plasticity within the cerebellar cortex. The current set of experiments test this hypothesis by examining the effects of CB1R agonist infusion directly into the cerebellar cortex on eyeblink conditioning sessions. Cannabinoid drugs were infused into the base of the primary fissure, which has been shown to be an eyeblink microzone in mice and is

necessary for EBC in rats (Heiney et al., 2014; Steinmetz and Freeman, 2014). We also examined the effects of systemic administration of WIN55,212-2 on Purkinje cell plasticity during EBC.

Methods

Subjects

The subjects were 152 male Long-Evans rats (250-300 g). The rats were housed in the animal colony in Spence Laboratories of Psychology at the University of Iowa (Iowa City, IA). All rats were maintained on a 12 h light/dark cycle and given *ad libitum* access to food and water.

Surgery

One week before training, rats were removed from their home cages and anesthetized with isoflurane. After the onset of anesthesia, the rats were fitted with differential electromyography (EMG) electrodes (stainless steel) implanted into the upper left orbicularis oculi muscle. The reference electrode was a silver wire attached to a stainless steel skull screw. The EMG electrode leads terminated in gold pins in a plastic connector. A bipolar stimulating electrode (Plastics One, Roanoke, VA) for delivering the periorbital stimulation US was implanted subdermally, caudal to the left eye. A cannula or hyperdrive was additionally implanted in the left cerebellar cortex. A 23 gauge guide cannula was implanted at the base of the primary fissure in the cerebellar cortex. A 30 gauge stylet was inserted into the guide cannula and extended 0.5 mm from the end of the guide. A custom-made hyperdrive array fitted with an electronic interface board (Neuralynx) was implanted over the cerebellar cortex in separate groups of rats. The skull surface was marked for the tetrode bundle and drilled out with a square drill bit. Skull

fragments were carefully removed along with the dura matter for the drill hole under visual guidance. The bundle of tetrodes were lowered to the surface of the brain and sealed with low viscosity silicon (Kwik-Sil; World Precision Instruments). A reference tetrode was lowered dorsal to the anterior interpositus nucleus but ventral to the cerebellar cortex. Each independently moveable tetrode was composed of four nichrome wires (12 µm diameter; Kanthal Palm Coast), twisted and partially melted together to form a tetrode. Each tetrode was gold plated to reduce final impedance to 250-400 k measured at 1 kHz (Neuralynx Nano-Z). The stereotaxic coordinate taken from bregma for the cannula and hyperdrive was 11.0 mm posterior, 2.4 mm lateral, and 4.0 mm ventral to the skull surface. The plastic connector housing the EMG electrode leads, bipolar stimulating electrodes, the guide cannula or hyperdrive, and skull screws were secured to the skull with Osteobond copolymer bone cement.

Infusion Procedure

Before each infusion, the stylet was removed from the guide cannula and replaced with a 30 gauge infusion cannula that extended 1.0 mm beyond the guide cannula. The infusion cannula was connected to polyethylene tubing (PE 10), which was connected to a 10 µl gas tight syringe (Hamilton, Reno, NV). The syringe was placed in an infusion pump (Harvard Apparatus, Holliston, MA), and 0.5 µl of each drug or vehicle (pH = 7.4) was infused over 5 minutes at a rate of 6.0 µl/h. After the infusion, the infusion cannula was left for 3 mins in order to allow diffusion of the drug. Following this time, the infusion cannula was removed and replaced with the 30 gauge stylet.

Pharmacological Manipulations of Cannabinoid Receptors

The CBR agonist WIN55,212-2([R]-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone), agonist ACEA (Arachidonyl-2'-chloroethylamide), antagonist SR141716A (5-(4-Chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide), or vehicle was administered intracranially or subcutaneously to rats 30 min before each daily infusion session. WIN55,212-2 binds with high affinity to CB1R and CB2R (Pertwee 1997; Howlett et al. 2002). WIN55,212-2 was administered intracranially at a concentration of 10 µg/µL, 1.0 µg/µL , and 0.1 µg/µL for Experiment 1 and at 10 µg/µL for Experiments 2-4. ACEA is a CB1R preferring agonist and binds at about 1000x higher affinity to CB1R than CB2R. ACEA was administered at 3.2 µg/µL in order to produce similar binding as 10 µg/µL WIN55,212-2 (Hillard et al., 1999). SR141716A, a selective CB1R antagonist, was administered at 1 µg/µL similar to previous reports (Trezza et al., 2012). Subcutaneous injections of WIN55,212-2 were administered at 3 mg/kg based on previous studies from our laboratory that produced marked deficits in learning (Steinmetz and Freeman, 2010). WIN55,212-2 and ACEA was dissolved in a vehicle of 1:1:18 solution of ethanol, cremaphor, and saline. SR141716A was dissolved in a vehicle of 1:1:18 solution of ethanol, tween 80, and saline. WIN55,212-2 and ACEA was purchased from Sigma/RBI. SR141716A was a generous gift from NIDA Drug Supply Program (Rockville, MD).

Recording and Purkinje Cell Isolation.

The tetrode bundle was placed over the cerebellar cortex and tetrodes were advanced to within 1.0 mm of the target during surgery using stereotaxic guidance.

Tetrodes were then lowered slowly to the target over several days while changes in neuronal activity were observed. The head stage (Neuralynx) was connected to a motorized commutator to allow the rats to move during training sessions. After stable units were obtained, eyeblink training was commenced along with neural recording. Neuronal signals were first preamplified at unity-gain. The signals were then amplified between 2000 and 20,000 times and bandpass filtered (0.3–6.0 kHz; Neuralynx). Neural signals that exceed a channel amplitude threshold were digitized and stored at 32 kHz (Digital Lynx; Neuralynx). Waveform characteristics were plotted as a scatterplot of one of the electrodes versus another in the energy, peak, and valley planes. Individual units formed clusters of points on these different presentations, and boundaries of these plots were defined under visual inspection with an interactive spike sorting software (MClust, A.D. Redish). An automatic cluster cutting program was used (Klusta-Kwik, K. Harris) to identify possible clusters followed by additional manual identification of single units. Criteria for Purkinje cell identification was based on previous reports examining Purkinje cell activity *in vivo* with tetrodes (de Solages et al., 2008), which included the presence of simple and complex spikes, and spontaneous firing rates between 20-150 Hz. Additionally, we employed two measures that indicate the amount of separation the clusters had from one another and from background activity on the same tetrode: L-ratio and isolation distance (Schmitzer-Torbert, et al., 2005). An isolation distance greater than 7 and an L-ratio of less than 1.5 were used as filters.

Single-unit Data Analysis.

Peristimulus histograms of unit activity were created for both CS, US, and post-US periods. CS periods were divided into four equal blocks and each block were divided

into bins. Analysis of the unit activity for each block during the CS (400 ms) and US (100 ms) for all trials was compared with an equal pre-CS period (400 ms) using z-scores (Ng and Freeman, 2012). Different response types were classified based on the results of the z-score analysis. The Wilcoxon signed rank matched pairs test was used to compare the activity of each neuron for each session during the CS and US periods for CR and non-CR trials to identify differences in neural responding that are correlated with learning. Population responses were analyzed with z-scores using repeated measures analysis of variance (ANOVA).

Apparatus

The conditioning apparatus consisted of four small-animal sound-attenuating chambers (BRS/LVE, Laurel, MD). Within each sound-attenuating chamber was a small-animal operant chamber (BRS/LVE) in which the rats were kept during conditioning. One wall of the operant chamber was fitted with two speakers that independently produce tones of up to 120 dB (sound pressure level) with a frequency range of 1000-9000 Hz. An exhaust fan on one of the walls provided a 65 dB masking noise. The tone CS used in training was a 2000 Hz pure tone (85 dB). The electrode leads from the rat's headstage were connected to peripheral equipment by lightweight cables that allowed the rat to move freely during conditioning. For the tetrode recording study, the electrode leads were fed through a motorized commutator that connected to peripheral equipment. A desktop computer was connected to the peripheral equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs, Raleigh, NC). One circuit was used to deliver the shock stimulus through a stimulus isolator (model 365A; World Precision Instruments, Sarasota, FL). Another circuit amplified

differentially (gain, 2000; sampling rate, 250 Hz), filtered (500-5000 Hz), and integrated (time constant, 20 ms) EMG activity. The intensity of the shock US was set for eliciting a discrete eyeblink (typical range of final current intensity, 2.5-3.5 mA; 60 Hz; constant current).

Conditioning Procedure

The rats were allowed to adapt to the training environment for 5 min before each training session. For the cannula studies rats underwent three different types of sessions. A pre-training session measured spontaneous eyelid activity in which EMG recordings were collected 100 times for 400 ms each trial, approximating a conditioning session. A second pre-training session consisted of 100 CS (400 ms; 2 kHz; 85 dB) and 90 US (25 ms shock) unpaired presentations. Sessions 3-12 consisted of paired training sessions in which 10 blocks of nine paired CS–US presentations and 1 CS-alone probe trial were presented. CS-alone probe trials were used to accurately measure the timing and amplitude of the CR without interferences from the US. For the tetrode recording experiment, rats underwent 10 trials to record spontaneous activity followed by 10 unpaired presentations of the CS and US during pre-training. Following pre-training, rats were given 5 sessions of paired training in which subcutaneous injections of WIN55,212-2 were made 30 min prior to each session. Following session 5, rats were given additional training until they reached criterion (at or above 80% CRs). Rats then underwent 2 retention tests in which 50 trials were given followed by administration of vehicle or WIN55,212-2, a 30 min break, and then the remaining 50 trials. The order of vehicle and WIN55,212-2 administration was counterbalanced. For both cannula and recording experiments the CS was a 400-ms tone (2 kHz; 85 dB) which terminated with a

25-ms US. The US intensity was adjusted in each rat to elicit eyelid closure and slight head movement (range =2.5–3.5 mA). CRs were defined as EMG activity that exceeded a threshold of 0.4 units (amplified and integrated units) above the baseline mean during the CS period. EMG responses that exceeded the threshold during the first 80 msec of the CS period were defined as startle responses to the CS. On CS-alone probe trials, the duration for scoring CRs will be extended beyond the CS to the end of the trial period (1.0 sec). URs were defined as responses that crossed the threshold after the onset of the US.

Histology

After completion of the recording experiment, a small lesion was made to mark the tip of each tetrode with 4 s of 10 µA current through one of the tetrode wires. The rats were euthanized with a lethal injection of sodium pentobarbital (150 mg/kg) and transcardially perfused with physiological saline followed by 10% neutral buffered formalin. After perfusion, the brains were cryo-protected in a 30% sucrose in formalin solution, and subsequently sectioned at 50 µm with a sliding microtome. Sections were then stained with thionin. The location of the cannula and tetrode placements were then verified using a light microscope (Leica DMLS, Wetzlar, Germany) and a stereotaxic brain atlas (Paxinos and Watson, 2007).

Results

Cannula placements were verified with light microscopy. Cannulae were targeted at the base of the primary fissure (eyeblink conditioning microzone) for Experiments 1-4, anterior interpositus nucleus for Experiment 4, and medial cerebellar cortex for Experiment 4 (Figure 5, 6). Ten animals were removed from analysis for cannula placements that were anterior to the targeted region.

Experiment 1: Dose-Response Within the Cerebellar Cortex

Previous work from our laboratory demonstrated that systemic administration of WIN55,212-2 at different concentrations resulted in a dose-dependent deficit in acquisition of EBC (Steinmetz and Freeman, 2010). If this effect is localized to the cerebellar cortex there should be a dose-dependent deficit in EBC with cerebellar cortical infusions of WIN55,212-2. To test this WIN55,212-2 (0.1 µg/µl, 1.0 µg/µl, or 10.0 µg/µl) or vehicle was infused into the eyeblink conditioning microzone of the cerebellar cortex prior to the first 7 sessions. During the first pre-training session, rats were placed into the conditioning chambers while spontaneous EMG activity was collected (Figure 7, SP). The next pre-training session consisted of unpaired presentations of the CS and US to assess non-associative blinking to the tone CS (Figure 7, UP). Separate one-way ANOVAs indicated no significant differences between the groups for either the SP or UP sessions. Taken together, these results indicate that differing doses of WIN55,212-2 did not change the rate of spontaneous or non-associative responding to the CS.

Over the next 10 sessions, rats were given 90 CS-US pairings and 10 CS-alone presentations daily to examine acquisition of the eyeblink CR. Rats were given infusions of WIN55,212-2 (0.1 µg/µl, 1.0 µg/µl, or 10.0 µg/µl) or vehicle prior to the first 5 of these sessions. During the last 5 sessions of training animals did not receive infusions in order to examine learning following cessation of infusions (Figure 7). Animals given infusions of vehicle showed acquisition of the CR with asymptotic learning of approximately 90% on session 5. Rats given 0.1 µg/µl of WIN55,212-2 showed a similar acquisition curve as the vehicle treated rats with asymptotic learning slightly lower,

around 80%. In contrast, the 1.0 µg/µl and 10.0 µg/µl groups showed more marked impairments with the maximum levels of learning at around 50% and 20%, respectively. A 4 (Group) x 2 (Half; Infusion vs No Infusion) x 5 (Session) repeated measures ANOVA yielded a significant three-way interaction, $F(12, 112) = 8.285$, $P < 0.001$. Post-hoc tests (Tukey's Honestly Significant Difference [HSD]) indicated that rats given 0.1 µg/µl of WIN55,212-2 exhibited significantly fewer CRs on session 3 than the vehicle trained animals ($p < 0.01$). Rats given 1.0 µg/µl of WIN55,212-2 were significantly impaired on sessions 2-8 as compared to controls rats ($p<0.01$). Rats infused with 0.1 µg/µl of WIN55,212-2 were impaired during sessions 2-9 compared to the vehicle treated animals ($p<0.01$) and sessions 2 through 7 as compared to the 0.1 µg/µl group. Rats receiving 10.0 µg/µl of WIN55,212-2 exhibited significantly fewer CRs during sessions 2-9 as compared to the vehicle and 0.1 µg/µl groups and during sessions 3-8 as compared to the 1.0 µg/µl group. These results indicate that intracerebellar cortical WIN55,212-2 produced a dose-dependent impairment in associative learning similar to previous experiments with subcutaneous injections.

The CR amplitude from all trials was also compared between groups using a 5 (Session) x 2 (Half) x 4 (drug concentration) repeated measures ANOVA. No significant two- or three-way interactions were found but there were significant main effects of Session [$F(4, 112) = 2.355$, $P = 0.048$], Half [$F(1, 112) = 27.793$, $P < 0.001$], and Group [$F(3, 28) = 3.290$, $P = 0.035$]. Post-hoc tests found that the group infused with 10 µg/µl had significantly lower amplitude CRs than the vehicle group. The other WIN55,212-2 groups did not significantly differ from the vehicle group. The main effects of session and half indicate that CR amplitude increased with training over sessions and was higher

during the no infusion sessions when learning had increased. Two components of CR timing, onset and peak latencies, from all trials were compared across the doses. A 4 (Group) x 2 (Half) x (Session) repeated measures ANOVA for CR onset latency revealed no significant interactions or main effects. CR peak latency was also compared for the groups, no interactions were revealed but there was a main effect of Session [$F(4, 112) = 2.736$, $P = 0.003$] and Half [$F(1, 112) = 52.455$, $P < 0.001$]. Potential drug effects on the US and UR were examined during the unpaired session and all paired sessions to determine whether WIN55,212-2 altered sensitivity to the US or performance of the UR. Separate ANOVAs revealed no significant group differences in the US intensity or UR amplitude during unpaired or paired training. Thus, the intensity of the US delivered and the amplitude of the eyeblink reflex did not differ due to drug administration, suggesting that the drug did not disrupt the response or sensitivity to the shock US.

Experiment 2: Comparison of CB1R Activation across the Cerebellar Cortex

In Experiment 1, infusions of WIN55,212-2 were made into the eyeblink conditioning microzone, based upon previous work from our laboratory which showed that this area of the cerebellar cortex is essential for acquisition of EBC (Steinmetz and Freeman, unpublished). However, CB1Rs are located densely throughout the cerebellar cortex and sparsely in the deep nucleus (Suarez et al., 2008). We tested whether or not the effects of WIN55,212-2 on EBC are localized to the EBC microzone. Animals were implanted with a cannula in the EBC microzone, central vermis, or anterior interpositus nucleus ipsilateral to the conditioned eye and then infused with WIN55,212-2 prior to the

first 7 sessions. The infusion schedule and behavioral procedures were the same as Experiment 1

As in Experiment 1, there were no effects of WIN55,212-2 on spontaneous or non-associative responding to the CS (Figure 8, SP/UP). Infusion of WIN55,212-2 into the EBC microzone impaired EBC (~30% on session 5) to the same degree as in Experiment 1 (Figure 8). Infusions into either the anterior interpositus nucleus or central vermis resulted in no significant differences from the vehicle group. A 4 (Group) x 2 (Half) x 5 (Session) repeated measures ANOVA revealed a significant three-way interaction, $F(12,81)=10.786$, $p<.001$. Post-hoc tests confirmed that WIN55,212-2 into the EBC microzone impaired CR percentage relative to the vehicle group on sessions 2-8; whereas administration into either of the other areas did not result in differences from the vehicle group. These results shows that the effects of WIN55,212-2 during EBC are localized to the EBC microzone within the cerebellar cortex.

Measures of CR performance (CR amplitude and latency) were examined next in order to further determine whether or not the effects of WIN455/212-2 are localized anatomically. A repeated measures ANOVA for the CR amplitude data revealed main effects of Session [$F(4,25)=12.608$,$P<.001$], Half [$F(1,28)=26.572$,$P<.001$], and Group [$F(3,28)=4.488$, $P=.011$]. Post-hoc test showed that WIN55,212-2 infused into the EBC microzone decreased CR amplitude, but had no effects when infused into the anterior interpositus nucleus or central vermis. ANOVAs for both CR onset and peak latency revealed main effects of Session [$F(4,25)=3.890$, $P=.014$] and Half [$F(1,28)=17.228$, $p<.001$], respectively. No group-related impairments were found for the CR onset latency, CR peak latency, UR amplitude, or US sensitivity data.

Experiment 3: SR141716A within the Cerebellar Cortex and Systemic WIN,212-2

As mentioned previously, CB1Rs are located throughout the mammalian brain, with the highest density of the receptors within the cerebellum (Herkenham et al., 1990; 1991). However, there are receptors in other areas that have been shown to modulate eyeblink conditioning such as sensory cortex, amygdala, and hippocampus (Lee and Kim, 2004; Steinmetz, Harmon, and Freeman 2013). We have conducted experiment thus far examining cannabinoid receptors in the cerebellar cortex; however, it is possible that CB1R activation in other areas is additionally providing a modulatory effect. In the current experiment, we infused the CB1R antagonist SR141716A into the cerebellar cortex prior to systemic injections of the CB1R agonist WIN55,212-2. Thus, SR141716A will only block CB1Rs within the cerebellar cortex and WIN55,212-2 will bind to receptors throughout the rest of the brain. If an impairment is observed, it would suggest that areas outside the cerebellar cortex are also involved. Animals received infusions of either SR141716A or vehicle 30 mins before subcutaneous injection of WIN55,212-2 or vehicle. The behavioral procedures were the same as Experiments 1 and 2.

As in Experiments 1 and 2, no significant drug effects were found during the pre-training sessions. Intracerebellar infusions of SR141716A or vehicle were made prior to subcutaneous injections of WIN55,212-2 or vehicle for the first 5 sessions of CS-US paired trials (Figure 9). Peripheral administration of WIN55,212-2 impaired CR percentage (~ 30% at session 5), similar to previous findings involving subcutaneous injections. When SR141716A was infused into the cerebellar cortex prior to the administration of WIN55,212-2 CRs returned to control-like levels. A repeated measures

ANOVA conducted for 4 (Group) x 2 (Half) x 5 (Session) revealed a significant three-way interaction, F(12,81) =2.455, p= .009. Post-hoc tests indicated that the WIN55,212-2 group was impaired on sessions 2-8 as compared to both the control group and the group that received SR141716A prior to WIN55,212-2. No other significant differences were found for the CR percentage data. Thus, blocking CB1R agonist activity only within the cerebellar cortex is enough to rescue the effects of peripheral CB1R activation.

Drug effects on additional measures of the CR (amplitude and latency) were also examined. A repeated measures ANOVA conducted for the CR amplitude data found significant main effects of Half, [F(1,28)=12.579, P=.001], Session [F(4,25)=6.712, p=.001], and Group [F(3,28)=3.092, p=.043] but no significant two- or three-way interactions. Post-hoc tests found a significant decrease in CR amplitude for the WIN55,212-2 group as compared to the other groups. This decrease in CR amplitude was reversed by SR141716A administration. There were no significant effects for either CR onset or peak latency. UR amplitude and US sensitivity were examined for the groups and no significant effects were found.

Experiment 4: Receptor Specificity

WIN55,212-2 binds with a similar efficacy to both CB1Rs and CB2Rs. CB1Rs are densely located in the cerebellar cortex; however, a moderate level of CB2Rs are also located proximal to Purkinje cells (Herkenham et al, 1990; 1991). Data from our laboratory found that when the selective CB1R antagonist SR141716A was administered subcutaneously prior to WIN55,212-2, learning was similar to controls. Additionally, WIN55,212-2 has been the only CB1R agonist tested with cerebellar-dependent learning. We used the CB1R preferring agonist ACEA to test if similar results would occur with

another agonist the is more selective for CB1Rs than WIN55,212-2. Rats received two infusions prior to each of the first 7 sessions. An hour before training rats received either vehicle or the CB1R antagonist SR141716A (1.0 µg/µl). Thirty minutes later a second infusion occurred of either WIN55,212-2 (10.0 µg/µl), ACEA (3.2 µg/µl), or vehicle. The behavioral procedures were the same as in Experiment 1.

Separate one-way ANOVAs revealed no significant effects between the groups during either pre-training session. These results indicate that multiple infusions of CB1R antagonists or agonists do not change the rate of spontaneous or non-associative responding to the CS.

Infusions of either WIN55,212-2 or ACEA resulted in severe impairments in learning, with CR percentage at the end of training at approximately 30% and 20%, respectively. When infusions of SR141716A were made prior to either WIN55,212-2 (Figure 10A) or ACEA (Figure 10B) the impairment was blocked, resulting in learning that resembled vehicle treated animals. A 4 (Group) 2 x (Half) x 5 (Session) repeated measures ANOVA yielded a significant three-way interaction, $F(20, 168) = 12.784$, $P < 0.001$. Post-hoc tests revealed that the WIN55,212-2 and ACEA treated groups were impaired compared to vehicle treated animals during Sessions 2-9. Administration of SR141716A alone did not significantly change CR percentage. Additionally, the performance of rats administered SR141716A prior to WIN55,212-2 or ACEA did not differ from the vehicle group, but did from both the WIN55,212-2 and ACEA groups (Sessions 1-9). These results indicate that EBC impairments are not specific to WIN55,212-2. Also, a CB1R selective antagonist blocked the effects of the CB1R

agonists, indicating that the impairments in learning were caused by drug action on CB1Rs, not on CB2Rs.

WIN55,212-2 and ACEA administration into the cerebellar cortex decreased CR amplitude during acquisition. When SR141716A was administered prior to either WIN55,212-2 or ACEA thee the CR amplitude deficit was blocked. A repeated measures ANOVA found a significant Group x Half x Session interaction, $F(20, 168)=1.699$, $p=.038$. Post-hoc tests confirmed that the WIN55,212-2 and ACEA groups exhibited lower CR amplitudes on sessions 4-6. SR141716A administration completely rescued this impairment. For CR onset latency, significant main effects of Half $F(1,42)=7.524, P=.009$, and Session, $F(4,39)=2.951, P=.032$, were found, which indicate that CR onsets became earlier over the sessions and were earlier for the no infusion sessions than the infusion sessions. These results are consistent with previous reports of eyeblink conditioning. No significant differences were revealed with peak latency, US sensitivity, or UR amplitude.

Experiment 5: Tetrode Recordings of Purkinje Cells Following WIN55,212-2 Administration

Eyeblink Conditioning

Animals received subcutaneous injections of WIN55,212-2 or vehicle prior to the first 5 daily sessions of paired CS-US training. Systemic WIN55,212-2 produced marked deficits in learning with levels of CRs at approximately 30% on session 5 (Figure 11). A 2 (Group) x 5 (Session) repeated measures ANOVA indicated a significant two-way interaction $F(4,28) = 9.128$, $p<.001$. Post-hoc tests showed that the WIN55,212-2 group exhibited significantly fewer CRs on sessions 2-5 relative to the vehicle group. Animals

were then trained to reach a criterion of 80% CRs before retention tests. All of the vehicle treated animals reached the criterion by session 5 and were immediately moved to retention training. The WIN55,212-2 treated animals required an average of 4 (± 0.71) additional sessions without drug administration to reach criterion. For retention tests, animals were given 50 trials with no injections and then 50 trials with either vehicle or WIN55,212-2 injection (order of first treatment counterbalanced) ANOVAs examining the effects of WIN55,212-2 and vehicle administration during retention found no significant effects (Figure 11).

Tetrode Placements

Only tetrodes located in the base of the primary fissure (EBC microzone) were analyzed. Figure 12 shows an example of tetrode placements located in the EBC microzone. Purkinje cells were identified by the presence of distinguishable simple and complex spike activity and spontaneous firing rates between 20-150 Hz. These criteria are similar to past reports of Purkinje cells recordings using tetrodes (de Solages et al., 2008). These criteria yielded 280 Purkinje cells. To ensure quality of the single neuron isolation we applied isolation distance and L-ratio criteria. These measures are indicative of how well separated spikes of one cluster are from other clusters on the same tetrode (Schmitzer-Torbert, et al., 2005). An isolation distance greater than 7 and an L-ratio of less than 1.5 were used as filters. These criteria eliminated 124 cells, leaving 156 cells for analysis. There was no significant difference between L-ratios or isolation distances between the WIN55,212-2 and vehicle groups.

Single Purkinje Cell Activity

Purkinje cells were classified based on their simple spike activity during CS presentations as compared to the pre-CS period. The three classifications of Purkinje cells were increased, decreased, or no difference in simple spike activity. Table 2 shows Purkinje cell numbers and percentages for data collected in each group across sessions. Examples of Purkinje cells with decreased and increased simple spike activity during the CS are shown in Figures 15 and 16. All comparisons of unit response types within and between groups were made with the $\chi 2$ test at the $p < 0.05$ significance level. The percentage of decreasing Purkinje cells increased across sessions from 11% on Session 1 to 53% on Session 5 in the vehicle group (Figure 13). The percentage of decreasing Purkinje cells also increase in the WIN55,212-2 group from 0% on Session 1 to 30% on Session 5. However, there was a significant difference between the vehicle and WIN55,212-2 groups during Session 3 (26% vs. 5%) and Session 5 (53% vs. 30%). There were no significant differences within or between groups for the Purkinje cells that increased their activity during the CS (Figure 13). These data suggest that the induction of Purkinje cell LTD is impaired when WIN55,212-2 is administered.

The percentage of Purkinje cells that showed decreases or increases in activity during the CS did not differ between retention tests (vehicle or WIN55,21-2) in this within subject design and did not differ from session 5 of the vehicle treated animals (Figure 14). Decreasing Purkinje cells were the majority of the recordings obtained during Session 5 (53%), the WIN55,212-2 retention session (60%), and the vehicle retention session (50%). Increasing Purkinje cells made up a smaller percentage during Session 5 (30%), the WIN55,212-2 retention session (33%), and the vehicle retention

session (38%). The neuronal results during retention tests are therefore consistent with the behavior results; that is, neither the percentage of CRs or Purkinje cell activity differed on vehicle and WIN55,212-2 retention sessions. These findings suggest that WIN55,212-2 impairs the induction of Purkinje cell plasticity but has no effects on its maintenance.

Purkinje Cell Population Activity

The average activity of the Purkinje cell types across the sessions and groups was examined to determine if WIN55,212- altered the overall pattern and magnitude of activity following CS onset. Differences in the magnitude of the neuronal responses to the CS were analyzed across CS intervals with repeated measures ANOVAs of the normalized activity (Figure 17). There were no decreasing cells found for Session 1 for the WIN55,212-2 group so the analysis was not conducted during this session. There were no significant differences between the groups during Session 3 for the decreasing cells. However, on session 5 there was a significant Group x Bin interaction, $F(39, 741)= 1.725$, $p = .004$. Post-hoc tests revealed that the Purkinje cells in the WIN55,212-2 group had significantly decreases earlier during the CS (Bins 5-11; 50-110 ms following CS onset) than the Purkinje cells from the vehicle group (Bins 32-40; 320-420 ms following CS onset). For increasing Purkinje cells, there were no significant interactions for any of the sessions, but there was a significant main effect of Bin for Session 3 [$F(39, 546) = 3.100$, $p <.001$] and Session 5 [$F(39, 507) = 6.708$, $p <.001$], which was due to an increase in activity during the CS (Figure 18). There were no significant interactions or main effects for the Purkinje cells that did not change from baseline. These results indicate that WIN55,212-2 administration altered the activity of decreasing cells as

compared to Purkinje cells recorded during vehicle treatment: The timing of the decrease was earlier in the CS and the amplitude was lower. WIN55,212-2 did not alter the timing or amplitude of Purkinje cells with increased activity during the CS. These results indicate a selective change in the magnitude of LTD induction and timing when WIN55,212-2 is administered.

During retention, animals were given 50 trials of training prior to being administered WIN55,212-2 or vehicle. Decreasing Purkinje cells did not significantly alter their activity following either WIN55,212-2 or vehicle administration (Figure 19). There was a significant main effect of Bin for both the WIN55,212-2 [$F(39, 468) = 13.639$, $p < .001$] and vehicle [$F(39, 546) = 15.108$, $p < .001$] retention sessions. Additionally, there was a significant main effect of Bin during the WIN55,212-2 [$F(39, 468) = 11.601$, $p < .001$] and vehicle administration [$F(39, 546) = 10.795$, $p < .001$] retention sessions for the increasing Purkinje cells (Figure 20). There were no significant effects of WIN55,212-2 administration on the magnitude or temporal pattern of the increasing Purkinje cell activity during the retention tests. Similar to the single Purkinje cells results, administration of WIN55,212-2 did not alter Purkinje cell activity in either increasing or decreasing cells during retention tests.

CR-Related Purkinje Cell Activity

Purkinje cell activity was compared during trials in which a CR occurred and trials in which a CR did not occur. Wilcoxon signed rank tests were conducted to compare activity during CR and no-CR trials for each Purkinje cell. Examples of simple spike activity from Purkinje cells for CR and non-CR trials are displayed in Figure 21. There was a significant difference in the percentage of Purkinje cells showing greater

activity on CR trials relative to no-CR trials between the WIN55,212-2 group (15%) and the vehicle group (88%) on Session 5 ($p < 0.05$). The population of Purkinje cells was then subdivided into increasing, decreasing, and no change cells based upon the z-score analysis. Among decreasing Purkinje cells, 93% of the vehicle and 40% of the WIN55,212-2 cells differed in activity between CR and no-CR trials. Among increasing Purkinje cells, 100% of the vehicle and 0% of the WIN55,212-2 cells differed in activity between CR and no-CR trials. Thus, Purkinje cells recorded from vehicle treated animals exhibited significant differences in simple spike activity between CR and non-CR trials. In contrast, Purkinje cells recorded from WIN55,212-2 treated animals exhibited relatively few cells that had significant changes in simple spike activity between CR and non-CR trials.

The population activity during CR vs no-CR trials differed significantly in the vehicle and WIN55,212-2 groups.. CR vs Non-CR trials were compared statistically between the WIN55,212-2 and vehicle groups by a 2 (Group) x 2 (Trial Type; CR or non-CR) x 40 (Bin) ANOVA (Figure 22). A significant three-way interaction was found, $F(39,702)=2.370$, $p = .015$. Post-hoc tests found significant differences in vehicle treated animals between the trials on Bins 12-40 ($p <0.05$). For WIN55,212-2 treated animals, CR activity was decreased to a larger extent on Bins 10-11, 17, 20, 23, 26-27, 35. An ANOVA for the increasing Purkinje cells also found a significant Trial x Bins x Group interaction, $F(39,390)=3.785$, $p = .020$. Post-hoc tests revealed that increasing cells had significantly greater activity on CR trials than non-CR trials during Bins 14-40 (Figure 23). There were no significant differences in WIN55,212-2 treated cells between CR and non-CR trials. Thus, significant differences were found between CR and non-CR trials

for both increasing and decreasing Purkinje cells in vehicle treated animals. Purkinje cells recorded from WIN55,212-2 treated animals showed smaller differences between CR and non-CR trials for decreasing cells than the Purkinje cells from the vehicle treated animals, and no differences were found with increasing Purkinje cells.

Discussion

These experiments are the first to demonstrate that CB1Rs within the cerebellar cortex are important for the acquisition of eyeblink conditioning. We report here that similar to results from subcutaneous injections, infusions of WIN55,212-2 directly into the cerebellar cortex resulted in a dose-dependent decrement in acquisition. Administration of a CB1R antagonist within the cerebellar cortex blocked the effects of local and systemically administration CB1R agonists on eyeblink conditioning. The effects of CB1R agonists within the cerebellar cortex were specific to the eyeblink conditioning microzone and were not seen with infusions into the central vermis or the anterior interpositus nucleus. Plasticity formation, specifically the learning-related pauses in simple spike activity, was impaired in Purkinje cells within the eyeblink conditioning microzone. However, once plasticity formed, CB1R manipulation did not impair retention of eyeblink conditioning. These experiments are the first examination of the role of CB1Rs and Purkinje cells during learning *in vivo*.

Infusions of differing concentrations of cannabinoid agonists resulted in dose-dependent impairments in eyeblink conditioning, a result that was found with subcutaneous injections (Steinmetz and Freeman, 2010). Additionally, intracerebellar infusion of a CB1R antagonist administered prior to the CB1R agonists blocked the impairment. This blockage of the agonists-induced impairment in eyeblink conditioning

was found with intracerebellar or systemic administration of the agonist. Only administration into the EBC microzone within the cerebellum resulted in impaired learning. Taken together, these results indicate that CB1Rs within the EBC microzone of the cerebellar cortex are impaired from systemic administration. Localization of the site of action of CB1R agonists is an important first step toward elucidating the cellular mechanisms underlying the effects on plasticity.

As mentioned previously, the cerebellar cortex contains a high density of CB1Rs and a moderate level of CB2Rs (Herkenham 1990; 1991; Suarez et al., 2008). CB1Rs have been found to be critical in the formation of LTD in Purkinje cells, tested by bath application of a selective CB1R antagonist and in CB1R knockout mice *in vitro* (Safo and Regehr, 2005). Both of these manipulations leave CB2Rs intact thus indicating that CB2Rs are not important for LTD induction. Behavioral and neurophysiological studies have focused primarily on CB1R function in the cerebellum, whereas the role of CB2Rs within the cerebellum has remained elusive. We report here that infusions of a selective CB1R antagonist can block impairments caused by both a non-selective and a selective CB1R agonist. Interestingly, infusion of the CB1R antagonist into the EBC microzone with systemic injections of the non-selective CB1R/CB2R agonist also blocks the impairment from the agonist. Thus, not only do CB2Rs within the cerebellar cortex appear to not be important for normal cerebellar-dependent learning, but so do the CB2Rs throughout the brain. These results emphasize that CB1Rs are the driving force of the impairments produced through CB1R agonist administration and that CB2Rs are playing little to no role in EBC.

Learning of eyeblink conditioning involves the development of long-term depression (LTD) of parallel fiber to Purkinje cell synapses and long-term potentiation (LTP) of mossy fiber synapses within the anterior interpositus nucleus (Mauk & Donegan, 1997). The first step in learning, LTD of parallel fiber to Purkinje cell synapses, is Purkinje cells developing pauses in simple spike firing. The pauses occur within the CS period and then return to baseline during extinction training (Jirenhed et al., 2007). These pauses then allow the interpositus nucleus, which is tonically inhibited by Purkinje cells, to be released from inhibition and develop LTP at mossy fiber synapses (Pugh & Raman, 2008). Manipulations of cannabinoid receptors during *in vitro* recordings in Purkinje cells produce impaired LTD formation (Levenas et al., 1998; Safo and Regehr, 2005). Purkinje cells with learning-related pauses in simple spike activity were substantially less frequent in rats given WIN55,212 than in rats given the vehicle. Moreover, among the relatively few Purkinje cells that showed simple spike decreases after WIN55,212-2, the magnitude and timing of the simple spike pause was altered. In rats given WIN55,212-2, there were an increased number of Purkinje cells that did not change their firing patterns, but a similar proportion of Purkinje cells that exhibited a learning-related increased in simple spike activity. The effect of WIN55,212-2 on Purkinje cell activity *in vivo* is consistent with the partial impairment of LTD *in vitro* (Levenes et. al., 1998). Thus, the impairment in eyeblink conditioning may be caused by an impairment in induction of LTD in Purkinje cells. Additionally, there was no deficit in the percentage of Purkinje cells showing learning-related increases in simple spike activity or in the magnitude of these increases in activity following WIN55,212-2 administration, suggesting that the eyeblink conditioning deficit was specifically caused

by the impairment in Purkinje cell simple spike pauses and not by a general impairment in plasticity.

How CB1R activation disrupts LTD formation remains controversial and not well known. Administration of WIN55,212-2 *in vitro* results in decreased synaptic transmission at parallel fiber-to-Purkinje cell synapses by reducing glutamate release probability (Levenes et al., 1998). This result is most likely due to inhibition of presynaptic calcium channels in parallel fibers. Levens et al. (1998) proposed that the decreased release of glutamate would in turn lead to a lower activation of AMPA and metabotropic postsynaptic receptors. Another possible presynaptic mechanism would be through nitric oxide (NO) signaling to Purkinje cells. NO is produced by depolarization of parallel fibers during LTD induction (Crepel et al., 1994). Activation of CB1Rs would decrease NO production via Ca2+ entry into parallel fibers. This would lead to reduced LTD amplitudes, which may have caused the reduction in pauses of simple spike activity in Purkinje cells in the current study.

After eyeblink conditioning was well established administration of cannabinoid agonists within the cerebellum had no effect on behavioral memory or Purkinje cell activity during retention tests. Previous examinations have focused on CB1Rs in formation but not maintenance of the plasticity (Levenes, et al., 1998; Safo and Regehr, 2005). The behavioral and neurophysiological findings from the current study suggest that CB1R agonist administration does not affect already formed LTD or LTP *in vivo*. Taken together with the results discussed above, the induction of LTD formation in Purkinje cells needs normal CB1R function but maintenance does not. CB1R activation closes Ca2+ channels and reduces glutamate release from parallel fibers, which is

important in LTD induction (Safo and Regehr, 2005), but LTD can presumably be maintained or even strengthened by reduced glutamate release after induction. The absence of a post-induction effect of CB1R agonists on LTD might explain the absence of an effect of agonists on CRs and Purkinje cell pauses in simple spikes during retention tests.

Most of the previous experiments examining single Purkinje cell activity during eyeblink conditioning have focused on the activity following asymptotic learning. However, Jirenhed et al. (2007) examined Purkinje cell activity during acquisition, extinction, and reacquisition in decerebrated ferrets. They reported that early in training Purkinje cells develop pauses in simple spike activity to the CS. However, these experiments did not record any behavioral responses by the animals thus making it difficult to make a causal connection between the behavior and the pauses in Purkinje cell activity. Here we examined the activity of Purkinje cells during acquisition of the conditioned response. The ratio of decreasing to increasing Purkinje cells following eyeblink conditioning depends on the electrode location within the cerebellar cortex. For example, one experiment reported a 2:1 ratio of decreasing to increasing cells within the anterior lobe, whereas a 1:2 ratio was found in lobule HVI (Green and Steinmetz, 2005). Here we recorded from the base of the primary fissure, between the lobules recorded from in the Green and Steinmetz (2005) study and we report a 1:2 ratio of decreasing to increasing cells following training. In addition, we showed an increase in the percentage of Purkinje cells with simple spike pauses as conditioning developed. There was also an increase in the percentage of Purkinje cells that showed an increase in simple spike activity with conditioning, but the activity of these Purkinje cells was not affected by

WIN55,212-2, even though learning was impaired. Thus, the increase in the prevalence of Purkinje cells with decreases in simple spike activity during the CS is the mechanism underlying the development of the eyeblink CR.

We tested the hypothesis that *in vivo* disruptions of cerebellum-dependent eyeblink conditioning in human and non-human mammals by cannabinoid agonists is the result of impaired LTD formation within the cerebellar cortex. We localized the effects of cannabinoid agonists to the area of the cerebellar cortex that is necessary for eyeblink conditioning. Next, we found that fewer Purkinje cells exhibited pauses in simple spike activity during the CS that were impaired by a cannabinoid agonist. These findings suggest that WIN55,212-2 causes impairments to LTD formation pre-synaptically at parallel fiber to Purkinje cell synapses. Interestingly, we report that following learning WIN55,212-2 did not impair LTD maintenance suggesting that pre-synaptic processes aren't involved in maintenance. These experiments provide evidence that normal CB1R receptor function plays an important role in the acquisition of plasticity within the cerebellum *in vivo*. This additionally establishes the first evidence concurrently *in vivo* of disrupted behavioral and neurophysiological acquisition of cerebellar learning due to cannabinoid administration.

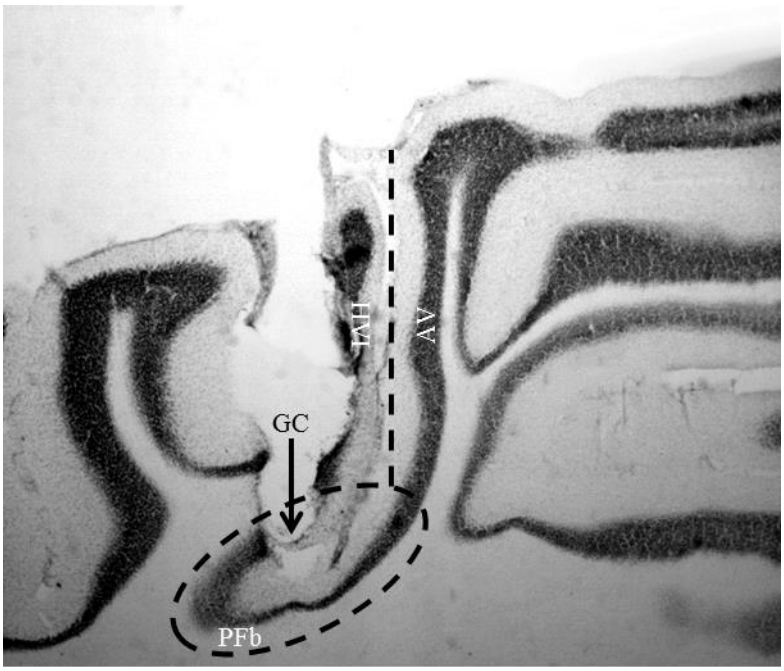

Figure 5. Coronal sections of the cerebellum showing representative cannula placements for eyeblink conditioning microzone of the cerebellar cortex.

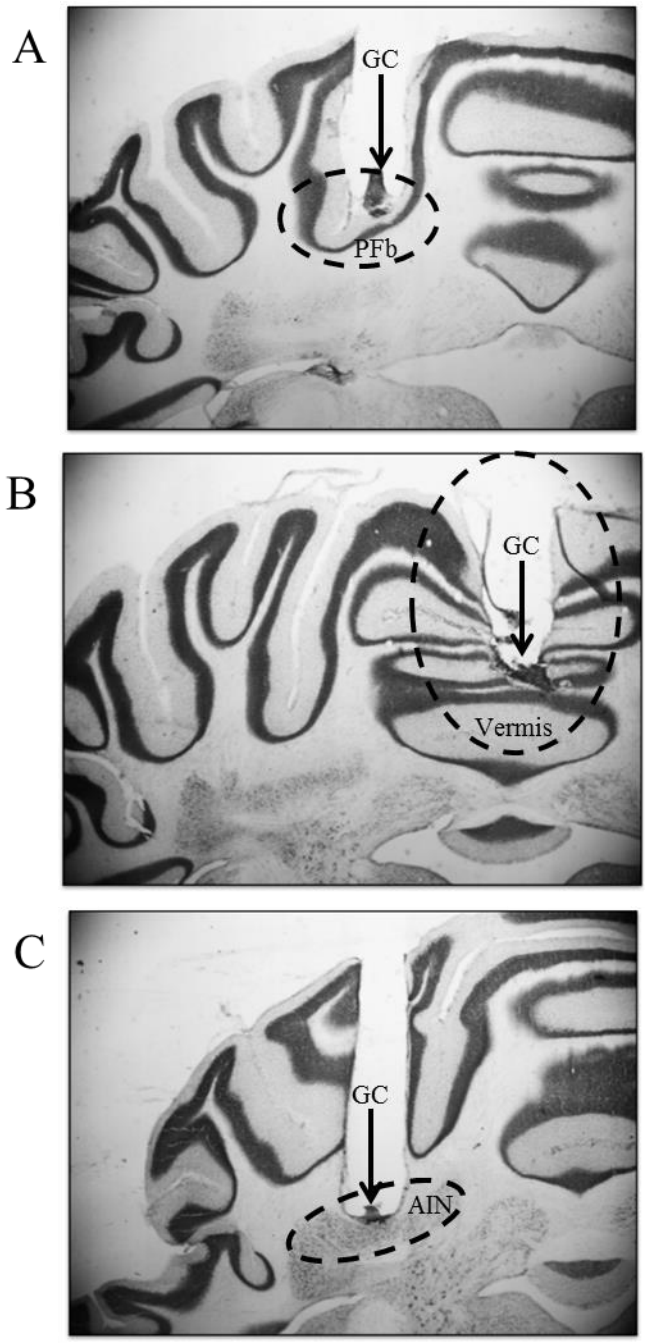

Figure 6. Coronal sections of the cerebellum showing representative cannula placements for **A:** eyeblink conditioning microzone of the cerebellar cortex, **B:** central vermis, and **C:** anterior interpositus nucleus.

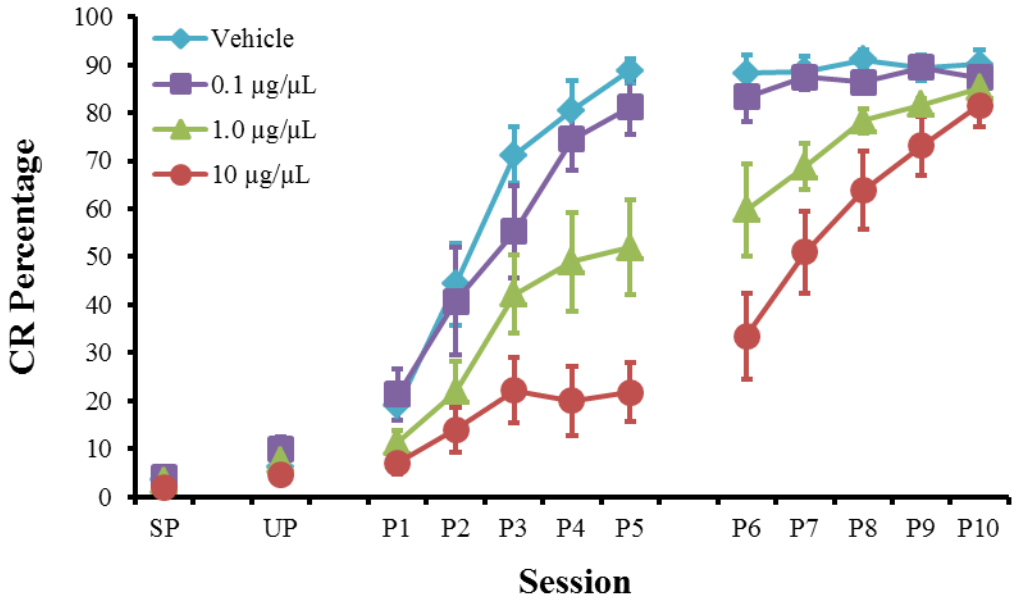

Figure 7. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with the CB1R agonist WIN55,212-2 infusions (SP, UP, P1-P5) of differing concentrations into the base of the primary fissure of the cerebellar cortex. Rats received infusions of either 0.1 µg/µL, 1.0 µg/µL, 10.0 µg/µL or vehicle. No infusions were given on P6-P10.

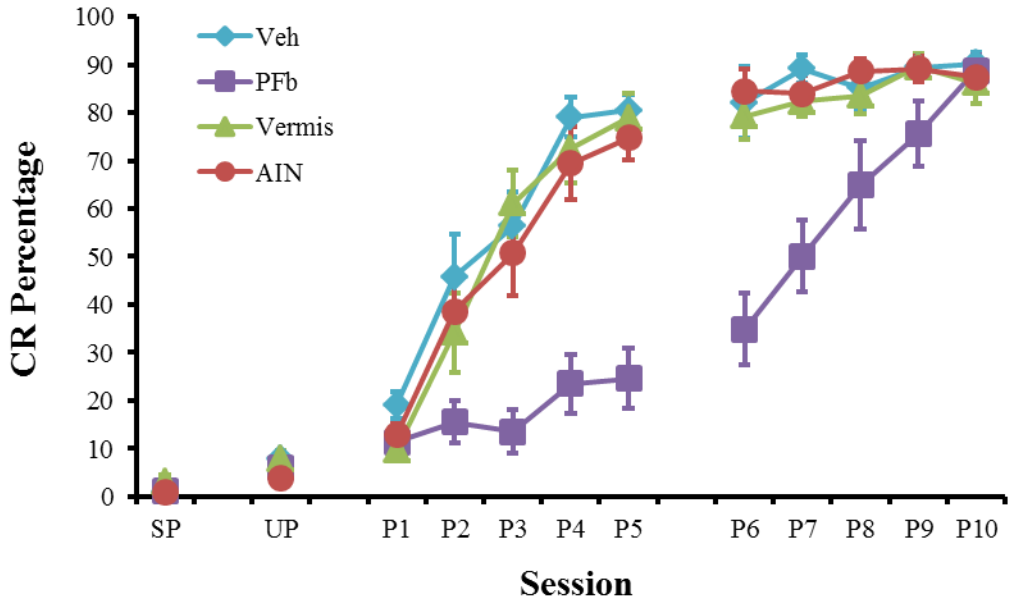

Figure 8. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with the CB1R agonist WIN55,212-2 infusions (SP, UP, P1-P5) into either the EBC microzone of the cerebellar cortex (PFb), central vermis (vermis), or the anterior interpositus nucleus (AIN). No infusions were given on P6-10.

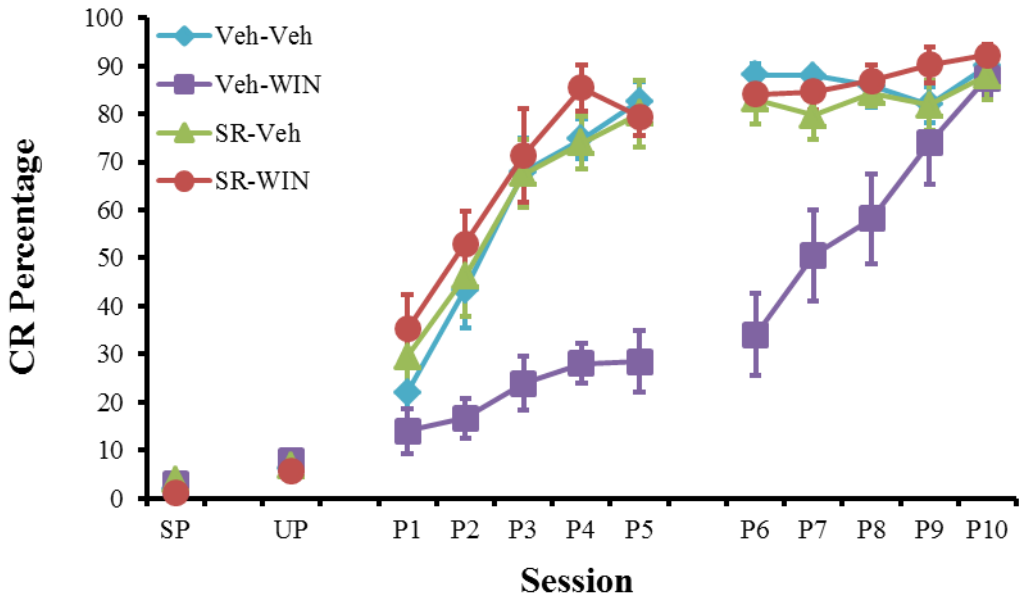

Figure 9. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with the CB1R antagonist SR141716A or vehicle infusions into the cerebellar cortex and the CB1R agonist WIN55,212-2 or vehicle injections subcutaneously. Rats received infusions of the infusion into the cerebellar cortex 30 mins prior to the subcutaneous injection. Infusions and injections were made during SP, UP, and P1-P5. No infusions were given on P6-P10.

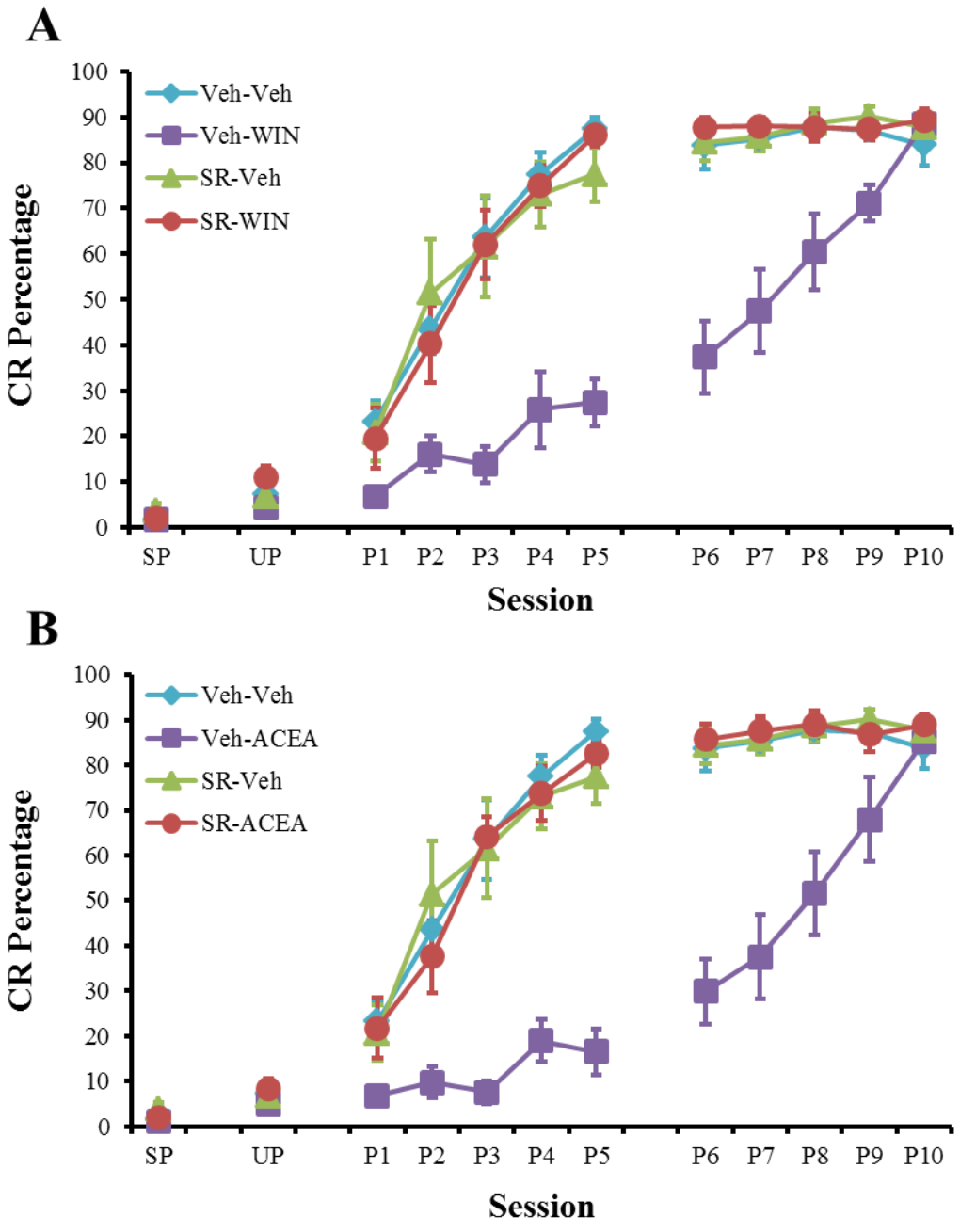

Figure 10. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with **A**: the CB1R agonist WIN55,212-2 or **B**: the CB1R agonist ACEA infusions during SP, UP, and P1-P5. SR141716A, a selective Cb1R antagonist, was administered prior to either WIN55,212-2, ACEA, or vehicle.

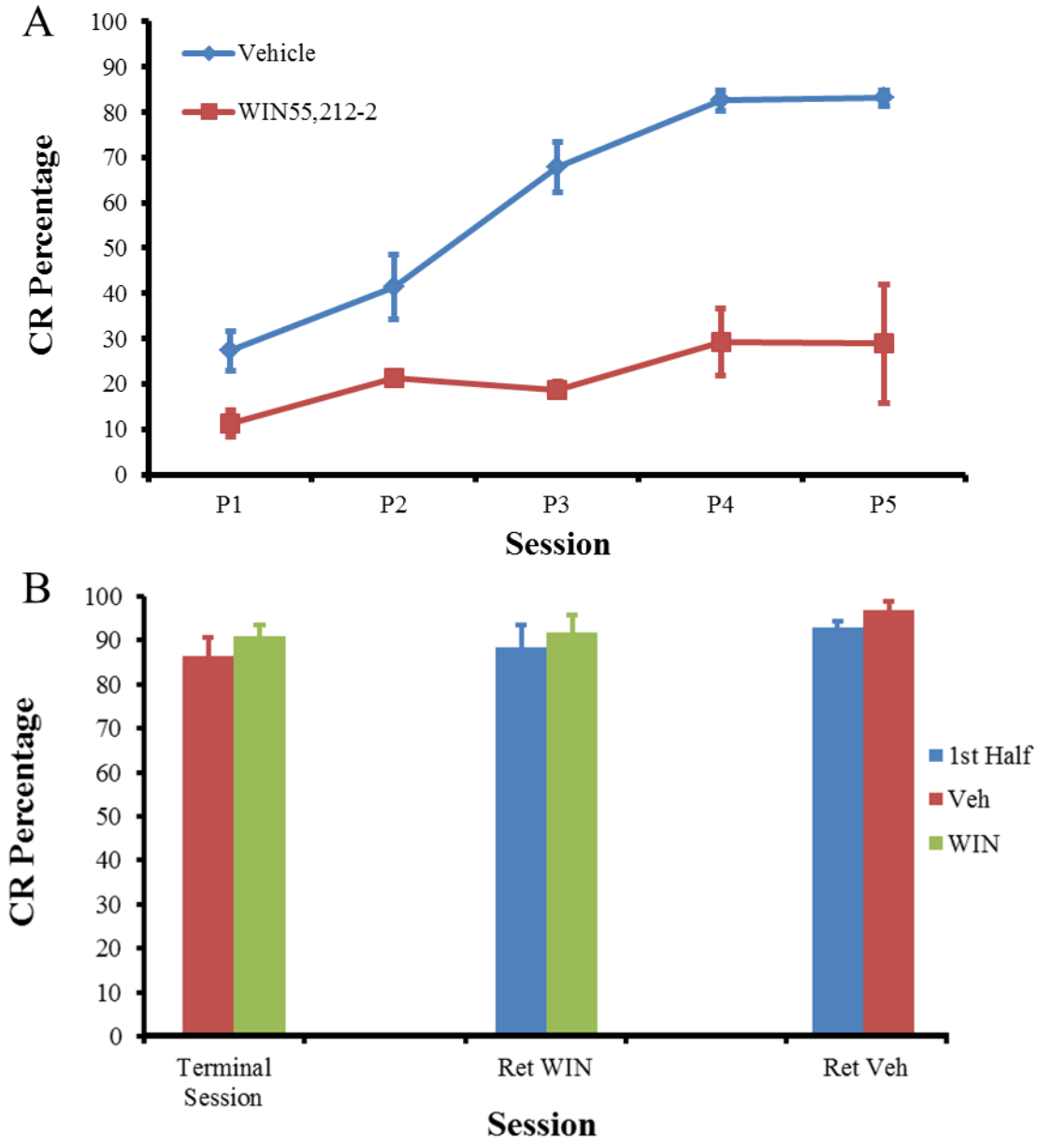

Figure 11. **Top:** Mean ± SE conditioned response (CR) percentages for rats administered subcutaneous injections of vehicle or the CB1R agonist WIN55,212-2 (3 mg/kg) subcutaneously (n = 5 per group). **Bottom:** Percentage CRs for the last session prior to retention (terminal session), and then two sessions of retention in which WIN55,212-2 or vehicle was administered for 50 trials after receiving 50 trials.

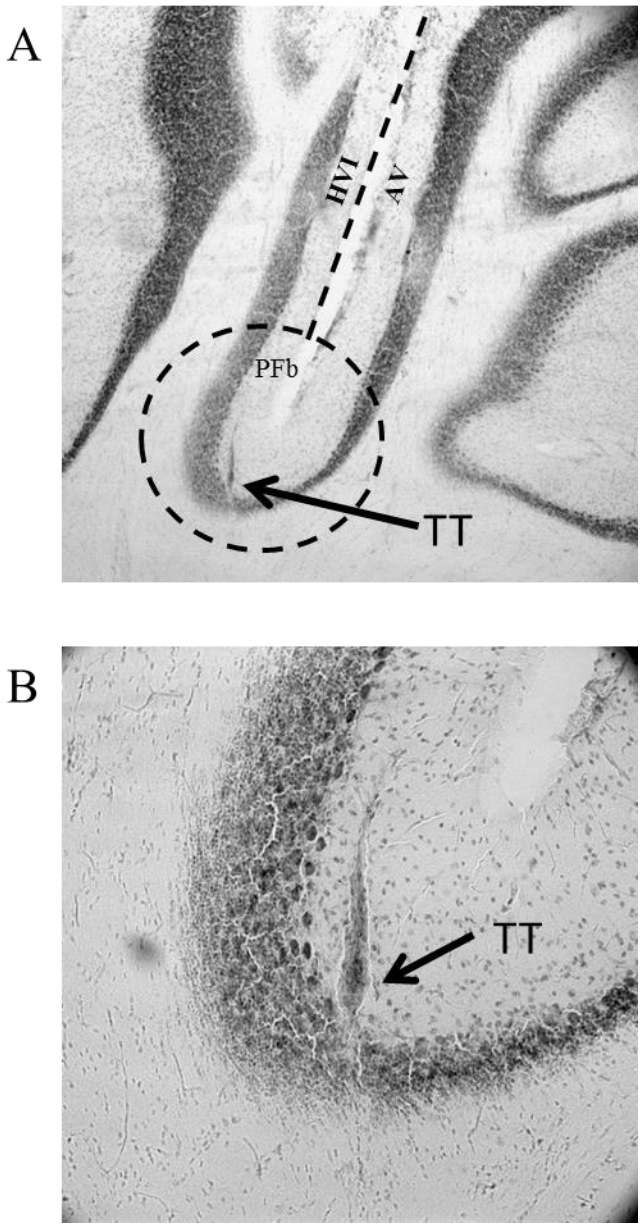

Figure 12. Coronal section of the cerebellar cortex showing a tetrode placement in the Purkinje cell layer for 2.5x (A) and 20x (B) magnification.

	Vehicle		WIN55,212-2	
	n	%	n	%
Session 1				
Increasing	3	18	2	20
Decreasing	2	12	0	0
No Change	12	70	8	80
Session 3				
Increasing	9	48	7	39
Decreasing	5	26	1	6
No Change	5	26	10	55
Session 5				
Increasing	9	30	6	23
Decreasing	16	53	8	31
No Change	5	17	12	46

Table 2. Summary of cell numbers and percentages of Purkinje cells divided based on their activity.

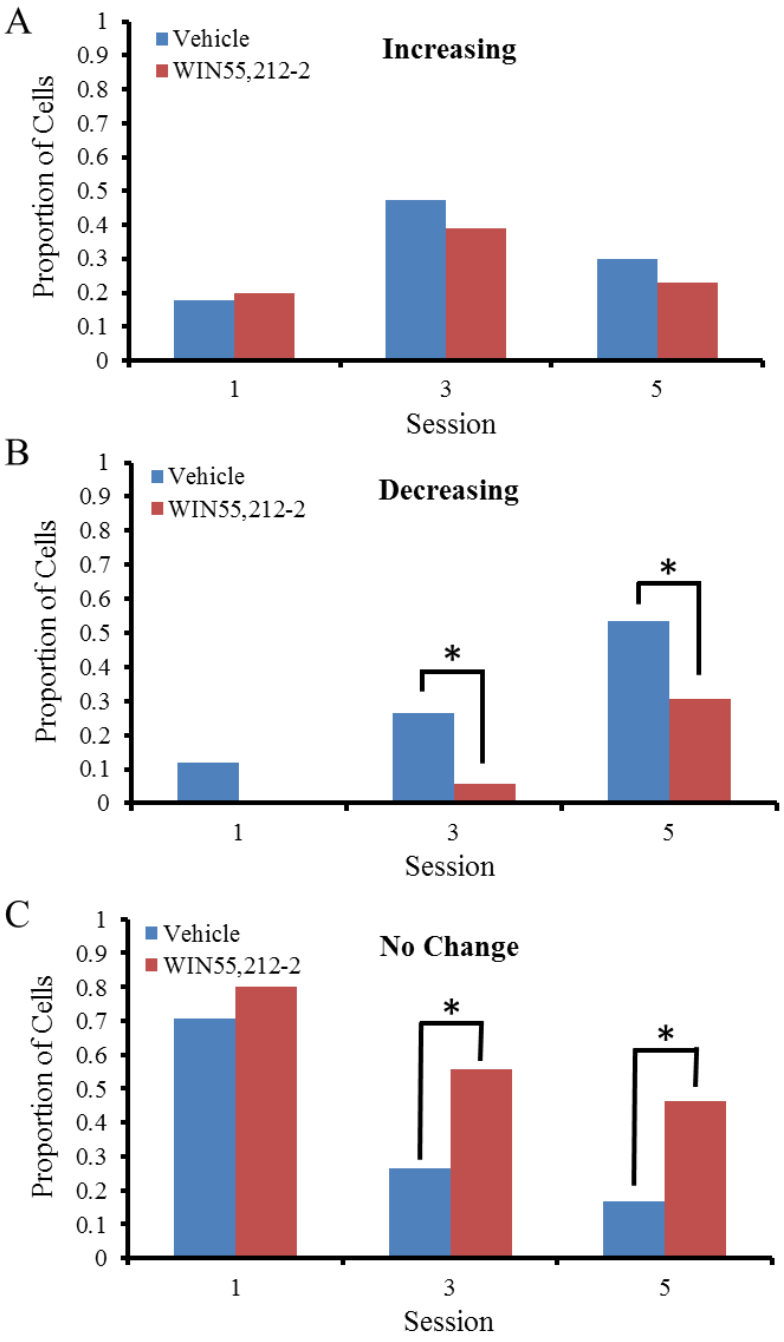

Figure 13. Proportion of cells during each session (1 ,3, and 5) for increasing cells (A), decreasing cells (B), and cells that did not significantly change from baseline (C). Asterisks (*) denote significant differences.

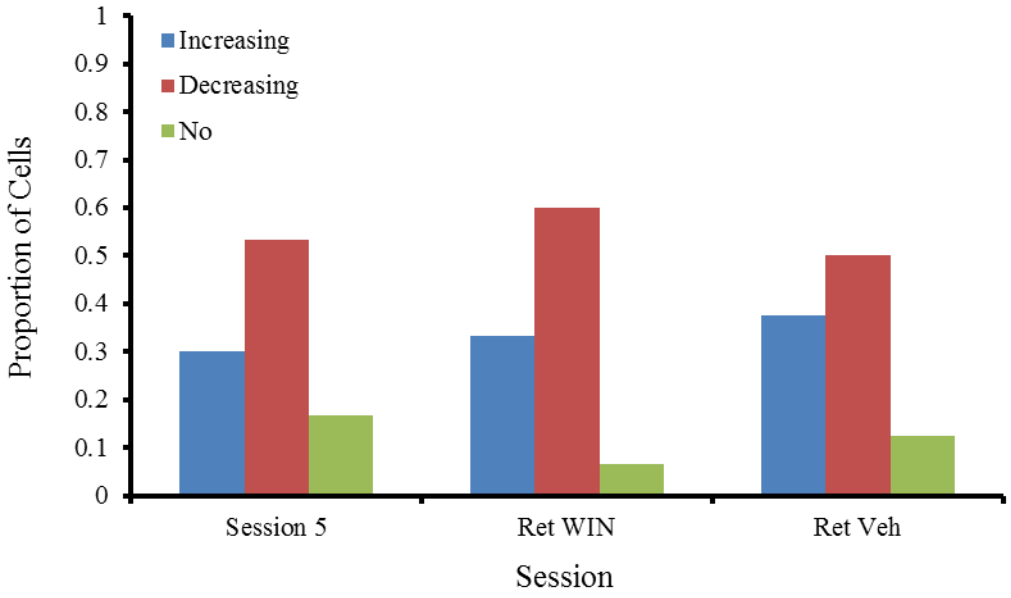

Figure 14. Proportion of cells during the session prior to retention (terminal session), and the retention sessions with WIN55,212-2 and vehicle.

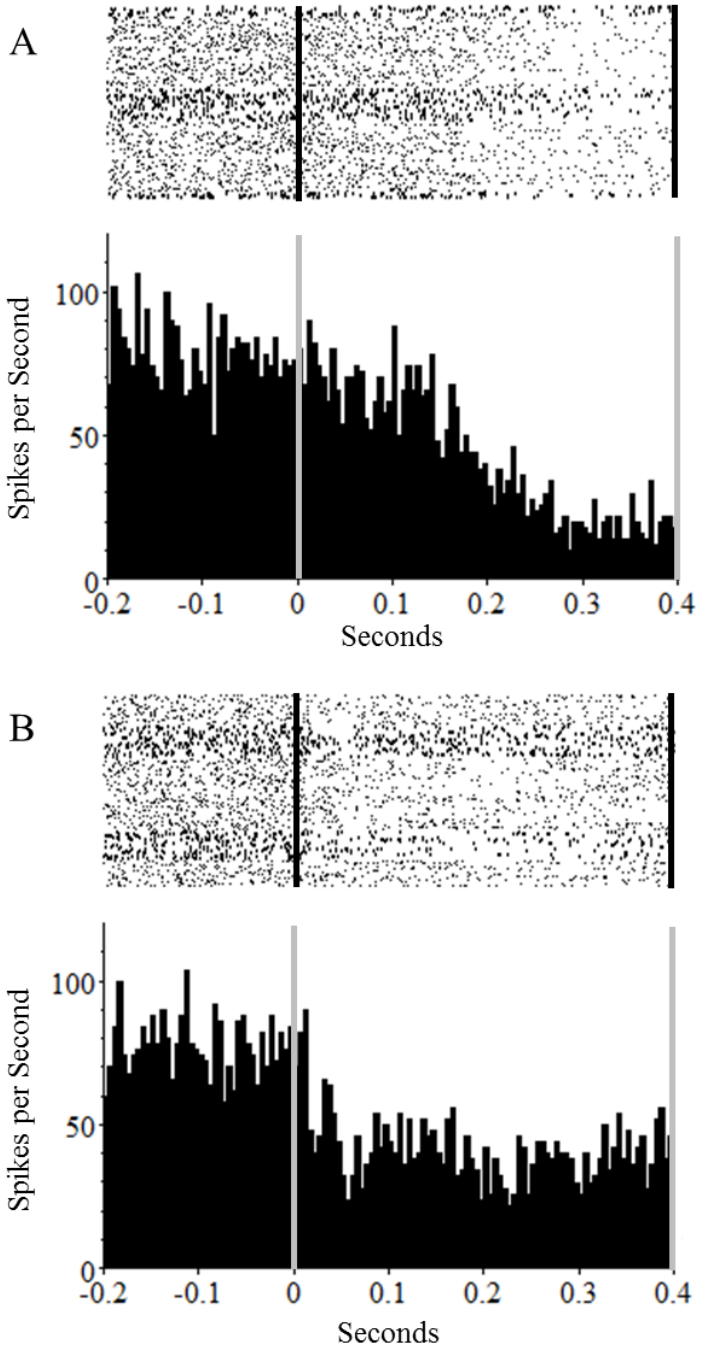

Figure 15. Histograms and raster plots of single unit activity of Purkinje cells on Session 5 for the Vehicle group (top) and WIN55,212-2 group (bottom) for decreasing cells.

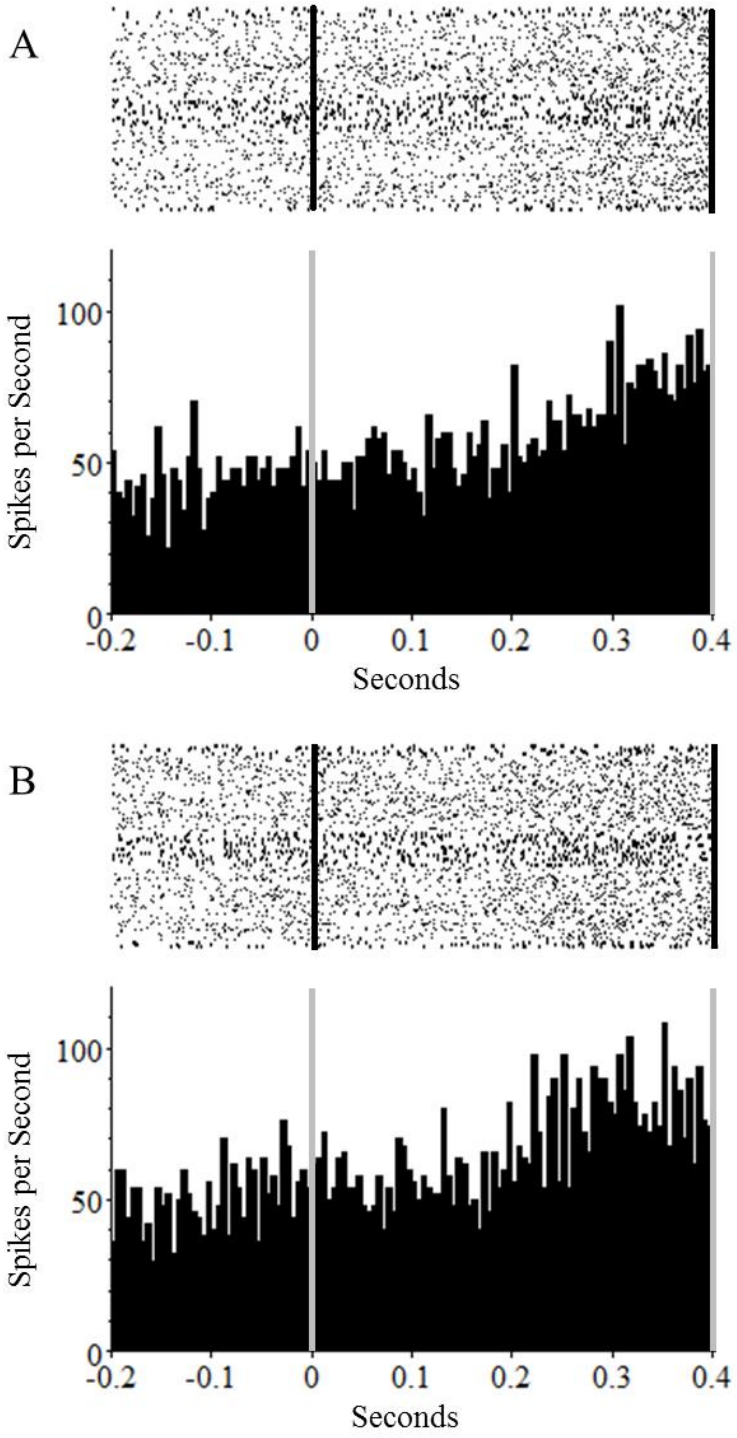

Figure 16. Histograms and raster plots of single unit activity of Purkinje cells on Session 5 for the Vehicle group (top) and WIN55,212-2 group (bottom) for increasing cells.

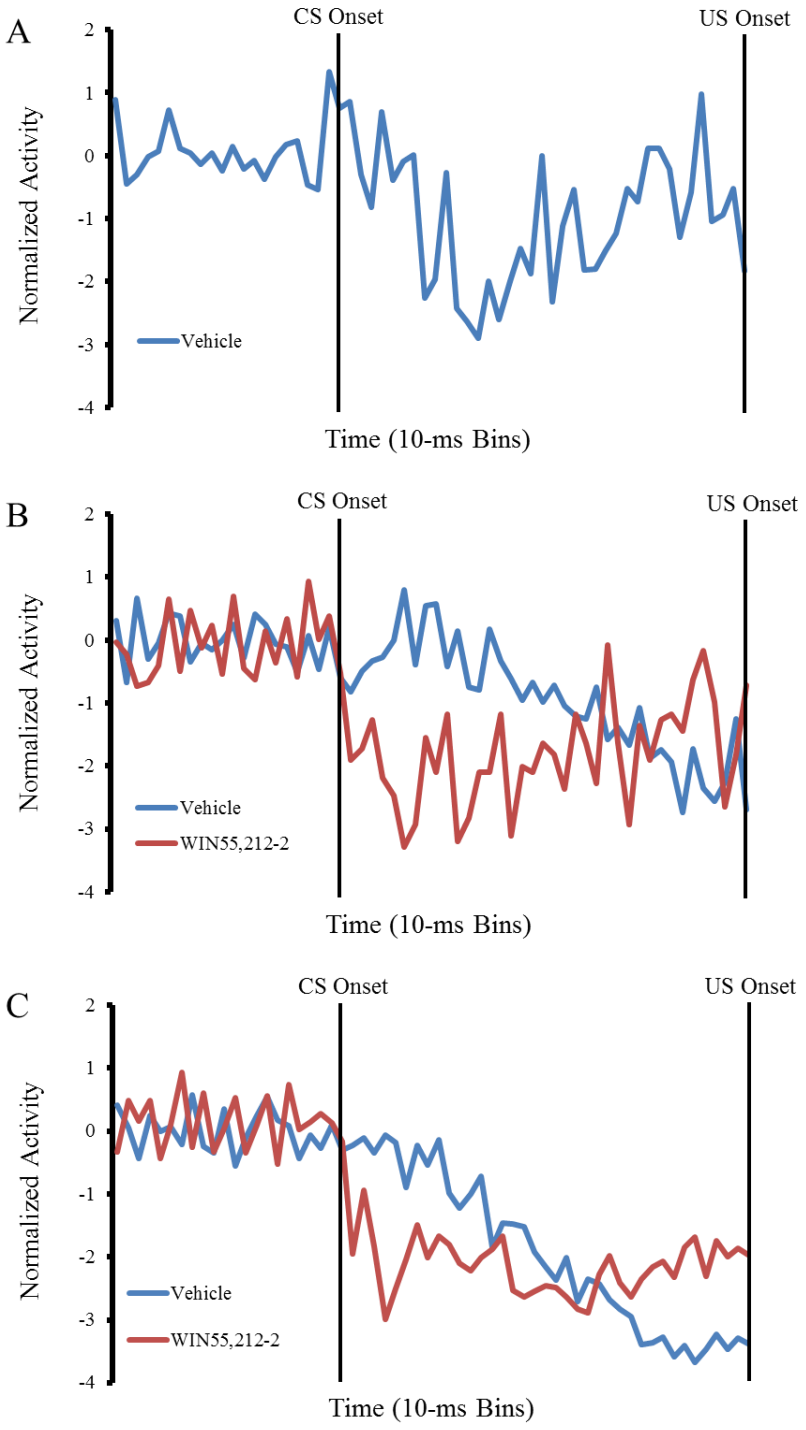

Figure 17. Average activity of decreasing units for Session 1(A), Session 3 (B), and Session 5 (C) of cells receiving Vehicle (blue line) or WIN55,212-2 (red line).

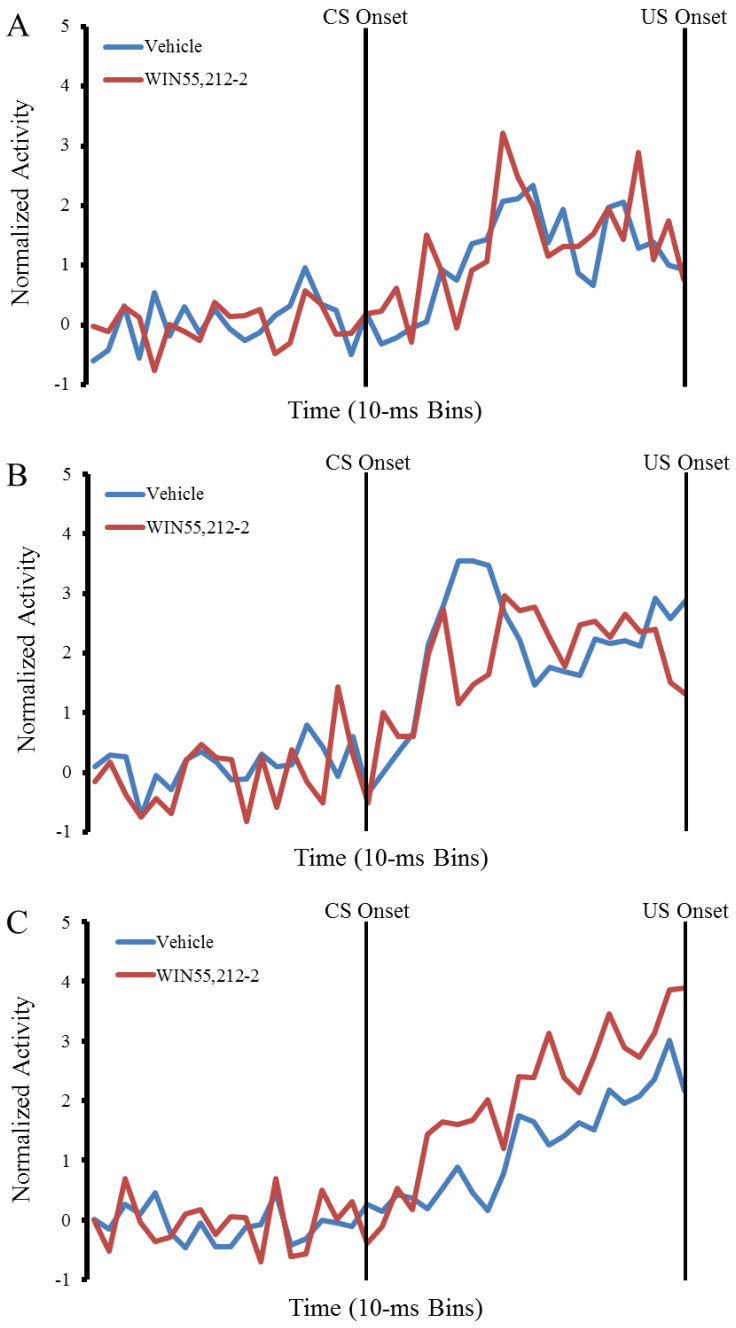

Figure 18. Average activity of increasing units for Session 1(A), Session 3 (B), and Session 5 (C) of cells receiving Vehicle (blue line) or WIN55,212-2 (red line).

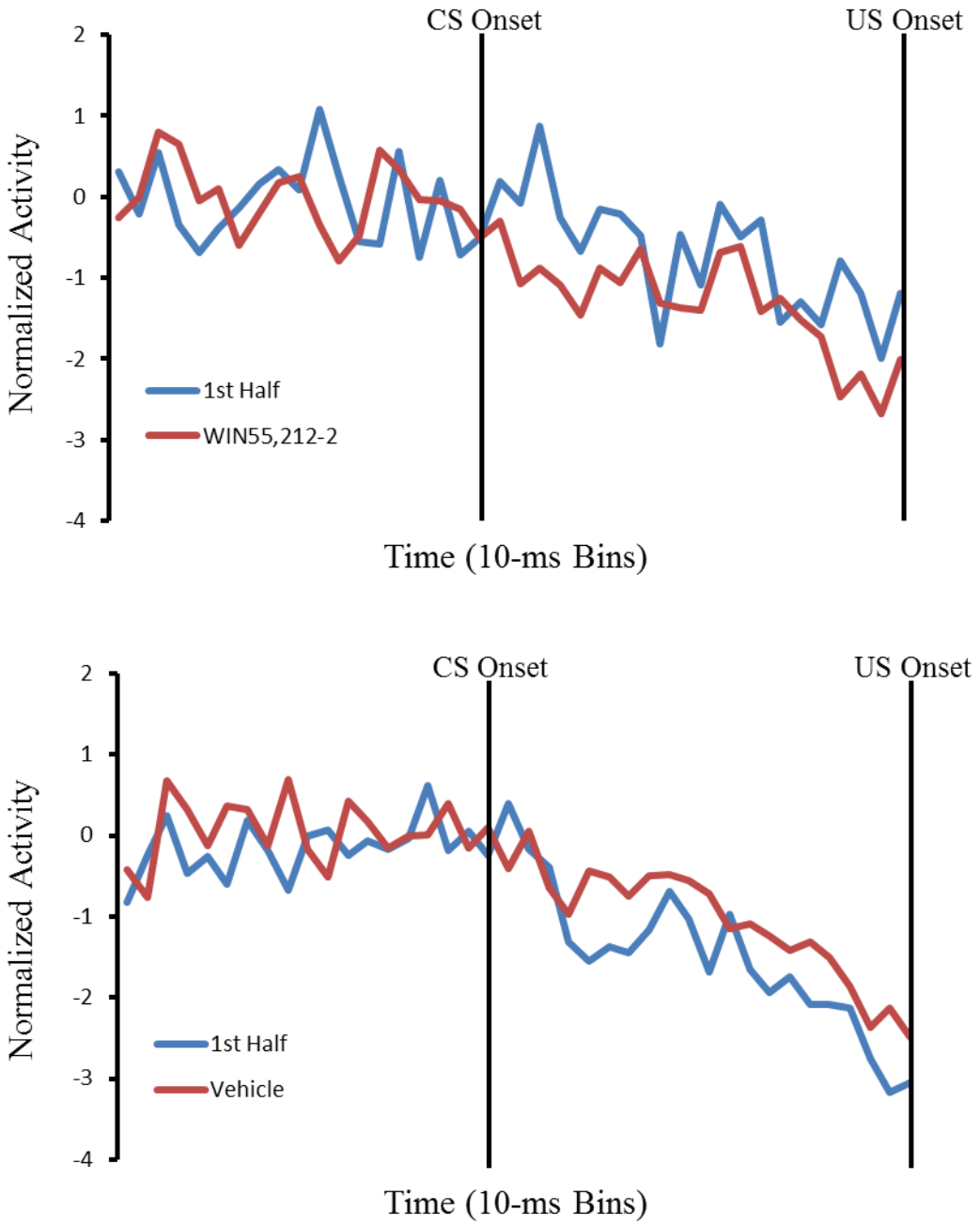

Figure 19. Average activity of decreasing cells during retention during the first 50 trials (blue line) and after WIN55,212-2 administration (top; red line) or vehicle (bottom; red line).

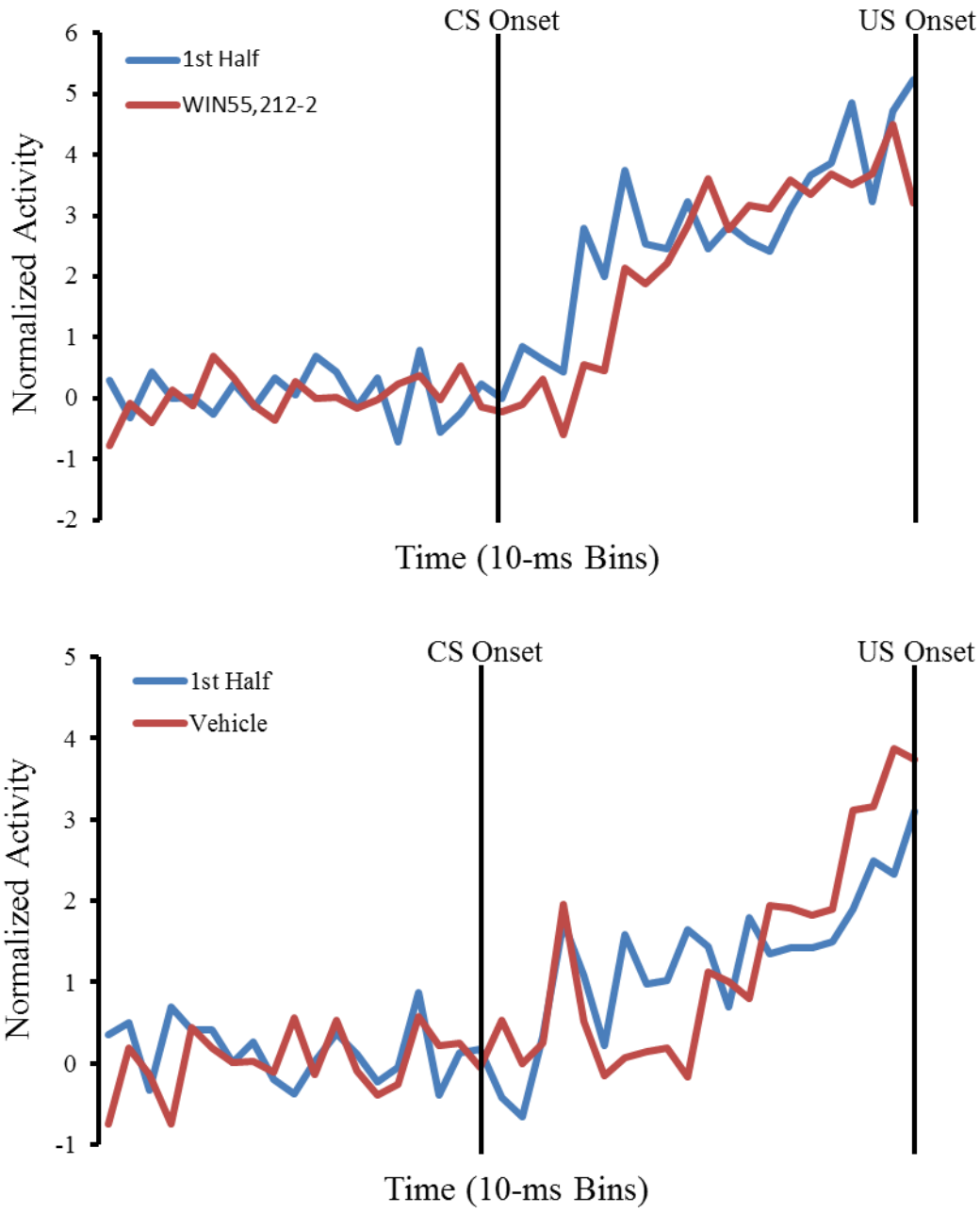

Figure 20. Average activity of increasing cells during retention during the first 50 trials (blue line) and after WIN55,212-2 administration (top; red line) or vehicle (bottom; red line).

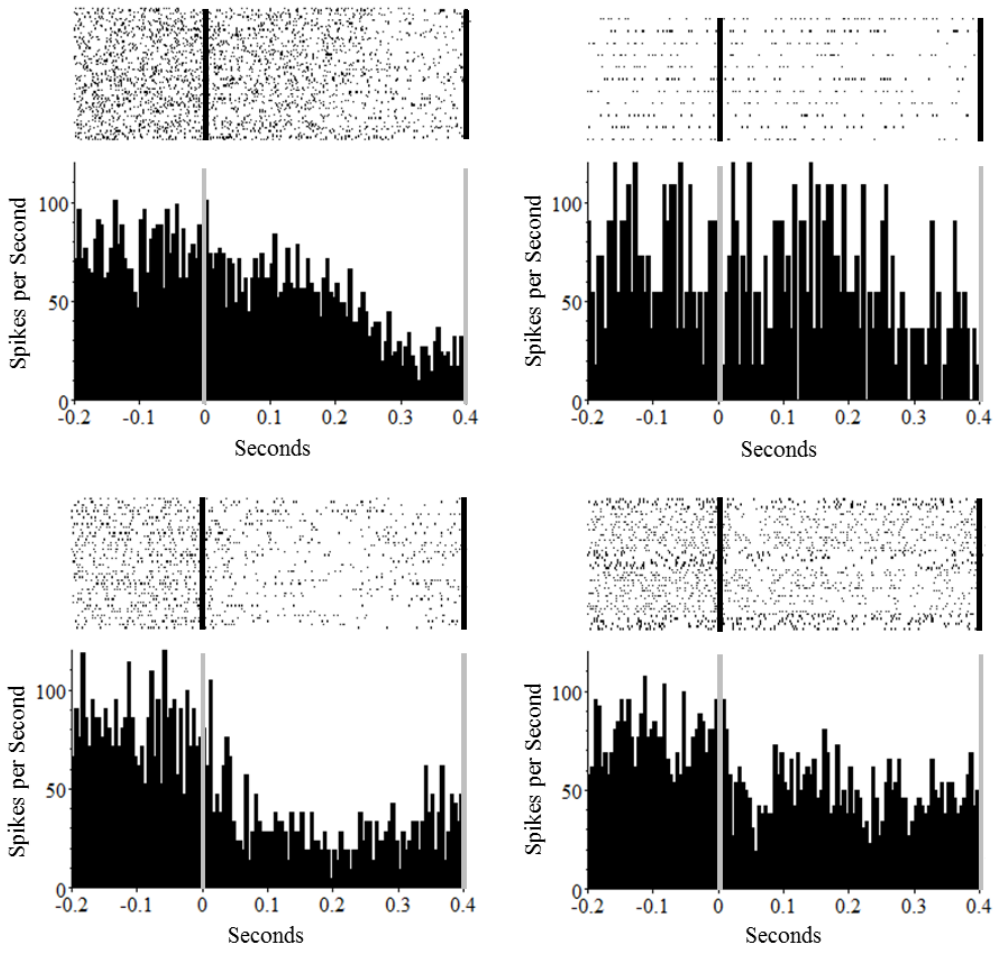

Figure 21. Histograms and raster plots of single unit activity of Purkinje cells on Session 5 for the Vehicle group CR trials (top left) and non-CR trials (top right) and WIN55,212-2 group CR trials (bottom left) and non-CR trials (bottom right)..

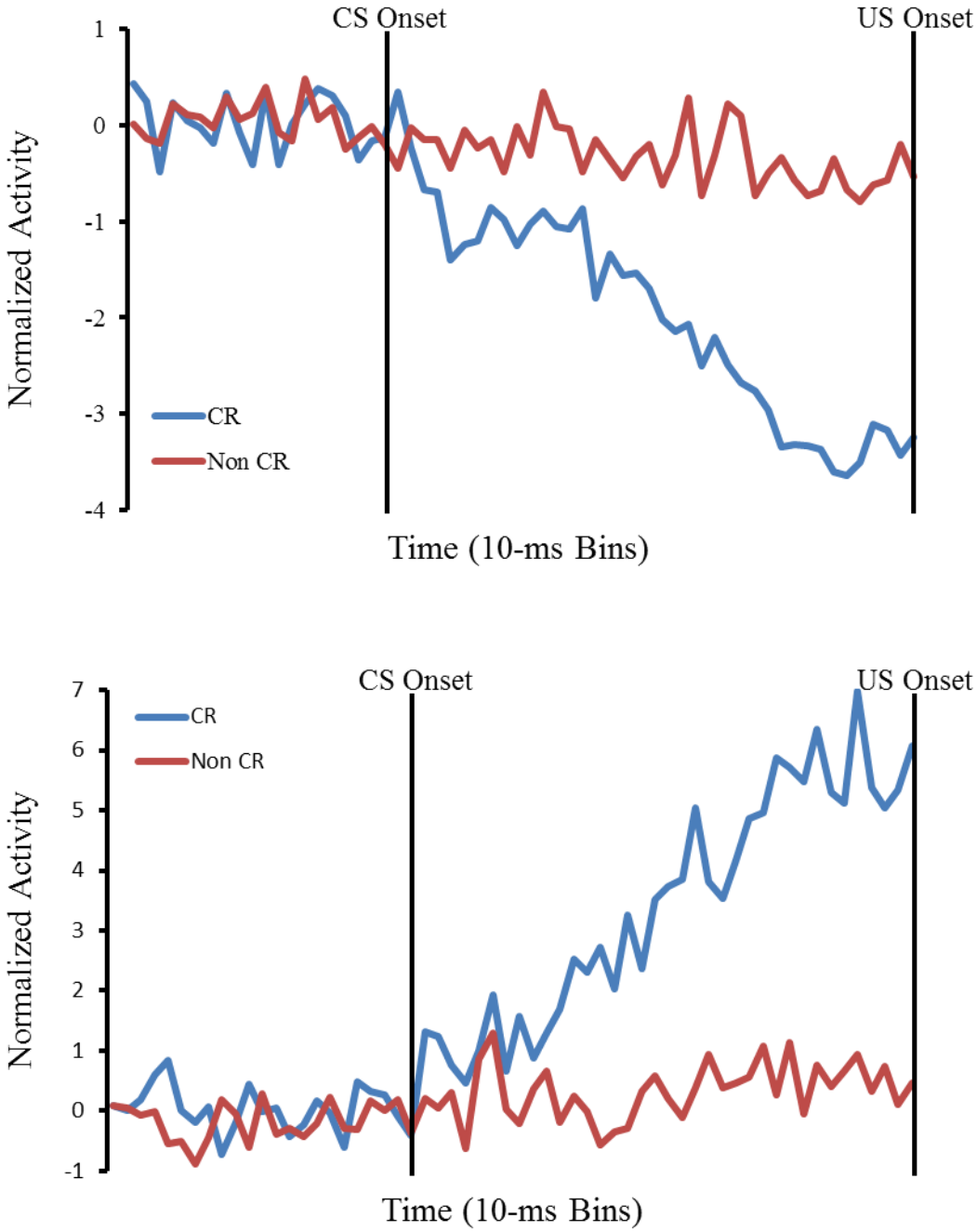

Figure 22. CR (blue line) vs Non CR (red line) trials in vehicle treated animals for decreasing (top) or increasing (bottom) cells for Session 5.

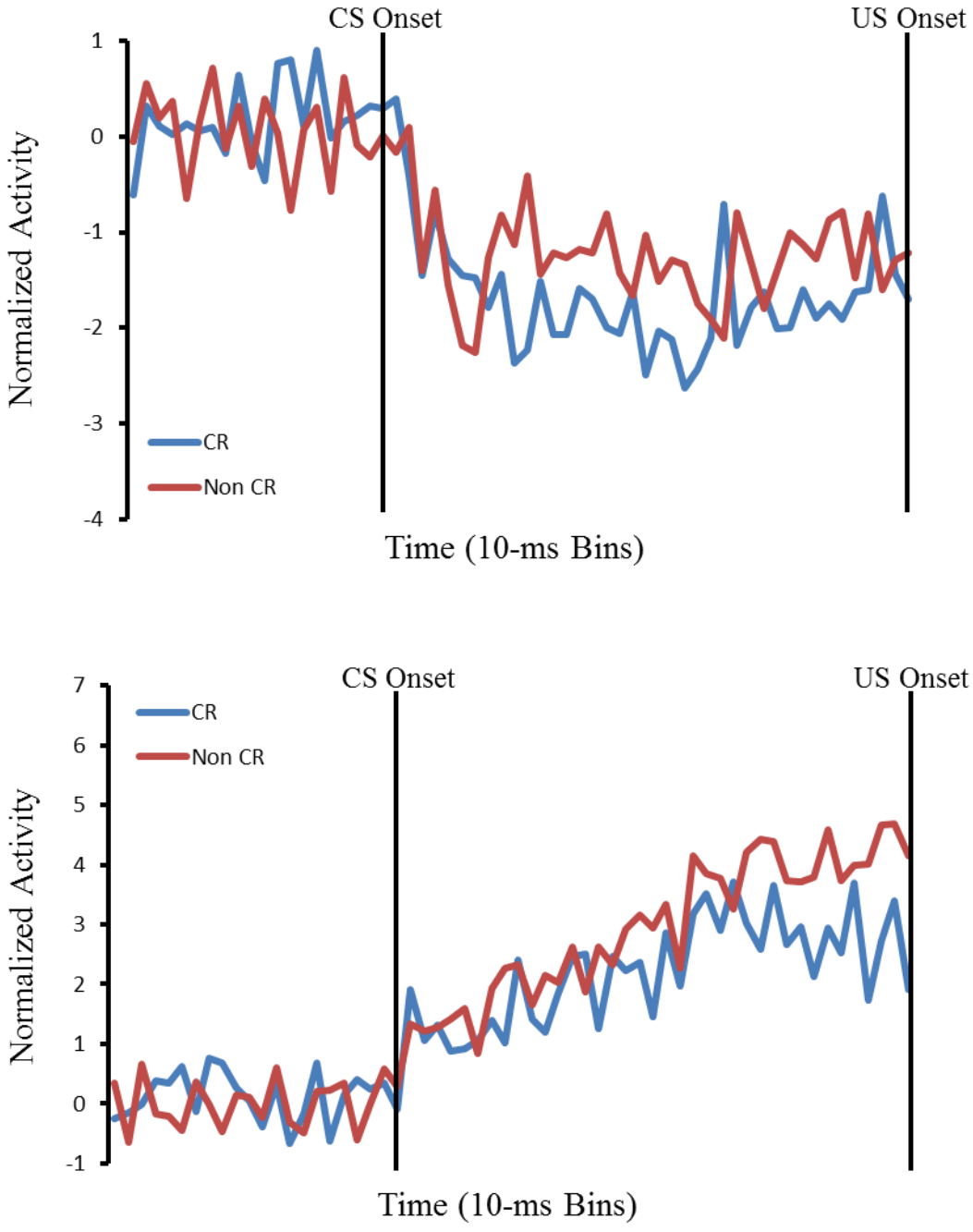

Figure 23. CR (blue line) vs Non CR (red line) trials in WIN55,212-2 treated animals for decreasing (top) or increasing (bottom) cells for Session 5.

CHAPTER 3: INTRACEREBELLAR CANNABINOID ADMINISTRATION IMPAIRS DELAY BUT NOT TRACE EYEBLINK CONDITIONING

Cannabinoid-1 receptors (CB1R) are the most abundant G-coupled receptors found throughout the mammalian brain with their highest densities within the cerebellum (Herkenham et al., 1990; 1991). The cerebellum traditionally has been thought to be only important for fine motor control, posture, and gait. However, this traditional conceptualization of the cerebellum as a motor control structure has been updated and it has been implicated in a wide range of non-motor higher cognitive functions including attention, language, working memory, and learning (Strick, Dum, and Fiez, 2009). Additionally, the cerebellum is important in temporal operations such as time perception, which has been repeatedly reported to be disturbed with acute cannabis intoxication (Hicks et al., 1984; Mathew et al., 1998; O'Leary et al., 2003). A paradigm that has been employed to probe cerebellar function in both human and non-human mammals is eyeblink conditioning (EBC). EBC involves the presentation of a conditioned stimulus (CS; e.g. a tone) that is paired with an unconditioned stimulus (US; e.g. a shock) which elicits an unconditioned response (UR) prior to training. After repeated CS-US pairings, a conditioned response (CR) occurs prior to the onset of the US. Two paradigms, known as delay and trace EBC, are widely used to examine the neural substrates and mechanisms involved in learning. In delay EBC, the CS terminates with the onset of the US; whereas in trace EBC following the presentation of the CS a stimulus free "trace" interval occurs prior to the onset of the US.

The neural circuitry involved in acquisition of delay EBC has been localized within the cerebellum to the anterior interpositus nucleus and cerebellar cortex (Thompson and Steinmetz, 2009; Freeman and Steinmetz, 2011). Areas outside the cerebellum, including the hippocampus, amygdala, and sensory cortex, have been shown to contribute to the rate of learning but not to be necessary for learning to occur (Neufeld and Mintz, 2001; Lee and Kim, 2004; Steinmetz, Harmon, and Freeman, 2013). Trace EBC has also been shown to require the anterior interpositus nucleus but also actively recruits other areas such as the hippocampus, primary sensory cortex, and prefrontal cortex for learning (Woodruff-Pak and Disterhoft, 2008).

Studies examining global CB1R dysfunction in humans and non-human mammals have examined both delay and trace EBC in order to understand the role of cannabinoids in learning. CB1R manipulations, via CB1R agonist administration, CB1R knockout, or chronic cannabis use, results in impaired delay EBC but intact trace EBC (Kishimoto and Kano, 2006; Skosnik et al., 2007; Edwards et al., 2008; Steinmetz and Freeman, 2010; 2013; Steinmetz et al., 2013). The contrast in effects due to CB1R manipulations lead to the hypothesis that cerebellar cortical dysfunction resulted in the selective impairment in delay EBC. Additionally, mice with genetic alterations targeted towards cerebellar cortex functioning have repeatedly shown intact trace EBC but impaired delay EBC (Kishimoto et al., 2001a, b, c; Brown et al., 2010). Recently, we examined the role of CB1Rs in trace EBC by administering the CB1R agonist WIN55,212-2 systemically while manipulating CS and trace durations. We reported that CB1Rs become involved in trace EBC when the CS duration was greater than the trace interval. When the trace interval was larger than the CS duration CB1Rs are not involved (Steinmetz and Freeman, 2013). We also

reported that CB1Rs are more involved in long delay (750 ms CS) then delay (250 ms CS) EBC.

We attributed the effects of systemic WIN55,212-2 on EBC to altered cerebellar cortical function but had no direct evidence that CB1Rs in other brain areas did not play a role. In order to examine the role of the cerebellar cortical CB1Rs in EBC, the current study infused the CB1R agonist WIN55,212-2 into the cerebellar cortex during delay (250 ms CS), long delay (750 ms CS), and trace (250 ms CS; 500 ms trace interval). The base of the primary fissure (eyeblink conditioning microzone) was targeted for infusions of WIN55,212-2 for two reasons. First, we previously investigated the role of different areas within the cerebellar cortex in EBC and found that lesions or inactivations of this area severely disrupted learning (Steinmetz and Freeman, in prep). Second, we found that delay EBC is impaired by infusion of CB1R agonists into this area of the cortex (Chapter 2). Administration of WIN55,212-2 into the cerebellar cortex impairs the formation of LTD leaving intact LTP formation (Chapter 2). Thus, this study will test whether LTD formation within the cerebellar cortex is necessary for the learning of trace conditioning.

Methods

Subjects

The subjects were 54 male Long-Evans rats (250-300 g). The rats were housed in the animal colony in Spence Laboratories of Psychology at the University of Iowa (Iowa City, IA). All rats were maintained on a 12 h light/dark cycle and given *ad libitum* access to food and water.

Surgery

One week before training, rats were removed from their home cages and anesthetized with isoflurane. At the onset of anesthesia, the rats were fitted with differential electromyography (EMG) electrodes (stainless steel) implanted into the upper left orbicularis oculi muscle. The reference electrode was a silver wire attached to a stainless steel skull screw. The EMG electrode leads terminated in gold pins in a plastic connector. A bipolar stimulating electrode (Plastics One, Roanoke, VA) for delivering the shock US was implanted subdermally, caudal to the left eye. A 23 gauge guide cannula was implanted at the base of the primary fissure ipsilateral to the trained eye (Figure 1). A 30 gauge stylet was inserted into the guide cannula and extended 0.5 mm from the end of the guide. The stereotaxic coordinates taken from bregma for the cannula were 11.0 mm posterior, 3.0 mm lateral, and 3.2 mm ventral from skull surface. The plastic connector housing the EMG electrode leads, bipolar stimulating electrode, the guide cannula, and skull screws were secured to the skull with Osteobond copolymer bone cement.

Infusion procedure

Before the WIN55,212-2 infusions, the stylet was removed from the guide cannula and replaced with a 30 gauge infusion cannula that extended 1.0 mm beyond the guide cannula. The infusion cannula was connected to polyethylene tubing (PE 10), which was connected to a 10 µl gas tight syringe (Hamilton, Reno, NV). The syringe was placed in an infusion pump (Harvard Apparatus, Holliston, MA), and 0.5 µl of WIN55,212-2 (10 µg/µl, pH = 7.4) or vehicle was infused over 5 minutes at a rate of 6.0

µl/h. After the infusion, the cannula was left for 3 min in order to allow diffusion of the drug. The infusion cannula was then removed and replaced with the 30 gauge stylet.

Pharmacological activation of CB1 receptors

The CB1 agonist WIN55,212-2([R]-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone) were administered intracranially to rats 30 min before each daily training session. WIN55,212-2 binds with high affinity to CB1R and CB2R (Pertwee 1997; Howlett et al. 2002). WIN55,212-2 was administered intracranially at a dose of 10 µg/µL. WIN55,212-2 was dissolved in a vehicle of 1:1:18 solution of ethanol, cremaphor, saline. WIN55,212-2 was purchased from Sigma/RBI.

Apparatus

The conditioning apparatus consisted of two small-animal sound-attenuating chambers (BRS/LVE, Laurel, MD). Within each sound-attenuating chamber was a small-animal operant chamber (BRS/LVE) in which the rats were kept during conditioning. One wall of the operant chamber was fitted with two speakers that independently produce tones of up to 120 dB (sound pressure level) with a frequency range of 1000-9000 Hz. An exhaust fan on one of the walls provided a 65 dB masking noise. The tone CS used in training was a 2000 Hz pure tone (85 dB). The electrode leads from the rat's headstage was connected to peripheral equipment by lightweight cables that allowed the rat to move freely during conditioning. A desktop computer was connected to the peripheral equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs, Raleigh, NC). One circuit was used to deliver the

shock stimulus through a stimulus isolator (model 365A; World Precision Instruments, Sarasota, FL). Another circuit amplified differentially (gain, 2000; sampling rate, 250 Hz), filtered (500-5000 Hz), and integrated (time constant, 20 ms) the EMG activity. The intensity of the periorbital stimulation US was set for eliciting a discrete eyeblink (typical range of final current intensity, 2.5-3.5 mA; 60 Hz; constant current).

Conditioning procedure

Rats recovered from surgery for 1 week prior to the initiation of training. All rats completed 12 consecutive daily sessions of training. The first session measured spontaneous blink activity in which EMG recordings were collected 100 times, approximating a conditioning session. The collection period was equal to the presentation of the CS or CS + trace interval used for conditioning. Session 2 consisted of 100 CS (2 kHz; 85 dB) and 90 US (25 ms) unpaired presentations. The CS presentation during the unpaired session was equal to the CS presented during training sessions. Paired training sessions occurred in which 10 blocks of 9 paired CS–US presentations and 1 CS-alone probe trial were presented. CS-alone probe trials were used to accurately measure the timing and amplitudes of the CR without interferences of the US. Three paradigms were employed (Figure 24): delay (250 ms CS), long delay (750 ms CS) and trace (250 ms CS, 500 ms trace) EBC. All CSs were 2 kHz and 85 dB. These paradigms were chosen to equate the CS duration (delay vs. trace) or the interstimulus interval (ISI; long delay vs. trace) to better compare the paradigms. The US intensity was adjusted in each rat to elicit a blink and slight head movement (range = 2.5 – 3.5 mA). CRs were defined as EMG activity that exceeded a threshold of 0.4 units (amplified and integrated units) above the baseline mean during the CS period after 80 ms. EMG responses that exceeded the

threshold during the first 80 ms of the CS period were defined as startle responses. On CS-alone probe trials, the duration for scoring CRs was extended beyond the CS to the end of the trial period (0.5 s). URs were defined as responses that crossed the threshold after the onset of the US. The rats were given either vehicle or WIN55,212-2 (10 µg/µL) infusions into the cerebellar cortex 30 minutes before each infusion sessions (spontaneous blink session, unpaired session, and 5 paired CS-US sessions). Rats then received 5 paired CS-US sessions without infusions to examine effects of the drug following treatment.

Histology

After training, the rats were euthanized with a lethal injection of sodium pentobarbital (150 mg/kg) and transcardially perfused with physiological saline followed by 10% neutral buffered formalin (Surgipath, Richmond, IL). After perfusion, the brains were cryo-protected in a 30% sucrose in formalin solution, and subsequently sectioned at 50 µm with a sliding microtome. Sections were then stained with thionin. The location of the cannula placements were verified using a light microscope (Leica DMLS, Wetzlar, Germany) and a stereotaxic brain atlas (Paxinos and Watson, 2007).

Results

Cannula placement was verified using a light microscope and stained slides. An example of a cannula placement is presented in Figure 25. Cannula placement was consistent between the groups and across experiments. Six animals were removed from analysis due to placements being either too ventral (n=3), anterior (n=2), or posterior (n=1).Rats were given daily infusions of WIN55,212-2 or vehicle into the cerebellar

cortex prior to the first 7 sessions. Rats completed one of three different paradigms, delay (250 ms CS; Experiment 1), long delay (750 ms CS; Experiment 2), or trace (250 ms CS; 500 ms trace; Experiment 3) EBC.

Experiment 1: Delay Conditioning

The effects of intracerebellar infusion of WIN55,212-2 on delay conditioning were examined first to compare with the effects of systemic administration in previous studies (Steinmetz & Freeman, 2010; 2013). Prior to acquisition training animals were given two pre-training sessions examining spontaneous EMG activity and unpaired CS/US presentations. Separate one-way ANOVAs found no significant effects of WIN55,212-2 administration into the cerebellar cortex during either pre-training session. Animals then received 10 sessions of CS-US paired training; the first 5 sessions involved infusions of WIN55,212-2 or vehicle (1^{st} Half) followed by 5 sessions without infusions (2^{nd} Half). WIN55,212-2 administered locally into the cerebellar cortex resulted in significantly decreased CR percentages as compared to controls (Figure 26A). A 2 (Half) x 5 (Session) x 2 (Group) repeated measures ANOVA found a significant three way interaction, $F(4,11)=5.174$, $p = .014$. Post-hoc tests (Tukey's Honestly Significant Differences) found that WIN55,212-2 administration resulted in fewer CRs on Sessions 2-6 as compared to vehicle treated animals. There was no significant savings (difference between P5 and P6) for the WIN55,212-2 group indicating that WIN55,212-2 was not blocking expression of the CR. Additionally, there was a significant difference between P1 and P5 of the WIN55,212-2 group, suggesting learning did occur during the sessions. Administration of WIN55,212-2 into the cerebellar cortex also resulted in smaller CR amplitude (Figure 26B). A significant three-way interaction was found for the CR

amplitude data, F(4,11)=1.764, p = .035. The CR amplitude was smaller on Sessions 4 and 5 when WIN55,212-2 was administered. Analysis of CR timing found a significant Session x Half interaction for the peak latency data, F(4,11)=9.185, p < .001, but no other significant effects. Measurements of the UR amplitude and sensitivity to the US were examined in order to see if WIN55,212-2 administration impaired UR functioning. No significant effects were found with UR amplitude or US intensity. These results, taken together, indicate that delay conditioning was impaired when WIN55,212-2 was administered prior to each training session. The deficit was not due to altered blink properties or responding to the US.

Experiment 2: Long Delay Conditioning

Long delay conditioning (750 ms CS) is more impaired by systemic administration of WIN55,212-2 than delay conditioning (Steinmetz and Freeman, 2013) and was expected to be more impaired with intracerebellar infusions as well. The procedures were the same as the first experiment except that the CS was 750 ms.WIN55,212-2 administration did not alter spontaneous blinks or unpaired responding, as verified by one-way ANOVAs. Similar to subcutaneous injections, WIN55,212-2 administration resulted in a significant impairment in acquisition of long delay conditioning (Figure 27A). A repeated measures ANOVA revealed a significant three way interaction, F(4,12)=9.489, p = .001. Post-hoc tests found that the impairment was evident during Sessions 2-9. Learning following the last administration of WIN55,212-2 was slow, but did reach the same asymptote as the vehicle treated animals by Session 10. There was no significant savings (differences between P5 and P6) or in learning (differences between P1 and P5) in the WIN55,212-2 group, indicating that expression of

the response was not blocked and that no significant learning occurred during the drug sessions. CR amplitude was examined using a repeated measures ANOVA (Figure 27B); no significant interactions were found but there were significant main effects of Group [$F(1,12)=5.732$, $p = .034$], Session [$F(1,12)=3.562$, $p = .013$], and Half $F(1,12)=34.000$, $p <.001$. It was found, through post-hoc tests, that WIN55,212-2 treated animals produced significantly lower amplitude CRs than the controls. There were no significant effects for CR latency, UR amplitude, or US sensitivity. WIN55,212-2 administration impaired the formation of learning as compared to vehicle treated animals, which was not the result of non-associative blinking or US processing dysfunction.

Experiment 3: Trace Conditioning

Systemic administration of CB1R agonists does not impair trace EBC in humans or rats (Edwards, et al., 2007; Steinmetz and Freeman 2013). The third experiment examined whether or not trace conditioning is impaired when WIN55,212-2 is infused directly into the cerebellar cortex. The procedures were the same as in the first two experiments except that there was a 500 ms trace interval after the 250 ms CS. Infusions occurred during sessions P1-P5 (1st Half) and not during sessions P6-P10 (2nd Half). Spontaneous and unpaired responding were not affected by WIN55,212-2 (Figure 28A). During CS-US training, trace conditioning was not impaired by WIN55, 212-2. There was a significant Half x Session interaction, $F(4,12)=9.845$, $p < .001$, which was due to more CRs produced in the sessions without infusions and also during the last 2 sessions of each half (P4/5 and P9/10). Additionally, WIN55,212-2 did not impair CR amplitude during trace conditioning. Once again, there was a significant Half x Session interaction, $F(4,11)=4.347$, $p =.024$, indicating that CR amplitude increased across the sessions

(Figure 28B). Peak latency significantly decreased over the sessions, $F(1,13)=11.333$, $p = .005$. However, WIN55,212-2 administration did not affect either CR onset or peak latency. The UR amplitude and US levels administered did not differ between groups significantly. These results indicate that WIN55-212-2 administration into the cerebellar cortex did not alter trace conditioning.

Discussion

The current study examined the role of cerebellar cortical CB1Rs in eyeblink conditioning (EBC) as a function of delay and trace conditioning. Animals were given infusions of the CB1R agonist WIN55,212-2 or vehicle into the cerebellar cortex before training in delay (250-ms CS), long-delay (750-ms CS) or trace (250-ms CS, 500-ms trace) EBC. We report here that delay and long-delay but not trace conditioning were impaired with WIN55,212-2 administration,. The impairment to long-delay was greater than to delay. These results indicate that CB1Rs within the cerebellar cortex are important for the acquisition of cerebellar-dependent delay conditioning but not forebrain-dependent trace conditioning.

EBC with a trace interval exceeding 250 ms requires an intact hippocampus in rats, whereas short-delay EBC does not (Weiss et al., 1999). Previous work has indicated that systemic manipulations of CB1Rs impair delay EBC, but not a standard trace (250 ms CS, 500 ms trace) EBC, in humans and rodents (Edwards et al., 2008; Kishimoto & Kano, 2006; Skosnik et al., 2008; Steinmetz & Freeman, 2010, 2011; 2013; Steinmetz et al., 2012). Here we report intact trace conditioning even when the administration of the CB1R agonist is directly into the cerebellar cortex.

In a previous study we reported that learning-related pauses in Purkinje cell activity during the CS were impaired following administration of WIN55,212-2 *in vivo*. These Purkinje cell pauses are thought to be caused by LTD at parallel fiber synapses (Jirenhed et al., 2007). Two previous experiments also highlighted the importance of cannabinoid receptors during LTD formation within Purkinje cells. First, administration of WIN55,212-2 *in vitro* resulted in decreased LTD formation by decreasing the probability of release of glutamate (Levenes et al., 1998). Additionally, CB1R knockout mice and endocannabinoid inhibition impaired LTD formation *in vitro* (Safo and Regehr, 2005). In the current experiment it is expected that LTD formation is impaired; thus indicating that LTD formation in Purkinje cells is not needed for forebrain-dependent trace conditioning. This has been previously shown with mice with genetic manipulations to LTD formation (Kishimoto et al., 2001a, b, c; Brown et al., 2010).

The cerebellar cortical role in differing lengths of CSs has not received little attention from researchers. Vogel et al. (2009) blocked cerebellar cortex inputs to the interpositus nucleus during optimal (250 ms CS) or non-optimal CSs. It was reported that this blockage had a larger impact on non-optimal CS duration. Our findings from this study, and subcutaneous injections, support the view that longer non-optimal CS durations require more cerebellar cortical inputs to the anterior nucleus (Steinmetz and Freeman, 2013). Reports from lesions of the cerebellar cortex show that learning is possible without much of the cerebellar cortex (Lavond and Steinmetz, 1987; Steinmetz and Freeman, unpublished). However, these studies have only used only CS durations that fall in the optimal zone of eyeblink conditioning. We hypothesize that CS duration that are in the non-optimal range would require sustained cerebellar cortical input.

The current study found that cerebellar-dependent delay conditioning was impaired following CB1R agonist administration but not forebrain-dependent trace conditioning. This study used infusions into the cerebellar cortex whereas previous experiments used systemic manipulations. Long delay conditioning was more impaired than delay conditioning, a result we previously showed with subcutaneous injections. We hypothesize that Purkinje cell LTD is more critical for delay conditioning than trace conditioning and that it is particularly critical when the ISI is relatively long. Future studies should examine the differences in LTD formation between delay, long delay, and trace conditioning with CB1R dysfunction.

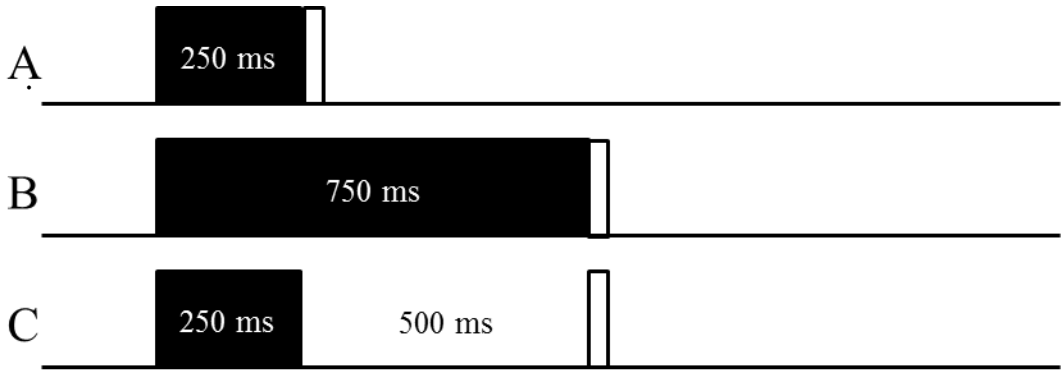

Figure 24. Schematic representation of trial types for the 3 paradigms. Animals received either delay (A: 250-ms CS), long-delay (B: 750-ms CS), or trace (C: 250-ms CS, 500-ms trace) eyeblink conditioning. Open rectangles represent US presentation.

Figure 25. Coronal section of the cerebellum showing representative cannula placements located at the anterior lobe/lobule HVI subdivision.

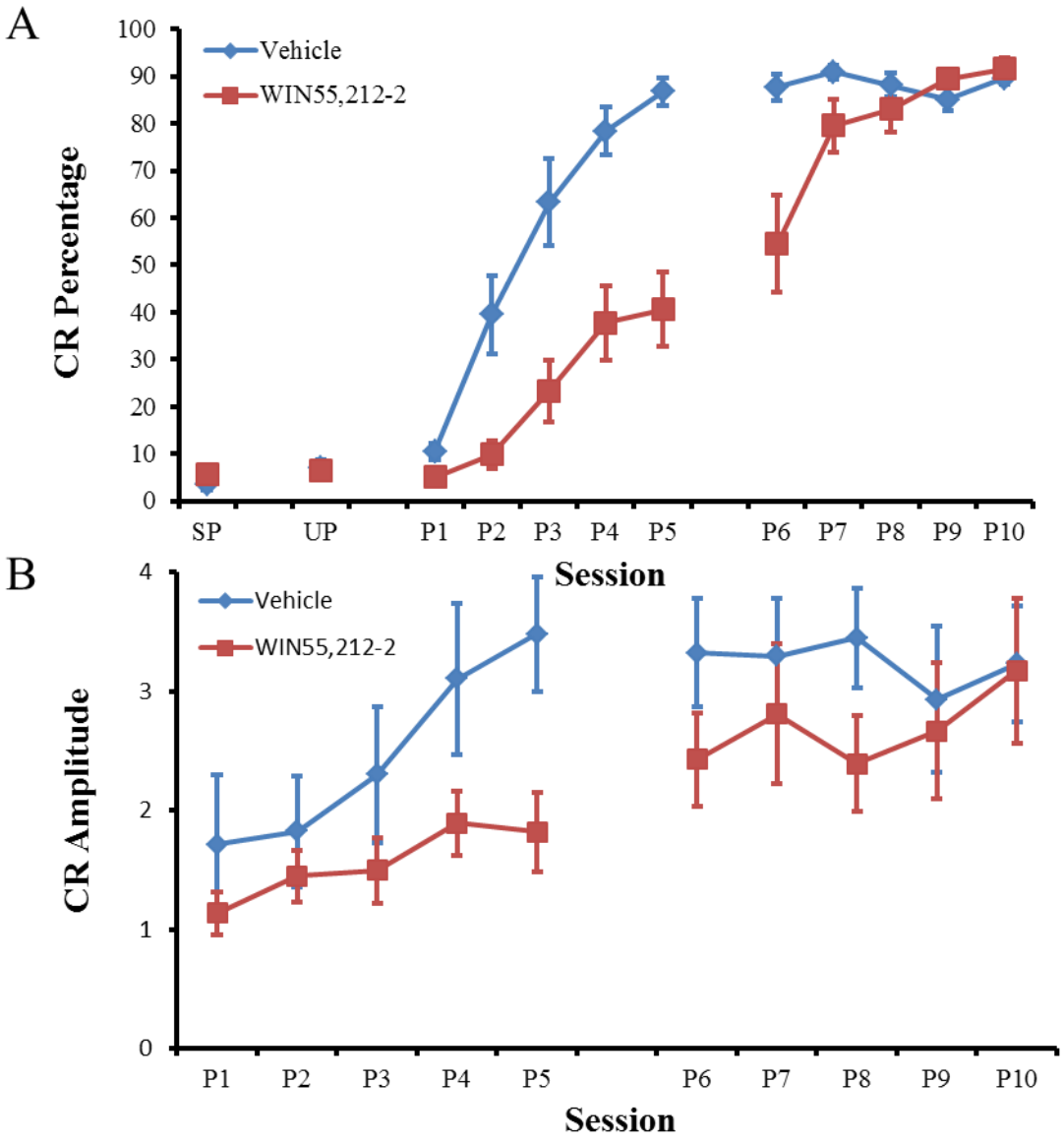

Figure 26. Mean ± SE conditioned response (CR) percentage (A) and amplitudes (B) for rats (n = 8 per group) given training with WIN55,212-2 infusions into the cerebellar cortex (SP, UP, P1-P5) of during delay (250 ms CS) conditioning.

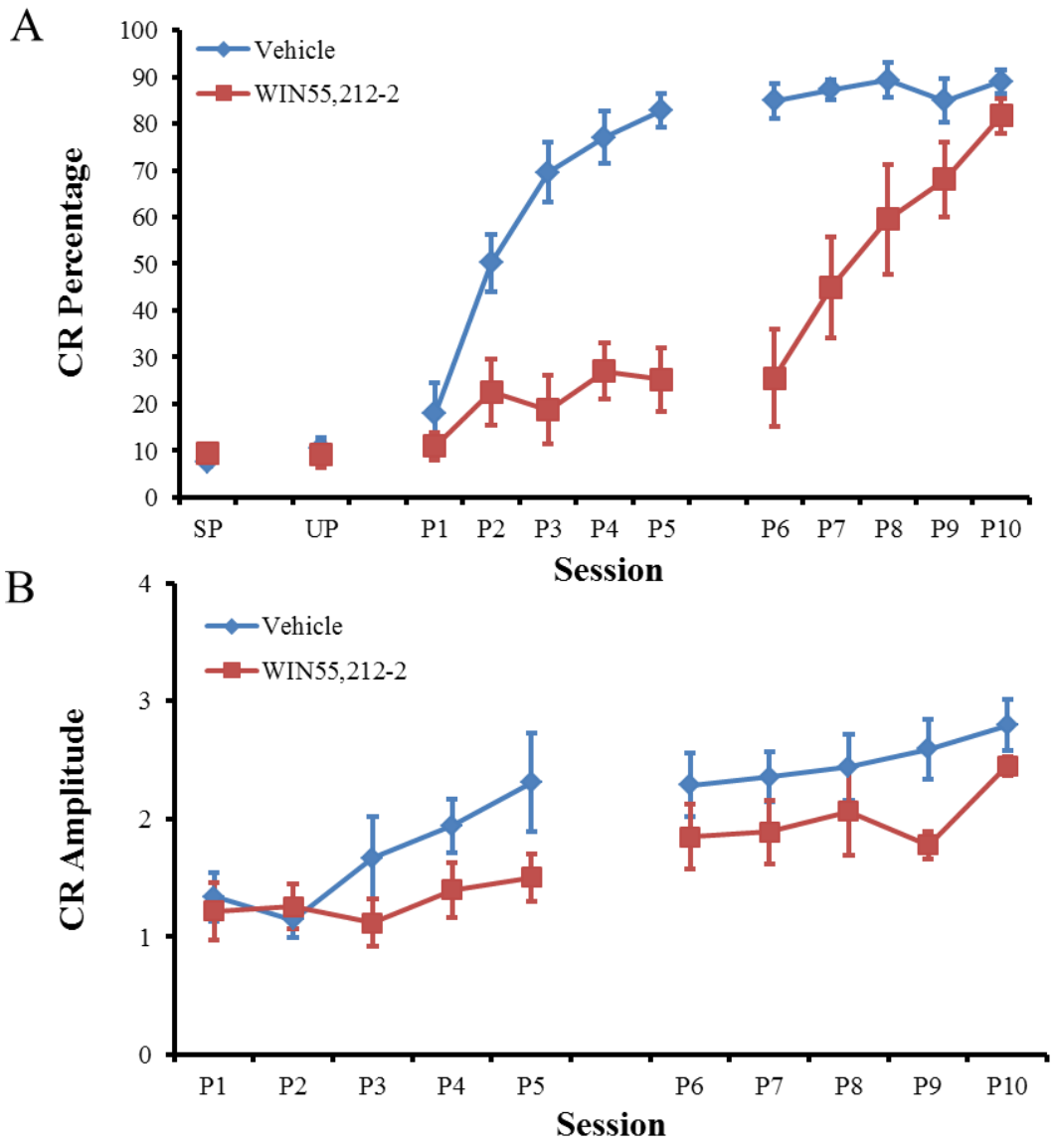

Figure 27. Mean ± SE conditioned response (CR) percentage (A) and amplitudes (B) for rats (n = 8 per group) given training with WIN55,212-2 infusions into the cerebellar cortex (SP, UP, P1-P5) of during long delay (750 ms CS) conditioning.

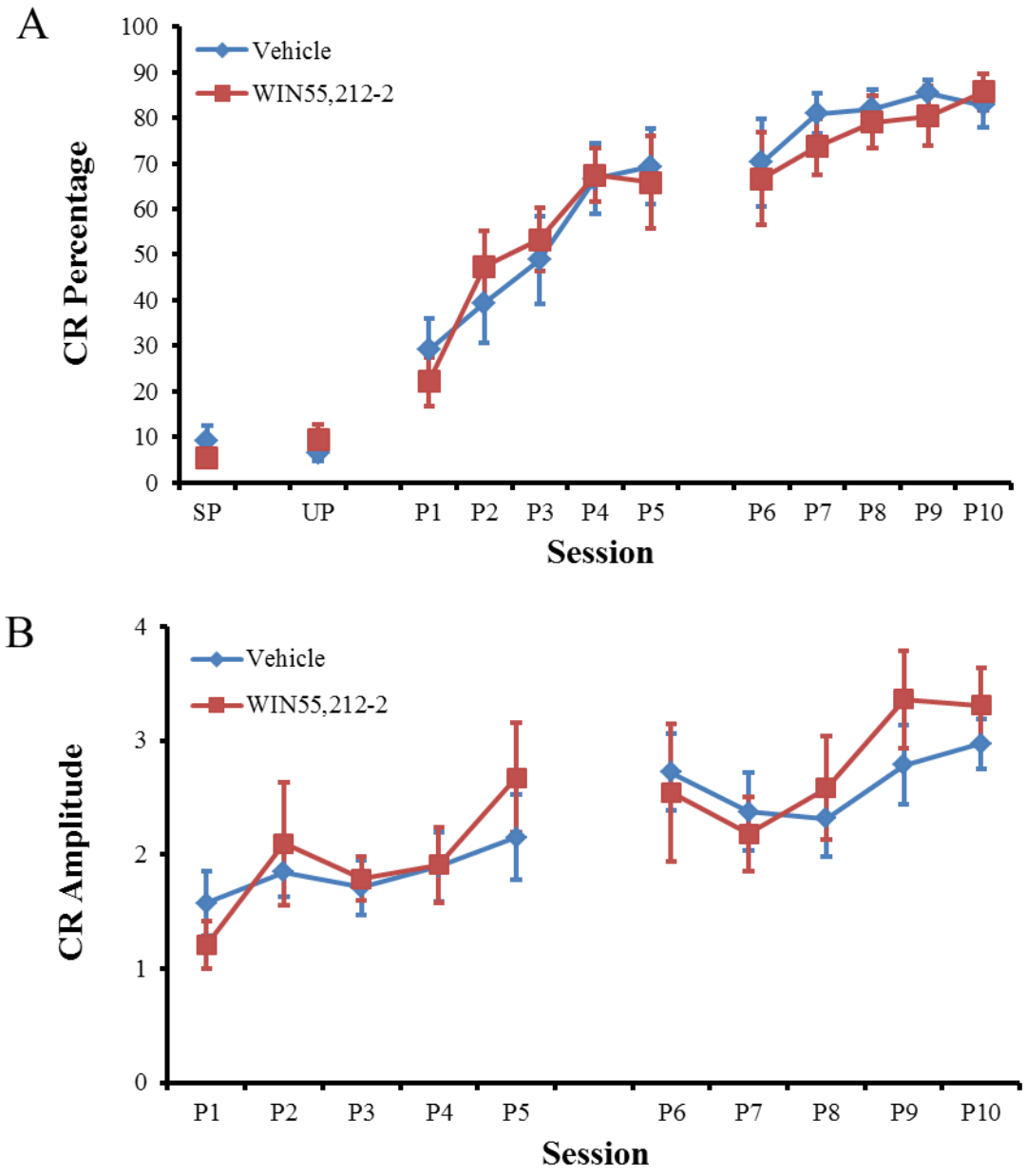

Figure 28. Mean ± SE conditioned response (CR) percentage (A) and amplitudes (B) for rats (n = 8 per group) given training with WIN55,212-2 infusions into the cerebellar cortex (SP, UP, P1-P5) of during trace (250 ms CS; 500 ms trace) conditioning.

CHAPTER 4. CANNABINOID AGONIST ADMINISTRATION WITHIN THE CEREBELLAR CORTEX IMPAIRS ACQUISITION AND ENHANCES CONSOLIDATION OF EYEBLINK CONDITIONING

Cannabis saltiva remains the most widely used psychoactive drug in the United States with rates increasing over the past decade (Substance Abuse Mental Health Services Administration, 2009). The major active ingredient in cannabis is delta-9 THC, which binds to cannabinoid-1 (CB1R) and cannabinoid-2 receptors (CB2R; Gaoni and Mechoulam, 1964; Devane et al., 1988). CB1Rs are the most abundant G-protein coupled receptor in the mammalian brain with the highest density of receptors within the cerebellar cortex (Herkenham et al., 1990; 1991). Two endogenous cannabinoids (endocannabinoids) have been discovered, 2-Arachidonoylglycerol (2-AG) and Anandamide. Cannabinoid receptors have many diverse roles throughout the brain but more recently have been implicated in synaptic plasticity during learning and memory. CB1Rs within the cerebellar cortex are important for the induction of long-term depression (LTD) in parallel fiber synapses with Purkinje cells *in vitro* (Lévénés, et al., 1998; Safo and Regher, 2005; van Beugen, Nagaraja, and Hansel, 2006). Systemic or genetic CB1R manipulations in humans and non-human mammals have been shown to result in decrements in the rate of eyeblink conditioning, a type of associative learning that depends on the cerebellum (Kishimoto and Kano, 2006; Skosnik et al., 2007; Edwards et al., 2008; Steinmetz and Freeman, 2010; 2011; 2013). The hypothesis drawn from these studies is that the decrement in acquisition of eyeblink conditioning is the result of impaired LTD formation within Purkinje cells.

Less is known about the role of cerebellar cannabinoid receptors in memory consolidation. Studies examining the role of CB1Rs and endocannabinoids during consolidation have been conducted mostly in the hippocampus, amygdala, and prefrontal cortex (Wise, Thorpe, and Lichtman, 2009; Tan et al., 2011). Each of these areas is important in memory consolidation for different tasks but contain moderate levels of CB1Rs as compared to the cerebellar cortex. To date, there are no studies examining the role of CB1Rs during memory consolidation within the cerebellum. As mentioned above, the cerebellar cortex contains the highest density of CB1Rs and also is an important site of plasticity within the brain (Safo and Regehr, 2005). Thus, it is important to investigate the role of cannabinoid receptors and endocannabinoids in memory formation and consolidation in order to further understand the basic mechanisms underlying memory.

The current study employed cerebellum-dependent delay eyeblink conditioning to examine the role of CB1Rs and endocannabinoids during both learning and memory consolidation. Eyeblink conditioning involves the presentation of a conditioned stimulus (CS) that does not elicit eyelid closure prior to learning, which terminates with an unconditioned stimulus (US) that does elicit eyelid closure. After repeated CS-US pairings an adaptively timed eyelid closure conditioning response (CR) emerges prior to the onset of the US. The cerebellum, specifically the cerebellar cortex and anterior interpositus nucleus, is essential for acquisition and retention of the CR (McCormick & Thompson, 1984). The current study examined the role of cannabinoids in memory consolidation after learning eyeblink conditioning. Rats received 5 daily sessions of eyeblink conditioning in which a CB1R agonist (WIN55,212-2), CB1R antagonist (SR141716A), monoacylglycerol lipase inhibitor (JZL-184; increases endocannabinoid

levels), or diacylglycerol lipase inhibitor (THL; decreases endocannabinoid levels) was infused before or after each session. Cannula were targeted to the base of the primary fissure in the cerebellar cortex. This area, termed the EBC microzone, was previously examined as the major location implicated in the acquisition of delay EBC (Steinmetz and Freeman, in prep). It was hypothesized that administration of WIN55,212-2 and JZL-184 would impair eyeblink conditioning when administered before or after training sessions.

Methods

Subjects

The subjects were 113 male Long-Evans rats (250-300 g). The rats were housed in the animal colony in Spence Laboratories of Psychology at the University of Iowa (Iowa City, IA). All rats were maintained on a 12 h light/dark cycle and given *ad libitum* access to food and water.

Surgery

One week before training, rats were removed from their home cages and anesthetized with isoflurane. At the onset of anesthesia, the rats were fitted with differential electromyography (EMG) electrodes (stainless steel) implanted into the upper left orbicularis oculi muscle. The reference electrode was a silver wire attached to a stainless steel skull screw. The EMG electrode leads terminated in gold pins in a plastic connector. A bipolar stimulating electrode (Plastics One, Roanoke, VA) for delivering the shock US was implanted subdermally, caudal to the left eye. A 23 gauge guide cannula was implanted dorsal to the division of the anterior lobe and lobule HVI of the cerebellar

cortex. A 30 gauge stylet was inserted into the guide cannula and extended 0.5 mm from the end of the guide. The stereotaxic coordinates taken from bregma for the cannula was 11.4 mm posterior, 3.0 mm lateral, and 3.2 mm ventral. The plastic connector housing the EMG electrode leads, bipolar stimulating electrode, the guide cannula, and skull screws were secured to the skull with Osteobond copolymer bone cement.

Infusion Procedure

Before each infusion, the stylet was removed from the guide cannula and replaced with a 30 gauge infusion cannula that extended 1.0 mm beyond the guide cannula. The infusion cannula was connected to polyethylene tubing (PE 10), which was connected to a 10 µl gas tight syringe (Hamilton, Reno, NV). The syringe was placed in an infusion pump (Harvard Apparatus, Holliston, MA), and 0.5 µl of each drug was infused over 5 minutes at a rate of 6.0 µl/h. After the infusion, the infusion cannula was left in place for 3 mins in order to allow diffusion of the drug. Following this time, the infusion cannula was removed and replaced with the 30 gauge stylet.

Apparatus

The conditioning apparatus consisted of two small-animal sound-attenuating chambers (BRS/LVE, Laurel, MD). Within each sound-attenuating chamber was a small-animal operant chamber (BRS/LVE) in which the rats were kept during conditioning. One wall of the operant chamber is fitted with two speakers that independently produce tones of up to 120 dB (sound pressure level) with a frequency range of 1000-9000 Hz. An exhaust fan on one of the walls provided a 65 dB masking white noise. The tone CS used in training was a 2000 Hz pure tone (85 dB). The electrode leads from the rat's headstage was connected to peripheral equipment by lightweight cables that allowed the rat to move freely during conditioning. A desktop computer was connected to the peripheral

equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs, Raleigh, NC). One circuit was used to deliver the shock stimulus through a stimulus isolator (model 365A; World Precision Instruments, Sarasota, FL). Another circuit amplified differentially (gain, 2000; sampling rate, 250 Hz), filtered (500-5000 Hz), and integrated (time constant, 20 ms) EMG activity. The intensity of the shock US was set for eliciting a discrete eyeblink (2.5 mA; 60 Hz; constant current).

Conditioning Procedure

The rats were allowed to adapt to the training environment for 5 min before each training session. Paired training sessions occurred in which 5 blocks of nine paired CS–US presentations and 1 CS-alone probe trial were presented. Fewer trials were administered than previous studies in order to avoid within-session consolidation. The CS was a 400-ms tone (2 kHz; 85 dB) and terminated with a 25-ms shock US. The US intensity was set such that a rat would elicit eyelid closure and slight head movement (2.5 mA). CRs were defined as EMG activity that exceeds a threshold of 0.4 units (amplified and integrated units) above the baseline mean during the CS period after 80 msec. EMG responses that exceeded the threshold during the first 80 msec of the CS period were defined as startle responses to the CS. On CS-alone probe trials, the duration for scoring CRs was extended beyond the CS to the end of the trial period (1.0 sec). URs were defined as responses that crossed the threshold after the onset of the US. The rats were randomly assigned to receive either vehicle, WIN55,212-2 (10 µg/µL), SR141716A (1.0 µg/µL), JZL-184 (1 µM), or THL (1 µM) infusions into the cerebellar cortex. Additionally, these rats were randomly assigned to receive the infusions at different time points. These time points were immediately before the session (pre), immediately following the session (0 hr), 1 hour following the session (1 hr), 3 hours following the

session (3 hr), or 6 hours following the session (6 hr). Vehicle treated animals were randomly assigned to receive infusions at one of the time points before or after the sessions. These time points were used for both WIN55,212-2 and SR141716A. JZL-184 and THL administration were limited to pre, 1 hr, and 3 hr due to the longer receptor binding times compared to WIN55,212-2 and SR141716A. Rats received 5 session of training with these infusions.

Histology

After training, the rats were perfused with a lethal injection of sodium pentobarbital (150 mg/kg) and transcardially perfused with physiological saline followed by 10% neutral buffered formalin (Surgipath, Richmond, IL). After perfusion, the brains were cryo-protected in a 30% sucrose in formalin solution, and subsequently sectioned at 50 µm with a sliding microtome. Sections were then stained with thionin. The location of the cannula placements were verified using a light microscope (Leica DMLS, Wetzlar, Germany) and a stereotaxic brain atlas (Paxinos and Watson, 1998).

Pharmacological Manipulations of CB1Rs

The CB1R/CB2R agonist WIN55,212-2 ([R]-(+)-[2,3-Dihydro-5-methyl-3- **(**4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-napthalenylmethanone), CB1R antagonist SR141716A [5-(4-Chlorophenyl)-1- (2,4-dichloro-phenyl)- 4-methyl-*N*- (piperidin-1-yl)-1*H*-pyrazole-3-carboxamide], the monoacylglycerol lipase inhibitor JZL-184 [4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate] or the diacylglycerol lipase inhibitor THL [(*S*)-((*S*)-1-((2*S*,3*S*)-3-Hexyl-4-oxooxetan-2-yl)tridecan-2-yl) 2-formamido-4-methylpentanoate] was administered intracranially to rats. WIN55,212-2 and THL were dissolved in a vehicle of 1:1:18 solution of ethanol, cremaphor, saline and SR141716A and JZL-184 were dissolved in a

vehicle of 1:1:18 solution of ethanol, tween 80, saline. WIN55,212-2, JZL-184 and THL were purchased from Sigma/RBI. SR141716A was a generous gift from NIDA Drug Supply Program (Rockville, MD).

Results

Cannula placements were verified with light microscopy. A representative cannula placement is displayed in Figure 29. Four animals were removed from the analysis due to cannula placements that were outside of the targeted area of the cerebellar cortex.

Experiment 1: WIN55,212-2

It has been previously reported by our laboratory that WIN55,212-2 administration prior to each acquisition session impaired eyeblink conditioning (Steinmetz and Freeman, 2010); however, post-training administration of WIN55,212-2 during the memory consolidation phase has not been examined. To examine memory consolidation animals underwent 5 sessions of CS-US paired training with administration of the CB1R agonist WIN55,212-2 into the cerebellar cortex either immediately before training or at different interval after training. The post-training intervals at which WIN55,212-2 was infused included immediately following training (0 hr), 1 hr , 3 hr, or 6 hr post-training. In order to examine the effects of the administration of WIN55,212-2 on learning at the different time points a 6 (Group) x 5 (Session) repeated measures ANOVA was conducted for the CR percentage data (Figure 30A; B). A significant interaction was found, $F(4,32)=4.520$, $p < .001$ and post-hoc tests (Tukey-Kramer) indicated that WIN55,212-2 administered prior to each training session resulted in a

significant decrease in the percentage of CRs on Sessions 2-5 as compared to the vehicle group ($p<.01$). However, there was a significant increase in CRs during Sessions 2 and 3 when WIN55,212-2 was administered 1 hr following training as compared to the vehicle group. There were no other significant differences when WIN55,212-2 was given immediately following the session or at 3 or 6 hr following the session. In order to further examine the effects, each session of 50 trials was divided into 5 blocks of 10 trials (Figure 31). A 6 (Group) x 25 (Block) repeated measures ANOVA was conducted and found a significant interaction, $F(24,120)= 2.466$, $p <.001$. Post-hoc tests revealed that administration prior to each session resulted in decreased CRs during blocks 7-25. The enhancement in learning from administration 1 hr following the session was found as early as block 1 of the second session (block 6 overall). This enhancement was prevalent until block 12.

Additional measures of CRs, including CR amplitude and timing (onset latencies and peak latency), were examined to further understand the role of cannabinoid receptor activation during and following training. A significant Group x Session interaction was found, $F(5,35)=2.528$, $p = .047$ for the CR amplitude data (Figure 32A; B). Post-hoc tests indicated that WIN55,212-2 administered before the session decreased the amplitude of CRs during sessions 3-5. Similar to results for CR percentage, administration of WIN55,212-2 1 hr following training increased CR amplitude during session 2. No other significant differences were found for the CR amplitude data. CR timing was not altered for any of the groups, but main effects of sessions were found for both CR onset latency, $F(4,20)=7.528$, $p <.001$, and CR peak latency, $F(4,20)=3.957$, $p =.004$.

Experiment 2: SR141716A

Systemic administration of SR141716A has been reported to impair cerebellar learning, although the magnitude of the impairment was lower than with WIN55,212-2 (Kishimoto and Kano, 2005; Steinmetz and Freeman, 2010). Animals underwent 5 sessions of paired training with administration of SR141716A given either before or after each training session. The post-training intervals at which drug was administered were the same as with WIN55,212-2. A 6 (Group) x 5 (Session) repeated measures ANOVA was conducted for the CR percentage data (Figure 33A; B) and found a significant interaction, $F(4,31)=1.822$, $p =.041$. Post-hoc tests indicated that CR percentage was impaired relative to the vehicle group on sessions 3-5 when SR141716A was administered 1 hr following training ($p<.01$). There were no other significant differences when SR141716A was given immediately before or after 3 or 6 hr following the session. Block data (5 blocks of 10 trials) were also examined (Figure 34). A 6 (Group) x 25 (Block) repeated measures ANOVA was conducted and found a significant interaction, $F(24,120)= 1.416$, $p = .048$. Post-hoc tests revealed that administration of SR141716A 1 hr following the session caused a deficit during blocks 12-19 as compared to vehicle.

To further examine the role of SR141716A during acquisition and consolidation CR amplitude and timing were examined. There were no significant interactions or main effects involving the group factor. However, there was a main effect of Session for CR amplitude (Figure 35), $F(4,20)=11.600$, $p <.001$, onset latency, $F(4,20)=2.748$, $p = .046$, and peak latency, $F(4,20)=17.167$, $p <.001$, which indicate that all groups showed changes across training sessions.

Experiment 3: JZL-184

Endocannabinoids have been shown to be important in the induction of synaptic plasticity in Purkinje cells *in vitro* (Safo and Regehr, 2005). However it is not known if endocannabinoids are necessary for cerebellum-dependent learning *in vivo*. To examine the role of endocannabinoids in acquisition and consolidation of cerebellum-dependent learning the monoacylglycerol lipase (MAGL) inhibitor JZL-184 was infused into the cerebellar cortex before 5 consecutive sessions of eyeblink conditioning either prior to the session or following the session, at 1 hr or 3 hr post training. MAGL is the primary enzyme necessary for the degradation of the endocannabinoid 2-arachidonoylglycerol (2-AG), the primary endocannabinoid in the cerebellum. Thus, inhibition of MAGL results in an increase in 2-AG levels within the cerebellar cortex. A 4 (Group) x 5 (Session) repeated measures ANOVA was conducted for the CR percentage data and found a significant interaction, $F(4,21)=4.219$, $p < .001$ (Figure 36A; B). Post-hoc tests revealed that when JZL-184 was administered prior to training there were significantly fewer CRs during Sessions 2-5 as compared to the vehicle group ($p<.01$). In contrast, there was a significant increase in CR percentage during Session 2 when JZL-184 was administered 1 hr following training. There were no significant group differences when JZL-184 was given 3 hr following the session. Block data were examined to further understand how endocannabinoids are involved in acquisition and consolidation (Figure 37). Each session was broken down into 5 blocks containing 10 trials resulting in 25 blocks. A 6 (Group) x 25 (Block) repeated measures ANOVA was conducted and found a significant interaction, $F(24,72)= 3.192$, $p < .001$. Post-hoc tests revealed that JZL-184 administration prior to the session caused a deficit during blocks 9-25, whereas when

administered 1 hr following training an enhancement occurred during blocks 6-10 as compared to the vehicle group.

CR amplitude and latency (onset and peak) were examined across the 5 sessions. A significant Group x Session interaction was found for the CR amplitude data, $F(4,12)=2.069$, $p =.026$ (Figure 38A; B). Post-hoc tests revealed a decrease in CR amplitude during sessions 4 and 5 when JZL-184 was administered prior to each session and an increase in CR amplitude during sessions 2 and 3 when administered 1 hr following each session. There was a significant main effect of session for CR peak latency, $F(4,12)=6.181$, $p <.001$, which reflected a change across sessions for all groups. No significant effects were found for CR onset latency.

Experiment 4: THL

In contrast to JZL-184, administration of THL results in a decrease in 2-AG levels. THL inhibits 2-AG formation via blocking diacylglycerol lipase (DAGL). In order to test the effects of THL during acquisition and consolidation infusions occurred either before or after (1 hr or 3 hr) 5 consecutive CS-US paired training sessions. A 6 (Group) x 5 (Session) repeated measures ANOVA was conducted for CR percentage and found a significant interaction, $F(4,21)=2.638$, $p =.007$ (Figure 39A; B). Post-hoc tests indicated that THL administration prior to training produced a reduction in CR percentage on Sessions 2-5 as compared to the vehicle group ($p<.01$). Additionally, there was a significant decrease in CR percentage during sessions 2-5 when THL was administered 1 hr following training. There were no significant group differences when THL was given immediately following the session or 3 hr following the session. In order to further

examine the effects of THL each session of 50 trials was divided into 5 blocks of 10 trials (Figure 40). A 4 (Group) x 25 (Block) repeated measures ANOVA was conducted and found a significant interaction, $F(24,72)= 2.855$, $p < .001$. Post-hoc tests indicated that THL administration prior to each session and 1 hr following each session resulted in a deficit in CR percentage during blocks 9-25 and 12-25 respectively.

Additional measures of the CR, amplitude and latency, were examined for the 5 CS-US training sessions. For CR amplitude, a significant Group x Session interaction was found, $F(4,12)=2.093$, $p =.024$ (Figure 41). Lower amplitude CRs were evident in Sessions 4 and 5 in the group given THL prior to each session and in the group given THL 1 hr following each session. An ANOVA conducted for CR peak latency revealed a main effect of Session, $F(4,12)=2.805$, $p =.030$, which was due to a change in latency across sessions. There were no significant group differences for the CR onset latency data.

Discussion

This is the first study to report that cannabinoid receptor activation results in both impairments and enhancements in acquisition and memory consolidation within the same brain area. Similar to previous findings reported from our laboratory CB1R agonist administration before each training session impaired acquisition of eyeblink conditioning. We extended these findings to show that increasing or decreasing endocannabinoid levels within the cerebellar cortex also resulted in impaired acquisition of eyeblink conditioning when done before each training session. However, an increase in learning was found with cannabinoid receptor activation or endocannabinoid enhancement 1 hr following each

training session. The reverse, a decrement in learning, occurred if cannabinoid receptors were blocked or endocannabinoids were decreased 1 hr after each training session. The time-course of these consolidation effects occurred within a relatively small window. Manipulations made either immediately following the session or 3 or 6 hr after the session did not affect memory consolidation. These results indicate a dynamic role for cannabinoid receptors and endocannabinoids in consolidation within the cerebellum.

Manipulations of cannabinoid receptors, via agonist/antagonist action or removal of the receptors in knockout mice, result in impaired *in vivo* cerebellar learning (Kishimoto and Kano 2006; Steinmetz and Freeman, 2010). The current set of experiments replicated and extended these results. We report that manipulating cannabinoid receptor activity within the cerebellar cortex by either a CB1R agonist (WIN55,212-2), increasing endocannabinoid levels (JZL), or decreasing endocannabinoid levels (THL) resulted in impaired acquisition if administered prior to training. These results indicate that maintaining normal cannabinoid receptor function and endocannabinoid levels is important in establishing cerebellar learning. Both cannabinoid receptors and endocannabinoids have been reported to be important for the induction of LTD in the cerebellar cortex *in vitro* (Levenes, et al., 1998*;* Safo and Regehr, 2005*).* The current findings are the first to indicate that normal endocannabinoid function *in vivo* is important for cerebellar learning. Cannabinoid receptors are important in the formation of LTD within Purkinje cells (Lévénés, et al., 1998; Safo and Regehr, 2005). Induction of LTD in the cerebellar cortex is hypothesized as an important early step in the acquisition of eyeblink conditioning (Mauk & Donegan, 1997); thus, manipulations of cannabinoid receptors and endocannabinoids are hypothesized to impair cerebellum-dependent

learning by impairing LTD formation.

The role of cannabinoid receptors during consolidation of memories in areas such as the hippocampus, amygdala, and prefrontal cortex has been studied, but not in the cerebellum, even though this area has the highest density of cannabinoid receptors (Herkenham et al., 1990; 1991). In the current study we manipulated cannabinoid receptor activity and endocannabinoid levels within the cerebellar cortex during memory consolidation with post-training infusions. Infusing WIN55,212-2 or JZL into the cerebellar cortex after training sessions resulted in enhanced learning, whereas the administration of SR141716A and THL impaired learning. The post-training effects of these manipulations are hypothesized to affect the mechanisms underlying long-lasting Purkinje cell LTD. There are two potential locations within the cerebellar cortex that are involved in the mechanisms underlying LTD within Purkinje cells. First, activation of cannabinoid receptors have been shown to be important in the induction of LTD and LTP in parallel fiber to Purkinje cell synapses (Safo and Regehr, 2005). Cannabinoid receptor activation has been reported to cause AMPA receptor endocytosis within the ventral tegmental area (VTA) that resulted in LTD (Liu et al., 2010). Thus, CB1R activation during the consolidation phase could be increasing AMPA receptor endocytosis in Purkinje cells which in turn would increase LTD formation. Second, CB1R activation has been reported to modulate K+ channels on interneurons (Diana and Marty, 2003). This increase of K+ channels on interneurons has been shown to enhance GABA release onto Purkinje cells and also learning of delay eyeblink conditioning (Williams et al., 2012). It is thus hypothesized that CB1R activation leads to enhanced consolidation through modulation of interneurons, parallel fibers, or both.

This is the first study to show that post-training manipulations of cannabinoid receptors or endocannabinoid levels within the cerebellar cortex alter memory consolidation. Consolidation underlying eyeblink conditioning was facilitated by enhancement of cannabinoid receptor action and impaired by reducing cannabinoid receptor action when given 1 hour after training. The time window for these effects was relatively narrow, with cannabinoid effects at 1 hour after training, but not after 0, 3, or 6 hours. Furthermore, this is the first study to show that cannabinoid activation impairs and enhances memory within the same brain area. The only thing separating the impairment and the enhancement is the time of the infusion. Thus, cannabinoid and endocannabinoid manipulations within the cerebellar cortex made at discrete time points resulted in either impairments (before training) or enhancements (following training) in learning.

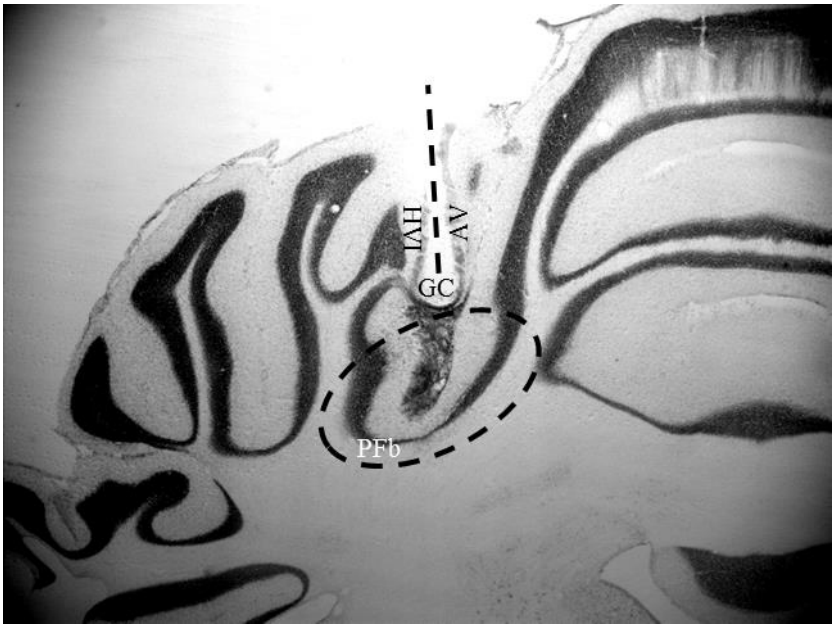

Figure 29. Coronal sections of the cerebellum showing representative cannula placements for eyeblink conditioning microzone (primary fissure base; PFb) of the cerebellar cortex. HVI: lobule HVI, AV: anterior lobe, GC: guide cannula. Magnification is 2.5x.

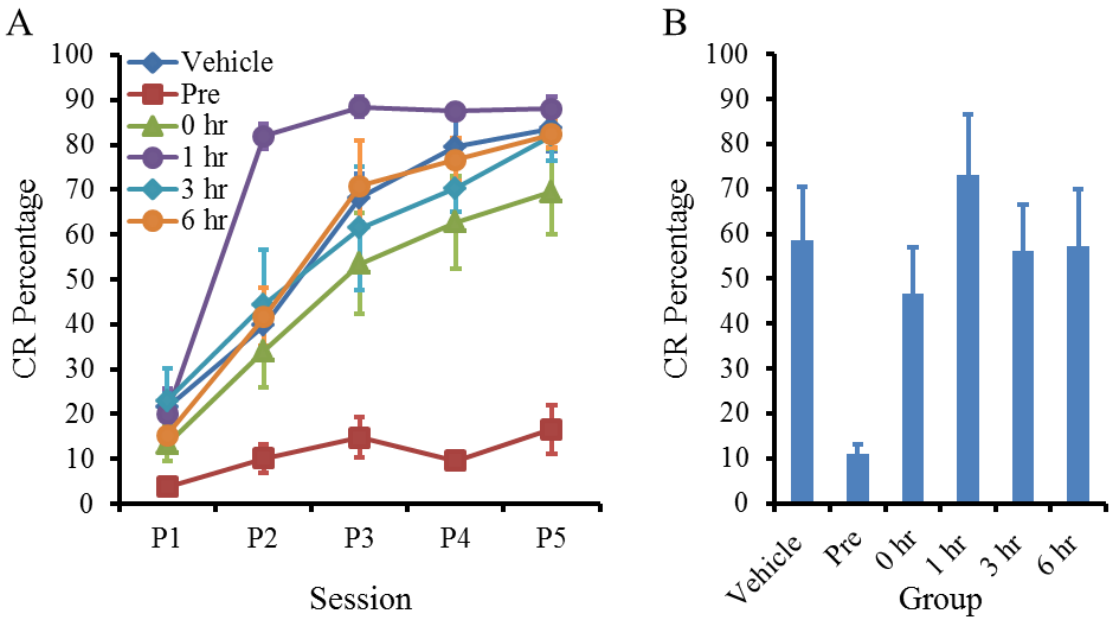

Figure 30. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with WIN55,212-2 infusions during 5 paired sessions (A**:** P1-P5). Infusions were made either were immediately before the session (pre), immediately following the session (0 hr), 1 hour following the session (1 hr), 3 hours following the session (3 hr), or 6 hours following the session (6 hr). **B:** Mean ± SE conditioned response (CR) percentages collapsed for P1-P5.

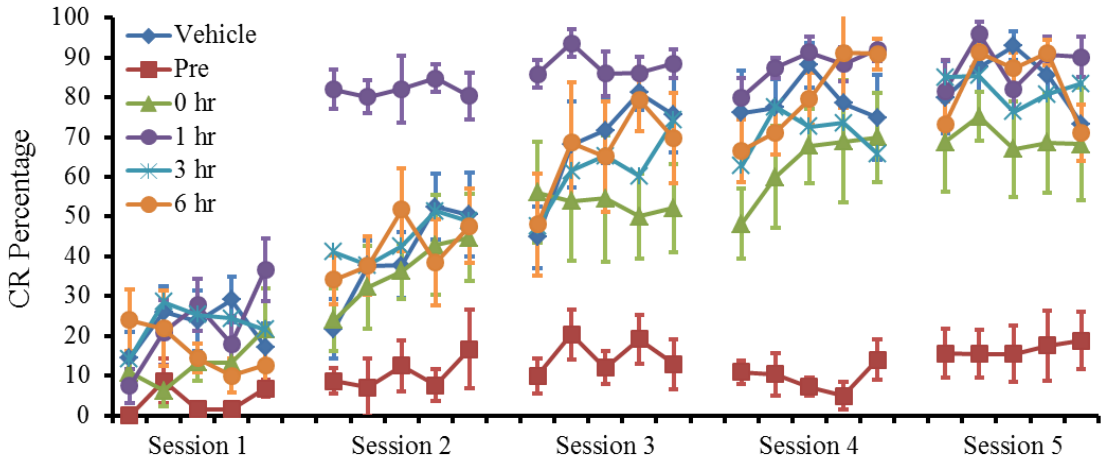

Figure 31.Block data (5 blocks of 10 trials) for Sessions 1-5 for animals administered either vehicle or WIN55,212-2 at differing time intervals.

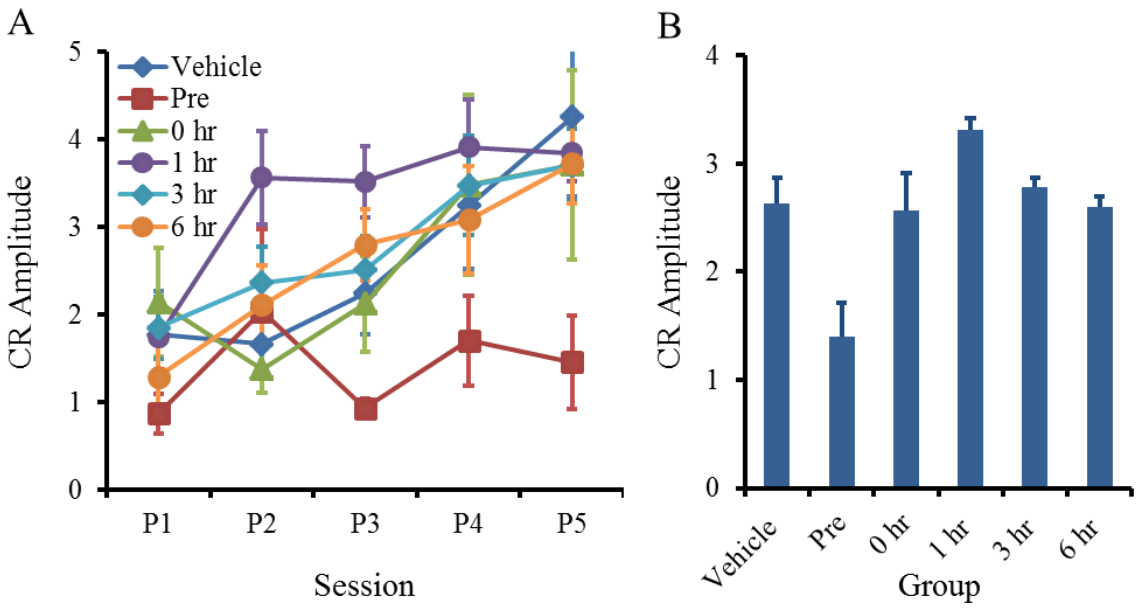

Figure 32. Mean ± SE conditioned response (CR) amplitudes for rats given training with WIN55,212-2 during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), immediately following the session (0 hr), 1 hour following the session (1 hr), 3 hours following the session (3 hr), or 6 hours following the session (6 hr). **B:** Mean ± SE conditioned response (CR) amplitudess collapsed for P1-P5.

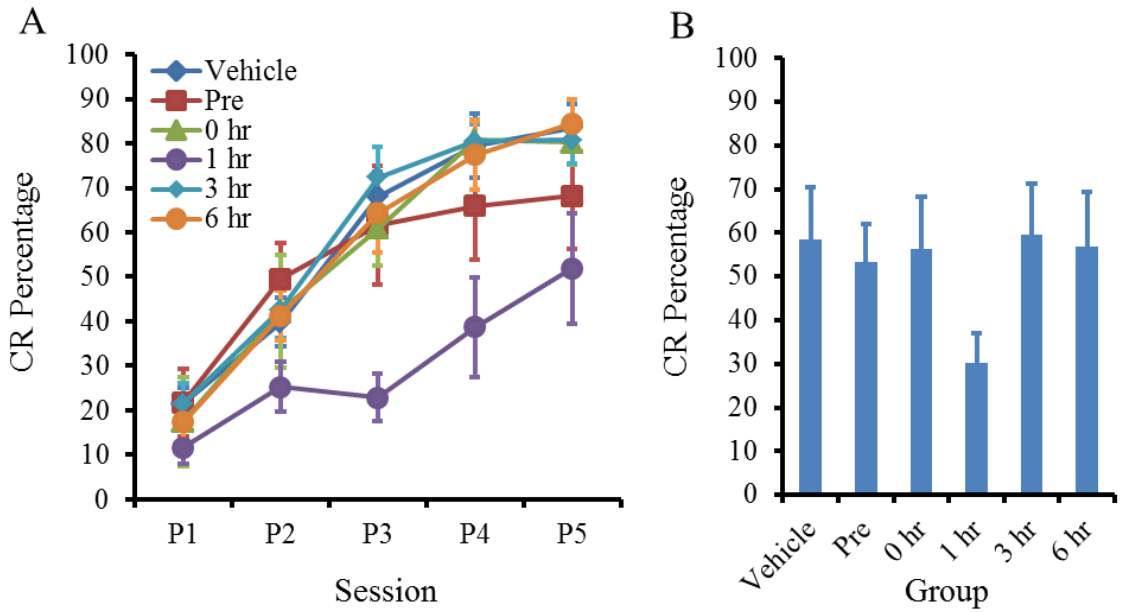

Figure 33. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with SR141716A infusions during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), immediately following the session (0 hr), 1 hour following the session (1 hr), 3 hours following the session (3 hr), or 6 hours following the session (6 hr). **B:** Mean ± SE conditioned response (CR) percentages collapsed for P1-P5.

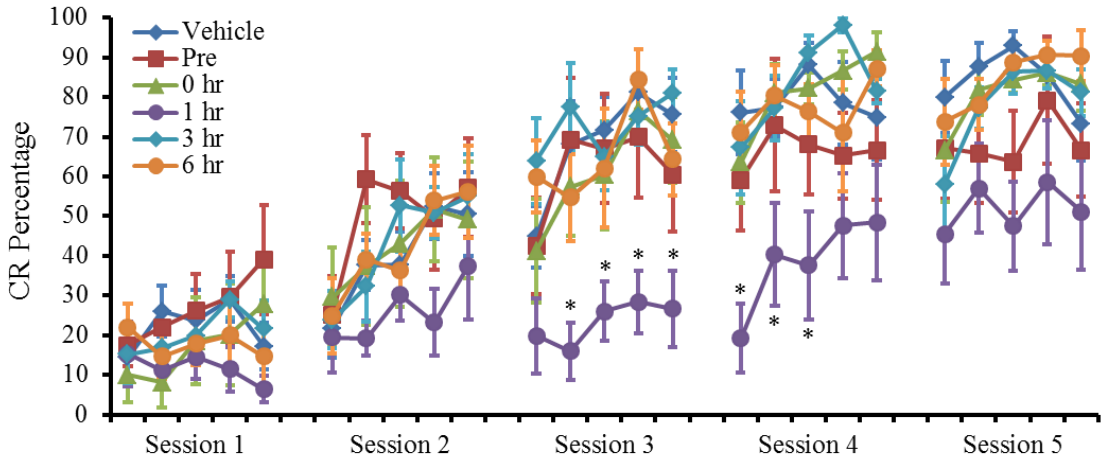

Figure 34. Block data (5 blocks of 10 trials) for Sessions 1-5 for animals administered either vehicle or SR141716A at differing time intervals. Asterisks (*) represent significant differences ($p < .05$).

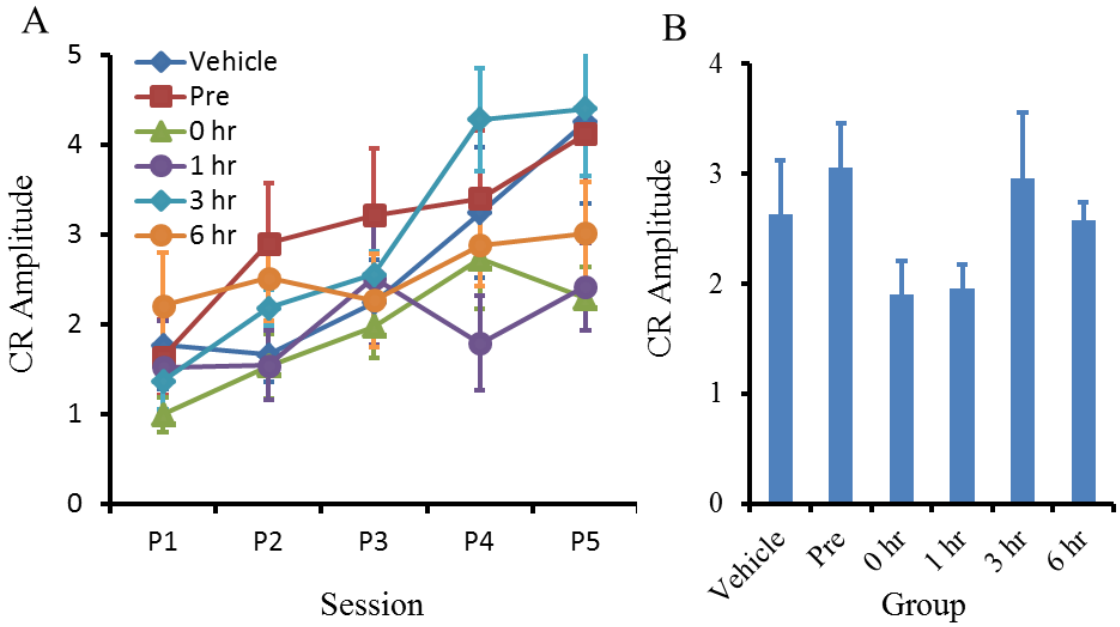

Figure 35. Mean ± SE conditioned response (CR) amplitudes for rats given training with SR141716A infusions during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), immediately following the session (0 hr), 1 hour following the session (1 hr), 3 hours following the session (3 hr), or 6 hours following the session (6 hr). **B:** Mean ± SE conditioned response (CR) amplitudes collapsed for P1-P5.

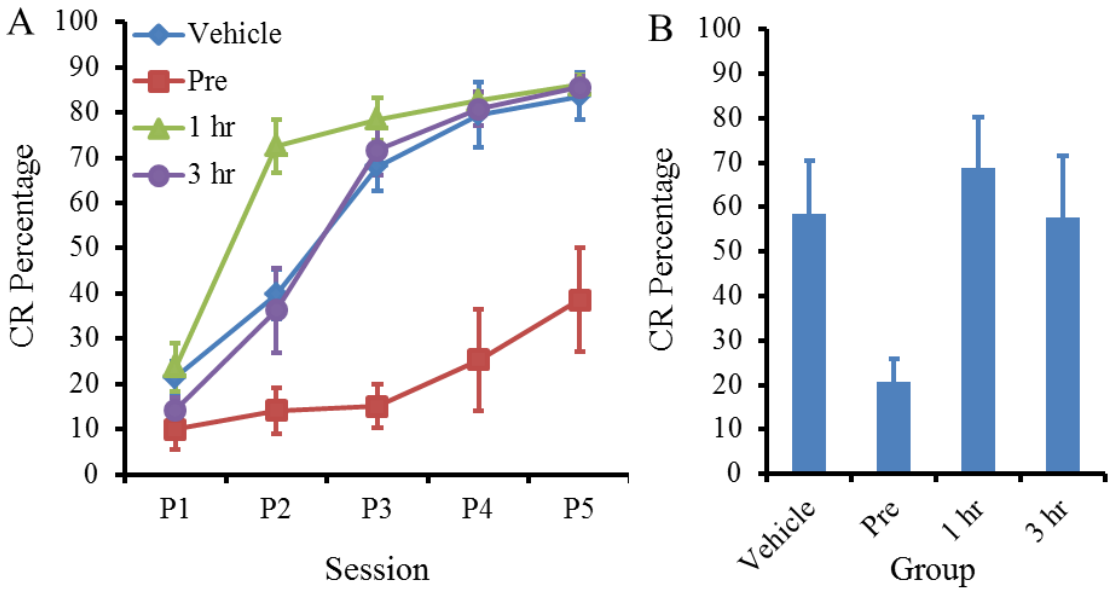

Figure 36. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with JZL-184 infusions during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), 1 hour following the session (1 hr), or 3 hours following the session (3 hr). **B:** Mean ± SE conditioned response (CR) percentages collapsed for P1-P5.

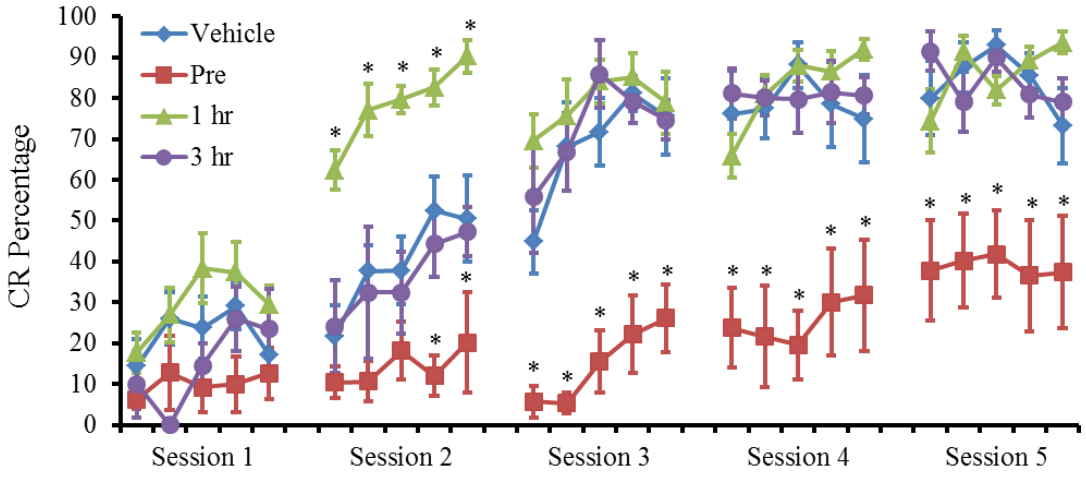

Figure 37. Block data (5 blocks of 10 trials) for Sessions 1-5 for animals administered either vehicle or JZL-184 at differing time intervals. Asterisks (*) represent significant differences ($p < .05$).

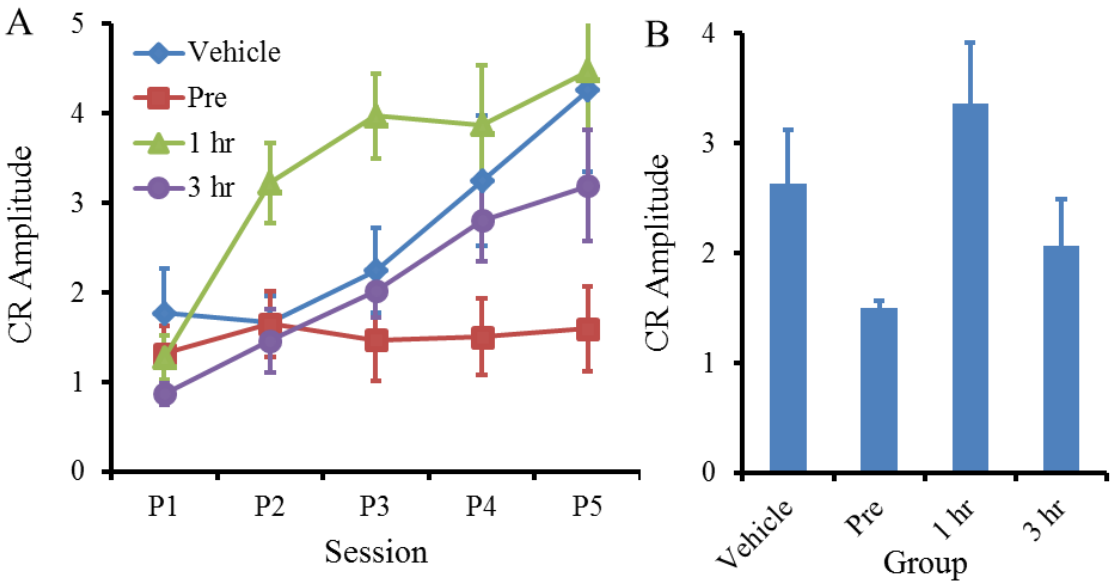

Figure 38. Mean ± SE conditioned response (CR) amplitudes for rats given training with JZL-184 during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), 1 hour following the session (1 hr), or 3 hours following the session (3 hr). B: Mean ± SE conditioned response (CR) amplitudes collapsed for P1-P5.

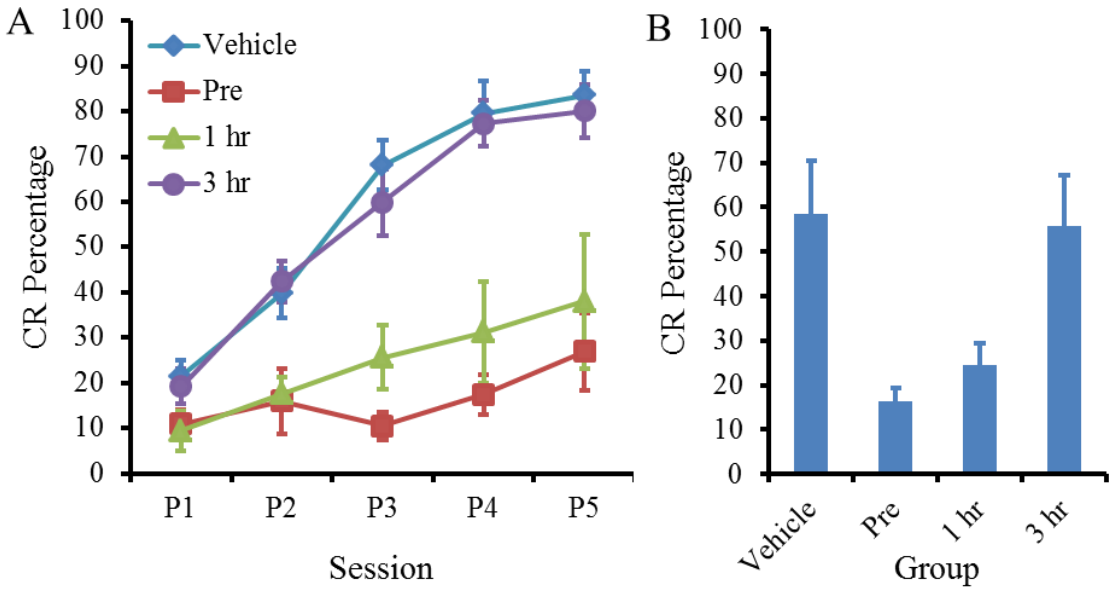

Figure 39. Mean ± SE conditioned response (CR) percentage for rats (n = 8 per group) given training with THL infusions during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), 1 hour following the session (1 hr), or 3 hours following the session (3 hr). B: Mean ± SE conditioned response (CR) percentages collapsed for P1-P5.

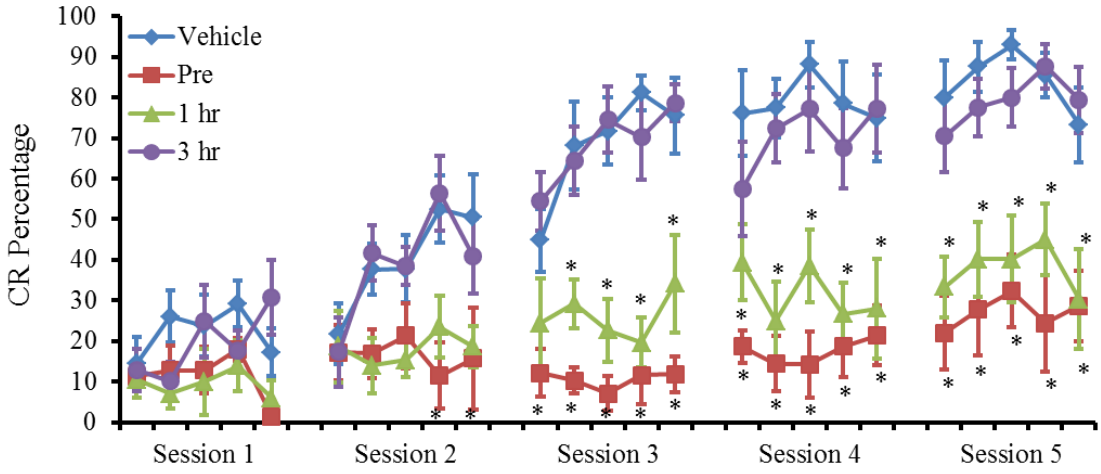

Figure 40. Block data (5 blocks of 10 trials) for Sessions 1-5 for animals administered either vehicle or THL at differing time intervals. Asterisks (*) represent significant differences ($p < .05$).

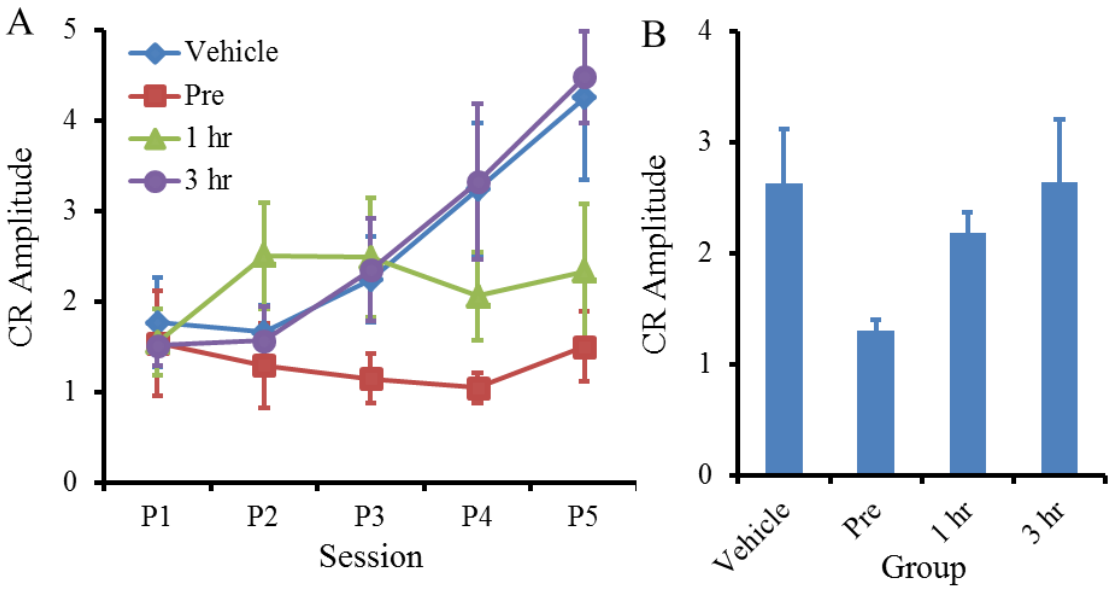

Figure 41. Mean ± SE conditioned response (CR) amplitudes for rats given training with THL during 5 paired sessions (**A:** P1-P5). Infusions were made either were immediately before the session (pre), 1 hour following the session (1 hr), or 3 hours following the session (3 hr). B: Mean ± SE conditioned response (CR) amplitudes collapsed for P1-P5.

CHAPTER 5. GENERAL DISCUSSION

Cannabinoid receptors are the most highly expressed G-protein coupled receptors in the mammalian brain. The receptor is expressed at low to moderate levels throughout the brain but the highest levels lie within the cerebellar cortex (Herkenham et al., 1990; 1991). Examination of the role of these receptors in learning and memory consolidation has focused traditionally on the hippocampus and amygdala, areas which contain moderate levels of the receptors as compared to the cerebellum. The focus in these experiments is on the effects of cannabinoid compounds on cerebellum-dependent learning. Previous examinations looking at the impact of CB1Rs on cerebellar learning have employed eyeblink conditioning. The first such study used CB1R knockout mice that were trained on delay or trace eyeblink conditioning. It was reported that delay conditioning was impaired while trace conditioning was not, leading to the hypothesis that cerebellar cortical CB1Rs are necessary for eyeblink conditioning (Kishimoto and Kano, 2006). These results were than extended to show that systemic CB1R agonist administration resulted in deficits in delay but not trace conditioning (Steinmetz and Freeman, 2010; 2013). Additionally, *in vitro* manipulations of CB1R manipulations impair LTD formation (Levenes et al., 1998; Safo and Regehr, 2005). Thus, the hypothesis is that CB1R manipulations impair LTD formation within the cerebellar cortex, which then results in behavioral deficits observed.

In Chapter 2 we first localized the effects of CB1R systemic administration to the cerebellar cortex. We first report that differing concentrations of the CB1R agonist WIN55,212-2 resulted in a dose-dependent impairment in learning, which is similar to results previously reported using subcutaneous injections (Steinmetz and Freeman, 2010). We next examined if other areas within the cerebellum, specifically the vermis and the anterior interpositus nucleus, exhibited deficits with infusions of CB1R agonists. Only

cannula directed to the eyeblink conditioning microzone resulted in impairments. WIN55,212-2 binds to both CB1Rs and CB2Rs with similar affinities. Thus, it was important to understand if the impairment was due to CB1Rs or CB2Rs. To test this we infused the selective CB1R antagonist SR141716A into the cerebellar cortex prior to either intracerebellar or systemic administration of WIN55,212-2. SR141716A blocked the effects of WIN55,212-2, indicating the role of CB1Rs and not CB2Rs in the impairment. Importantly, an additional CB1R selective agonist ACEA was administrated, produced impairments similar to WIN55,212-2, and was blocked by SR141716A administration. Taken together, these results indicate that intracerebellar infusions of cannabinoid agonists produce the same deficits as subcutaneous injections and the eyeblink conditioning microzone is therefore the site of actions for impairments produced by systemic administration of CB1R agonists.

The second part of Chapter 2 examined the formation and maintenance of learning-related changes in Purkinje cell activity following CB1R agonist or vehicle injections. Previous reports have shown that normal CB1R function is necessary for LTD formation (Levenes et al., 1998; Safo and Regehr, 2005). LTD formation is also an important first step in learning of eyeblink conditioning and causes Purkinje cells to produce pauses in simple spike activity during the CS (Medina and Mauk, 2000; Green and Steinmetz, 2005). Tetrode recordings were made during 5 sessions of paired training and during retention tests (following 80% CRs). We reported significantly reduced LTD in Purkinje cells when WIN55,212-2 was administered during acquisition. Additionally, the Purkinje cell LTD had an earlier onset and a smaller amplitude than the LTD in vehicle treated animals. No differences were found with Purkinje cells showing learning-related increases in simple spike firing. During retention WIN55,212-2 did not alter the activity of any of the cell types. Thus, CB1R agonist-induced impairments were specific to LTD formation within Purkinje cells during acquisition of eyeblink conditioning.

These results indicate an important role in normal CB1R function during acquisition of LTD but not in retention of eyeblink conditioning.

Forebrain-dependent trace eyeblink conditioning has been reported to not be altered when CB1R agonists are injected subcutaneously or in CB1R knockout mice (Kishimoto and Kano, 2006; Steinmetz and Freeman, 2013). Chapter 3 examined CB1R infusions into the cerebellar cortex during delay, long delay, and trace eyeblink conditioning. Similar to previous reports, trace conditioning was unimpaired by intracerebellar CB1R infusions. This suggests that forebrain-dependent trace eyeblink conditioning it is not reliant on LTD induction within Purkinje cells, which has been reported previously (Kishimoto et al., 2001a;b;c). Additionally, WIN55,212-2 infusions impaired long delay conditioning more than delay conditioning. These results add to a growing set of results showing significant differences between different CS durations and mechanisms or brain areas involved. The visual cortex and cerebellar cortex have been implicated to play a larger role in the learning to non-optimal long delay more than optimal delay conditioning (Vogel at al., 2009; Steinmetz, Harmon, and Freeman, 2013). Thus, the current results suggest that LTD formation and CB1Rs maybe more important during non-optimal long delay than optimal delay conditioning.

The involvement of CB1Rs in consolidation of cerebellum-dependent learning has not been examined; research has focused on the amygdala and hippocampus which contain moderate levels of the receptor. Endocannabinoids are critical for the induction of LTD in Purkinje cell and have not been examined in cerebellum-dependent learning (Safo and Regher, 2005). In Chapter 4 infusions of a CB1R agonist, CB1R antagonist, DAGL inhibitor, and a MAGL inhibitor were given before or after each training session. The infusions were given at different intervals after the session, ranging from 0 to 6 hours. Manipulations of endocannabinoids (DAGL and MAGL inhibitors) or WIN55,212-2 administration impaired acquisition when administered prior to the session.

Infusions resulting in endocannabinoid increases and WIN55,212-2 resulted in stronger memory when the activations occurred 1 hour following the session. The enhancement was observed in the first block of the second session, indicating an increase in between session consolidation. Decreases in endocannabinoids and CB1R antagonist administration impaired consolidation when administered 1 hour following training. The effects of cannabinoid enhancement or impairment were not seen after 1 hour. These results are the first to demonstrate a role for endocannabinoids in consolidation of cerebellar learning *in vivo*.

The current set of experiments demonstrates a dynamic role of CB1Rs during cerebellum-dependent learning. CB1Rs are important in the establishment of LTD in Purkinje cells. This LTD establishment is important for normal delay conditioning *in vivo*. However, we reported that forebrain-dependent trace eyeblink conditioning was unimpaired by local infusions that disrupt LTD formation. Thus, CB1Rs do not impair learning of all conditioning paradigms but maybe selective to paradigms in which LTD formation is important. Finally, consolidation is enhanced by CB1R activation post-training, specifically within a small temporal window.

.

REFERENCES

Adams, M. D., Earnhardt, J. T., Dewey, W. L., & Harris, L. S. (1976). Vasoconstrictor actions of delta8-and delta9-tetrahydrocannabinol in the rat. Journal of Pharmacology and Experimental Therapeutics, 196(3), 649-656.

Alba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., ... & Tonegawa, S. (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell, 79(2), 377-388.

Alba, A., Kano, M., Chen, C., Stanton, M. E., Fox, G. D., Herrup, K., & Tonegawa, S. (1994). Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell, 79(2), 377-388.

Albus, J. S. (1971). A theory of cerebellar function. Mathematical Biosciences, 10(1), 25-61.

Attwell, P. J., Cooke, S. F., & Yeo, C. H. (2002). Cerebellar function in consolidation of a motor memory. Neuron, 34(6), 1011-1020.

Bliss, T. V., & Collingridge, G. L. (1993). A synaptic model of memory: long-term potentiation in the hippocampus. Nature, 361(6407), 31-39.

Bolbecker, A. R., Mehta, C., Johannesen, J. K., Edwards, C. R., O'Donnell, B. F., Shekhar, A., Nurnburger, J. I., Steinmetz, J. E., & Hetrick, W. P. (2009). Eyeblink conditioning anomalies in bipolar disorder suggest cerebellar dysfunction. Bipolar disorders, 11(1), 19-32.

Bolbecker, A. R., Steinmetz, A. B., Mehta, C. S., Forsyth, J. K., Klaunig, M. J., Lazar, E. K.,Steinmetz, J. E., O'Donnell, B. F., & Hetrick, W. P. (2011). Exploration of cerebellar-dependent associative learning in schizophrenia: Effects of varying and shifting interstimulus interval on eyeblink conditioning. Behavioral neuroscience, 125(5), 687.

Bracha, V., Irwin, K. B., Webster, M. L., Wunderlich, D. A., Stachowiak, M. K., & Bloedel, J. R. (1998). Microinjections of anisomycin into the intermediate cerebellum during learning affect the acquisition of classically conditioned responses in the rabbit. Brain research, 788(1), 169-178.

Breivogel, C. S., & Childers, S. R. (2000). Cannabinoid agonist signal transduction in rat brain: comparison of cannabinoid agonists in receptor binding, G-protein activation, and adenylyl cyclase inhibition. Journal of Pharmacology and Experimental Therapeutics, 295(1), 328-336.

Brenowitz, S. D., & Regehr, W. G. (2005). Associative short-term synaptic plasticity mediated by endocannabinoids. Neuron, 45(3), 419.

Brown, K. L., Agelan, A., & Woodruff-Pak, D. S. (2010). Unimpaired trace classical eyeblink conditioning in Purkinje cell degeneration pcd mutant mice. Neurobiology of learning and memory, 93(3), 303-311.

Carlson, G., Wang, Y., & Alger, B. E. (2002). Endocannabinoids facilitate the induction of LTP in the hippocampus. Nature neuroscience, 5(8), 723-724.

Cason, H. (1922). The conditioned eyelid reaction. Journal of Experimental Psychology, 5(3), 153.

Caulfield, M. P., & Brown, D. A. (2002). Cannabinoid receptor agonists inhibit Ca current in NG108–15 neuroblastoma cells via a Pertussis toxin-sensitive mechanism. British journal of pharmacology, 106(2), 231-232.

Chapman, P. F., Steinmetz, J. E., Sears, L. L., & Thompson, R. F. (1990). Effects of lidocaine injection in the interpositus nucleus and red nucleus on conditioned behavioral and neuronal responses. Brain research, 537(1), 149-156.

Chen, L., Bao, S., & Thompson, R. F. (1999). Bilateral lesions of the interpositus nucleus completely prevent eyeblink conditioning in Purkinje cell-degeneration mutant mice. Behavioral neuroscience, 113(1), 204.

Chen, L., Bao, S., Lockard, J. M., Kim, J. K., & Thompson, R. F. (1996). Impaired classical eyeblink conditioning in cerebellar-lesioned and Purkinje cell degeneration (pcd) mutant mice. The Journal of neuroscience, 16(8), 2829-2838.

Chevaleyre, V., Heifets, B. D., Kaeser, P. S., Südhof, T. C., & Castillo, P. E. (2007). Endocannabinoid-mediated long-term plasticity requires cAMP/PKA signaling and RIM1α. Neuron, 54(5), 801-812.

Collins, D. R., Pertwee, R. G., & Davies, S. N. (2012). Prevention by the cannabinoid antagonist, SR141716A, of cannabinoid-mediated blockade of long-term potentiation in the rat hippocampal slice. British journal of pharmacology, 115(6), 869-870.

Cooke, S. F., Attwell, P. J., & Yeo, C. H. (2004). Temporal properties of cerebellar-dependent memory consolidation. The Journal of neuroscience, 24(12), 2934-2941.

Clarke, J. R., Rossato, J. I., Monteiro, S., Bevilaqua, L. R., Izquierdo, I., & Cammarota, M. (2008). Posttraining activation of CB1 cannabinoid receptors in the CA1 region of the dorsal hippocampus impairs object recognition long-term memory. Neurobiology of learning and memory, 90(2), 374-381.

Crepel, F., Audinat, E., Daniel, H., Hemart, N., Jaillard, D., Rossier, J., & Lambolez, B. (1994). Cellular locus of the nitric oxide-synthase involved in cerebellar long-term depression induced by high external potassium concentration. Neuropharmacology, 33(11), 1399-1405.

D'Hooge, R., & De Deyn, P. P. (2001). Applications of the Morris water maze in the study of learning and memory. Brain Research Reviews, 36(1), 60-90.

D'Souza, D. C., Sewell, R. A., & Ranganathan, M. (2009). Cannabis and psychosis/schizophrenia: human studies. European archives of psychiatry and clinical neuroscience, 259(7), 413-431.

Desmond, J. E., & Moore, J. W. (1991). Altering the synchrony of stimulus trace processes: Tests of a neural-network model. Biological cybernetics, 65(3), 161-169.

de Solages, C., Szapiro, G., Brunel, N., Hakim, V., Isope, P., Buisseret, P., & Léna, C. (2008). High-frequency organization and synchrony of activity in the Purkinje cell layer of the cerebellum. Neuron, 58(5), 775-788.

Devane, W. A., Dysarz, F. 3., Johnson, M. R., Melvin, L. S., & Howlett, A. C. (1988). Determination and characterization of a cannabinoid receptor in rat brain. Molecular Pharmacology, 34(5), 605-613.

Devane, W. A., Hanus, L., Breuer, A., Pertwee, R. G., Stevenson, L. A., Griffin, G., & Mechoulam, R. (1992). Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science (New York, NY), 258(5090), 1946.

Dornbush, R. L. (1974). The long-term effects of cannabis use. Marijuana: Effects on Human Behavior. New York: Academic.

Dornbush, R. L., Fink, M., & Freedman, A. M. (1971). Marijuana, memory, and perception. The American journal of psychiatry.

Edwards, C. R., Skosnik, P. D., Steinmetz, A. B., Vollmer, J. M., O'Donnell, B. F., & Hetrick, W. P. (2008). Assessment of forebrain-dependent trace eyeblink conditioning in chronic cannabis users. Neuroscience letters, 439(3), 264-268.

Egertová, M., & Elphick, M. R. (2000). Localisation of cannabinoid receptors in the rat brain using antibodies to the intracellular C-terminal tail of CB1. The Journal of comparative neurology, 422(2), 159-171.

Eggan, S. M., & Lewis, D. A. (2007). Immunocytochemical distribution of the cannabinoid CB1 receptor in the primate neocortex: a regional and laminar analysis. Cerebral Cortex, 17(1), 175-191.

Ekerot, C. F., & Kano, M. (1985). Long-term depression of parallel fibre synapses following stimulation of climbing fibres. Brain research, 342(2), 357-360.

Ferrari, F., Ottani, A., Vivoli, R., & Giuliani, D. (1999). Learning impairment produced in rats by the cannabinoid agonist HU 210 in a water-maze task. Pharmacology Biochemistry and Behavior, 64(3), 555-561.

Freeman Jr, J. H., Shi, T., & Schreurs, B. G. (1998). Pairing-specific long-term depression prevented by blockade of PKC or intracellular Ca2+. Neuroreport, 9(10), 2237-2241.

Freeman, J. H., & Steinmetz, A. B. (2011). Neural circuitry and plasticity mechanisms underlying delay eyeblink conditioning. Learning & Memory, 18(10), 666-677.

Freund, T. F., Katona, I., & Piomelli, D. (2003). Role of endogenous cannabinoids in synaptic signaling. Physiological Reviews, 83(3), 1017-1066.

Gaetani, S., Dipasquale, P., Romano, A., Righetti, L., Cassano, T., Piomelli, D., & Cuomo, V. (2009). The endocannabinoid system as a target for novel anxiolytic and antidepressant drugs. Int Rev Neurobiol, 85, 57-72.

Galante, M., & Diana, M. A. (2004). Group I metabotropic glutamate receptors inhibit GABA release at interneuron-Purkinje cell synapses through endocannabinoid production. The Journal of neuroscience, 24(20), 4865-4874.

Gaoni, Y., & Mechoulam, R. (1964). Isolation, structure, and partial synthesis of an active constituent of hashish. Journal of the American Chemical Society, 86(8), 1646-1647.

Garcia, K. S., Steele, P. M., & Mauk, M. D. (1999). Cerebellar cortex lesions prevent acquisition of conditioned eyelid responses. The Journal of Neuroscience, 19(24), 10940-10947.

Gerwig, M., Kolb, F. P., & Timmann, D. (2007). The involvement of the human cerebellum in eyeblink conditioning. The Cerebellum, 6(1), 38-57.

Glass, M., & Felder, C. C. (1997). Concurrent stimulation of cannabinoid CB1 and dopamine D2 receptors augments cAMP accumulation in striatal neurons: evidence for a Gs linkage to the CB1 receptor. The Journal of neuroscience, 17(14), 5327-5333.

Gonzalez, R. (2007). Acute and non-acute effects of cannabis on brain functioning and neuropsychological performance. Neuropsychology review, 17(3), 347-361.

Gormezano, I., Kehoe, E. J., & Marshall, B. S. (1983). Twenty years of classical conditioning with the rabbit. Progress in psychobiology and physiological psychology, 10, 197-275.

Gormezano, I., Schneiderman, N., Deaux, E., & Fuentes, I. (1962). Nictitating membrane: classical conditioning and extinction in the albino rabbit. Science, 138(12), 33-34.

Green, J. T., & Steinmetz, J. E. (2005). Purkinje cell activity in the cerebellar anterior lobe after rabbit eyeblink conditioning. Learning & Memory, 12(3), 260-269.

Harvey, J. A., Land, T., & McMaster, S. E. (1984). Anatomical study of the rabbit's corneal-VIth nerve reflex: Connections between cornea, trigeminal sensory complex, and the abducens and accessory abducens nuclei. Brain research, 301(2), 307-321.

Heiney, S. A., Kim, J., Augustine, G. J., & Medina, J. F. (2014). Precise Control of Movement Kinematics by Optogenetic Inhibition of Purkinje Cell Activity. The Journal of Neuroscience, 34(6), 2321-2330.

Henry, D. J., & Chavkin, C. (1995). Activation of inwardly rectifying potassium channels (GIRK1) by co-expressed rat brain cannabinoid receptors in Xenopus oocytes. Neuroscience letters, 186(2), 91-94.

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., & Rice, K. C. (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. The Journal of neuroscience, 11(2), 563-583.

Herkenham, M., Lynn, A. B., Little, M. D., Johnson, M. R., Melvin, L. S., De Costa, B. R., & Rice, K. C. (1990). Cannabinoid receptor localization in brain. Proceedings of the national Academy of sciences, 87(5), 1932-1936.

Hicks, R. E., Gualtieri, C. T., Mayo Jr, J. P., & Perez-Reyes, M. (1984). Cannabis, atropine, and temporal information processing. Neuropsychobiology, 12(4), 229-237.

Hillard, C. J., Manna, S., Greenberg, M. J., DiCamelli, R., Ross, R. A., Stevenson, L. A., & Campbell, W. B. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). Journal of Pharmacology and Experimental Therapeutics, 289(3), 1427-1433.

Howlett, A. C. (2002). The cannabinoid receptors. Prostaglandins & other lipid mediators, 68, 619-631.

Howlett, A. C. (2005). Cannabinoid receptor signaling. Cannabinoids, 53-79.

Howlett, A. C., Barth, F., Bonner, T. I., Cabral, G., Casellas, P., Devane, W. A., Devane, W. A., Felder, C. C., Herkenham, M., Mackie, K., Martin B. R., Mechoulam, R., & Pertwee, R. G. (2002). International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. Pharmacological Reviews, 54(2), 161-202.

Hunault, C. C., Mensinga, T. T., Böcker, K. B., Schipper, C. M. A., Kruidenier, M., Leenders, M. E., de Vries, I., & Meulenbelt, J. (2009). Cognitive and psychomotor effects in males after smoking a combination of tobacco and cannabis containing up to 69 mg delta-9-tetrahydrocannabinol (THC). Psychopharmacology, 204(1), 85-94.

Ito, M., & Kano, M. (1982). Long-lasting depression of parallel fiber-Purkinje cell transmission induced by conjunctive stimulation of parallel fibers and climbing fibers in the cerebellar cortex. Neuroscience letters, 33(3), 253-258.

Ito, M. (1993). Synaptic plasticity in the cerebellar cortex and its role in motor learning. The Canadian journal of neurological sciences. Le journal canadien des sciences neurologiques, 20, S70-4.

Iversen, L. (2003). Cannabis and the brain. Brain, 126(6), 1252-1270.

Jager, G., Kahn, R. S., Van Den Brink, W., Van Ree, J. M., & Ramsey, N. F. (2006). Long-term effects of frequent cannabis use on working memory and attention: an fMRI study. Psychopharmacology, 185(3), 358-368.

Jirenhed, D. A., Bengtsson, F., & Hesslow, G. (2007). Acquisition, extinction, and reacquisition of a cerebellar cortical memory trace. The Journal of neuroscience, 27(10), 2493-2502.

Jirenhed, D. A., & Hesslow, G. (2011). Learning stimulus intervals—adaptive timing of conditioned Purkinje cell responses. The Cerebellum, 10(3), 523-535.

Karachot, L., Shirai, Y., Vigot, R., Yamamori, T., & Ito, M. (2000). Rapidly turned over protein maintains metabotropic synaptic transmission in Purkinje cells. Neuroreport, 11(13), 2903-2906.

Kim, J., Isokawa, M., Ledent, C., & Alger, B. E. (2002). Activation of muscarinic acetylcholine receptors enhances the release of endogenous cannabinoids in the hippocampus. The Journal of neuroscience, 22(23), 10182-10191.

Kishimoto, Y., & Kano, M. (2006). Endogenous cannabinoid signaling through the CB1 receptor is essential for cerebellum-dependent discrete motor learning. The Journal of neuroscience, 26(34), 8829-8837.

Kishimoto, Y., Hirono, M., Sugiyama, T., Kawahara, S., Nakao, K., Kishio, M., Katsuki, M., Yoshioka, T., & Kirino, Y. (2001c). Impaired delay but normal trace eyeblink conditioning in PLC [beta] 4 mutant mice. Neuroreport, 12(13), 2919-2922.

Kishimoto, Y., Kawahara, S., Fujimichi, R., Mori, H., Mishina, M., & Kirino, Y. (2001b). Impairment of eyeblink conditioning in GluRδ2-mutant mice depends on the temporal overlap between conditioned and unconditioned stimuli. European Journal of Neuroscience, 14(9), 1515-1521.

Kishimoto, Y., Kawahara, S., Suzuki, M., Mori, H., Mishina, M., & Kirino, Y. (2001a). Classical eyeblink conditioning in glutamate receptor subunit δ2 mutant mice is impaired in the delay paradigm but not in the trace paradigm. European Journal of Neuroscience, 13(6), 1249-1253.

Kreitzer, A. C., & Regehr, W. G. (2001a). Retrograde inhibition of presynaptic calcium influx by endogenous cannabinoids at excitatory synapses onto Purkinje cells. Neuron, 29(3), 717-727.

Kreitzer, A. C., & Regehr, W. G. (2001b). Cerebellar depolarization-induced suppression of inhibition is mediated by endogenous cannabinoids. The Journal of neuroscience: the official journal of the Society for Neuroscience, 21(20), RC174.

Krupa, D. J., & Thompson, R. F. (1995). Inactivation of the superior cerebellar peduncle blocks expression but not acquisition of the rabbit's classically conditioned eye-blink response. Proceedings of the National Academy of Sciences, 92(11), 5097-5101.

Krupa, D. J., & Thompson, R. F. (1997). Reversible inactivation of the cerebellar interpositus nucleus completely prevents acquisition of the classically conditioned eye-blink response. Learning & Memory, 3(6), 545-556.

Krupa, D. J., Thompson, J. K., & Thompson, R. F. (1993). Localization of a memory trace in the mammalian brain. Science, 260, 989-989.

Lange, W. (1975). Cell number and cell density in the cerebellar cortex of man and some other mammals. Cell and Tissue Research, 157(1), 115-124.

Lavond, D. G., & Steinmetz, J. E. (1989). Acquisition of classical conditioning without cerebellar cortex. Behavioural brain research, 33(2), 113-164.

Lavond, D. G., Lincoln, J. S., McCormick, D. A., & Thompson, R. F. (1984). Effect of bilateral lesions of the dentate and interpositus cerebellar nuclei on conditioning of heart-rate and nictitating membrane/eyelid responses in the rabbit. Brain research, 305(2), 323-330.

Lee, T., & Kim, J. J. (2004). Differential effects of cerebellar, amygdalar, and hippocampal lesions on classical eyeblink conditioning in rats. The Journal of neuroscience, 24(13), 3242-3250.

Levenes, C., Daniel, H., & Crépel, F. (1998). Long-term depression of synaptic transmission in the cerebellum: cellular and molecular mechanisms revisited. Progress in neurobiology, 55(1), 79-91.

Li, H. L. (1973). An archaeological and historical account of cannabis in China. Economic Botany, 28(4), 437-448.

Liu, Z., Han, J., Jia, L., Maillet, J. C., Bai, G., Xu, L., & Zhang, X. (2010). Synaptic neurotransmission depression in ventral tegmental dopamine neurons and cannabinoid-associated addictive learning. PloS one, 5(12), e15634.

Lichtman, A. H., & Martin, B. R. (1996). Δ 9-Tetrahydrocannabinol impairs spatial memory through a cannabinoid receptor mechanism. Psychopharmacology, 126(2), 125-131.

Lichtman, A. H., Dimen, K. R., & Martin, B. R. (1995). Systemic or intrahippocampal cannabinoid administration impairs spatial memory in rats. Psychopharmacology, 119(3), 282-290.

Linden, D. J., Dickinson, M. H., Smeyne, M., & Connor, J. A. (1991). A long-term depression of AMPA currents in cultured cerebellar Purkinje neurons. Neuron, 7(1), 81-89.

Linden, D. J. (1994). Input-specific induction of cerebellar long-term depression does not require presynaptic alteration. Learning & Memory, 1(2), 121-128.

Llano, I., Leresche, N., & Marty, A. (1991). Calcium entry increases the sensitivity of cerebellar Purkinje cells to applied GABA and decreases inhibitory synaptic currents. Neuron, 6(4), 565-574.

Mackie, K., & Hille, B. (1992). Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells. Proceedings of the National Academy of Sciences, 89(9), 3825-3829.

Mackie, K., Devane, W. A., & Hille, B. E. R. T. I. L. (1993). Anandamide, an endogenous cannabinoid, inhibits calcium currents as a partial agonist in N18 neuroblastoma cells. Molecular pharmacology, 44(3), 498-503.

Mackie, K., Lai, Y., Westenbroek, R., & Mitchell, R. (1995). Cannabinoids activate an inwardly rectifying potassium conductance and inhibit Q-type calcium currents in AtT20 cells transfected with rat brain cannabinoid receptor. The Journal of neuroscience, 15(10), 6552-6561.

Maćkowiak, M., Chocyk, A., Dudys, D., & Wedzony, K. (2009). Activation of CB1 cannabinoid receptors impairs memory consolidation and hippocampal polysialylated neural cell adhesion molecule expression in contextual fear conditioning. Neuroscience, 158(4), 1708-1716.

Maejima, T., Hashimoto, K., Yoshida, T., Aiba, A., & Kano, M. (2001). Presynaptic inhibition caused by retrograde signal from metabotropic glutamate to cannabinoid receptors. Neuron, 31(3), 463-475.

Maejima, T., Oka, S., Hashimotodani, Y., Ohno-Shosaku, T., Aiba, A., Wu, D., Waku, K., Sugiura, T., & Kano, M. (2005). Synaptically driven endocannabinoid release requires Ca2+-assisted metabotropic glutamate receptor subtype 1 to phospholipase C β4 signaling cascade in the cerebellum. The Journal of neuroscience, 25(29), 6826-6835.

Marsicano, G., Wotjak, C. T., Azad, S. C., Bisogno, T., Rammes, G., Cascio, M. G., Hermann, H., & Lutz, B. (2002). The endogenous cannabinoid system controls extinction of aversive memories. Nature, 418(6897), 530-534.

Mathew, R. J., Wilson, W. H., G Turkington, T., & Coleman, R. E. (1998). Cerebellar activity and disturbed time sense after THC. Brain research, 797(2), 183-189.

Mauk, M. D., & Donegan, N. H. (1997). A model of Pavlovian eyelid conditioning based on the synaptic organization of the cerebellum. Learning & Memory, 4(1), 130-158.

Mauk, M. D., Steinmetz, J. E., & Thompson, R. F. (1986). Classical conditioning using stimulation of the inferior olive as the unconditioned stimulus. Proceedings of the National Academy of Sciences, 83(14), 5349-5353.

McCormick, D. A., & Thompson, R. F. (1984). Neuronal responses of the rabbit cerebellum during acquisition and performance of a classically conditioned nictitating membrane-eyelid response. The Journal of neuroscience, 4(11), 2811-2822.

McCormick, D. A., Guyer, P. E., & Thompson, R. F. (1982). Superior cerebellar peduncle lesions selectively abolish the ipsilateral classically conditioned nictitating membrane/eyelid response of the rabbit. Brain Research, 244(2), 347-350.

McCormick, D. A., Lavond, D. G., Clark, G.A., Kettner, R. E., Rising, C. E., & Thompson, R. F. (1981). The engram found? Role of the cerebellum in classical conditioning of nictitating membrane and eyelid responses. Bulletin of the Psychonomic Society.

Mechoulam, R., & Hanuš, L. (2000). A historical overview of chemical research on cannabinoids. Chemistry and physics of lipids, 108(1), 1-13.

Mechoulam, R., Ben-Shabat, S., Hanus, L., Ligumsky, M., Kaminski, N. E., Schatz, A. R., & Vogel, Z. V. I. (1995). Identification of an endogenous 2-monoglyceride, present in canine gut, that binds to cannabinoid receptors. Biochemical pharmacology, 50(1), 83-90.

Medina, J. F., & Mauk, M. D. (2000). Computer simulation of cerebellar information processing. nature neuroscience, 3, 1205-1211.

Melis, M., Perra, S., Muntoni, A. L., Pillolla, G., Lutz, B., Marsicano, G., ... & Pistis, M. (2004). Prefrontal cortex stimulation induces 2-arachidonoyl-glycerol-mediated suppression of excitation in dopamine neurons. The Journal of neuroscience, 24(47), 10707-10715.

Miller, L. L., & Cornett, T. L. (1978). Marijuana: Dose effects on pulse rate, subjective estimates of intoxication, free recall and recognition memory. Pharmacology Biochemistry and Behavior, 9(5), 573-577.

Misner, D. L., & Sullivan, J. M. (1999). Mechanism of cannabinoid effects on long-term potentiation and depression in hippocampal CA1 neurons. The Journal of neuroscience, 19(16), 6795-6805.

Nagy, I., White, J. P., Paule, C. C., & Köfalvi, A. (2008). An Historical Introduction to the Endocannabinoid and Endovanilloid Systems. Cannabinoids and the Brain, 3-13.

Neufeld, M., & Mintz, M. (2001). Involvement of the amygdala in classical conditioning of eyeblink response in the rat. Brain research, 889(1), 112-117.

Nogueron, M. I., Porgilsson, B., Schneider, W. E., Stucky, C. L., & Hillard, C. J. (2008). Cannabinoid receptor agonists inhibit depolarization-induced calcium influx in cerebellar granule neurons. Journal of neurochemistry, 79(2), 371-381.

Nolan, B. C., & Freeman, J. H. (2006). Purkinje cell loss by OX7-saporin impairs acquisition and extinction of eyeblink conditioning. Learning & Memory, 13(3), 359-365.

Nowicky, A. V., Teyler, T. J., & Vardaris, R. M. (1987). The modulation of long-term potentiation by delta-9-tetrahydrocannabinol in the rat hippocampus in vitro. Brain research bulletin, 19(6), 663-672.

Ng, K. H., & Freeman, J. H. (2012). Developmental changes in medial auditory thalamic contributions to associative motor learning. The Journal of Neuroscience, 32(20), 6841-6850.

Ohno-Shosaku, T., Maejima, T., & Kano, M. (2001). Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. Neuron, 29(3), 729-738.

Ohno-Shosaku, T., Tsubokawa, H., Mizushima, I., Yoneda, N., Zimmer, A., & Kano, M. (2002). Presynaptic cannabinoid sensitivity is a major determinant of depolarization-induced retrograde suppression at hippocampal synapses. The Journal of neuroscience, 22(10), 3864-3872.

Ohyama, T., Nores, W. L., Medina, J. F., Riusech, F. A., & Mauk, M. D. (2006). Learning-induced plasticity in deep cerebellar nucleus. The Journal of neuroscience, 26(49), 12656-12663.

Ong, W. Y., & Mackie, K. (1999). A light and electron microscopic study of the CB1 cannabinoid receptor in the primate spinal cord. Journal of neurocytology, 28(1), 39-45.

O'Leary, D. S., Block, R. I., Turner, B. M., Koeppel, J., Magnotta, V. A., Ponto, L. B., & Andreasen, N. C. (2003). Marijuana alters the human cerebellar clock. Neuroreport, 14(8), 1145-1151.

Pamplona, F. A., & Takahashi, R. N. (2006). WIN 55212-2 impairs contextual fear conditioning through the activation of CB1 cannabinoid receptors. Neuroscience letters, 397(1), 88-92.

Pamplona, F. A., Prediger, R. D., Pandolfo, P., & Takahashi, R. N. (2006). The cannabinoid receptor agonist WIN 55,212-2 facilitates the extinction of contextual fear memory and spatial memory in rats. Psychopharmacology, 188(4), 641-649.

Paton, G. S., Pertwee, R. G., & Davies, S. N. (1998). Correlation between cannabinoid mediated effects on paired pulse depression and induction of long term potentiation in the rat hippocampal slice. Neuropharmacology, 37(9), 1123-1130.

Pavlov, I. P. (1927). Conditioned reflexes: An investigation of the physiological activities of the cerebral cortex.

Paxinos, G., & Watson, C. (2007). The rat brain in stereotaxic coordinates. Academic press.

Pertwee, R. G. (1997). Pharmacology of cannabinoid CB1 and CB2 receptors. Pharmacology & therapeutics, 74(2), 129.

Pertwee, R. G. (1999). Pharmacology of cannabinoid receptor ligands. Current medicinal chemistry, 6, 635-664.

Pitler, T. A., & Alger, B. E. (1992). Postsynaptic spike firing reduces synaptic GABAA responses in hippocampal pyramidal cells. The Journal of neuroscience, 12(10), 4122-4132.

Pugh, J. R., & Raman, I. M. (2008). Mechanisms of potentiation of mossy fiber EPSCs in the cerebellar nuclei by coincident synaptic excitation and inhibition. The Journal of Neuroscience, 28(42), 10549-10560.

Riedel, G., & Davies, S. N. (2005). Cannabinoid function in learning, memory and plasticity. Cannabinoids, 445-477.

Robbe, D., Alonso, G., Chaumont, S., Bockaert, J., & Manzoni, O. J. (2002). Role of p/q-Ca2+ channels in metabotropic glutamate receptor 2/3-dependent presynaptic long-term depression at nucleus accumbens synapses. The Journal of neuroscience, 22(11), 4346-4356.

Rosenfield, M. E., & Moore, J. W. (1983). Red nucleus lesions disrupt the classically conditioned nictitating membrane response in rabbits. Behavioural brain research, 10(2), 393-398.

Russo, E. B. (2007). History of cannabis and its preparations in saga, science, and sobriquet. Chemistry & biodiversity, 4(8), 1614-1648.

Ryberg, E., Larsson, N., Sjögren, S., Hjorth, S., Hermansson, N. O., Leonova, J., Elebring, T., Nilsson, K., Drmota, T., & Greasley, P. J. (2009). The orphan receptor GPR55 is a novel cannabinoid receptor. British journal of pharmacology, 152(7), 1092-1101.

Safo, P. K., & Regehr, W. G. (2005). Endocannabinoids control the induction of cerebellar LTD. Neuron, 48(4), 647-659.

Safo, P. K., Cravatt, B. F., & Regehr, W. G. (2006). Retrograde endocannabinoid signaling in the cerebellar cortex. The Cerebellum, 5(2), 134-145.

Sañudo-Peña, M., Romero, J., Seale, G. E., Fernandez-Ruiz, J. J., & Walker, J. M. (2000). Activational role of cannabinoids on movement. European journal of pharmacology, 391(3), 269-274.

Sañudo-Peña, M., Tsou, K., & Walker, J. M. (1999). Motor actions of cannabinoids in the basal ganglia output nuclei. Life sciences, 65(6), 703-713.

Segev, A., & Akirav, I. (2011). Differential effects of cannabinoid receptor agonist on social discrimination and contextual fear in amygdala and hippocampus. Learning & Memory, 18(4), 254-259.

Schmitzer-Torbert, N., Jackson, J., Henze, D., Harris, K., & Redish, A. D. (2005). Quantitative measures of cluster quality for use in extracellular recordings. Neuroscience, 131(1), 1-11.

Schneiderman, N., & Gormezano, I. (1964). Conditioning of the nictitating membrane of the rabbit as a function of CS-US interval. Journal of comparative and physiological psychology, 57(2), 188.

Schreurs, B. G. (1988). Stimulation of the spinal trigeminal nucleus supports classical conditioning of the rabbit's nictitating membrane response. Behavioral neuroscience, 102(1), 163.

Schreurs, B. G., & Alkon, D. L. (1993). Rabbit cerebellar slice analysis of long-term depression and its role in classical conditioning. Brain research, 631(2), 235-240.

Siegelbaum, S. A., & Kandel, E. R. (1991). Learning-related synaptic plasticity: LTP and LTD. Current opinion in neurobiology, 1(1), 113-120.

Shibuki, K., Gomi, H., Chen, L., Bao, S., Kim, J. J., Wakatsuki, H., Wu, Y., & Itohara, S. (1996). Deficient cerebellar long-term depression, impaired eyeblink conditioning, and normal motor coordination in GFAP mutant mice. Neuron, 16(3), 587-599.

Shinoda, Y., Sugihara, I., Wu, H. S., & Sugiuchi, Y. (2000). The entire trajectory of single climbing and mossy fibers in the cerebellar nuclei and cortex. Progress in brain research, 124, 173-186.

Skosnik, P. D., Edwards, C. R., O'Donnell, B. F., Steffen, A., Steinmetz, J. E., & Hetrick, W. P. (2007). Cannabis use disrupts eyeblink conditioning: evidence for cannabinoid modulation of cerebellar-dependent learning. Neuropsychopharmacology, 33(6), 1432-1440.

Slipetz, D. M., O'Neill, G. P., Favreau, L., Dufresne, C., Gallant, M., Gareau, Y., & Metters, K. M. (1995). Activation of the human peripheral cannabinoid receptor results in inhibition of adenylyl cyclase. Molecular pharmacology, 48(2), 352-361.

Shigemoto, R., Abe, T., Nomura, S., Nakanishi, S., & Hirano, T. (1994). Antibodies inactivating mGluR1 metabotropic glutamate receptor block long-term depression in cultured Purkinje cells. Neuron, 12(6), 1245-1255.

Steinmetz, A. B., & Freeman, J. H. (2010). Central cannabinoid receptors modulate acquisition of eyeblink conditioning. Learning & Memory, 17(11), 571-576.

Steinmetz, A. B., & Freeman, J. H. (2011). Retention and extinction of delay eyeblink conditioning are modulated by central cannabinoids. Learning & Memory, 18(10), 634-638.

Steinmetz, A. B., & Rice, M. L. (2010). Cerebellar-dependent delay eyeblink conditioning in adolescents with Specific Language Impairment. Journal of neurodevelopmental disorders, 2(4), 243-251.

Steinmetz, A. B., Edwards, C. R., Vollmer, J. M., Erickson, M. A., O'Donnell, B. F., Hetrick, W. P., & Skosnik, P. D. (2012). Examining the effects of former cannabis use on cerebellum-dependent eyeblink conditioning in humans. Psychopharmacology, 1-9.

Steinmetz, A. B., Harmon, T. C., & Freeman, J. H. (2013). Visual cortical contributions to associative cerebellar learning. Neurobiology of learning and memory, 104, 103-109.

Steinmetz, A. B. & Freeman, J. H. (2013). Differential effects of the cannabinoid agonist WIN55, 212–2 on delay and trace eyeblink conditioning. Behavioral neuroscience, 127(5), 694.

Steinmetz, A. B. & Freeman, J. H. (in prep). Localization of the cerebellar cortical zone mediating acquisition of eyeblink conditioning in rats.

Steinmetz, J. E., Lavond, D. G., & Thompson, R. F. (1989). Classical conditioning in rabbits using pontine nucleus stimulation as a conditioned stimulus and inferior olive stimulation as an unconditioned stimulus. Synapse, 3(3), 225-233.

Steinmetz, J. E., Logue, S. F., & Steinmetz, S. S. (1992). Rabbit classically conditioned eyelid responses do not reappear after interpositus nucleus lesion and extensive post-lesion training. Behavioural brain research, 51(1), 103-114.

Stella, N., & Piomelli, D. (2001). Receptor-dependent formation of endogenous cannabinoids in cortical neurons. European journal of pharmacology, 425(3), 189-196.

Stella, N., Schweitzer, P., & Piomelli, D. (1997). A second endogenous cannabinoid that modulates long-term potentiation. Nature, 388(6644), 773-778.

Strick, P. L., Dum, R. P., & Fiez, J. A. (2009). Cerebellum and nonmotor function. Annual review of neuroscience, 32, 413-434.

Suárez, J., Bermúdez-Silva, F. J., Mackie, K., Ledent, C., Zimmer, A., Cravatt, B. F., & de Fonseca, F. R. (2008). Immunohistochemical description of the endogenous cannabinoid system in the rat cerebellum and functionally related nuclei. The Journal of comparative neurology, 509(4), 400-421.

Substance Abuse and Mental Health Services Administration.(2009). Results from the 2008 National Survey on Drug Use and Health: National findings.(Office of Applied Studies, NSDUH Series H–36, DHHS Publication No. SMA 09–4434). Rockville, MD: SAMHSA. Rockville, MD.

Substance Abuse and Mental Health Services Administration.(2011). Results from the 2010 National Survey on Drug Use and Health: National findings.(Office of Applied Studies, NSDUH Series H–36, DHHS Publication No. SMA 09–4434). Rockville, MD: SAMHSA. Rockville, MD.

Sugihara, I., Wu, H. S., & Shinoda, Y. (2001). The entire trajectories of single olivocerebellar axons in the cerebellar cortex and their contribution to cerebellar compartmentalization. The Journal of Neuroscience, 21(19), 7715-7723.

Tarald, O. K. (1977). Effects of marijuana on human reaction time and motor control. Perceptual and motor skills, 45(3), 935-939.

Tart, C. T. (1971). On being stoned. Palo Alto, Calif: Science and Behavior Books.

Terranova, J. P., Storme, J. J., Lafon, N., Perio, A., Rinaldi-Carmona, M., Le Fur, G., & Soubrie, P. (1996). Improvement of memory in rodents by the selective CB1 cannabinoid receptor antagonist, SR 141716. Psychopharmacology, 126(2), 165-172.

Thompson, R. F., & Steinmetz, J. E. (2009). The role of the cerebellum in classical conditioning of discrete behavioral responses. Neuroscience, 162(3), 732-755.

Trezza, V., Damsteegt, R., Manduca, A., Petrosino, S., Van Kerkhof, L. W., Pasterkamp, R. J., & Vanderschuren, L. J. (2012). Endocannabinoids in amygdala and nucleus accumbens mediate social play reward in adolescent rats. The Journal of Neuroscience, 32(43), 14899-14908.

Tsou, K., Brown, S., Sanudo-Pena, M. C., Mackie, K., & Walker, J. M. (1997). Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience, 83(2), 393-411.

Twitchell, W., Brown, S., & Mackie, K. (1997). Cannabinoids inhibit N-and P/Q-type calcium channels in cultured rat hippocampal neurons. Journal of Neurophysiology, 78(1), 43-50.

Van der Want, J. J. L., Wiklund, L., Guegan, M., Ruigrok, T., & Voogd, J. (2004). Anterograde tracing of the rat olivocerebellar system with phaseolus vulgaris leucoagglutinin (PHA-L). Demonstration of climbing fiber collateral innervation of the cerebellar nuclei. The Journal of comparative neurology, 288(1), 1-18.

van Ham, J. J., & Yeo, C. H. (1996). Trigeminal inputs to eyeblink motoneurons in the rabbit. Experimental neurology, 142(2), 244-257.

Varma, N., Carlson, G. C., Ledent, C., & Alger, B. E. (2001). Metabotropic glutamate receptors drive the endocannabinoid system in hippocampus. The Journal of neuroscience: the official journal of the Society for Neuroscience, 21(24), RC188.

Varvel, S. A., & Lichtman, A. H. (2002). Evaluation of CB1 receptor knockout mice in the Morris water maze. Journal of Pharmacology and Experimental Therapeutics, 301(3), 915-924.

Vogel, R. W., Amundson, J. C., Lindquist, D. H., & Steinmetz, J. E. (2009). Eyeblink conditioning during an interstimulus interval switch in rabbits (Oryctolagus cuniculus) using picrotoxin to disrupt cerebellar cortical input to the interpositus nucleus. Behavioral neuroscience, 123(1), 62.

Weiss, C., Bouwmeester, H., Power, J. M., & Disterhoft, J. F. (1999). Hippocampal lesions prevent trace eyeblink conditioning in the freely moving rat. Behavioural brain research, 99(2), 123-132.

Wiley, J. L., & Martin, B. R. (2002). Cannabinoid pharmacology: implications for additional cannabinoid receptor subtypes. Chemistry and physics of lipids, 121(1), 57-63.

Wilson, R. I., & Nicoll, R. A. (2001). Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature, 410(6828), 588-592.

Woodruff-Pak, D. S., Lavond, D. G., & Thompson, R. F. (1985). Trace conditioning: abolished by cerebellar nuclear lesions but not lateral cerebellar cortex aspirations. Brain research, 348(2), 249-260.

Woodruff-Pak, D. S., & Disterhoft, J. F. (2008). Where is the trace in trace conditioning? Trends in neurosciences, 31(2),105-12.

Yeo, C. H., & Hardiman, M. J. (1992). Cerebellar cortex and eyeblink conditioning: a reexamination. Experimental Brain Research, 88(3), 623-638.

Yeo, C. H., Hardiman, M. J., & Glickstein, M. (1984). Discrete lesions of the cerebellar cortex abolish the classically conditioned nictitating membrane response of the rabbit. Behavioural brain research, 13(3), 261-266.

Yeo, C. H., Hardiman, M. J., & Glickstein, M. (1986). Classical conditioning of the nictitating membrane response of the rabbit. Experimental Brain Research, 63(1), 81-92.

Yim, T. T., Hong, N. S., Ejaredar, M., McKenna, J. E., & McDonald, R. J. (2008). Post-training CB1 cannabinoid receptor agonist activation disrupts long-term consolidation of spatial memories in the hippocampus. Neuroscience, 151(4), 929-936.