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Original Article

Time Course of the Development and Loss of Delta-9-tetrahydrocannabinol Tolerance: Effects on Hypothermia and Spontaneous Locomotor Activity in Mice

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Deregulation of cannabis use is gradually expanding in Europe and the United States. However, the biological processes driving tolerance to delta-9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive component of cannabis, remain unclear. Thus, this study aimed to investigate the mechanisms and time course of tolerance development and loss to Δ^9-THC in mice. Male ICR mice (7 weeks old) were administered Δ^9-THC once daily for 3 days and then divided into three groups according to the washout period (3-, 10-, and 17-day washout groups). After each washout, changes in body temperature and locomotor activity were measured following re-exposure to Δ^9-THC. Furthermore, the mRNA expression levels of CB_1 and CB_2 receptors in the brain were evaluated using real-time PCR. On day 1, significant hypothermia and reduced spontaneous locomotor activity were observed in the Δ^9-THC-treated mice compared with the vehicle-treated mice. Tolerance to the hypothermic and locomotor-suppressing effects of Δ^9-THC developed on days 2 and 3, respectively, and dissipated after 3 and 11 days of washout, respectively. These differences in the rates of tolerance development and recovery may reflect distinct underlying mechanisms. No significant changes in receptor mRNA expression were observed. These findings highlight the complexity of Δ^9-THC tolerance and its potential implications for long-term cannabis use.

Key words: delta-9-tetrahydrocannabinol, cannabis, tolerance, locomotor, hypothermic

Cannabis (*Cannabis sativa*) is a widely cultivated psychoactive plant that has been used for centuries for medicinal, industrial, and recreational purposes. In recent years, global deregulation of cannabis has accelerated. As of June 26, 2025, 40 U.S. states had legalized cannabis for medical use and 24 had legalized it for recreational use. Under the federal Controlled Substances Act, cannabis remains classified as a Schedule I substance; however, in 2024, the U.S. Drug Enforcement Administration proposed reclassifying it as Schedule III. This reclassification would recognize its medical use and likely promote broader acceptance and utilization. Nonetheless, cannabis is associated with major social issues and health concerns, including physical and psychological dependence and escalating use driven by drug tolerance [1].

Cannabis contains approximately 100 cannabinoids,

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including delta-9-tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol, and cannabigerol. Δ^9-THC, the principle psychoactive component, produces diverse pharmacological effects such as hypothermia, learning and memory impairments, aggressive behavior, and anxiety reduction, and has also been reported to exert neuroprotective effects against stroke [2-6]. Repeated exposure to Δ^9-THC leads to tolerance [7], suggesting that regular cannabis users may increase their consumption to overcome the diminished effects.

In Japan, exposure to Δ^9-THC is expected to increase with intentional medical use and accidental ingestion from CBD-containing products. Elucidating the mechanisms underlying the development of Δ^9-THC tolerance is thus increasingly important. Δ^9-THC exerts its effects primarily through cannabinoid receptor type 1 (CB_1), and repeated exposure stimulation can attenuate its efficacy, potentially contributing to dependence and other adverse effects. As the legal and medical landscape surrounding cannabis-derived substances continues to evolve, elucidating the biological basis of Δ^9-THC tolerance is critical for ensuring safe and effective use of these compounds. Such knowledge could also inform regulatory decisions, guide therapeutic practices, and contribute to public health strategies regarding cannabis use.

However, the processes underlying Δ^9-THC-induced tolerance — particularly the roles of cannabinoid receptors such as CB_1 and CB_2 — remain unclear. This study therefore aimed to explore the mechanisms and time course of the development and loss of Δ^9-THC tolerance in mice, focusing on changes in body temperature and spontaneous locomotor activity. A clearer understanding of the timeline and molecular mechanisms of tolerance formation may help prevent the adverse consequences of cannabis abuse while facilitating the appropriate therapeutic use of cannabinoid-based drugs.

Materials and Methods

Animals. Male ICR mice (7-8 weeks old; 25-35g; CLEA Japan, Tokyo) were used in this study. The mice were housed under controlled temperature (23 ± 2°C) and humidity conditions ($60\% \pm 2\%$) with a 12-hour light/dark cycle (lights on from 7 : 00 AM to 7 : 00 PM) in groups of six per cage (plastic, 30 cm $\times$35 cm$\times$17 cm). All procedures involving animals were conducted in accordance with the guidelines of the Animal Care and Use Committee of Fukuoka University.

Drug administration. Cannabis was obtained from Professors H. Tanaka and S. Morimoto of Fukuoka University, and Δ^9-THC was isolated from cannabis by Professors J. Kinjo and R. Tuchihashi of Fukuoka University. The drug was emulsified in 4% dimethyl sulfoxide and 1% Tween 80 (Difco Laboratories, Detroit, MI, USA) and then diluted with 0.9% NaCl (Wako, Tokyo). Δ^9-THC was administered intraperitoneally at a concentration of 10 mg/kg.

Experimental design. The mice were divided into four groups of six each. One group served as the vehicle-treated control group, and three groups were treated with Δ^9-THC. The Δ^9-THC-treated mice were further divided into three groups based on washout duration: 3-day (3 d-washout), 10-day (10 d-washout), and 17-day (17 d-washout) groups. All groups received the drugs once daily for the first 3 days, followed by a washout period starting on the 4 th day. This experimental schedule is shown in Fig. 1A.

Body temperature measurements. Rectal temperatures were measured 15 min after the administration of vehicle or Δ^9-THC (10 mg/kg) (Fig. 1B). The rectal temperature of each mouse was measured using a digital laboratory thermometer (BAT-12 ; Physitemp Instruments, Clifton, NJ, USA). Drug administration and rectal temperature measurements were conducted from days 1 to 3 for all groups, on day 7 in the vehicle and 3 d-washout groups, on day 14 in the vehicle and 10 d-washout groups, and on day 21 in the vehicle and 17 d-washout groups.

Locomotor studies. Baseline locomotor activity was measured in all groups a day prior to the first drug administration to control for individual variability. Immediately after rectal temperature measurement, each mouse was placed alone in a plastic cage (18 cm $\times$35 cm$\times$17 cm) and allowed to move freely. Locomotor activity was recorded for 15 min using infrared sensors that detected and counted beam interruptions caused by the mouse's movement. Measurements were conducted according to the same schedule as body temperature (Fig. 1B).

Real-time PCR. The mRNA expression levels of the cannabinoid receptors CB_1 and CB_2, which are targets of Δ^9-THC, were measured by real-time PCR. Whole-brain samples were collected at the endpoint of

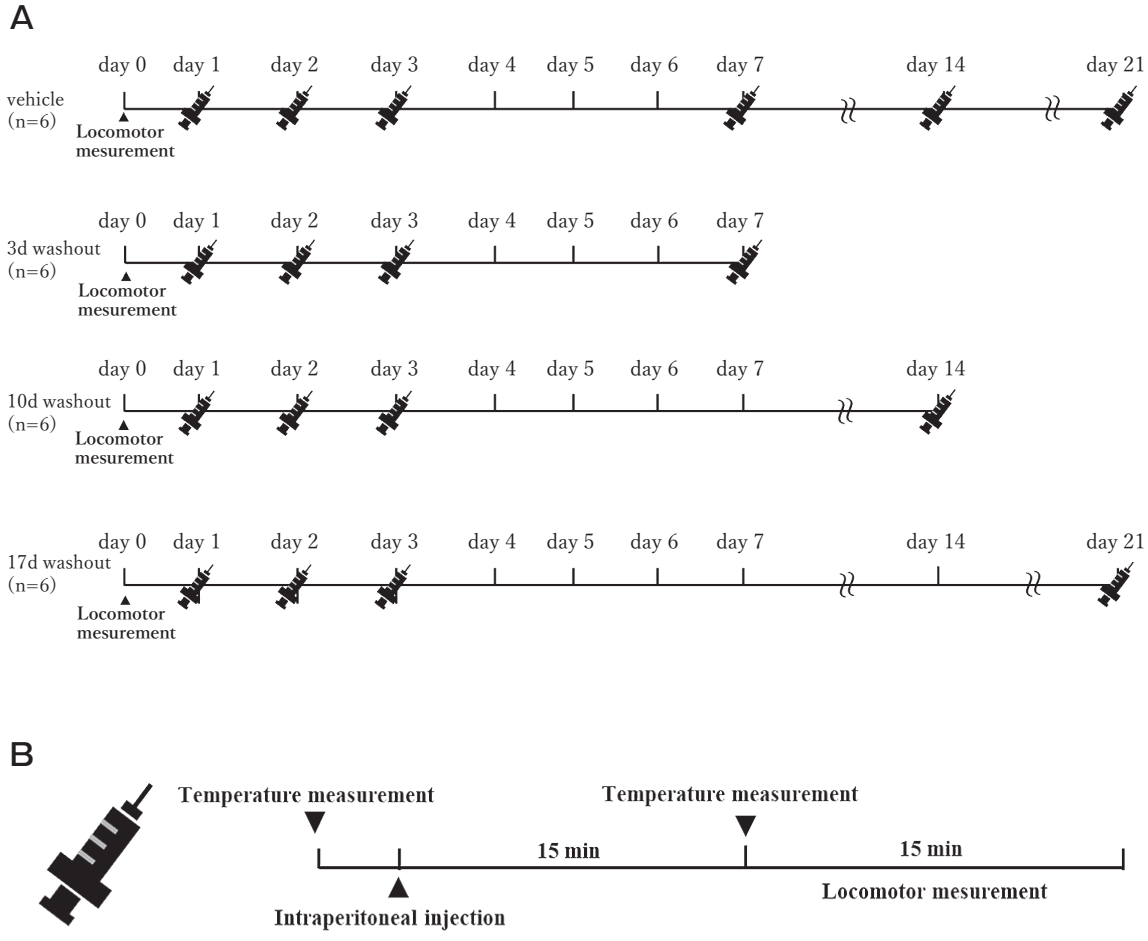

Fig. 1 Experimental schedule for measurements of locomotor activity and body temperature. (A) Experimental timeline for each group. Syringe symbols denote intraperitoneal drug injections, during which body temperature and locomotor activity were measured simultaneously. (B) Detailed timeline of injections and measurement schedule.

each course and on day 3. Samples were immersed in phenol solution and stored at −80°C. After all the experiments were completed, each brain was homogenized using a 23 G needle. mRNA was extracted from the homogenized samples using Tri Reagent (Cosmo Bio, Tokyo), and cDNA was synthesized using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). The resulting cDNA solution was mixed with Thunderbird SYBR qPCR Mix (TOYOBO) for qPCR analysis. Primers for CB_1 and CB_2 receptors were designed based on previous reports [8]. The sequences of the forward and reverse primers are listed in Table 1. DNA amplification was quantified using the LightCycler 96 system (Roche Diagnostics, distributed by Japan Genetics Inc., Tokyo).

Statistical analysis. Sample size was determined using G*Power ver. 3.1.9.4 software (Heinrich Heine University, Düsseldorf, Germany) based on effect sizes from a pilot study. All data are presented as the mean ± standard error of the mean. Between-group comparisons were conducted using Student's *t*-test, and PCR results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer post-hoc tests. Within-group comparisons of body temperature and locomotor activity were performed using repeated-measures ANOVA followed by Bonferroni post-hoc tests. All statistical analyses were conducted using JMP version 12.0.1 (SAS Institute, Tokyo). A p-value of < 0.05 was considered statistically significant.

Table 1 List of gene primer sequences

Gene	Sequences (5'->3')	Amplicon size (bp)
Cnr1	Forward primer 5'-CTGATGTTCTGGATCGGAGTC-3' Reverse primer 5'-GCATCATCATTCACACCTCAGA-3'	143
Cnr2	Forward primer 5'-TGACAAATGACACCCAGTCTTCT-3' Reverse primer 5'-AAGGAGTACATGATCCTGAGCAGT-3'	148
Gapdh	Forward primer 5'-AGGTCGGTGTGAACGGATTTG-3' Reverse primer 5'-TGTAGACCATGTAGTTGAGGTCA-3'	126

Results

Body temperature studies. Across all groups, the mean body temperature of the mice before drug administration was 37.5±0.9°C, within the normal range. Within-group comparisons over time revealed no significant differences in body temperature in the vehicle-treated group. Compared with the values on day 1, the mice in the Δ^9-THC-treated group showed significant differences in body temperature on days 2 and 3 ($p<0.0001$), although no significant differences were detected on days 7, 14, and 21. Subsequent between-group comparisons revealed that on day 1, the body temperature of the mice in the Δ^9-THC-treated group was significantly lower than that of the mice in the vehicle-treated group (−2.5°C from baseline for the Δ^9-THC-treated group vs. −0.50°C for the vehicle-treated group; $p=0.0004$). These findings suggest that administration of Δ^9-THC promotes hypothermia. Based on the standard body temperatures of the mice, administration of Δ^9-THC caused hypothermia. However, on days 2 and 3, body temperature in the Δ^9-THC-treated group was not significantly lower than that in the vehicle-treated group (day 2 : −0.54°C vs. −0.16°C from baseline, $p=0.5$; day 3 : −0.40°C vs. +0.35°C, $p=0.2$). These data indicated that tolerance to the hypothermic effect of Δ^9-THC developed on day 2. On day 7, after a 3-day washout period from day 4, body temperature in the Δ^9-THC-treated group was significantly lower than that in the vehicle-treated group (−2.3°C vs. −0.70°C from baseline, $p=0.0003$). The hypothermic effect was also observed on days 14 (after a 10-day-washout period from day 4) and 21 (after a 17-day-washout period from day 4), although no significant difference in the magnitude of the hypothermic effect was observed among the different washout periods (Fig. 2).

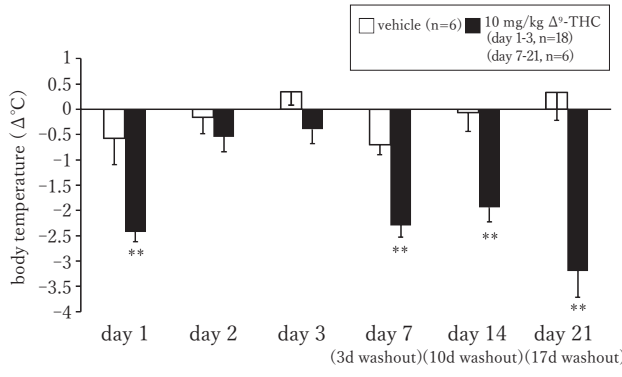

Fig. 2 Effects of chronic administration and withdrawal of Δ^9-THC on hypothermic response. Quantitative analysis of rectal temperature following Δ^9-THC administration (n=6 or 18 per group). Between-group comparisons were analyzed using Student's *t*-test, and within-group time-course comparisons were performed using repeated-measures ANOVA followed by Bonferroni *post-hoc* tests. $^{**}p<0.01$ vs. vehicle, $^{\dagger\dagger}p<0.01$ vs. day 1 in the same group.

Locomotor studies. Within-group comparisons over the observational period (from day 1 to day 21) revealed no significant day-to-day differences in the locomotor activity of the mice in either the vehicle- or Δ^9-THC-treated group. Similarly, we detected no significant differences in the spontaneous locomotor activity of the mice in the two groups on day 0 (data not shown). On days 1 and 2, the spontaneous locomotor activity of the mice in the Δ^9-THC-treated group was significantly lower than that of the mice in the vehicle-treated group (day 1 : 259±32 vs. 468±50 counts per 15 min, $p=0.003$; on day 2 : 278±20 vs. 413±39 counts per 15 min, $p=0.004$). Administration of Δ^9-THC decreased the locomotor activity of the mice. However, on day 3, the locomotor activity in the Δ^9-THC-treated group was not significantly lower than that in the vehi-

cle-treated group (267 ± 34 vs. 390 ± 27 counts, $p = 0.06$). These data indicated that the suppressive effect of Δ^9-THC on spontaneous locomotor activity disappeared on day 3. On day 7, after a 3-day washout period from day 4, the locomotor activity in the Δ^9-THC-treated group was not significantly lower than that in the vehicle-treated group (295 ± 70 vs. 383 ± 38 counts, $p = 0.3$). The spontaneous locomotor activity decreased again on days 14 (after a 10-day washout period from day 4) and 21 (after a 17-day washout period from day 4). These data indicated that the spontaneous locomotor-suppressing effect of Δ^9-THC, after having disappeared, reemerged by day 14 (Fig. 3).

CB_1 and CB_2 gene expression. The mRNA levels of CB_1 and CB_2 did not differ significantly among the four groups (Fig. 4).

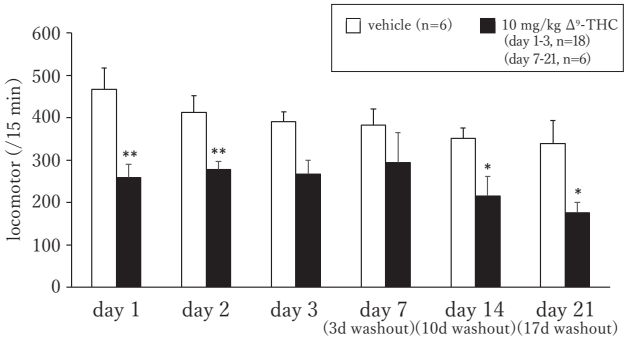

Fig. 3 Effects of chronic administration and withdrawal of Δ^9-THC on reduced spontaneous locomotor activity. Quantitative analysis of infrared beam interruptions as a measure of locomotor activity following Δ^9-THC administration (n = 6 or 18 per group). Data are presented as mean ± SEM and were analyzed using Student's *t*-test. $^*p < 0.05$, $^{**}p < 0.01$ vs. vehicle.

Discussion

To understand the mechanisms underlying tolerance formation to Δ^9-THC, we focused on two of its effects, hypothermia and reduced locomotor activity. Our results showed that tolerance to the hypothermic effect of Δ^9-THC developed and dissipated more rapidly than tolerance to its locomotor-suppressing effect.

Δ^9-THC exerts its psychoactive and physiological effects through CB_1 receptors in the endocannabinoid system (ECS) and regulates immune responses via CB_2 receptors. CB_1 receptors are primarily found in the central nervous system [9], whereas CB_2 receptors are predominantly expressed in immune cells [10]. These receptors, along with the endocannabinoids anandamide and 2-arachidonoylglycerol, constitute the ECS [11]. When animals are subjected to external stress or aging, the functionality of the ECS diminishes, potentially leading to various diseases [12]. Therefore, understanding tolerance development caused by cannabis administration is crucial for preventing health risks among cannabis users. In this study, we used Δ^9-THC at a dose of 10 mg/kg a level commonly employed in animal experiments. Numerous studies worldwide have investigated the effects of Δ^9-THC on behavior and metabolism [13], sex differences [14], and the autonomic nervous system [15], typically using the same dose. We therefore adopted this standard dosage. Additionally, several studies explored the effects of Δ^9-THC withdrawal [16, 17]. In those studies, the withdrawal period was set at around 21-28 days. This

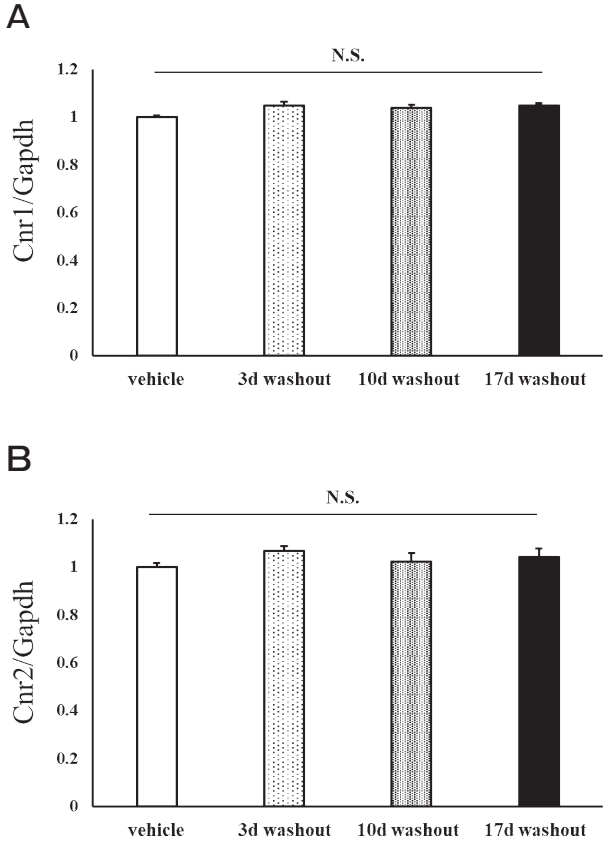

Fig. 4 mRNA expression levels of CB_1 (**A**) and CB_2 (**B**) in the whole brain. Quantitative analysis of CB_1 (**A**) and CB_2 (**B**) receptor mRNA expression in whole-brain samples collected at the end of each experimental course (day 7, 14, or 21 after the start of the experiment; n = 6 per group). N.S. = not significant.

period is considered sufficient for Δ^9-THC to be completely eliminated from the body, ensuring no impact on behavior or mental state. However, cannabis users who have developed dependence may resume use before its effects have fully dissipated. Therefore, we established shorter withdrawal periods (*i.e.*, 3, 10, and 17 days) than those set in previous studies.

We investigated the time required for the development and loss of tolerance to the hypothermic and locomotor-reducing effects of Δ^9-THC. Our results showed that tolerance to the hypothermic effects of Δ^9-THC was established by day 2 after administration and disappeared after a 3-day washout period. In contrast, tolerance to the locomotor-suppressing effect appeared later, on day 3, and persisted after a 3-day washout but disappeared after 10 days. This study is the first to delineate the timeline of Δ^9-THC tolerance development and loss in mice based on body temperature and locomotor activity.

The reason tolerance to the locomotor-suppressing effect of Δ^9-THC developed and disappeared later than tolerance to its hypothermic effect remains unclear, but several possibilities can be considered. First, the two physiological effects are thought to be mediated by distinct brain regions and neural circuits. The hypothermic effect is mainly induced in the hypothalamus, particularly in the preoptic area of the anterior hypothalamus, and likely involves by a relatively simple pathway [18]. By contrast, locomotor suppression depends on a more complex motor control system centered on the basal ganglia, including the striatum and substantia nigra, where CB_1 receptors are highly expressed [19]. Moreover, the rate of intracellular mechanisms such as CB_1 receptor desensitization and internalization may differ among brain regions. Functional downregulation of CB_1 receptors in the hypothalamus tends to occur rapidly with short-term administration, whereas in motor-related regions, it may require more time and produce more persistent plastic changes. Such region-specific differences in neural plasticity may underlie the differing timelines of tolerance development and loss.

Previous studies in humans suggest that cannabis tolerance may develop at different rates depending on the pharmacological function involved; however, the factors underlying such differences in tolerance formation remain unclear [1]. Ourfindings indicate that tolerance to Δ^9-THC also varies by pharmacological effect. Specifically, we found that tolerance to the locomotor-suppressing effects of this compound developed more slowly and less completely than tolerance to its hypothermic effect. This observation suggests that motor impairments relevant to daily activities, such as driving or working at heights, may persist during long-term cannabis use and thus require continued vigilance. Future studies examining tolerance across different pharmacological activities, including appetite stimulation and anxiolytic effects, could help ensure the safe and effective therapeutic use of cannabis-based therapies.

Recent research on cannabinoid receptors has shown that CB_1 receptor knockout mice exhibit increased mortality rates [20]. Additionally, mutations in the CB_1 receptor may play a role in the development of tolerance to cannabinoids [21]. Based on these reports, we hypothesized that the development and dissipation of Δ^9-THC tolerance observed in this study would be associated with CB_1 and CB_2 receptor expression levels. To test this hypothesis, we collected whole-brain samples from each of the vehicle, 3 d-washout, 10 d-washout, and 17 d-washout groups after the completion of the experiments and examined the mRNA expression levels of these receptors. Our results revealed no significant differences in mRNA expression levels among the groups (Fig. 4). Furthermore, previous reports suggested that repeated administration of Δ^9-THC decreases the CB_1 receptor density and coupling efficiency in the brains of rats and mice [22, 23]. Based on these findings, we evaluated the expression of CB_1 and CB_2 mRNA on day 3, when both body temperature and locomotor activity tolerance could be observed; however, no significant differences were observed between the vehicle and Δ^9-THC-treated groups (Fig. 5). These findings suggest that the development of Δ^9-THC tolerance is not associated with changes in the overall mRNA expression levels of cannabinoid receptors in the brain.

The ECS activates various receptors besides CB_1 and CB_2 through endogenous cannabinoids. Many cannabinoids act as agonists for TRPV1 [24, 25]. Additionally, CB_1 receptors co-localize with TRP channels in sensory and central nervous systems [26], whereas CB_2 receptors co-localize with these channels in sensory neurons and osteoclasts [27, 28]. This highlights a strong functional relationship between cannabinoids and TRP channels, with recent discoveries indicating that Δ^9-THC

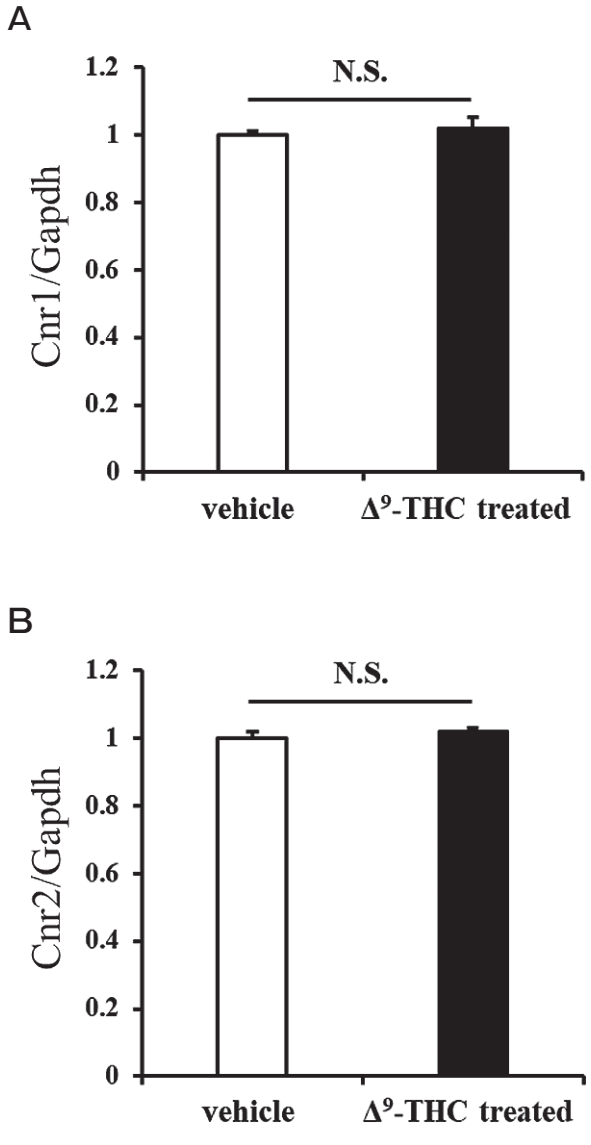

Fig. 5 mRNA expression levels of CB_1 and CB_2 in the whole brain on day 3. Quantitative analysis of CB_1 and CB_2 receptor mRNA expression. Whole-brain samples were collected on day 3 following intraperitoneal drug administration of Δ^9-THC (n = 6) or vehicle (n = 3). N.S. = not significant. The primer sequences for the *Cnr1* gene were forward 5'-CTGATGTTCTGGATCGGAGTC-3' and reverse 5'-GCATCATCATTCACACCTCAGA-3'. For the *Cnr2* gene, the forward and reverse primers were 5'-GGAAGTGCTTGGTTCTGTCAACATA-3' and 5'-TGCAGGAATTCACAGCTGTGG-3', respectively.

affects TRPV2, TRPV3, TRPV4, TRPA1, and TRPM8 [25]. Furthermore, several cannabinoids function as agonists for GPR55 [29, 30] and PPAR [31] receptors. In the present study, the differing durations for the development and loss of tolerance to the physiological effects of Δ^9-THC suggest that receptors other than CB_1 and CB_2 are involved.

The main limitation of this study is that we did not directly examine the molecular mechanisms underlying the observed differences in tolerance timelines. Given that we detected no significant changes in CB_1 or CB_2 receptor mRNA expression, it is conceivable that post-transcriptional mechanisms contributed to the observed effects. In addition, mRNA levels were assessed in whole-brain homogenates, which could have masked region-specific changes. Distinct alterations in receptor expression might therefore occur in brain regions involved in the control of thermoregulation or motor control. Consequently, to better elucidate the mechanisms associated with tolerance development induced by Δ^9-THC, future studies should assess receptor expression at the protein level and include functional assays. Another limitation concerns behavioral variability in the vehicle-treated group, where locomotor activity showed a gradual decline over the observational period. This pattern likely reflects exploratory behavior, which is commonly seen in rodents exposed to novel environments or stimuli. Although we strived to minimize these confounding effects by conducting habituation and handling, these procedures could not completely eliminate the influence of exploratory activity. Consequently, we cannot fully exclude the possibility that the exploratory behaviors observed in the vehicle-treated mice influenced the outcomes of the locomotor assessments. Nevertheless, the results in the Δ^9-THC-treated mice suggest that Δ^9-THC itself may modulate innate exploratory behaviors. Future experiments should therefore include refined habituation protocols, adjusted Δ^9-THC dosing schedules, and optimized behavioral assessment procedures to achieve more precise evaluation of these behavioral effects.

In conclusion, tolerance to the hypothermic effect of Δ^9-THC developed and dissipated more rapidly than tolerance to its locomotor-suppressing effect. These findings enhance our understanding of the differing mechanisms of Δ^9-THC tolerance and the potential health risks associated with long-term cannabis use.

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