Published in final edited form as:

Behav Brain Res. 2020 January 27; 378: 112258. doi:10.1016/j.bbr.2019.112258.

Intracerebellar Cannabinoid Administration Impairs Delay but not Trace Eyeblick Conditioning

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Abstract

Intracerebellar administration of cannabinoid agonists impairs cerebellum-dependent delay eyeblink conditioning (EBC) in rats. It is not known whether the cannabinoid-induced impairment in EBC is found with shorter interstimulus intervals (ISI), longer ISIs, or with trace EBC. Moreover, systemic administration of cannabinoid agonists does not impair trace EBC, suggesting that cannabinoid receptors within the cerebellum are not involved in trace EBC. To more precisely assess the effects of cannabinoids on cerebellar learning mechanisms the current study examined the effects of the cannabinoid agonist WIN55,212-2 (WIN) infusion into the area of the cerebellar cortex necessary for EBC (the eyeblink microzone) in rats during short delay (250 ms CS), long delay (750 ms CS), and trace (250 ms CS, 500 ms trace interval) EBC. WIN was infused into the eyeblink microzone 30 min before pretraining sessions and five EBC training sessions, followed by five EBC training sessions without infusions to assess recovery from drug effects and savings. WIN had no effect on spontaneous blinks or non-associative responses to the CS or US during the pretraining sessions. Short and long delay EBC were impaired by WIN but trace EBC was unaffected. The results indicate that trace EBC is mediated by mechanisms that are resistant to cannabinoid agonists.

Keywords

cannabinoid; learning; cerebellum; eyeblink conditioning; Purkinje cell

1. Introduction

Eyeblink conditioning (EBC) is an associative learning task used extensively to examine cerebellar function in both human and non-human mammals [1-4]. EBC involves the presentation of a conditioned stimulus (CS) that does not elicit eyelid closure before training, such as a tone or light, paired with an unconditioned stimulus (US) that elicits an eyelid closure unconditioned response prior to training such as a corneal air puff or

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periorbital shock [5,6]. After repeated CS-US pairings, a conditioned response (CR) occurs prior to the onset of the US. The CR is part of a constellation of synchronized defensive responses [7], which include eyelid closure, eyeball retraction, nictitating membrane movement (rabbit and cat), head turn, pinna movement, forelimb movement, body turning, and these responses are then followed by freezing in rodents [8]. Acquisition and retention of the CR depend on the intermediate cerebellum (ipsilateral to the trained eye), including the anterior interpositus nucleus and the cortex at the base of the primary fissure (the eyeblink microzone) and cerebellar projections to the red nucleus [9-19].

Simple spike activity in Purkinje cells decreases within the CS period during EBC [20-23]. The dynamics of the pauses in Purkinje cell activity are highly correlated with the kinematics of the CR [21]. Purkinje cell pauses then release the deep cerebellar nuclei (e.g., the anterior interpositus nucleus) from inhibition, resulting in an increase in neuronal firing and plasticity within the nuclei [3,24-32]. This increase in cerebellar nucleus activity is the inverse of the Purkinje cell pauses and activates the red nucleus [33,34], which in turn activates motor neurons in the facial nucleus that cause eyelid closure [35]. Learning-specific pauses in Purkinje cell activity have been attributed to long-term depression (LTD) of parallel fiber synapses with Purkinje cells [21,36,37]. Other synaptic and non-synaptic mechanisms might underlie or contribute to learning-specific pauses in Purkinje cells as well [23,38-40]. Even though the precise mechanisms underlying learning-specific pauses in Purkinje cells are currently being debated, there is abundant evidence that this plasticity occurs within the cerebellar cortex [9-11,23,41,42].

The cerebellar cortex has a high density of type-1 cannabinoid receptors (CB1R) on presynaptic terminals [43,44]. Agonist activation of CB1Rs causes a reduction in neurotransmitter release from parallel fiber terminals and other terminals [45-47]. Cannabinoid agonists could therefore affect LTD and other plasticity mechanisms within the cerebellar cortex and thereby affect EBC. Indeed, systemic administration of cannabinoid agonists impairs EBC in humans and rats [48,49]. The EBC deficits are caused by cannabinoid action within the cerebellar cortex, as determined with microinfusions of cannabinoid agonists into the eyeblink microzone during EBC in rats [50,51]. These studies also demonstrated that cannabinoid-induced impairments in EBC occur through agonist action on CB1Rs [51]. Control conditions in these studies including assessments of spontaneous blinks, non-associative responses to the CS, non-associative responses to the US, and sensitivity to the US indicate that the cannabinoid-induced deficit in EBC is a learning impairment and not caused by sensory or motor deficits [49-53]. The results of these studies indicate that cannabinoid agonist cause a dose-dependent deficit in EBC and this impairment is due to alteration of plasticity mechanisms within the cerebellar cortex.

Cannabinoid-induced deficits in EBC are well documented in humans and rodents; however, a variant of the EBC task, *trace* EBC, is not impaired by cannabinoid agonists [53-55]. In *trace* EBC, there is a 250 -500 ms stimulus free “trace” interval between the end of the CS and the start of the US, whereas the EBC tasks used in studies described above used *delay* EBC in which there is no trace interval between the CS and US. This seemingly trivial difference in EBC tasks, in fact, results in the recruitment of additional brain areas including the sensory cortex, medial prefrontal cortex and hippocampus [56-64]. One interpretation of

the absence of a trace conditioning impairment with cannabinoid agonists is that trace and delay EBC are mediated by different mechanisms, with the *trace* EBC mechanism being immune to cannabinoid action. An alternative possibility is that systemic cannabinoid administration results in compensation for deficits in cerebellar cortical plasticity by non-cerebellar brain areas. The central issue addressed in the current study is whether cannabinoid action localized within the eyeblink microzone of the cerebellar cortex impairs *trace* EBC and whether this impairment would be as severe as the impairment in *delay* EBC.

Another important issue addressed in this study is whether intracerebellar infusions of cannabinoid agonists would affect delay EBC with a relative short or long inter-stimulus interval (ISI). Previous studies examining the effects of intracerebellar agonists on EBC used a delay EBC task with a 400 ms ISI [50,51], but robust EBC in rats can be established with ISIs as short as 250 ms or as long as 750 ms [53,63]. It is therefore crucial to determine the generality of the cannabinoid-induced deficit in delay EBC by examining the effects of intracerebellar cannabinoid agonist infusions on short and long delay EBC.

The current study examined the effects of cannabinoid agonist administration into the eyeblink microzone of the cerebellar cortex in rats during short delay, long delay, and trace EBC, with CS duration and ISI equated between groups (Fig. 1). The cannabinoid agonist WIN55,212-2 (WIN) or vehicle was infused into the eyeblink microzone 30 min before two pretraining sessions and five EBC training sessions with short delay (250 ms CS), long delay (750 ms CS), or trace (250 ms CS, 500 ms trace interval) tasks. The rats were then given five EBC training sessions without infusions to assess recovery from drug effects and savings.

2. Material and methods

2.1. Animals

The subjects were 55 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. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa and comply with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

2.2. Surgery

One week before training, rats were anesthetized with isoflurane and then 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 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. 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 bone cement.

2.3. Infusion procedure

Before the 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.

2.4. Drug

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) was infused into the cerebellar cortex 30 min before the first 7 daily training sessions. WIN55,212-2 binds to CB1 and CB2 receptors [65,66]. WIN55,212-2 was infused at a dose of 10 µg/µL. This dose was chosen based on dose-response analyses in previous studies (Steinmetz & Freeman, 2016; Steinmetz & Freeman, 2018). The vehicle was a 1:1:18 solution of ethanol, cremaphor, saline. WIN55,212-2 was purchased from Sigma/RBI.

2.5. Conditioning apparatus

The conditioning apparatus consisted of 8 small-animal sound-attenuating chambers (BRS/LVE, Laurel, MD). Within each sound-attenuating chamber was a small-animal chamber (BRS/LVE) in which the rats were kept during training. One wall of the 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 via a commutator 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). The US was delivered through a stimulus isolator (model 365A; World Precision Instruments, Sarasota, FL). The EMG activity was recorded differentially, amplified (2000 X), filtered (500-5000 Hz), and integrated (time constant, 20 ms).

2.6. Conditioning procedures

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 tasks were used (Fig. 1): short delay (250 ms CS), long delay (750 ms CS) and trace (250 ms CS, 500 ms trace) conditioning. 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). 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. 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 of the first 7 daily training sessions (spontaneous blink session, unpaired session, and 5 paired CS-US sessions). Rats then received 5 paired CS-US sessions without infusions to examine recovery from drug effect and savings.

2.7. 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 was verified using a light microscope (Leica DMLS, Wetzlar, Germany).

2.8. Data Analysis

The dependent variables were CR percentage, amplitude, onset latency, and peak latency. Amplitude and latency measures are typically taken from CS-alone probe trials to avoid the UR, but we used all trials to analyze these variables since the incidence of CRs was very low in some of the drug conditions (see results below), resulting in numerous rats with missing data for CS-alone trials.

All dependent variables were analyzed across CS-US paired sessions and phases of training with linear mixed effects modeling (R, version 3.6.0), which circumvents some of the limitations of repeated measures ANOVA for learning data including the assumption of independent observations and intolerance to missing data [67]. Failure to account for the non-independence of trial, block, or session data in a learning experiment can increase the probability of a Type I error (false positive). Mixed effects modeling accounts for the non-independence of the learning data, yielding a lower Type I error rate. Models included fixed effects for Group (Saline, WIN), Condition (Short Delay, Long Delay, Trace), Phase (Infusion Sessions 1-5, Non-infusion Sessions 6-10), and Session (1-10). Random effects included Intercept, Phase, and Session for each rat. The CR percentage data were best fit by a quadratic function, which was added to the models. The CR amplitude, onset latency, and peak latency were best fit by a simple linear function; the model results therefore do not

include the quadratic function for these variables. Satterthwaite approximations to degrees of freedom were used for the mixed effects analyses. The t statistic is presented for comparisons of learning curves for two groups because there are only two data points (slope or quadratic function) and the F statistic is presented for interactions involving more than two groups. Data from the pretraining sessions were analyzed separately by ANOVA rather than mixed effects modeling because each rat had a single data point for each pretraining session.

3. Results

3.1. Cannula Placements

Cannula placement was consistent between the groups, across experiments, and with previous studies targeting the eyeblink microzone in the cerebellar cortex ipsilateral to the conditioned eye [17,50,51]. An example of a cannula placement is presented in Fig. 2. Six rats were removed from the subsequent data analyses due to placements being either too ventral (n = 3; 1 long delay WIN, 1 long delay vehicle, and 1 trace vehicle), anterior (n = 2; 1 short delay WIN, 1 trace WIN), or posterior (n = 1; long delay vehicle). The number of rats per group after histological verification was as follows: short delay (vehicle = 8, WIN = 8); long delay (vehicle = 9, WIN = 8); and trace (vehicle = 8, WIN = 8).

3.2. Behavioral Data

Prior to CS-US paired training the rats were given two pretraining sessions examining spontaneous blinks and responses to unpaired CS/US presentations. Separate one-way ANOVAs found no effects of WIN administration on spontaneous blink rate, or on the frequency or amplitude of responses to the CS and US, indicating that WIN had no effects on sensory responses or motor performance. The results from the pretraining sessions are consistent with all of our previous studies with systemic and intracerebellar administration of cannabinoid agonists [49-53].

In contrast to the pretraining sessions, there were substantial effects of WIN during CS-US paired training. CR percentage was severely impaired in the short delay (Fig. 3) and long delay (Fig. 4) EBC conditions but not in the trace EBC condition (Fig. 5). The short delay WIN group showed an increase in CR percentage during the training sessions with WIN infusions (P1-P5) and rapidly reached an asymptote comparable to the vehicle control group during the noinfusion sessions (P6-P10). In contrast, the long delay WIN group showed very little increase in CR percentage during the infusion sessions (P1-P5) and required five training sessions after the cessation of infusions (P6-P10) to reach the asymptotic level of CRs in the vehicle group. WIN did not have any effect on trace EBC during infusion or non-infusion sessions.

The CR percentage data were first analyzed with an omnibus linear mixed effects model that included fixed effects for Group (Saline, WIN), Condition (Short Delay, Long Delay, Trace), Phase (Infusion Sessions 1-5, Non-Infusion Sessions 6-10), and Session (linear and quadratic functions within Phase). Random effects included Intercept, Phase, and Session. The omnibus analysis of CR percentage yielded an interaction of the Group, Condition,

Phase, and Session (quadratic function) variables, $F(2, 325.39) = 5.4469$, $P = 0.004712$, indicating that the learning curves differed as a function of group, condition, phase and sessions within phases. To explore this interaction further, separate analyses were conducted for the short delay, long delay, and trace EBC conditions (see sections 3.3 - 3.5).

The CR amplitude data were best fit by linear functions rather than the quadratic functions used for the CR percentage data. The model yielded a Phase X Session X Group X Condition interaction, $F(2, 379.1) = 4.3196$, $P = 0.014$, indicating that the CR amplitude differed as a function of group, condition, phase and sessions within phases. As with the CR percentage data, separate analyses of the CR amplitude data were conducted for the short delay, long delay, and trace EBC conditions (see sections 3.3 - 3.5).

The analyses of CR onset latency and peak latency data found interactions of the Phase, Session, and Group factors, but no interactions involving the Condition factor (onset latency, $F(1, 378.51) = 6.9525$, $P = 0.008715$; peak latency, $F(1, 379.05) = 4.3435$, $P = 0.03782$). For both measures, the interaction was caused by a higher CR latency during the infusion sessions in the WIN groups relative to the vehicle groups. There were also main effects of Condition for CR onset and peak latency, which were caused by substantially lower CR latencies in the short delay group relative to the long delay and trace groups, which was expected since the ISI for the short delay group was 500 ms shorter than the ISI for the other groups.

3.3. Short Delay Conditioning

WIN administered locally into the cerebellar cortex resulted in a substantial impairment in associative learning during the infusion sessions (Fig. 3, P1-P5). After the cessation of infusions, the WIN group showed rapid learning and reached the same asymptote as the controls (Fig. 3, P6-P10). These observations are supported by a linear mixed effects model with fixed effects for Phase, Session, and Group and random effects for Intercept, Session and Phase which yielded a Phase X Session (quadratic function) X Group interaction, $t(119.88) = 3.296$, $P = 0.001292$ and a Phase X Session (linear function) X Group interaction, $t(119.88) = 3.698$, $P = 0.000329$, indicating that the learning curves between the groups differed across sessions and phases. Follow up models analyzed the CR percentage data for each phase separately. The model for the infusion sessions yielded a Session X Group interaction, $t(62.0) = 3.993$, $P = 0.000175$, which was due to the higher CR percentage across sessions (higher slope) in the control group relative to the WIN group (Fig. 3, P1-P5). There was also a Session X Group interaction for the non-infusion sessions, $t(62.0) = 5.432$, $P = 0.000000989$, which reflected asymptotic CR percentage in the control group, resulting in a minimal slope, and an increase in CRs across sessions in the WIN group, resulting in a higher slope (Fig. 3, P6-P10). The analyses of the CR percentage data indicate that WIN infusion into the EBC microzone caused a reversible deficit in short delay EBC.

A WIN-related deficit was also observed in the amplitude of the CR. A mixed effects model with the same fixed and random effects as the model for CR percentage (see description above) yielded a Phase X Session X Group interaction, $t(124.0) = 2.375$, $P = 0.019088$, which was caused by a higher slope for CR amplitude curves across sessions in the vehicle

group relative to the WIN group during the infusions sessions, but not during the non-infusion sessions.

3.4. Long Delay Conditioning

Long delay conditioning (750 ms CS) is more impaired by systemic administration of WIN than short delay conditioning [53] and was expected to be more impaired with intracerebellar infusions as well. Similar to subcutaneous injections, WIN administration resulted in a severe impairment in acquisition of long delay conditioning (Fig. 4). A mixed effects model with fixed effects for Phase, Session, and Group and random effects for the intercept and Session yielded a Phase X Session X Group interaction, $t(132.0) = 7.386$, $P = 0.00000000154$, which reflected a higher slope in the vehicle group relative to the WIN group during the infusion sessions, whereas the WIN group showed a higher slope during the non-infusion sessions as they learned relative to the vehicle group which was at asymptote (Fig. 4). A follow up analysis of the infusion sessions found a Session X Group interaction, $t(66) = 4.803$, $P = 0.00000935$, resulting from the increase in CR percentage in the vehicle group relative to the WIN group (Fig. 4, P1-P5). Analysis of the non-infusion sessions also yielded a Session X Group interaction, $t(66.0) = 5.912$, $P = 0.000000131$, resulting from the increase in CR percentage in the WIN group and the flat asymptotic CR percentage in the vehicle group (Fig. 4, P6-P10). The results of the CR percentage analyses indicate that WIN severely impaired acquisition of long delay EBC, but rats given WIN during initial training were able to acquire long delay EBC at a normal rate after the cessation of infusions.

WIN did not significantly affect the amplitude of the CR in long delay EBC.

3.5. Trace Conditioning

Systemic administration of CB1R agonists does not impair trace EBC in humans or rats [53,54]. In the current study, trace conditioning was not impaired by intracerebellar WIN. The mixed effects models were run that included fixed effects for Phase, Session (linear and quadratic functions), and Group and random effects for intercept, Phase, and Session, which did not find any effects related to the Group factor. There were significant effects of Session and a Phase X Session interaction, which simply reflect the increase in CR percentage in both groups and the leveling off of CR percentage during the non-infusion sessions (Fig. 5).

WIN administration did not affect CR amplitude in trace EBC.

4. Discussion

Delay and long delay eyeblink conditioning were impaired with administration of the cannabinoid agonist WIN55,212-2 into the eyeblink microzone of the cerebellar cortex (Fig. 3, 4). Long delay EBC was more impaired than short delay EBC. In contrast, trace EBC was unaffected. The results indicate that cannabinoid agonists impair delay EBC by affecting mechanisms within the cerebellar cortex, whereas trace EBC is mediated by mechanisms that are resistant to cannabinoid agonist action.

Previous research found that systemic manipulations of CB1Rs impair delay EBC, but not trace EBC in humans and rodents [48,49,52-55]. In the current study, trace EBC was unimpaired even when the CB1R agonist was infused directly into the area of the cerebellar cortex necessary for EBC. The mechanisms underlying trace conditioning's immunity to cannabinoid agonists are unknown but some researchers have concluded that trace conditioning simply does not require the cerebellar cortex [68]. Findings from a recent study, however, cast doubt upon the hypothesis that cerebellar cortex is not necessary for trace EBC [69].

Delay EBC is associated with pauses in Purkinje cell simple spike activity within the eyeblink microzone during the CS; these pauses in simple spike activity develop in parallel with the CR and the dynamics of the pauses correlate highly with CR kinematics [21]. Pauses in Purkinje cell activity release the deep cerebellar nuclei from inhibition, which is reflected in a CR-related increases in neuronal firing [25,27,28,30,70]. The increase in DCN neuronal firing is also highly correlated with the kinematics of the CR and drives the activity of efferent motor neurons that mediate the CR [21]. Thus, Purkinje cell pauses ultimately cause CR production.

The pauses in simple spike activity have been attributed to long-term depression (LTD) of parallel fiber synapses with Purkinje cells [21,36,37]. The CR-related pause in simple spike activity could also be caused by other synaptic and non-synaptic plasticity mechanisms [23,38-40]. Induction of parallel fiber LTD, and presumably other plasticity mechanisms, is impaired by cannabinoid agonists such as WIN [46]. The absence of a trace EBC deficit with WIN infusions into the cerebellar cortex in the current study might therefore suggest that cerebellar Purkinje cell plasticity is not necessary for trace EBC; however, this conclusion is not consistent with the findings of a recent study which demonstrated Purkinje cell simple spike pauses within the eyeblink microzone of the cerebellar cortex during trace EBC [69]. As with delay EBC, pauses in Purkinje cell simple spike activity, within the same area of the cortex, were highly correlated with the kinematics of the CR. It is therefore unlikely that trace EBC depends on a plasticity mechanism that is completely distinct from the mechanism underlying delay EBC. The more likely explanation is that trace conditioning depends on a plasticity mechanism similar to that of delay conditioning but insensitive to cannabinoid agonists.

How might the plasticity mechanism underlying trace EBC be more resistant to the effects of cannabinoids than the mechanism underlying delay EBC? *In vitro* studies of LTD demonstrate that stronger parallel fiber stimulation, recruiting more parallel fibers, results in increased intracellular calcium by activation of voltage-gated calcium channels, which supports stronger LTD and heterosynaptic LTD at unstimulated synapses [71-73]. The resistance of trace EBC to cannabinoids might therefore be related to the recruitment of more parallel fibers relative to delay EBC. Consistent with this speculation are findings showing that the cerebellar cortex receives mossy fiber inputs from both the CS and the medial prefrontal cortex during trace EBC [59,60,74]. The effects of these inputs may summate, resulting in LTD or another plasticity mechanism that is robust enough to support pauses in simple spike activity, even in the presence of reduced glutamate release from parallel fibers. Another possibility is that the mossy fiber inputs from the medial PFC are

stronger, recruited in higher numbers, or fire at a higher frequency than the CS-related mossy fiber inputs and are therefore less affected by the activation of the presynaptic CB1Rs. As a result, there is sufficient glutamate release from parallel fibers activated by the medial PFC to support Purkinje cell plasticity in trace conditioning. Lastly, an extracerebellar area such as the prefrontal cortex or amygdala might compensate for the reduction in parallel fiber glutamate release by boosting mossy fiber input to the cerebellum. The forebrain compensation could be triggered by an error signal from reduced cerebellar output and thus reduced feedback to the PFC or amygdala relative to non-drugged conditions. These proposed mechanisms are plausible but additional research is needed to identify the mechanism(s) underlying the immunity of trace conditioning to cannabinoid agonists.

Short delay EBC (250 ms ISI) was impaired by WIN in the current study but less so than long delay (750 ms ISI) EBC (Fig. 3, 4) or the intermediate delay EBC (400 ms ISI) used in previous studies [51]. The difference in the efficacy of WIN on short and long delay conditioning might be mediated by differences in the degree to which the CR depends on cerebellar cortical plasticity. Purkinje cell simple spike recordings show a less precise correlation between pauses in simple spike activity and the CR in short delay conditioning relative to long delay conditioning [21]. Moreover, short delay CRs in mice have a short-latency amygdala component as well as a longer-latency cerebellar component [75]. The short-latency component of the CR might be a fear potentiated startle response. The amygdala may therefore partially mediate learning and expression of CR-like responses in the short delay condition.

The current study extends previous research on the effects of cannabinoid agonists on cerebellar learning by showing differential effects of intracerebellar infusions of WIN55,212-2 on short delay, long delay, and trace EBC. Infusions were made into the eyeblink microzone of the cerebellar cortex, which is crucial for learning and expression of eyeblink CRs. The absence of an impairment in trace EBC with WIN infusions suggests that it is mediated by mechanisms that are immune to or can compensate for cannabinoid action. Additional research to determine why trace EBC is not impaired by cannabinoid agonists may have the additional benefit of revealing differences in the fundamental mechanisms underlying delay and trace conditioning.

Acknowledgements

This research was supported by National Institutes of Health grants MH080005 and NS088567.

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Highlights

- Intracerebellar infusion of WIN55,212-2 impaired delay eyeblink conditioning
- Trace conditioning was unaffected by intracerebellar WIN
- Long delay conditioning was more impaired by WIN than short delay conditioning
- Cerebellar cortical mechanisms of trace conditioning are resistant to cannabinoids

Fig. 1.

Schematic representation of the trial types for A) short delay, B) long delay, and C) trace conditioning. Rats received either short delay with a 250 ms CS, long delay with a 750 ms CS, or trace conditioning with a 250 ms CS and a 500 ms time gap between the CS and US (750 ms interstimulus interval). The CS did not overlap with the US in the delay conditioning tasks.

Fig. 2.

A representative coronal section of the cerebellum showing a cannula placement located within the eyeblink microzone of the cerebellar cortex (red outline). The arrow points to the track left by the cannula.

Fig. 3.

Mean $\pm$ SE conditioned response (CR) percentage for rats given WIN or vehicle (Veh) infusions into the eyeblink microzone of the cerebellar cortex 30 min before a pretraining session to assess spontaneous blinking (SP), a second pretraining session to assess non-associative responses to unpaired presentations of the CS and US (UP), and the first five CS-US paired sessions (P1-P5) of short delay conditioning. No infusions were given for the last five CS-US paired conditioning sessions (P6-P10).

Fig. 4.

Mean $\pm$ SE conditioned response (CR) percentage for rats given WIN or vehicle (Veh) infusions into the eyeblink microzone of the cerebellar cortex 30 min before a pretraining session to assess spontaneous blinking (SP), a second pretraining session to assess non-associative responses to unpaired presentations of the CS and US (UP), and the first five CS-US paired sessions (P1-P5) of long delay conditioning. No infusions were given for the last five CS-US paired conditioning sessions (P6-P10).

Fig. 5. Mean $\pm$ SE conditioned response (CR) percentage for rats given WIN or vehicle (Veh) infusions into the eyeblink microzone of the cerebellar cortex 30 min before a pretraining session to assess spontaneous blinking (SP), a second pretraining session to assess non-associative responses to unpaired presentations of the CS and US (UP), and the first five CS-US paired sessions (P1-P5) of trace conditioning. No infusions were given for the last five CS-US paired conditioning sessions (P6-P10).