Central cannabinoid receptors modulate acquisition of eyeblink conditioning

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Delay eyeblink conditioning is established by paired presentations of a conditioned stimulus (CS) such as a tone or light, and an unconditioned stimulus (US) that elicits the blink reflex. Conditioned stimulus information is projected from the basilar pontine nuclei to the cerebellar interpositus nucleus and cortex. The cerebellar cortex, particularly the molecular layer, contains a high density of cannabinoid receptors (CB1R). The CB1Rs are located on the axon terminals of parallel fibers, stellate cells, and basket cells where they inhibit neurotransmitter release. The present study examined the effects of a CB1R agonist WIN55,212-2 and antagonist SR141716A on the acquisition of delay eyeblink conditioning in rats. Rats were given subcutaneous administration of 1, 2, or 3 mg/kg of WIN55,212-2 or 1, 3, or 5 mg/kg of SR141716A before each day of acquisition training (10 sessions). Dose-dependent impairments in acquisition were found for WIN55,212-2 and SR141716A, with no effects on spontaneous or nonassociative blinking. However, the magnitude of impairment was greater for WIN55,212-2 than SR141716A. Dose-dependent impairments in conditioned blink response (CR) amplitude and timing were found with WIN55,212-2 but not with SR141716A. The findings support the hypothesis that CB1Rs in the cerebellar cortex play an important role in plasticity mechanisms underlying eyeblink conditioning.
mechanisms underlying the acute effects of cannabis use on associative learning in humans.

The current study used a selective CB1R agonist WIN55,212-2 and antagonist SR141716A to examine the role of the CB1R in acquisition of dEBC. Rats were given subcutaneous administration of 1, 2, or 3 mg/kg of WIN55,212-2 in Experiment 1 and 1, 3, or 5 mg/kg of SR141716A in Experiment 2. In both experiments, drug administration was followed by an assessment of spontaneous blinking (session 1), unpaired CS/US training (session 2), and paired CS–US training (sessions 3–12).

Results

Experiment 1: WIN55,212-2

The CB1R agonist WIN55,212-2 (1, 2, and 3 mg/kg) or the vehicle (control) was injected subcutaneously prior to each session. During the first session, the rats were placed into the conditioning chambers while spontaneous blinks were collected (Fig. 1, sp). A one-way analysis of variance (ANOVA) confirmed that there were no significant differences between the groups. During the following session, the rats were given unpaired presentations of the CS and US to assess nonassociative blinking to the tone CS (Fig. 1, up). A one-way ANOVA revealed no significant effects between the groups. Taken together, these results indicate that the varying doses of WIN55,212-2 did not change the rate of spontaneous blinking or nonassociative blinking to the tone CS.

Over the next 10 sessions, rats were given 90 CS–US pairings and 10 CS-alone presentations daily to examine acquisition of the eyelink CR. Rats given injections of the vehicle showed acquisition of the CR with asymptotic learning around 90%. Rats given 1 mg/kg showed a similar acquisition curve with asymptotic learning slightly lower, around 85%. However, the 2 and 3 mg/kg groups showed more marked impairments with the maximum levels of learning at around 80% and 50%, respectively. A 4 (group) × 10 (session) repeated-measures ANOVA yielded an interaction of the group and session factors, $F_{(27,66)} = 4.301, P < 0.001$. Post hoc tests (Tukey’s honestly significant difference [HSD]) revealed that rats given 2 mg/kg of WIN55,212-2 were significantly impaired on sessions 3 to 7 relative to the control rats, $P < 0.05$. Rats given 3 mg/kg were significantly impaired on sessions 2 through 10 compared with controls rats, $P < 0.01$, and compared with the 1 mg/kg (sessions 2–10, $P < 0.01$) and 2 mg/kg (sessions 2–10, $P < 0.01$) groups. These results indicate that WIN55,212-2 produced a dose-dependent impairment in associative learning.

The amplitudes of the CR from CS-alone trials were also compared between groups using a repeated-measures ANOVA (Fig. 2), which yielded a group × session interaction, $F_{(27,66)} = 3.751, P < 0.001$. Post hoc tests revealed larger CR amplitudes for the controls compared with the 3 mg/kg group (sessions 4–10; $P < 0.01$), 2 mg/kg group (sessions 4–9; $P < 0.01$), and 1 mg/kg group (sessions 5–8; $P < 0.05$).

Two components of CR timing, onset, and peak latencies from CS-alone trials were compared across the doses. A repeated-measures ANOVA for CR onset latencies (Fig. 3) revealed a significant interaction between the group and session factors, $F_{(27,66)} = 1.726, P = 0.017$. Post hoc tests indicated significantly later CR onsets for the 3 mg/kg group in sessions 4–10 when compared with controls, $P < 0.05$. No significant differences were found with the remaining groups. CR peak latencies were also compared for the groups but no main effects or interactions were revealed.

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 eyelink reflex did not differ, suggesting that the drug did not disrupt the response or sensitivity to the shock US.

Experiment 2: SR141716A

SR141716A, a CB1R antagonist, was injected subcutaneously at doses of 1, 3, or 5 mg/kg or the vehicle prior to each session. As in Experiment 1, spontaneous blinks and blinks during unpaired training were examined prior to CS–US conditioning (Fig. 4). Separate one-way ANOVAs found no significant effects relating to groups for the percentage of spontaneous blinks or responses to the CS during unpaired training. These results indicate that, like WIN55,212-2, SR141716A did not alter spontaneous blinking or nonassociative responding to the CS.
terminals (Diana et al. 2002), terminals (Breivogel et al. 2004; Kawamura et al. 2006) and to mGluR1 activation, which binds to CB1Rs on granule cell axon terminals (Herkenham et al. 1990; Ong et al. 1998; Lee et al. 2000). Purkinje cells release 2-AG following significant main effect for the group factor, ANOVAs revealed no significant group differences for US intensity and UR amplitude. Further tests showed that the 3 mg/kg (P < 0.05) and 5 mg/kg (P < 0.01) groups significantly differed from the controls indicating that there were no drug-related deficits in acquisition of dEBC with WIN55,212-2 and SR141716A may, therefore, be caused by impairments in cerebellar cortical LTD.

The results of the current study suggest that activating CB1Rs with WIN55,212-2 has more of an effect on cerebellar learning than blocking them with SR141716A. Rats given WIN55,212-2 had reduced CR percentages, reduced amplitudes, and longer onsets, whereas rats given SR141716A showed no impairment other than CR percentage, and the maximum CR percentage deficit in the 3 and 5 mg/kg groups was less severe than the deficit seen in the 3 mg/kg WIN55,212-2 group. It is possible that a higher dose of SR141716A would result in deficits similar to those produced by 3 mg/kg of WIN55,212-2. However, the 5 mg/kg group did not differ from the 3 mg/kg group for the CR percentage measure suggesting that further increases in the SR141716A dose may not produce a substantially greater impairment. The reduction in glutamate release from parallel fibers with WIN55,212-2 resulting in what has been termed depolarization-induced suppression of excitation (Kreitzer and Regehr 2001a) and depolarization-induced suppression of inhibition (Kreitzer and Regehr 2001b), respectively. WIN55,212-2 and SR141716A impair induction of parallel fiber-Purkinje cell LTD in vitro (Levène et al. 1998; Safo and Regehr 2005). LTD at parallel fiber–Purkinje cell synapses is hypothesized to produce pauses in Purkinje cell spiking that release neurons in the interpositus nucleus from inhibition (Ito and Kano 1982; Batini and Billard 1985). The disinhibition of interpositus neurons causes an increase in cerebellar output and may promote induction of mossy fiber–interpositus long-term potentiation (Mauk and Donegan 1997; Medina et al. 2000). Evidence for cerebellar cortical LTD during learning comes from studies that recorded Purkinje cell activity extracellularly during dEBC, showing pauses in simple spike activity when CRs occur (Hesslow and Ivarsson 1994; Green and Steinmetz 2005; Jirenhed et al. 2007). Decreasing glutamate release at parallel fiber–Purkinje cell synapses with WIN55,212-2 may impair induction of LTD in vivo by attenuating postsynaptic depolarization that occurs when CS and US inputs co-occur. Increasing glutamate release with SR141716A may also impair LTD in vivo, but to a lesser degree, by increasing background synaptic noise and thereby decreasing the signal-to-noise ratio for CS-specific parallel fiber inputs. Deficits in LTD with SR141716A could also be related to the increase in GABA release from basket and stellate cells. The impairments in acquisition of dEBC with WIN55,212-2 and SR141716A may, therefore, be caused by impairments in cerebellar cortical LTD.

Over the next 10 sessions, rats received 90 CS–US pairings and 10 CS alone presentations. Rats were administered SR141716A or vehicle 30 min prior to each daily session (Fig. 4, s1–s10). A repeated-measures ANOVA for the CR percentage data yielded a significant main effect for the group factor, F(3,28) = 7.17, P < 0.001, but the group x session interaction was not significant. Further tests showed that the 3 mg/kg (P < 0.05) and 5 mg/kg (P < 0.01) groups significantly differed from the controls in average percent CRs over the course of the 10 sessions. Thus, the groups differed in the overall amount of learning, but the rate at which they learned did not differ. Other CR measures including amplitude (Fig. 5), onset latency (Fig. 6) and peak latency did not differ between groups.

Potential drug effects on sensitivity to the US and UR performance were examined by analyzing US intensity and UR amplitude during the unpaired and all paired sessions. Separate ANOVAs revealed no significant group differences for US intensity or UR amplitude.

**Discussion**

Subcutaneous injections of the CB1R agonist WIN55,212-2 and antagonist SR141716A produced a dose-dependent impairment in acquisition of dEBC. Rats given WIN55,212-2 were more impaired than rats given SR141716A. For both drugs, the conditioning deficit was the result of not learning the CS–US association and not due to changes in spontaneous blinking or nonassociative blinking to the tone. Also, US and UR measures did not differ between the groups that received the drugs compared with controls indicating that there were no drug-related deficits in sensitivity to the US or performance of the response.

The cerebellum contains one of the highest densities of CB1Rs in the mammalian brain (Herkenham et al. 1990; Ong and Mackie 1999), with the highest levels in the cerebellar molecular layer (Eggan and Lewis 2007; Suárez et al. 2008). Purkinje cells appear to be the only cerebellar neuron type to synthesize, metabolize, and release endocannabinoids (Egertová et al. 1998; Tsou et al. 1998; Lee et al. 2000). Purkinje cells release 2-AG following mGluR1 activation, which binds to CB1Rs on granule cell axon terminals (Breivogel et al. 2004; Kawamura et al. 2006) and to GABAergic stellate and basket cell terminals (Diana et al. 2002), resulting in what has been termed depolarization-induced suppression of excitation (Kreitzer and Regehr 2001a) and depolarization-induced suppression of inhibition (Kreitzer and Regehr 2001b), respectively. WIN55,212-2 and SR141716A impair induction of parallel fiber-Purkinje cell LTD in vitro (Levène et al. 1998; Safo and Regehr 2005). LTD at parallel fiber–Purkinje cell synapses is hypothesized to produce pauses in Purkinje cell spiking that release neurons in the interpositus nucleus from inhibition (Ito and Kano 1982; Batini and Billard 1985). The disinhibition of interpositus neurons causes an increase in cerebellar output and may promote induction of mossy fiber–interpositus long-term potentiation (Mauk and Donegan 1997; Medina et al. 2000). Evidence for cerebellar cortical LTD during learning comes from studies that recorded Purkinje cell activity extracellularly during dEBC, showing pauses in simple spike activity when CRs occur (Hesslow and Ivarsson 1994; Green and Steinmetz 2005; Jirenhed et al. 2007). Decreasing glutamate release at parallel fiber–Purkinje cell synapses with WIN55,212-2 may impair induction of LTD in vivo by attenuating postsynaptic depolarization that occurs when CS and US inputs co-occur. Increasing glutamate release with SR141716A may also impair LTD in vivo, but to a lesser degree, by increasing background synaptic noise and thereby decreasing the signal-to-noise ratio for CS-specific parallel fiber inputs. Deficits in LTD with SR141716A could also be related to the increase in GABA release from basket and stellate cells. The impairments in acquisition of dEBC with WIN55,212-2 and SR141716A may, therefore, be caused by impairments in cerebellar cortical LTD.

The results of the current study suggest that activating CB1Rs with WIN55,212-2 has more of an effect on cerebellar learning than blocking them with SR141716A. Rats given WIN55,212-2 had reduced CR percentages, reduced amplitudes, and longer onsets, whereas rats given SR141716A showed no impairment other than CR percentage, and the maximum CR percentage deficit in the 3 and 5 mg/kg groups was less severe than the deficit seen in the 3 mg/kg WIN55,212-2 group. It is possible that a higher dose of SR141716A would result in deficits similar to those produced by 3 mg/kg of WIN55,212-2. However, the 5 mg/kg group did not differ from the 3 mg/kg group for the CR percentage measure suggesting that further increases in the SR141716A dose may not produce a substantially greater impairment. The reduction in glutamate release from parallel fibers with WIN55,212-2
Central CB1Rs modulate EBC

Cannabis sativa is one of the most widely abused psychoactive substances. In the United States alone, over 94 million individuals (approximately 40% of the population) have used cannabis at least once in their lifetime (Substance Abuse Mental Health Services Administration 2004). Thus, understanding the short- and long-term effects of cannabinoids has important social implications. Translational paradigms, such as eyelink conditioning, allow for advances in elucidating the mechanisms of cannabinoids action (Skosnik et al. 2008). More work on CB1R function including localizing the drug effects within the cerebellum, examining effects on the retention of learning and extinction, and possible long-term effects is needed to gain a more comprehensive understanding of the role of cannabinoid receptors in cerebellar learning.

Materials and Methods

Subjects

Subjects were 64 male Long-Evans rats, 250–350 g at the beginning of the experiment. The rats were housed in Spence Laboratories of Psychology at the University of Iowa with a 12-h 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 electromyograph (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) for delivering the shock US was implanted subdurally, caudal to the left eye.

Apparatus

The conditioning apparatus consisted of four small-animal sound-attenuation chambers (BRS/LVE). Within each sound-attenuation chamber was a small-animal operant chamber (BRS/LVE) 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

Studying the mechanisms and involvement of the cannabinoid system in cerebellar learning is of great applied importance.

administration may produce a more severe impairment in Purkinje cell LTD than increasing glutamate and GABA release with SR141716A administration.

Deficits in dEBC in the current study could be at least partially accounted for by alterations of CB1R function outside of the cerebellar cortex. One obvious candidate is the cerebellar nuclei which have CB1Rs and are necessary for producing the eyelink response (Herkenham et al. 1991; Thompson and Steinmetz 2009). The CB1Rs are very dense in the hippocampus and disrupting hippocampal activity can impair delay conditioning (Solomon et al. 1983). Disruption of auditory system function could also impair acquisition as well (Halverson and Freeman 2006). Low levels of CB1Rs are found in the red nucleus (Herkenham et al. 1991), but alterations in magnocellular neuronal function could impair CR performance by affecting excitatory input to the facial motor nucleus, which produces the blink response (Trigo et al. 1999). However, previous studies of eyelink conditioning in human cannabis users, knockout mice, and mice administered a CB1R antagonist suggest that the effects of CB1R manipulations on conditioning are selective for cerebellar cortical function (reviewed in Edwards and Skosnik 2007). Trace eyelink conditioning, which requires the hippocampus, auditory system, cerebellar deep nuclei, and red nucleus, is completely unaffected by manipulations of CB1Rs that impair dEBC (Kishimoto and Kano 2006; Edwards et al. 2008). Moreover, auditory-evoked field potentials elicited by the CS are normal in human cannabis users who show dEBC impairments (Skosnik et al. 2008). The absence of a deficit in trace conditioning despite impaired cerebellar cortical function is consistent with recent studies indicating that the cerebellar cortex is not necessary for trace conditioning in rodents (Woodruff-Pak and Disterhoft 2008). The findings from the human and mouse studies, although indirect, suggest that the deficits seen in the current study with manipulations of CB1R function are most likely due to alterations in cerebellar cortical function. This issue can only be resolved, however, by using localized infusions of WIN55,212-2 and SR141716A into the cerebellar cortex. A challenge for this approach is that the areas of the cerebellar cortex that contribute to acquisition of eyelink conditioning have not been identified in rats (Nolan and Freeman 2006).
were connected to peripheral equipment. Computer software controlled the delivery of stimuli and the recording of eyelid EMG activity (JSA Designs). The shock US (2–3.5 mÀ, DC constant current) was delivered through a stimulus isolator (Model number 365A, World Precision Instruments). EMG activity was recorded differentially, filtered (500–5000 Hz) and integrated by equipment (JSA Designs) as described in other reports (Nicholson and Freeman 2002; Freeman et al. 2005).

Conditioning procedures

Rats recovered from surgery for 1 wk prior to the initiation of training. All rats completed 12 consecutive daily sessions of training. Session 1 measured spontaneous blink activity in which EMG recordings were collected 100 times for 400 msec each trial, approximating a conditioning session. Session 2 consisted of 100 CS (400 msec; 2 kHz) and 100 US (25 msec shock) unpaired presentations. Sessions 3–12 were the acquisition phase of the experiment in which 10 blocks of nine paired CS–US presentations and 1 CS-alone probe trial were presented. The CS was a 400-msec tone (2 kHz; 85 dB). The CS terminated with a 25-msec shock US. The shock intensity was adjusted in each rat to elicit a blink and slight head movement (range = 2.0–3.5 mA). CRs were defined as EMG activity that exceeded a threshold of 0.4 units (amplified and integrated units) above the baseline units 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.

Pharmacological activation/blockade of CBR1 receptors

The CBR1 agonist WIN55,212-2 (R)-(+)-[3-(3-Dihydro-5-methyl-3-(4-morpholinoethyl)pyrrol-1,2,3-de]-1-4-benzoxazin-6-yl]-1-naphthalenylmethanone and antagonist SR141716A (N-piperidino-5-[4-chlorophenyl]-1-[2,4-dichlorophenyl]-4-methyl-3-pyrazole carboxamide) were administered subcutaneously to rats 30 min before each daily training session. WIN55,212-2 binds with high affinity to CB1R and very weakly to CB2R, whereas SR141716A binds exclusively to CB1R (Pertwee 1997; Howlett et al. 2002). WIN55,212-2 was administered at doses of 1, 2, or 3 mg/kg and SR141716A was administered at doses of 1, 3, and 5 mg/kg. WIN55,212-2 was dissolved in a vehicle of 1:1:18 solution of ethanol, cremaphor, saline; and SR141716A was dissolved in a vehicle of 1:1:18 solution of ethanol, Tween 80, and saline. WIN55,212-2 was purchased from Sigma/RBI and SR141716A was a generous gift from the NIH Drug Supply Program (Rockville, MD). The doses of WIN55,212-2 and SR141716A were chosen based on previous studies (Suzuki et al. 2004; Kishimoto and Kano 2006; Dissnayake et al. 2008).

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