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Differential Effects of the Cannabinoid Agonist WIN55,212-2 on Delay and Trace Eyeblick Conditioning

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Abstract

Central cannabinoid-1 receptors (CB1R) play a role in the acquisition of delay eyeblick conditioning but not trace eyeblick conditioning in humans and animals. However, it is not clear why trace conditioning is immune to the effects of cannabinoid receptor compounds. The current study examined the effects of variants of delay and trace conditioning procedures to elucidate the factors that determine the effects of CB1R agonists on eyeblick conditioning. In Experiment 1 rats were administered the cannabinoid agonist WIN55,212-2 during delay, long delay, or trace conditioning. Rats were impaired during delay and long delay but not trace conditioning; the impairment was greater for long delay than delay conditioning. Trace conditioning was further examined in Experiment 2 by manipulating the trace interval and keeping constant the conditioned stimulus (CS) duration. It was found that when the trace interval was 300 ms or less WIN55,212-2 administration impaired the rate of learning. Experiment 3 tested whether the trace interval duration or the relative durations of the CS and trace interval were critical parameters influencing the effects of WIN55,212-2 on eyeblick conditioning. Rats were not impaired with a 100 ms CS, 200 ms trace paradigm but were impaired with a 1000 ms CS, 500 ms trace paradigm, indicating that the duration of the trace interval does not matter but the proportion of the interstimulus interval occupied by the CS relative to the trace period is critical. Taken together the results indicate that cannabinoid agonists affect cerebellar learning the CS is longer than the trace interval.

Keywords

cannabinoid receptor; cannabis; cerebellum; cerebellar cortex; eyeblick conditioning; THC

Cannabis is one of the most widely used psychoactive substances in the world with around 94 million individuals in the United States alone having used cannabis at least once in their lifetime (SAMHSA 2006). Additionally, consumption rates of cannabis have been increasing substantially during the past 25 years (UNODC 2006). The principal psychoactive constituent in cannabis, Δ^9 -tetrahydrocannabinol (Gaoni & Mechoulam, 1964), affects the brain via the action of central cannabinoid receptors-1 (CB1R; Devane et al., 1988). CB1Rs are one of the most abundant G-protein coupled receptors in the brain, with high densities in the basal ganglia, cerebellum, and hippocampus (Herkenham et al., 1990; Eggen & Lewis, 2007). The highest density of receptors is within the cerebellar cortex, proximal to Purkinje cells. These receptors within the cerebellum have been postulated to be necessary for the induction of long-term depression, a synaptic plasticity mechanism critical for motor learning (Safo & Regher, 2005). Thus, studying the role of

CB1Rs during motor learning tasks is critical to the understanding the action of cannabinoid drugs like cannabis.

A motor learning task that has been widely employed to investigate the neural correlates and mechanisms underlying learning is eyeblink conditioning (EBC). EBC involves the presentation of a conditioned stimulus (CS; e.g., tone) that is repeatedly paired with an unconditioned stimulus (US; e.g., periorbital shock) that elicits an unconditioned response (UR) prior to training. Following repeated CS-US pairings a conditioned response (CR) develops that occurs prior to US onset. Two forms of EBC have traditionally been employed, termed delay and trace EBC. Delay EBC involves the CS terminating with the US; whereas trace EBC has a “trace” free interval between the CS and US. The neural circuitry underlying delay EBC includes the cerebellar cortex, anterior interpositus nucleus, and underlying brainstem nuclei (Freeman & Steinmetz, 2011). Trace EBC has been demonstrated to recruit the prefrontal cortex, hippocampus, and sensory cortex in addition to the cerebellum (Weiss & Disterhoft, 2011; Woodruff-Pak & Disterhoft, 2008). However, there remains debate about the exact role of the cerebellar cortex in trace EBC (Woodruff-Pak & Disterhoft, 2008).

Studies examining delay EBC have reported that damage to the cerebellar cortex, either by lesions or inactivation, prior to acquisition severely impairs the rate of learning (Lavond & Steinmetz, 1986; Chen, et al., 1996). The involvement of the cerebellar cortex has also been reported to differ depending on CS duration, with longer CSs requiring more input from the cerebellar cortex (Vogel et al., 2009). However, given enough training it appears that learning can occur without the cerebellar cortex (Chen et al., 1996; Lavond & Steinmetz, 1986; Nolan & Freeman, 2006). The involvement of the cerebellar cortex during trace EBC is not well understood in comparison to delay EBC. Results from mice with genetic modifications targeting the cerebellar cortex have shown intact trace EBC but impaired delay EBC (Kishimoto et al., 2001a, b, c; Brown et al., 2010). However, these studies have not manipulated training parameters such as the CS and trace interval durations to understand the role of the cerebellar cortex in EBC.

Previous work has indicated a role for CB1Rs in the acquisition, retention, and extinction of delay EBC, but not for trace EBC (Skosnik et al., 2007; Edwards et al., 2008; Kishimoto & Kano, 2006; Steinmetz & Freeman, 2010; 2011; Steinmetz et al., 2011). These studies have reported deficits during delay conditioning using optimal ISIs (around 300–400 ms) but have not examined non-optimal ISIs (greater than 500). Previous studies have reported similar levels of learning between experimental and control groups during trace EBC in humans (Edwards et al., 2008) and CB1R knockout mice (Kishimoto & Kano, 2006). However, it is not clear why trace conditioning is immune to the effects of cannabinoid receptor manipulations. The trial duration, trace interval, or the interaction of the CS and trace interval durations could account for the contributions of the CB1Rs.

Based on previous studies showing impaired delay EBC with cannabinoid agonist administration (Steinmetz & Freeman, 2010; 2011), the current study used the selective CB1R agonist WIN55,212-2 to examine the role of the CB1Rs in acquisition of delay and trace EBC. Experiment 1 examined the effects of systemic WIN55,212-2 on acquisition of delay (250 ms CS), long delay (750 ms CS), and trace (250 ms CS; 500 ms trace) EBC. The long delay group also completed 5 days of training without drug to examine if WIN55,212-2 interferes with acquisition or expression of learning. Experiment 2 manipulated the duration of the trace interval (100, 300, 500, and 700) while keeping the CS duration constant (250 ms) between groups. This manipulation examined if the duration of the trace interval is a critical parameter influencing the effects of WIN55,212-2 on EBC. Finally, Experiment 3 examined whether the relative durations of the CS and trace interval (100 ms CS, 200 trace

vs. 1000 ms CS, 500 trace) is a factor influencing the effects of WIN55,212-2 on EBC. In all three experiments, rats were given daily subcutaneous administration of WIN55,212-2 (3 mg/kg). The sequence of sessions was as follows: spontaneous blinking (session 1), unpaired CS/US training (session 2), and paired CS-US training (sessions 3–12).

Materials and Methods

Subjects

Subjects were 136 male Long-Evans rats, 250–300 g at the beginning of the experiment. The rats were housed in Spence Laboratories of Psychology at the University of Iowa with a 12 hour light-dark cycle and were given ad libitum access to food and water.

Surgery

One week prior to training, rats were removed from their home cage 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 shock US was implanted subdermally, caudal to the left eye.

Apparatus

The conditioning apparatus consisted of four small-animal sound attenuation chambers (BRS/LVE, Laurel, MD). Within each sound-attenuation chamber was a small animal operant chamber (BRS/LVE, Laurel, MD) where the rats were located during conditioning. One wall of the operant chamber was fitted with two speakers in which the CS was presented. The electrode leads from the rat's headstage were connected to peripheral equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs, Raleigh, NC). The shock US (2–3 mA, DC constant current) was delivered through a stimulus isolator (Model number 365A, World Precision Instruments, Sarasota FL). EMG activity was recorded differentially, filtered (500–5000 Hz) and integrated by equipment (JSA Designs, Raleigh, NC).

Conditioning procedures

Rats recovered from surgery for 1 week prior to the initiation of testing. 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 shock) unpaired presentations. The CS duration during the unpaired session was equal to the CS presented during training sessions. Sessions 3–12 constituted the acquisition phase of the experiment in which 10 blocks of 9 paired CS-US presentations and 1 CS alone probe trial were presented. The long delay group completed 5 more sessions that were identical as sessions 3–12. For Experiment 1 three paradigms were employed (Figure 1): delay (250 ms CS), long delay (750 ms CS) and trace (250 ms CS, 500 ms trace). 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. In Experiment 2 the CS duration was constant at 250 ms and the trace interval prior to US onset was set to 100, 300, 500 or 700 ms (Figure 1). This manipulation allowed for the examination of the what role the trace interval has in the effects of WIN55,212-2 while the CS duration remained constant. Experiment 3 consisted of two paradigms (Figure 1), relatively greater trace (RGT;

100 ms CS, 200 trace) and relatively greater CS (RGC; 1000 ms CS, 500 ms trace). These two paradigms allowed for the examination of two different ratios of CS duration to trace duration. The RGT group had a 1:2 CS duration to trace duration ratio whereas the RGC had a 2:1 CS duration to trace duration ratio. The trace intervals were set to values that produced impaired and unimpaired performance in Experiment 2, respectively. The shock intensity was adjusted in each rat to elicit a blink and slight head movement (range = 2.0 – 3.0 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

Pharmacological activation/blockade 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-naphthalenylmethanone] or vehicle was administered subcutaneously to rats 30 min before each daily training session. WIN55,212-2 binds with high affinity to CB1R and to CB2R (Howlett et al., 2002; Pertwee, 1997). WIN55,212-2 was administered at a concentration of 3 mg/kg based on previous research indicating severe impairments during delay EBC (Steinmetz & Freeman, 2010). WIN55,212-2 was dissolved in a vehicle of 1:1:18 solution of ethanol, cremaphor, saline and was purchased from Sigma/RBI (St. Louis, MO).

Results

Experiment 1: Effects of WIN55,212-2 on Delay, Long Delay, and Trace EBC

Rats received daily injections of WIN55,212-2 (3 mg/kg) or vehicle prior to 12 daily sessions. Rats were randomly assigned to complete one of three paradigms: delay (250 ms CS), long delay (750 ms CS), or trace (250 ms CS, 500 ms trace) EBC. These paradigms were matched on CS duration (delay and trace) and also interstimulus interval (long delay and trace) for comparison. In Session 1 rats were placed into the testing chamber and spontaneous blink activity was collected for 100 “trials” (Figure 2, SP); the duration of the activity collected in each trial was equal to the duration of the CS or CS + trace interval that was given during paired trials (i.e., 250 ms for the delay group). Session 2 consisted of 100 CS and 90 US unpaired presentations (Figure 2, UP); the CS and US were the same duration during paired training. Separate One-Way Analysis of Variance (ANOVA) indicated that neither WIN55,212-2 nor the length of the collection window altered spontaneous blink activity or blinking during unpaired presentations of the CS. Thus, the differences between the paradigms (length of CS or trace interval) did not significantly increase the amount of spontaneous or non-associative blinks.

Sessions 3–12 consisted of 90 CS-US pairings and 10 CS-alone probe trials per session (Figure 2, P1-10). A 2 (Drug [WIN55,212-2 or Vehicle]) $\times$ 3 (Paradigm [delay, long delay, or trace]) $\times$ 10 (Session) repeated measures ANOVA was conducted to examine differential effects of WIN55,212-2 administration on acquisition of delay, long delay, and trace EBC. A Drug $\times$ Paradigm $\times$ Session interaction was revealed [$F_{(18, 66)} = 6.407, p < .001$]. Post-hoc tests (Tukey-Kramer) indicated significant differences between the WIN55,212-2 and vehicle administration groups during delay (Sessions 2–9; $p < .05$) and long delay (Sessions 2–10; $p < .01$) conditioning, but not for the trace paradigm. The delay vehicle condition also exhibited more CRs than the long delay vehicle group (Sessions 3–6; $p < .05$) and the trace vehicle group (Sessions 2–4; $p < .05$); whereas CR levels were similar between the long delay and trace groups for the vehicle conditions. Rats receiving WIN55,212-2

administration exhibited more CRs during trace EBC than delay EBC (Session 2–6; $p < .01$) and long delay EBC (Sessions 2–10; $p < .01$). Additionally, the rats receiving WIN55,212-2 during delay EBC exhibited more CRs than those receiving WIN55,212-2 during long delay EBC (Sessions 5–10; $p < .01$).

CR amplitudes were also examined using a 2 (Drug [WIN55,212-2 or Vehicle]) $\times$ 3 (Paradigm [delay, long delay, or trace]) $\times$ 10 (Session) repeated measures ANOVA. A Drug $\times$ Paradigm $\times$ Session interaction was revealed [$F_{(18,66)} = 1.884$, $p = .046$]. Post-hoc tests (Tukey-Kramer) indicated significant impairments in WIN55,212-2 compared to vehicle administered groups during delay EBC ($p < .05$; Sessions 3–5) and long delay EBC ($p < .05$; Sessions 4–9) but no group differences in trace EBC. Rats administered vehicle during delay EBC had larger CR amplitudes compared to vehicle rats in long delay EBC ($p < .05$; Sessions 3–10) and trace EBC ($p < .05$; Sessions 3–10); no differences were observed between long delay and trace conditioning. Similarly, rats administered WIN55,212-2 had larger CR amplitudes during delay EBC compared to rats administered WIN55,212-2 during long delay EBC ($p < .05$; Sessions 5–10) and trace EBC ($p < .05$; Sessions 3–10); however, trace conditioned WIN55,212-2 rats had larger CR amplitudes than long delay WIN55,212-2 treated rats ($p < .05$; Sessions 5, 7–9).

Two timing parameters, CR onset and CR peak latency, were examined during CS-alone trials across the 10 training sessions. For CR onset latencies a 2 (Drug [WIN55,212-2 or Vehicle]) $\times$ 3 (Paradigm [delay, long delay, or trace]) $\times$ 10 (Session) repeated measures ANOVA was conducted and revealed no significant 3 or 2 way interactions. Repeated measures ANOVAs for CR peak latencies did not have a significant Drug $\times$ Paradigm $\times$ Session interaction but did have a Paradigm $\times$ Session interaction [$F_{(18,66)} = 2.584$, $p = .003$] but no Drug $\times$ Session interaction. Post-hoc tests revealed significantly earlier peak latencies in the delay condition than the long delay or trace paradigms ($p < .01$); the long delay and trace paradigms did not differ significantly.

UR amplitudes and US intensities were examined during the unpaired session and paired training. These analyses were conducted in order to examine potential effects of the drug and paradigm on UR amplitude. Separate ANOVAs revealed no significant differences between US intensity and UR amplitudes with regards to paradigm and/or drug administration.

In order to examine whether WIN55,212-2 impaired learning or the expression of learning, rats in the long delay paradigm completed 5 additional sessions of paired CS-US training without administration of WIN55,212-2 or vehicle. Rats that were previously treated with WIN55,212-2 showed a gradual, albeit slow, increase in learning following cessation of administration (Figure 2B, Sessions 11–15). The WIN55,212-2 group was first compared to the vehicle group during their last 5 days of training (without injections). A 2 (Drug) $\times$ 5 (Session) repeated measures ANOVA revealed a significant difference between the groups [$F_{(4,6)} = 5.768$, $p = .030$]. The WIN55,212-2 group had significantly fewer CRs ($p < .01$; Sessions 1–5) as compared to vehicle rats as revealed by a post-hoc test (Tukey-Kramer). No significant differences were found with CR onset latencies, CR peak latencies, CR amplitudes, or UR amplitudes. The WIN55,212-2 group was also compared to the vehicle administered group's first 5 sessions of paired CS-US training (Figure 2B P1-5). A 2 (Drug) $\times$ 5 (Session) repeated measures ANOVA revealed a significant interaction Drug $\times$ Session [$F_{(4,9)} = 5.636$, $p = .015$]. Post-hoc tests (Tukey Kramer) revealed that following WIN55,212-2 treatment, rats exhibited significantly less CRs than the rats initially receiving vehicle treatment ($p < .05$; Sessions 4–5). This indicates that WIN55,212-2 treatment does not decrease the expression of the response but impairs learning. Also, prolonged WIN55,212-2 administration impaired the acquisition of learning as compared to rats initially trained with

the vehicle. No significant differences were found with CR onset latencies, CR peak latencies, CR amplitudes, or UR amplitudes.

In summary, rats administered WIN55,212-2 exhibited significantly fewer CRs than controls during delay and long delay EBC but not trace EBC. This impairment was significantly greater for the long delay paradigm as compared to the delay paradigm. Following cessation of WIN55,212-2 administration, rats in the long delay group showed a gradual, but significantly impaired, rate of acquisition when compared to the first sessions of the vehicle treated rats. This indicates that WIN55,212-2 does not impair the expression of the response but impedes learning. Additionally, prolonged exposure to WIN55,212-2 decreased the rate of CRs following cessation of administration.

Experiment 2: Effects of WIN55,212-2 on EBC with Different Trace Intervals

Rats in Experiment 1 treated with WIN55,212-2 exhibited no impairment during trace EBC. Experiment 2 manipulated the trace interval duration (100, 300, 500, or 700 ms) while keeping the CS duration the same (250 ms) between groups to determine if the effect of WIN55,212-2 on EBC is influenced by the duration of the trace interval. Similar to Experiment 1, rats underwent a day of spontaneous activity (Figure 3, SP) and a day of unpaired training (Figure 3, UP). The duration of the activity measured during the spontaneous activity was equal to the CS (250 ms) + trace interval (either 100, 300, 500, or 700 ms) used in training. All rats underwent unpaired training with a 250 ms CS and a 25 ms US. Separate one way ANOVAs were conducted and found that spontaneous and unpaired blink activity did not differ when WIN55,212-2 was administered or between the trace paradigms.

Rats were then administered either WIN55,212-2 or vehicle for 10 consecutive days while undergoing paired CS-US trace EBC (Figure 3, P1-10). A 2 (Drug [WIN55,212-2 or Vehicle]) $\times$ 4 (Paradigm [100, 300, 500, or 700 trace]) $\times$ 10 (Session) repeated measures ANOVA was conducted to examine effects of WIN55,212-2 during acquisition of the trace conditioning paradigms. A Drug $\times$ Paradigm $\times$ Session interaction was not significant; however, there was a Paradigm $\times$ Session, [$F_{(27,135)}=2.088$, $p=.013$], and a Drug $\times$ Session interaction, [$F_{(9,43)}=2.616$, $p=.028$]. Separate 2 (Drug) $\times$ 10 (Session) ANOVAs were conducted for each trace paradigm to further examine the effects of WIN55,212-2. A Drug $\times$ Session interaction was revealed for 100 ms trace [$F_{(5,9)}=2.112$, $p=0.34$] and 300 ms trace, [$F_{(5,9)}=2.223$, $p=.025$], but not for either the 500 or 700 trace paradigms. Post-hoc tests (Tukey-Kramer) revealed that vehicle treated rats learned significantly better for the 100 trace ($p<.05$; Sessions 2–10) and 300 trace paradigms ($p<.05$; Sessions 3–5) than WIN55,212-2 treated rats. These results indicate that when the CS is constant, WIN55,212-2 administration differentially impairs trace conditioning; the shorter the trace interval the greater the impairment.

CR amplitudes were examined with a similar 2 (Drug) $\times$ 4 (Paradigm) $\times$ 10 (Session) repeated measures ANOVA as mentioned above; no Drug $\times$ Paradigm $\times$ Session interaction was found. However, a Paradigm $\times$ Session interaction was revealed [$F_{(9,27)}=1.697$, $p=.032$], but no Drug $\times$ Session interaction. Post-hoc tests (Tukey-Kramer) found that the 700 trace paradigm had significantly lower CR amplitudes than the 100 ($p<.001$), 300 ($p<.001$), and 500 trace paradigms ($p<.007$).

CR timing was examined with CR onset and peak latencies that were collected during CS alone trials. An ANOVA for CR onset found no significant Drug $\times$ Paradigm $\times$ Session interaction but did reveal a Paradigm $\times$ Session interaction, [$F_{(9,27)}=2.386$, $p=.002$]. Post-hoc tests (Tukey-Kramer) showed significantly earlier timing for the 100 trace paradigm as compared to the 500 trace ($p<.001$) and 700 trace ($p<.001$) paradigms but no other

differences. CR peak latencies were also examined and found no Drug $\times$ Paradigm $\times$ Session interaction but a Paradigm $\times$ Session interaction [$F_{(9,27)} = 2.526$, $p < .001$]. Post-hoc tests (Tukey-Kramer) revealed significantly earlier CR peak latencies for the 100 trace paradigm as compared to the 500 trace ($p < .001$) and 700 trace paradigms ($p < .001$).

Possible effects on the UR and US sensitivity were tested by examining UR amplitudes during unpaired and paired training and also US intensity values. Separate one way ANOVAs were conducted and found no significant differences with UR amplitudes or intensities between vehicle and WIN55,212-2 administered rats.

Experiment 3: Effects of WIN55,212-2 on EBC as a Function of the Relative Durations of the CS and Trace Interval

Experiment 3 was conducted in order to determine what parameter of trace conditioning, either the duration of the trace interval or the proportion of the ISI taken up by the CS or trace interval, makes EBC vulnerable to disruption by WIN55,212-2. In order to examine this, rats were assigned to complete either RGT (100 ms CS, 200 ms trace) or RGC (1000 ms CS, 500 ms trace) trace conditioning paradigms. The RGT paradigm used a 1:2 CS to trace interval ratio, whereas the RGC paradigm used a 2:1 CS to trace interval ratio. If the duration of the trace interval is the critical factor, WIN55,212-2 should have no effect on the RGC paradigm (see Experiment 2). If however, the proportion of the ISI occupied by the CS is the critical factor, WIN55,212-2 should have no effect on the RGT paradigm even though conditioning with short trace intervals and a shorter ISI are impaired (see Experiments 1 and 2). Spontaneous (Figure 4 SP) and unpaired activity (Figure 4 UP) was measured between the conditions and did not differ between the groups.

Following the spontaneous and unpaired sessions, rats were trained for 10 consecutive sessions of CS-US pairings (Figure 3 P1-10). A 2 (Drug [WIN55,212-2 or vehicle]) $\times$ 2 (Paradigms [RGT or RGC]) $\times$ 10 (Session) repeated measures ANOVA was conducted to examine effects of WIN55,212-2 during acquisition of trace EBC. A Paradigm $\times$ Drug $\times$ Session interaction was revealed [$F_{(9,19)} = 1.971$; $p = .043$]. Post-hoc tests (Tukey-Kramer) indicated that WIN55,212-2 impaired acquisition of RGC trace ($p < .05$; Session 3–10) but not RGT EBC. Vehicle administered rats exhibited similar levels of learning between the paradigms; whereas WIN55,212-2 rats exhibited fewer CRs in the RGC paradigm as compared to WIN55,212-2 treated rats in the RGT paradigm ($p < .05$; Sessions 3–10).

CR amplitudes were also examined using a repeated measures ANOVA and no Drug $\times$ Paradigm $\times$ Session interaction was found. However there was a significant Paradigm $\times$ Session interaction [$F_{(9,18)} = 2.694$, $p = .017$]. Post-hoc tests (Tukey Kramer) revealed that rats in the RGT condition had larger CR amplitudes than rats that were trained in the RGC condition.

CR timing was examined using CR onset and peak latency values that were collected during CS alone trials. No significant Drug $\times$ Paradigm $\times$ Session interactions were found. As expected, there was a Paradigm $\times$ Session interaction for both CR onset [$F_{(9,18)} = 4.212$, $p = .004$] and CR peak latency [$F_{(9,18)} = 4.570$, $p = .002$]. Post-hoc tests (Tukey-Kramer) revealed that the RGT condition had earlier onset and peak latencies ($p < .001$) as compared to the RGC condition.

UR amplitudes and US sensitivity were tested in order to examine possible effects of WIN55,212-2. There were no significant differences found with UR amplitudes or US intensities, as reported separate one way ANOVAs.

Discussion

The current study is the first to systematically examine the role of CB1Rs in EBC as a function of delay and trace parameters. In Experiment 1 delay (250 ms CS) and long delay (750 ms CS) EBC were impaired by the CB1R agonist WIN55,212-2, whereas trace EBC (250 ms CS, 500 ms trace) was not impaired. Moreover, long delay EBC was more impaired than delay EBC. Experiment 2 kept the CS duration constant (250 ms) while manipulating the trace interval (100, 300, 500, or 700 ms). The degree of impairment was related to the duration of the trace interval, with shorter intervals resulting in more impairment than longer intervals. Finally, Experiment 3 examined two additional trace paradigms to examine whether the impairments in trace conditioning were due to the trace interval duration or an interaction between the relative durations of the CS and trace interval. Administration of WIN55,212-2 did not impair EBC in the relatively greater trace condition (RGT; 100 ms CS, 200 trace) but did impair learning in the relatively greater CS condition (RGC; 1000 ms CS, 500 ms trace). A summary of each condition is graphed in Figure 5.

EBC with a trace interval exceeding 250 ms requires an intact hippocampus whereas short delay EBC does not in rats (Weiss et al., 1999). Previous work has indicated that manipulations of CB1Rs impair delay EBC but not trace EBC in humans and rodents (Skosnik et al., 2007; Edwards et al., 2008; Kishimoto & Kano 2006; Steinmetz & Freeman, 2010; 2011; Steinmetz et al., 2011). The current results show that WIN55,212-2 administration impairs trace conditioning when the interval is 300 ms or less. These effects are the opposite of the effects of hippocampal lesions, indicating that the effects of WIN55,212-2 during EBC are not due to impaired hippocampal function but most likely reflect cerebellar cortical impairment. Our findings also suggest that while the hippocampus becomes less involved in EBC during shorter trace intervals, cerebellar CB1Rs become more involved in EBC.

The duration of the trace interval is not, however, the most important parameter for determining the effects of cannabinoids on EBC. Rats administered WIN55,212-2 prior to training using a 100 ms CS and a 200 ms trace interval were not impaired, whereas rats trained with a 1000 ms CS and a 500 ms trace interval were impaired. Interestingly, results from Experiment 1 and 2 showed that training with a 250 ms CS and 500 ms trace interval produced no significant impairments. Thus, if the trace interval is 500 ms, a 1000 ms CS, but not a 250 ms CS, results in impaired EBC with WIN55,212-2, indicating that the trace interval is not the critical parameter for determining the role of CB1Rs in EBC. These results suggest that CB1Rs, and the cerebellar cortex, are involved in trace conditioning when the CS duration is greater than the trace duration. It is not known if the opposite parametric relationship is true for the involvement of the hippocampus in EBC. Previous studies examining the role of the hippocampus during trace EBC kept the CS duration the same (around 250 ms) and manipulated the duration of the trace interval (Kim, Clark, & Thompson, 1995; Moyer, Deyo, & Disterhoft, 1990; Port et al., 1986; Solomon et al., 1986; Weiss et al., 1999).

The finding that WIN55,212-2 produced an impairment in EBC when the relative duration of the CS was longer than the trace interval is difficult to explain with current theories of associative learning or timing. It is clearly not the case that the CB1R agonist affected EBC when the CS is a better temporal predictor of the US than the trace interval because EBC was impaired when WIN55,212-2 was given before training with a 1000 ms CS and a 500 ms trace interval, but not when a 250 ms CS was presented with a 500 ms trace interval. Moreover, the results cannot be explained by deficits in CS or US processing since the same US was used throughout and a CS with the same intensity and duration yielded impaired or unimpaired EBC with WIN55,212-2 administration (i.e., 250 ms CS in Experiments 1 and

2). We hypothesize that deficits are produced by WIN55,212-2 in training conditions in which the cerebellar cortex normally engages in intensive temporal processing of the CS; temporal processing of the trace interval is presumed to be handled by the prefrontal cortex (Kalmbach et al., 2010; Siegel et al., 2012). An underlying assumption of this hypothesis is that temporal processing of the CS within the cerebellar cortex is more intensive during delay conditioning because the end of the CS is the best temporal predictor of the US, whereas the best temporal predictor of the US in trace conditioning is the end of the trace interval. However, the absence of WIN55,212-2 effects on CR timing seems to be incompatible with our hypothesis unless rats, unlike rabbits, do not require cerebellar cortical processing for CR timing. Further experimental work to examine the neural mechanisms underlying cannabinoid effects and the role of the rat cerebellar cortex in CR timing is clearly needed to address our hypothesis.

Administration of WIN55,212-2 impairs acquisition of EBC but not retention. In Experiment 1, rats that were assigned to the long delay EBC condition completed 5 additional sessions in which no administration of WIN55,212-2 or vehicle was given. These rats exhibited impairments in learning as compared to rats in the vehicle group. The rats in the long delay group given WIN55,212-2 also exhibited a deficit relative to the first 5 sessions of paired training in the long delay control group. This persistent deficit may be due to downregulation of CB1Rs, most likely within the cerebellar cortex. Repeated administration of CB1R agonists has been shown to cause downregulation of CB1Rs (Sim-Selley, 2003). The time course of this downregulation depends on the brain area examined; hippocampus and cerebral cortex exhibit these changes as quickly as 24 hours, whereas areas with higher receptor densities, such as the cerebellum, show a significant reduction in receptors after 3 days (Romero et al., 1998; Breivogel et al., 1999). The current study administered WIN55,212-2 for 12 consecutive days prior to cessation, enough time to cause downregulation of CB1Rs within the cerebellum. The downregulation hypothesis is consistent with the persistent deficit in EBC among human cannabis users after the cessation of self-administration (Steinmetz et al., 2011).

The current results indicate that alteration of cannabinoid receptor function impairs delay EBC and long delay EBC, but not trace EBC. The results are consistent with deficits observed in humans who are chronic users of cannabis. The most unexpected finding from the current study is that the duration of the CS relative to the duration of the trace interval influences whether cannabinoid agonists affect EBC. Thus, rats given a cannabinoid agonist are impaired in trace conditioning if the duration of the CS is longer than the trace interval. Our current hypothesis is that cannabinoid agonists disrupt cerebellar cortical temporal processing of the CS. A prediction of this hypothesis is that cannabinoids will produce deficits when intensive temporal processing of the CS is necessary.

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References

- Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, Vogt LJ, Sim-Selley LJ. Chronic Δ^9 -Tetrahydrocannabinol Treatment Produces a Time-Dependent Loss of Cannabinoid Receptors and Cannabinoid Receptor-Activated G Proteins in Rat Brain. *Journal of Neurochemistry*. 2002; 73:2447–2459. [PubMed: 10582605]
- Brown KL, Comalli DM, De Biasi M, Woodruff-Pak DS. Trace eyeblink conditioning is impaired in $\alpha 7$ but not in $\beta 2$ nicotinic acetylcholine receptor knockout mice. *Frontiers in Behavioral Neuroscience*. 2010; 4:1–9. [PubMed: 20126432]

- Chen L, Bao S, Lockard JM, Kim JK, Thompson RF. Impaired classical eyeblink conditioning in cerebellar-lesioned and Purkinje cell degeneration (pcd) mutant mice. *The Journal of Neuroscience*. 1996; 16:2829–2838. [PubMed: 8786457]
- Devane WA, Dysarz F, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. *Molecular Pharmacology*. 1988; 34:605–613. [PubMed: 2848184]
- Edwards CR, Skosnik PD, Steinmetz AB, Vollmer JM, O'Donnell BF, Hetrick WP. Assessment of forebrain-dependent trace eyeblink conditioning in chronic cannabis users. *Neuroscience Letters*. 2008; 439:264–268. [PubMed: 18534754]
- Eggan SM, Lewis DA. Immunocytochemical distribution of the cannabinoid CB1 receptor in the primate neocortex: a regional and laminar analysis. *Cerebral Cortex*. 2007; 17:175–191. [PubMed: 16467563]
- Freeman JH, Steinmetz AB. Neural circuitry and plasticity mechanisms underlying delay eyeblink conditioning. *Learning & Memory*. 2011; 18:666–677. [PubMed: 21969489]
- Gaoni Y, Mechoulam R. Isolation, structure, and partial synthesis of an active constituent of hashish. *Journal of the American Chemical Society*. 1964; 86:1646–1647.
- Herkenham M, Lynn AB, Little MD, Johnson MR, Melvin LS, de Costa BR, Rice KC. Cannabinoid receptor localization in brain. *Proceedings of the National Academy of Sciences*. 1990; 87:1932–1936.
- Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie M, Martin BR, Mechoulam R, Pertwee RG. International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacological Reviews*. 2002; 54:161–202. [PubMed: 12037135]
- Kalmbach BE, Ohya T, Mauk MD. Temporal patterns of inputs to cerebellum necessary and sufficient for trace eyelid conditioning. *Journal of neurophysiology*. 2010; 104:627–640. [PubMed: 20484534]
- Kim JJ, Clark RE, Thompson RF. Hippocampectomy impairs the memory of recently, but not remotely, acquired trace eyeblink conditioned responses. *Behavioral Neuroscience*. 1995; 109:195–203. [PubMed: 7619310]
- Kishimoto Y, Kawahara S, Suzuki M, Mori H, Mishina M, Kirino Y. Classical eyeblink conditioning in glutamate receptor subunit $\delta 2$ mutant mice is impaired in the delay paradigm but not in the trace paradigm. *European Journal of Neuroscience*. 2001a; 13:1249–1253. [PubMed: 11285022]
- Kishimoto Y, Kawahara S, Fujimichi R, Mori H, Mishina M, Kirino Y. Impairment of eyeblink conditioning in GluR $\delta 2$ -mutant mice depends on the temporal overlap between conditioned and unconditioned stimuli. *European Journal of Neuroscience*. 2001b; 14:1515–1521. [PubMed: 11722613]
- Kishimoto Y, Hirono M, Sugiyama T, Kawahara S, Nakao K, Kishio M, Kirino Y. Impaired delay but normal trace eyeblink conditioning in PLC [beta] 4 mutant mice. *Neuroreport*. 2001c; 12:2919–2922. [PubMed: 11588603]
- Kishimoto Y, Kano M. Endogenous cannabinoid signaling through the CB1 receptor is essential for cerebellum-dependent discrete motor learning. *Journal of Neuroscience*. 2006; 26:8829–8837. [PubMed: 16928872]
- Lavond DG, Steinmetz JE. Acquisition of classical conditioning without cerebellar cortex. *Behavioural Brain Research*. 1989; 33:113–164. [PubMed: 2765164]
- Moyer JR, Deyo RA, Disterhoft JF. Hippocampectomy disrupts trace eye-blink conditioning in rabbits. *Behavioral Neuroscience*. 1990; 104:243–252. [PubMed: 2346619]
- Nolan BC, Freeman JH. Purkinje cell loss by OX7-saporin impairs acquisition and extinction of eyeblink conditioning. *Learning & Memory*. 2006; 13:359–365. [PubMed: 16741286]
- Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacology & therapeutics*. 1997; 74:129. [PubMed: 9336020]
- Port RL, Mikhail AA, Romano AG, Steinmetz JE, Patterson MM. Retention and acquisition of classical trace conditioned responses by rabbits with hippocampal lesions. *Behavioral Neuroscience*. 1986; 100:745–752. [PubMed: 3778637]

- Romero J, Berrendero F, Manzanares J, Perez A, Corchero J, Fuentes JA, Fernandez- Ruiz JJ, Ramos JA. Time-course of the cannabinoid receptor downregulation in the adult rat brain caused by repeated exposure to D9-tetrahydrocannabinol. *Synapse*. 1998; 30:298–308. [PubMed: 9776133]
- Substance Abuse Mental Health Services Administration. Results from the 2005 National Survey on Drug Use and Health: National Findings NSDUH Series H-25. DHHS Pub. No. (SMA) 04-3964 SAMHSA. Rockville, MD: 2006.
- Safo PK, Regehr WG. Endocannabinoids control the induction of cerebellar LTD. *Neuron*. 2005; 48:647–659. [PubMed: 16301180]
- Siegel JJ, Kalmbach B, Chitwood RA, Mauk MD. Persistent activity in a cortical-to-subcortical circuit: bridging the temporal gap in trace eyelid conditioning. *Journal of Neurophysiology*. 2012; 107:50–64. [PubMed: 21957220]
- Sim-Selley LJ. Regulation of cannabinoid CB1 receptors in the central nervous system by chronic cannabinoids. *Critical Reviews in Neurobiology*. 2003; 15:91–119. [PubMed: 14977366]
- Skosnik PD, Edwards CR, O'Donnell BF, Steffen A, Steinmetz JE, Hetrick WP. Cannabis use disrupts eyeblink conditioning: evidence for cannabinoid modulation of cerebellar-dependent learning. *Neuropsychopharmacology*. 2008; 33:1432–1440. [PubMed: 17637608]
- Solomon PR, Vander Schaaf ER, Thompson RF, Weisz DJ. Hippocampus and trace conditioning of the rabbit's classically conditioned nictitating membrane response. *Behavioral Neuroscience*. 1986; 100:129–144. [PubMed: 3513798]
- Steinmetz AB, Edwards CR, Vollmer JM, Erickson MA, O'Donnell BF, Hetrick WP, et al. Examining the effects of former cannabis use on cerebellum-dependent eyeblink conditioning in humans. *Psychopharmacology*. 2011; 221:133–141. [PubMed: 22134474]
- Steinmetz AB, Freeman JH. Retention and extinction of delay eyeblink conditioning are modulated by central cannabinoids. *Learning & Memory*. 2011; 18:634–638. [PubMed: 21940395]
- Steinmetz AB, Freeman JH. Central cannabinoid receptors modulate acquisition of eyeblink conditioning. *Learning & Memory*. 2010; 17:571–576. [PubMed: 21030483]
- Vogel RW, Amundson JC, Lindquist DH, Steinmetz JE. Eyeblink conditioning during an interstimulus interval switch in rabbits (*Oryctolagus cuniculus*) using picrotoxin to disrupt cerebellar cortical input to the interpositus nucleus. *Behavioral Neuroscience*. 2009; 123:62–74. [PubMed: 19170431]
- UNODC. World drug report. 2006. Available at http://www.unodc.org/unodc/en/world_drug_report_2006.html
- Weiss C, Bouwmeester H, Power JM, Disterhoft JF. Hippocampal lesions prevent trace eyeblink conditioning in the freely moving rat. *Behavioural brain research*. 1999; 99:123–132. [PubMed: 10512579]
- Woodruff-Pak DS, Disterhoft JF. Where is the trace in trace conditioning? *Trends in Neuroscience*. 2008; 31:105–112.

Experiment 1

Experiment 2

Experiment 3

Figure 1.

Schematic representation of trial types in experiments 1, 2, and 3. In Experiment 1, animals received either delay (250 ms CS), long delay (750 ms CS), or trace (250 ms CS, 500 ms trace) eyeblink conditioning. Experiment 2 manipulated the trace interval (100, 300, 500, or 700 ms) while keeping the CS duration the same (250 ms). In Experiment 3 rats were assigned to either relatively greater trace (RGC; 100 ms CS, 200 ms trace) or relatively greater CS (1000 ms CS, 500 ms trace) trace conditioning paradigms.

Figure 2.

Mean $\pm$ SEM conditioned response (CR) percentage for rats injected with WIN55,212-2 (WIN) or vehicle (Veh) and then given delay (A), long delay (B), or trace (C) eyeblink conditioning. Spontaneous (SP) blinks and responses during unpaired (UP) training were assessed in the initial sessions followed by 10 sessions of paired (P) training. Rats in the long delay groups were also given 5 additional sessions of paired training without injections (P11-15).

Figure 3.

Mean $\pm$ SEM conditioned response (CR) percentage for groups of rats injected with with WIN55,212-2 (WIN) or vehicle (Veh) and then given the same conditioned stimulus duration (250 ms) but trace intervals of 100 (A), 300 (B), 500 (C) or 700 (D) ms. Spontaneous (SP) blinks and responses during unpaired (UP) training were assessed in the initial sessions followed by 10 sessions of paired (P) training.

Figure 4.

Mean $\pm$ SEM conditioned response (CR) percentage for groups of rats injected with with WIN55,212-2 (WIN) or vehicle (Veh) and then given either relatively greater trace (RGC; 100 ms CS, 200 ms trace) or relatively greater CS (1000 ms CS, 500 ms trace) trace conditioning paradigms. Spontaneous (SP) blinks and responses during unpaired (UP) training were assessed in the initial sessions followed by 10 sessions of paired (P) training.

Figure 5.

Summary of Experiments 1, 2, and 3 for vehicle (open bars) and WIN55,212-2 (hatched bars) treated rats. Values given are the average conditioned response (CR) percentage for paired sessions 1-10. Asterisks (*) denote significant differences.