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Cannabinoid-induced motor incoordination through the cerebellar CB_1 receptor in mice

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Abstract

Cannabinoids are known to impair motor function in humans and laboratory animals. We have observed dose-dependent motor incoordination in mice evaluated by rotorod following direct intracerebellar (icb) microinjection of synthetic cannabinoid agonists CP55,940 (5–25 μg) and HU-210 (1.56–6.25 μg), through permanently implanted stainless steel guide cannulas. The motor incoordination was marked at 15, 35 and 55 min post-microinjection. The motor incoordination elicited by HU-210 (6.25 μg) and CP55,940 (20 μg) was significantly blocked by the CB_1 receptor-selective antagonist SR141716A (25 μg icb), indicating mediation by a cerebellar CB_1 receptor. Further direct evidence of CB_1 mediation was obtained through a CB_1 receptor antisense/mismatch oligodeoxynucleotide approach (3 μg/12 h; total of six doses). Mice treated with intracerebellar antisense had a significantly diminished motor incoordination response to intracerebellar CP55,940 15 μg compared to mice that received intracerebellar mismatch or no prior treatment. Also, the response to intracerebellar CP55,940 in the CB_1 mismatch-treated mice did not differ from the mice that received only CP55,940. A separate study using a cerebellar tissue punching technique, following intracerebellar [^{3}H]-CP55,940 microinjection, confirmed that cannabinoid drug dispersion following microinjections was exclusively confined to the cerebellum. Microinjection of CP55,940 (20 μg) into the hippocampus, an area with a large density of CB_1 receptors, did not impair motor coordination. Taken together, these results indicate that cannabinoid-induced motor impairment occurs by activation of a CB_1 receptor in the cerebellum. The participation of other brain motor areas in cannabinoid-induced motor incoordination will require future study.

Keywords: Cannabinoids; CB_1 receptor; Motor incoordination; Mice; Cerebellum; Ataxia

1. Introduction

Marijuana is the most commonly abused illicit psychoactive drug in the US and is most often consumed by smoking the dried flowering leaves of the *Cannabis sativa* plant. Δ^9-Tetrahydrocannabinol (Δ^9-THC), the main psychoactive component, elicits behavioral effects such as euphoria, sedation, impaired memory and altered sense of time (Adams and Martin, 1996; Ameri, 1999). In addition to these effects, Δ^9-THC and synthetic cannabinoids, such as CP55,940 and HU-210, produce immobility, catalepsy, hyperreflexia, hypothermia and motor incoordination in animals to varying degrees depending on the dose and route of administration (Adams and Martin, 1996). Humans do not exhibit the same degree of gross motor incoordination as rodents and dogs presumably due to a significantly lower cannabinoid receptor density in the cerebellum (Adams and Martin, 1996; Herkenham et al., 1991b).

The central nervous system (CNS) actions of cannabinoids are reported to result predominantly by activation of brain CB_1 receptors (Ameri, 1999; Felder and Glass, 1998). The CB_1 receptor is a member of the superfamily of G-protein-coupled receptors. Activation of the CB_1 receptor leads to an inhibition of adenylate cyclase through a $G_{i/o}$ protein and a subsequent decrease in cAMP accumulation (Howlett et al., 1985). Additionally, activation of the CB_1 receptor results in an inhibition of N-type Ca^+ channels and modulation of the activity of A-type K^+ channels. These actions on ion channels are in line with the role of presynaptic CB_1 receptors acting to inhibit the release of neurotransmitters (Gifford et al., 1997; Levenes et al., 1998; Shen et al., 1996).

Autoradiography of [^{3}H]CP55,940 binding in mouse brain has provided evidence that high-density CB_1 receptors

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are localized within the molecular layer of the cerebellum on the axonal nerve terminals (parallel fibers) of the glutamatergic cerebellar granule cell neurons (Herkenham et al., 1991a). In vitro electrophysiological studies have indicated that cannabinoids may act through CB_1 receptors to inhibit glutamate release from cerebellar granule cell axonal terminals onto cerebellar Purkinje cell neurons (Levenes et al., 1998; Takahashi and Linden, 2000).

Systemic and intracerebroventricularly administered cannabinoid agonists have been demonstrated to produce motor impairment in laboratory animals (Lichtman and Martin, 1997). This effect presumably occurs via activation of the densely localized $G_{i/o}$-protein-linked CB_1 receptors in brain motor regions (Herkenham et al., 1991b). Our laboratory has recently reported the involvement of cerebellar CB_1 receptors on motor incoordination in mice produced by direct microinjection into the cerebellar cortex of Δ^9-THC, the principal psychoactive component of marijuana (Dar, 2000). This microinjection approach has been a very direct and established way to observe the action of various drugs on motor coordination at the level of the cerebellum and other brain motor areas. This technique has been utilized extensively in our laboratory particularly in regard to the interaction of alcohol and various cerebellar neurotransmitter systems on motor coordination (Dar, 1996, 1997).

In the current investigation, we intended to establish the involvement of cerebellar CB_1 receptors in motor incoordination induced by the synthetic cannabinoid agonists, HU-210 and CP55,940, in mice by a rotorod procedure. The CB_1 receptor antagonist, SR141716A, and CB_1 receptor antisense oligodeoxynucleotides were also used to firmly establish the role of the CB_1 subtype in cannabinoid-induced motor incoordination. In addition, [^{3}H]-CP55,940 was used to confirm that the microinjected cannabinoid drugs remained confined within the cerebellar tissue. Microinjection of CP55,940 into the hippocampus served as a control area to further support the specificity of the cerebellum as a key brain area involved in the motor incoordination produced by cannabinoids in mice.

The cerebellum served as a logical target to study the actions of cannabinoids on motor coordination because of the reported high density of CB_1 cannabinoid receptors in the molecular layer of mouse cerebellum and the region's well-known function in coordinated movements (Herkenham et al., 1991b). The region of our unilateral microinjections was the anterior lobe of the spinocerebellum, which is an area responsible for integrating afferent and efferent signals relating to the control of movement of the distal limbs (Guyton et al., 1996).

2. Materials and methods

Male CD-1 mice, purchased from Charles River Laboratories (Raleigh, NC), were 5–6 weeks old and weighed between 23 and 28 g at the time of experiments. The mice were maintained in a housing facility at 23–25°C with controlled humidity and kept on a 12-h light/dark cycle (08:00 h, lights on). Each animal was housed in its own individual plastic cage after being implanted with a permanent, indwelling stainless steel guide cannula (22 gauge, 10 mm length) by stereotaxic surgery. Mice had free access to tap water and commercial mouse chow. All experimental protocols were evaluated and approved by the animal care and use committee of East Carolina University.

2.1. Surgery

Mice were anesthetized with chloral hydate (450 mg/kg ip) prior to placement in a small animal stereotaxic frame (David Kopf Instruments, Tujunga, CA). The head of each mouse was trimmed of hair, swabbed with povidone iodine (Iodophor PVP; Clinipad, Rocky Hill, CT) and then wiped clean with an isopropyl alcohol swab. With the skull flat, a 2-cm-long mid-sagittal incision was made by sterile scalpel in order to expose the periostium. Cannulation of the cerebellum was performed aseptically according to the following stereotaxic coordinates of Slotnick and Leonard (1975): anterior–posterior (AP) −6.4 mm (from bregma); medial–lateral (ML) +0.8 mm; dorsal–ventral (DV) −1.0 mm from the skull surface. The guide cannula (22 gauge, 10 mm length) was lowered through a drilled craniotomy hole into the superficial layers of the anterior lobe region of the cerebellum. Durelon cement (ESPE, Norristown, PA) was used to anchor the cannula to the skull surface. A removable stainless steel wire plug was then placed inside the guide cannula to prevent occlusion. Post-surgically, each animal received 3000 U sc (benzathine/procaine penicillin G) Durapen (Vedco, St. Joseph, MO) to prevent possible infection and ketorolac tromethamine (Toradol) 2 mg/kg sc, twice 4 h apart, for analgesia. The hippocampus was cannulated using the same surgical procedure described above except that the stereotaxic coordinates used were: AL −1.8 mm; ML+2.0 mm; DV −1.4 mm from the skull surface. Animals were allowed to recover from surgery and anesthesia in their own individual cages for a minimum of 5 days before use in an experiment.

2.2. Drugs

Drug solutions were prepared the day of use in behavioral experiments. The following drugs were used in the present investigation: CP55,940 (a gift from Pfizer, Groton, CT), HU-210 (RBI, Natick, MA), SR141716A (Sanofi, Montpellier, France). The CB_1 receptor antisense and mismatch phosphorothioate-linked oligodeoxynucleotides were synthesized at Lineberger Cancer Center (Chapel Hill, NC) based on the sequences published by Edsall et al. (1996). The antisense oligo sequence was 5′-TCCGTCTAAGATCGACTT-3′ and the mismatch oligo sequence was 5′-ACCGGCTATTATCTACTG-3′.

Dimethylsulfoxide (100%) was used as the vehicle for CP55,940, HU-210 and SR141716A. The oligodeoxynucleotides were dissolved in sterile water. The [^{3}H]CP55,940 (sp. ac. 180 Ci/mmol) used in the dispersion study was obtained from NEN Life Sciences (Boston, MA). It was reconstituted in 100% DMSO after evaporation of the stock ethanol solution under N_2 gas.

2.3. Intracerebellar (icb) microinjection of drugs

Drugs were microinjected through PE-10 (Clay Adams, Parsippany, NJ) polyethylene tubing by a microinjection pump (Model 22; Harvard Apparatus South Natick, MA) fitted with a 25 μl Hamilton syringe. The sterile stainless steel injection cannula (30 gauge, 0.31 mm diameter) was fitted to the PE-10 tubing so that the total length of exposed cannula was 11 mm. This allowed for protrusion of the injector cannula 1 mm beyond the lower tip of the guide cannula. The volume of microinjection was 500 nl for HU-210, CP55,940 and SR141716A, while all other drugs were administered in a volume of 200 nl. The rate of microinjection was 500 nl/min for all microinjections. The injection cannula was left in place for an additional 1 min after the end of each microinjection. An air bubble separating drug solution and water in the tubing was monitored for continuous movement to indicate that blockage was not occurring. Intracerebellar microinjection of SR141716A, the CB_1 receptor antagonist, was microinjected 8 min prior to intracerebellar HU-210 or CP55,940.

2.4. Rotorod evaluation

Mice were evaluated for motor coordination using a standard mouse rotorod treadmill (Ugo Basile, Verese, Italy) set at a fixed speed of 24 rpm. As previously described, normal motor coordination was arbitrarily defined as the ability of a mouse to walk continuously on the rotorod without falling off, for 180 s (Dar et al., 1983). Mice were screened prior to intracerebellar microinjection to establish normal motor coordination and therefore acted as their own controls. The screening of mice was always performed the morning of an experiment typically 30 min prior to microinjections and subsequent rotorod evaluations. Any mouse unable to walk 180 s in three attempts during screening was excluded from the experiment. Greater than 98% of the mice were screened normally on the morning of the experiments and therefore fulfilled our arbitrarily selected criteria to walk 180 s on the rotorod. All motor coordination experiments were performed between 8 and 11 am.

The rotorod evaluation times used were 15, 35 and 55 min following the end of the microinjections. After evaluation on the rotorod at each time point, the mouse was placed back into its original cage until the next evaluation time. The shorter walking times on the rotorod indicated increasing degrees of motor incoordination.

The number of animals in each treatment group ranged from 6 to 12. A maximum group of five mice in a single rotorod experiment can be evaluated simultaneously on the rotorod because there are five separate walking lanes divided by the walls of the rotating treadmill. At least two separate experiments were performed for each treatment group.

2.5. Locomotor activity monitoring

Spontaneous locomotor activity was evaluated as previously described (Dar, 1997). Mice were evaluated for activity over a 60-min time period following microinjection of SR141716A or vehicle. As with rotorod evaluation, these experiments were performed between 8 and 11 am. Following intracerebellar microinjection, each mouse was brought into the locomotor activity monitoring laboratory and then placed into an individual activity monitoring unit ($42.2 \times 42.5 \times 20.5$ cm) and allowed 5 min of acclimation before measurements began. The monitoring room was sound (white noise)- and light (red 25 W light from one side only)-regulated. Data were tabulated using the Auto-Track System Opto-Verimax Activity monitoring system (Columbus Instruments, Columbus, OH) attached to a PC. A maximum of four mice per group were monitored per experiment. Seven mice (two groups) were evaluated on separate days in our current investigation. On day 1, mice received intracerebellar 100% DMSO vehicle (500 nl) and their activity was monitored as described above. On day 2, activity of the same groups of mice was recorded following intracerebellar SR141716A 25 μg.

2.6. Tissue-punch analysis of [^{3}H]-CP55,940 dispersion

The dispersion of intracerebellar [^{3}H]-CP55,940 was studied to establish whether the microinjected cannabinoids remained within a confined area of the cerebellar cortex. Mice ($n=3$) were microinjected (intracerebellar) with a solution containing [^{3}H]-CP55,940 0.2 μCi and cold CP55,940 20 μg. At 15 min post-microinjection, the time of peak incoordination, mice were killed by cervical dislocation followed by decapitation and the brain was quickly removed on a cold plate. The brain was immediately frozen by immersion into a dry ice–ethanol solution and then mounted onto a freezing stage, maintained at -12°C, and cut into 0.5-mm coronal sections with a sliding microtome (Model 860; American Optical, Buffalo, NY).

The method of punching was generally based on a modification of a tissue punching technique of Eik-Nes and Brizzee (1965). Punches were made 1 mm on the center from each other beginning at the central site of microinjection using a 3-cm-long thin-walled stainless steel tube (21 gauge; Small Parts, Miami, FL) with an internal diameter of 0.6 mm. A total of six punches were taken from each section of cerebellum and an additional punch from the frontal cortex served as background counts. Punched tissue was pushed through the thin-walled tubing

with a cold stainless steel wire (diameter 0.56 mm; Small Parts) directly into a scintillation vial containing 100 μl of 0.1 N NaOH. The scintillation vials were then incubated overnight (approximately 12 h) at 45°C in an Isotemp oven (Fischer Scientific, Pittsburgh, PA). Five milliliters of Cytoscint liquid scintillation fluid (ICN Biochemicals, Irvine, CA) was then added to each vial and the vials were counted by a Beckman LS9000 liquid scintillation system (Beckman Instruments, Fullerton, CA).

The remaining brain tissue from each punched coronal section was placed into a scintillation vial containing 500 μl of Scinti-Gest tissue solubilizer (Fischer Scientific). Five milliliters of scintillation fluid was added following a 48-h incubation at 45° and the vials were then counted by the liquid scintillation counter.

2.7. Histology

It was important to obtain confirmation of properly located microinjections. Consequently, all mice were injected with fast green dye at the end of the experiments and killed by cervical dislocation and decapitation under light ether anesthesia. The guide cannula and brain were carefully removed and the site of microinjection was then verified by examining the location of the dye in the superficial region of the cerebellar anterior lobe. Only mice verified to have correct cannula placement were included in data analysis. The cannulation success rate was in excess of 95% in these studies.

Additionally, several mice from each treatment group were used for coronal sectioning of the cerebellum by frozen microtome. Briefly, the brains of mice were quickly removed and frozen in a dry ice–ethanol mixture and then mounted for sectioning. The coronal sections were thaw-mounted onto glass slides and stained with cresyl violet. Examination under light microscope allowed for evaluation of any excessive gross tissue damage caused by either the cannulation or drug microinjections.

2.8. Statistical analysis of data

Motor incoordination data were analyzed by a two-way ANOVA using treatment and time as factors and repeated measures on time. A Newman–Keuls post-hoc test was performed whenever significance was found on treatment and/or time. Two-sided Student's *t* test was also used where appropriate. A one-sided *t* test was used to determine significant differences of rotorod times in treatment groups vs. the 180-s control value. A P value $< .05$ was taken as the level of significance in all statistical tests.

The rotorod data for various doses of HU-210 and CP55,940 at the 15-min time points were transformed to log dose and time on rotorod. The GraphPad Prism Computer software was utilized for regression analysis of the graded dose–response data in order to obtain an ED_{50} value for each agonist (Tallarida and Murray, 1981).

3. Results

3.1. Rotorod evaluation of motor incoordination

CP55,940 and HU-210 produced a dose-related increase in motor incoordination in mice, which was significant at all evaluation periods (Fig. 1A and B). The peak effect, which occurred 15 min post-microinjection, was not associated with any visible sedation or loss of righting reflex. The lowest dose of HU-210 (1.56 μg) produced walking times significantly less than the 180 s control value at all three evaluation times [$t(9) = 2.88$, $P < .01$; $t(9) = 2.53$, $P < .05$; $t(9) = 1.83$, $P < .05$]. No significant Treatment × Time interaction was noted for the various doses of HU-210

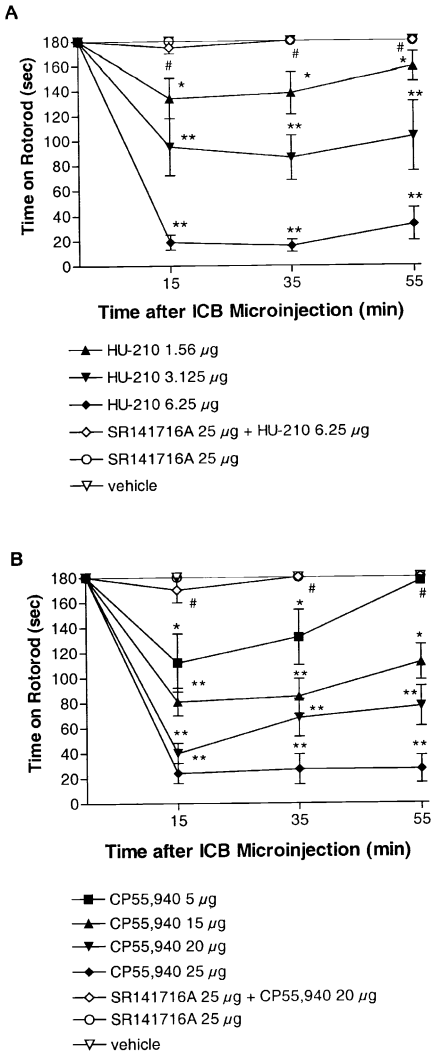

Fig. 1. Effect of intracerebellar microinjection of various doses of CB_1 agonists, (A) HU-210 (1.56–6.25 μg) and (B) CP55,940 (5–25 μg), on motor coordination in mice. Also shown is the effect of intracerebellar microinjection of CB_1 receptor antagonist, SR141716A (25 μg), 8 min prior to treatment with CB_1 agonist. Each point represents mean ± SEM of at least seven mice. * $P < .05$; ** $P < .01$, significantly different from 180-s control value; # $P < .0001$, significantly different from CP55,940 (20 μg) or HU-210 (6.25 μg).

[$F(4,150) = 0.2512$, $P = .9075$]. There was a significant treatment effect of various doses of HU-210 [$F(2,23) = 18.02$, $P < .0001$], with significantly increasing motor incoordination from lowest dose (1.56 μg) to highest dose (6.25 μg). No significant recovery from the 15-min peak motor incoordination was noted at the 55-min evaluation time for any dose of HU-210 ($P > .05$). Significant recovery with the highest dose began at approximately 75 min post-microinjection (data not shown).

The lowest CP55,940 dose (5 μg) produced walking times significantly different from the 180-s control value at the 15- [$t(5) = 2.96$, $P < .05$] and 35-min [$t(5) = 2.18$, $P < .05$] evaluation times. Significant CP55,940 dose effect [$F(3,46) = 14.31$, $P < .0001$] and time effect [$F(2,92) = 10.70$, $P < .0001$] were observed. Newman–Keuls post-hoc analysis indicated that the motor incoordination at 55 min was significantly less than at the 15-min time point for the 5, 15 and 20 μg doses ($P < .01$ to $P < .05$). Significant recovery did not occur at 55 min in the group treated with 25 μg of CP55,940 ($P = .057$). As with HU-210, no significant Treatment (dose) × Time interaction was noted with CP55,940 [$F(6,392) = 1.81$, $P = .11$]. This was fairly evident from the approximate parallelism of the curves for each dose of agonist over the three evaluation times (Fig. 1A and B).

The cannabinoid-induced motor incoordination was nearly abolished at the 15-min evaluation time by intracerebellar microinjection of the CB_1 selective antagonist SR141716A prior to microinjection of HU-210 [$t(13) = 18.46$, $P < .0001$] or CP55,940 [$t(24) = 7.05$, $P < .0001$] and completely blocked at the 35- and 55-min evaluation times (Fig. 1A and B). The residual motor incoordination observed at the 15-min time point was not significantly different from the 180-s control value for HU-210 and CP55,940 [$t(5) = 1.0$, $P = .18$; $t(9) = 1.0$, $P = .17$]. Intracerebellar SR141716A alone had no effect on motor coordination (Fig. 1A and B).

Fig. 2 shows the dose–response relationship comparison of the two cannabinoid agonists. The ED_{50} values calculated for HU-210 and CP55,940 were 2.85 μg (2.19–3.72) and 9.60 μg (6.78–13.58). The plotted values represent the peak responses, which occurred at the 15-min time period as demonstrated in Fig. 1A and B.

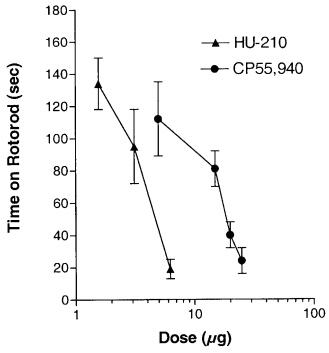

Fig. 2. Replotting of the HU-210 and CP55,940 dose–response data at the 15-min time point to derive and compare the ED_{50} values of each agonist on motor incoordination. The ED_{50} values and 95% confidence limits were 2.85 μg (2.19–3.72) for HU-210 and 9.60 μg (6.78–13.58) for CP55,940.

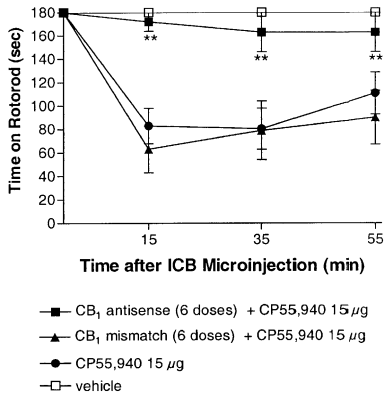

Fig. 3. Effect of intracerebellar pre-treatment with CB_1 antisense oligodeoxynucleotide and its mismatch on CP55,940-induced motor incoordination. Mice were treated with 3-day (every 12 h) intracerebellar CB_1 antisense or its mismatch. On day 4, 12 h after the last microinjection, intracerebellar CP55,940 (15 μg) was administered and mice were then evaluated on the rotorod. The 3-day antisense-treated mice showed significantly less motor incoordination when compared to mice treated with the 3-day CB_1 mismatch. * $P < .05$; ** $P < .01$, significantly different from the mismatch-treated group. Each point in the graph represents the mean ± SEM of at least seven mice.

Fig. 3 demonstrates a significant blockade of CP55,940-induced motor incoordination following 3-day (twice daily) CB_1 receptor antisense administration [$F(2,22) = 6.96$, $P < .01$]. Post-hoc analysis showed a significant difference in motor incoordination between the antisense and mismatch-treated groups at all three evaluation times ($P < .01$). A time effect was also observed [$F(2,40) = 3.63$, $P < .05$] (Fig. 3). There was no significant interaction between the CB_1 antisense-treated and CB_1 mismatch-treated groups and time [$F(4,132) = 2.36$, $P = .07$]. It is also important to note that the degree of motor incoordination in the CB_1 antisense + CP55,940 group was not statistically different from the control value of 180 s at any evaluation time [$t(6) = 0.36$, $P = .36$; $t(6) = 1.0$, $P = .18$; $t(6) = 1.0$, $P = .18$].

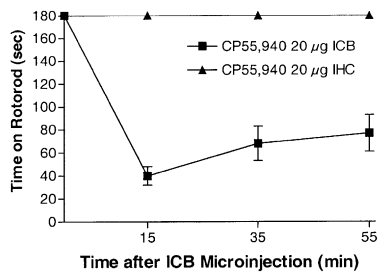

Fig. 4. The effect of microinjection of CP55,940 (20 μg) into the hippocampus. Microinjection of CP55,940 into the hippocampus (IHC) had no effect on motor coordination as opposed to that observed with the same dose by intracerebellar microinjection. Each point in the graph represents the mean ± SEM of seven mice.

3.2. Intrahippocampal microinjection of CP55,940

Intrahippocampal microinjection of CP55,940 20 μg did not induce motor incoordination at any time point after the microinjection (Fig. 4). The overall observed behavior of these mice in their cages was not grossly different from untreated mice.

3.3. Locomotor activity monitoring

Fig. 5 illustrates the results of intracerebellar microinjection of the CB_1 receptor antagonist, SR141716A, on spontaneous locomotor activity over a 60-min period. Intracerebellar SR141716A (25 μg) did not produce a significant change in spontaneous locomotor activity over this time period (Fig. 5A) compared to vehicle-treated mice [$t(12) = 0.87$, $P = .40$]. There was also no difference in locomotor activity at any 10-min interval between the two groups [$F(1,12) = 0.75$, $P = .40$] (Fig. 5B).

3.4. Dispersion of [^{3}H]-CP55,940 in the cerebellum

The dispersion of [^{3}H]-CP55,940 remained confined predominantly to the central site of the microinjection (Table 1). Based on the mean dispersion data of three mice, 31147 + 562 disintegrations per minute (dpm) of [^{3}H]-CP55,940 was found in the punch (0.6 mm diameter) corresponding to the central site of microinjection. Very low counts (< 300 dpm) were detected in the punch corresponding to the level of deep cerebellar nuclei and no counts were detected in the brainstem region. No counts above background were detected in coronal sections beyond 1.0 mm anterior or posterior of the central site of microinjection.

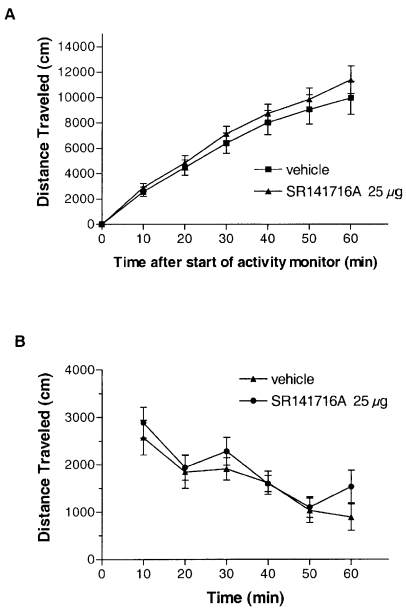

Fig. 5. Effect of intracerebellar microinjection of CB_1 receptor antagonist, SR141716A, on spontaneous locomotor activity. (A) Locomotor activity monitored over a 60-min period was not significantly different between the groups administered intracerebellar DMSO vehicle (500 nl) or intracerebellar SR141716A 25 μg ($P = .403$). (B) Locomotor activity illustrated per 10-min interval. ANOVA did not indicate any significant difference between the groups at any interval ($P = .404$). Each point represents the mean ± SEM of seven mice.

Table 1
Dispersion of [^{3}H] CP55,940 in the cerebellum following intracerebellar microinjection

Distance from bregma (mm)	− 5.4 dpm	− 5.9 dpm	− 6.4 dpm	− 6.9 dpm	− 7.4 dpm
Central site	31	706	32,003	13,743	38
1 mm dorsal	25	338	21,170	3078	0
1 mm lateral (left)	20	188	9119	37	7
1 mm lateral (right)	0	68	5743	345	0
1 mm ventral	0	572	1443	3136	10
Deep cerebellar nuclei	0	56	275	42	0
Remaining tissue	2362	15,137	146,219	16,750	223
Total recovered in section	2438	18,758	215,972	37,131	278

Dispersion of [^{3}H]-CP55,940 in a representative mouse cerebellum following intracerebellar microinjection of [^{3}H]-CP55,940 0.2 μCi + CP55,940 20 μg. The numbers represent dpm counted in the tissue plug from each punched 0.5 mm coronal section of cerebellar tissue. As evident from the table, the majority of dpm occurred in the punches corresponding to the coronal section at − 6.4 mm posterior of bregma. Within this coronal section, it is evident that the central site corresponding to the area just below the tip of the microinjection cannula contained the highest amount of CP55,940.

3.5. Histology

Fig. 6 illustrates the tract of the guide cannula in the anterior lobe of the cerebellum. The guide cannula and

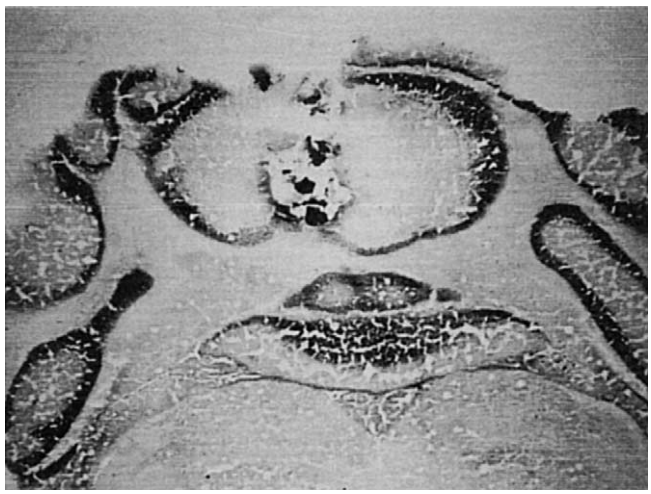

Fig. 6. A representative histologic photograph of a cerebellar coronal section demonstrating the location of the guide cannula in the cerebellum. The dark areas at the site of microinjection represent dye that was microinjected prior to removing the brain for sectioning and slide preparation.

drug microinjections produced minimal damage to the cerebellum as can be seen in this representative coronal section. The dark areas around the bottom of the cannula tract represent dye, which was microinjected prior to cannula removal.

4. Discussion

The data presented here demonstrate that CB_1 receptors in the mouse cerebellum are functionally involved in the motor incoordination induced by the synthetic cannabinoid agonists, HU-210 and CP55,940. The following evidence from our study support this statement. (1) The motor incoordination induced by intracerebellar HU-210 and CP55,940 was dose-dependent and reversible with mice recovering back to their normal coordination 1.5–3 h following intracerebellar microinjection of the two highest doses of the cannabinoid agonists (data not shown). (2) Intracerebellar SR141716A, a CB_1 receptor-selective antagonist, nearly abolished the motor incoordination elicited by HU-210 and CP55,940. (3) Intracerebellar microinjection of CB_1 receptor antisense oligodeoxynucleotides, but not its mismatch, significantly blocked the CP55,940-induced motor incoordination. (4) Intrahippocampal administration of CP55,940 did not induce motor incoordination despite the reported high density of CB_1 receptors in this brain region. (5) Intracerebellar microinjection of [^{3}H]-CP55,940 remained confined at the site of microinjection within the anterior lobe of the cerebellum.

Mice exhibited gross abnormalities in their movement following microinjection of the cannabinoid agonists CP55,940 and HU-210. We are aware that the rotorod test does have some limitations in the evaluation of motor incoordination. For example, mice that are heavily sedated or cataleptic from an administered drug may be unable to perform well on the rotorod. Conversely, mice that are in a state of hyperexcitability may jump off of the rotorod. If a drug produces these responses, then low rotorod times may not be a pure indicator of motor incoordination. Although not specifically quantified, intracerebellar administration of the cannabinoid agonists (HU-210 and CP55,940) and antagonists (SR141716A) did not visibly appear to cause sedation, hyperexcitability or catalepsy in the mice. The mice were freely mobile upon placement into their original cages between the rotorod evaluation periods.

In agreement with other reports, relatively high doses of microinjected cannabinoids (low microgram range) were necessary to elicit a pharmacological response (Dar, 2000; Lichtman et al., 1995, 1996; Martin et al., 1995). We speculate that this may result from difficulty of access to parallel fiber synapses due to distribution of these extremely lipid soluble compounds to the glial cells or partitioning into neuronal membranes following microinjections. The ED_{50} values (95% CL) for HU-210 and CP55,940 were 2.85 μg (2.19–3.72) and 9.60 μg (6.78–13.58), respectively (Fig. 2). The CP55,940 ED_{50} value was higher compared to the reported ED_{50} values of 2–3 μg following intracerebroventricular microinjection of CP55,940 when evaluating antinociception responses (Cook et al., 1995; Welch et al., 1995, 1998). However, the microinjections in our study were unilateral and targeted only one brain area involved in coordinating movements. In contrast, with microinjection by the intracerebroventricular route, many areas of the brain may be acted upon, which together can contribute to the observed response.

The extreme lipophilic property of cannabinoid agonists has led to much speculation in the past about the possibility of non-specific actions of cannabinoids on neuronal membranes as a mechanism of their behavioral effects. Various non-receptor-mediated actions of cannabinoids in vitro have been reported especially at high concentrations (Felder et al., 1992). We ruled out these non-specific actions in our present study by blocking the motor incoordination by intracerebellar microinjection of SR141716A, a CB_1 receptor selective antagonist, prior to microinjection of the cannabinoid agonists. There was some minor motor incoordination at the 15-min time point with each agonist, although the mean time on the rotorod was not significantly different from the control value of 180 s. Microinjection of SR141716A alone had no effect on motor coordination and caused no overt visible change in the behavior of the mice (Fig. 1A and B).

Although there has been a report of CB_2 receptor mRNA expression in mouse cerebellar granule cells (Skaper et al., 1996), no effect on brain binding of [^{3}H]-CP55,940 was observed in mice after oral or intracerebroventricular administration of the CB_2 receptor-selective antagonist, SR144528 (Rinaldi-Carmona et al., 1998). Furthermore, it seems unlikely that the CB_2 receptor was involved in the cannabinoid-induced motor incoordination in our study because there was virtually no residual motor incoordination in mice pre-treated with intracerebellar SR141716A, a CB_1 receptor-selective antagonist, just prior to CP55,940 or HU-210. However, CB_2 receptor involvement was not ruled out with a CB_2 receptor antagonist in our study.

SR141716A has been reported to be a CNS stimulant in some studies. For example, an increase in locomotor activity has previously been observed in mice following intracerebroventricular microinjection of this compound (Compton et al., 1996). This may have been due to blockade of endogenous cannabinoids at CB_1 receptors in brain motor regions or the possibility of SR141716A acting as an inverse agonist (Landsman et al., 1997) It was therefore necessary to determine whether intracerebellar SR141716A was acting as a general CNS stimulant in our study instead of simply blocking the actions of HU-210 and CP55,940 at the CB_1 receptor because it is conceivable that locomotor stimulation may allow enhanced performance on the rotorod. The results of our spontaneous locomotor activity monitoring did not indicate this to be the case because there was no significant increase in locomotor activity between mice treated with

SR141716A and vehicle (Fig. 5). Therefore, SR141716A most likely acted as a competitive antagonist at the CB_1 receptor, preventing the agonists, HU-210 and CP55,940, from activating $G_{i/o}$ proteins through the CB_1 receptor.

Further support for CB_1 receptor mediation was provided by the use of CB_1 antisense/mismatch oligos directed to the CB_1 receptor (Edsall et al., 1996). The blockade of CP55,940-induced motor incoordination by 72-h (six doses) intracerebellar CB_1 antisense pretreatment was significant (Fig. 3). It is likely that the antisense compound knocked down a significant number of functional CB_1 receptors in the area of microinjection, which then resulted in a significant attenuation of the effect of microinjected CP55,940. A smaller pool of CB_1 receptors could result in a decreased magnitude of activation of the $G_{i/o}$ proteins in the presence of a given concentration of cannabinoid agonist. The antisense abolishment of CP55,940-induced motor incoordination is consistent with previous reports demonstrating antisense abolishment of Δ^9-THC-induced motor incoordination (Dar, 2000) and CP55,940-induced antinociception (Edsall et al., 1996).

It was conceivable that a small amount of microinjected drug was exiting the cerebellum to act on other brain motor areas via the bloodstream. This did not appear to be the case because microinjection into a separate brain area, the hippocampus, did not produce motor incoordination in the mice (Fig. 4). Furthermore, because the hippocampus has a high density of CB_1 receptors, the lack of effect demonstrated that this area is not involved in motor incoordination by cannabinoids.

The drug remained predominantly at the microinjection site in the cerebellum as indicated by the tissue punch dispersion data of [^{3}H]-CP55,940 (Table 1). No counts above background were found in the brainstem and the counts in the region of the deep cerebellar nuclei were very low (<300 dpm). Our data thus support a role for CB_1 receptors in the superficial regions of cerebellar cortex in production of motor incoordination in response to cannabinoids. This is consistent with previous speculation about the cerebellar involvement in cannabinoid-induced motor incoordination (Childers and Breivogel, 1998). The cannabinoids microinjected in our study may be acting on presynaptic CB_1 receptors located on the glutamatergic parallel fiber terminals and/or one of the inhibitory interneuron populations such as the Basket cells. Anatomical localization studies have not ruled out the possibility of CB_1 receptors on these inhibitory GABAergic interneurons (Herkenham et al., 1991a; Mailleux and Vanderhaeghen, 1992). Future studies examining other regions by a microinjection approach will be required to evaluate whether other brain regions also contribute to cannabinoid-induced motor incoordination.

The localization and coupling efficiency of cannabinoid receptors may differ across species (Ameri, 1999). The more pronounced motor effects in laboratory animals compared to humans may be due to a higher density of CB_1 receptors in the cerebellum (Herkenham et al., 1991a). At the highest dose microinjected (25 μg), mice were grossly impaired and it was very evident in their inability to remain on the rotorod for more than 30 s. However, in humans, cannabinoid receptors are also localized in the cerebellum. Therefore, exposure to cannabinoids in the form of marijuana may impair motor function by a cerebellar mechanism. Also, as synthetic cannabinoids continue to be developed for potential therapeutic uses, it is important to understand mechanisms of potential side effects on motor coordination.

Our current study provided direct functional evidence of the previously speculated role of cerebellar CB_1 receptors in cannabinoid-induced motor incoordination. Further studies are necessary to elucidate the biochemical mechanisms of cannabinoid-induced motor incoordination in the cerebellum. The $G_{i/o}$ protein coupling of CB_1 receptors to inhibition of adenylate cyclase and N-type Ca^+ channels is well established (Adams and Martin, 1996). Therefore, it is conceivable that the cannabinoid agonists (HU-210 and CP55,940) microinjected in our study resulted in CB_1 receptor-coupled $G_{i/o}$-mediated inhibition of adenylate cyclase and subsequent decreased cAMP production. This signal transduction pathway has already been demonstrated to be necessary for A_1 receptor agonist enhancement of ethanol-induced motor incoordination (Dar, 1997). The inhibition of cAMP production by cannabinoids may interfere with neurotransmitter release by modulation of A-type potassium channels (Deadwyler et al., 1995). Additionally, the cAMP-independent inhibition of N-type Ca^+ channels through the CB_1 receptors may be involved in cannabinoid-induced motor incoordination following CB_1 receptor activation (Mackie and Hille, 1992). Overall, the interference with glutamatergic transmission at Purkinje cell synapses and/or GABA transmission by interneurons is likely to be the end result of cannabinoid interaction with cerebellar CB_1 receptors (Levenes et al., 1998) and this may account for the observed motor incoordination in mice following intracerebellar administration.

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