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# Chronic $\Delta^9$-tetrahydrocannabinol treatment produces antinociceptive tolerance in mice without altering Protein Kinase A activity in mouse brain and spinal cord

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**Abstract**

The present study investigated the effect of different levels of Δ-9-tetrahydrocannabinol ($\Delta^9$-THC) antinociceptive tolerance on Protein Kinase A (PKA) activity in mouse brain and spinal cord. To strengthen this investigation, a positive control was developed to demonstrate the assay utilized in this study was sensitive enough to detect an increase in PKA activity in the anatomical regions utilized in this study. The membrane-permeant and phosphodiesterase-resistant cAMP analog 8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS) was utilized for the development of this positive control and this compound produced an increase in PKA activity in several mouse brain regions (i.c.v.) and lumbar spinal cord (i.t.) following its administration. Models were then developed in which mice expressed either a 13-fold or 49-fold level of $\Delta^9$-THC antinociceptive tolerance following chronic treatment with 10 mg/kg $\Delta^9$-THC or 80 mg/kg $\Delta^9$-THC for 6.5 days. Basal and total cytosolic and particulate PKA activities were measured directly in homogenates from the striatum, hippocampus, cerebellum, cortex and lumbar spinal cord. Results from this study indicate that chronic exposure to $\Delta^9$-THC does not produce an increase in PKA activity in these mouse brain regions or spinal cord. Future work is needed to determine the role of PKA in cannabinoid tolerance in mice.


*Keywords:* Cannabinoid; THC; Antinociception; Tolerance; Protein Kinase A

## 1. Introduction

$\Delta^9$-Tetrahydrocannabinol ($\Delta^9$-THC) is the major psychoactive constituent of the hemp plant *Cannabis sativa* [1]. In humans, $\Delta^9$-THC produces numerous behavioral and pharmacological effects including euphoria, impairment of memory, tachycardia, impairment of motor function, and antinociception. These acute effects of $\Delta^9$-THC are mediated by binding to receptors in the central nervous system called $CB_1$ receptors. The $CB_1$ receptor is distributed throughout the brain and spinal cord with highest densities in the hippocampus, cerebellum, basal ganglia, cortex and striatum [2,3]. $CB_1$ receptors are coupled to inhibitory $G_{i/o}$ proteins that regulate the activity of multiple signal transduction pathways including inhibition of adenylate cyclase (AC), inhibition of N- and Q/P-type voltage-dependent calcium channels, activation of inwardly rectifying potassium channels, and activation of the MAP kinase system [4–8].

Chronic treatment with $\Delta^9$-THC results in the development of tolerance to most acute behavioral and pharmacological effects produced by $\Delta^9$-THC [9,10]. Several studies have attempted to identify the cellular adaptations underlying the development of tolerance to $\Delta^9$-THC. Results from these studies have provided significant evidence that the $CB_1$ receptor plays a major role in the production of cannabinoid tolerance. For instance, the development of tolerance to $\Delta^9$-THC is accompanied by downregulation of $CB_1$ receptors in most brain regions [11]. Moreover, this reduction in $CB_1$ receptor levels is not accompanied by parallel decreases in $CB_1$ receptor mRNA levels which indicates the primary action of $\Delta^9$-THC is on the $CB_1$ receptor protein [12]. Studies have also demonstrated that $CB_1$ receptors undergo desensitization following chronic treatment with $\Delta^9$-THC. Sim et al. [13] reported a reduction in agonist-stimulated [$^{35}$S]GTPγS

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binding across different rat brain regions following chronic treatment with $\Delta^9$-THC for 21 days. Thus, $CB_1$ receptor desensitization and downregulation appear to play a role in the development of tolerance to $\Delta^9$-THC.

At present, few studies have been done in brain to determine if adaptations occur at the effector level that may contribute to $\Delta^9$-THC tolerance. $\Delta^9$-THC acts acutely at $CB_1$ receptors which couple with the $G_{i/o}$ family of G-proteins to inhibit AC and reduce the cellular levels of cAMP and the activity of PKA. Theoretically, the disruption in $CB_1$ receptor-$G_{i/o}$-protein signaling produced by chronic cannabinoid exposure should result in an alteration in cannabinoid-mediated downstream cellular signaling cascades. More specifically, the desensitization/downregulation of the $CB_1$ receptor should result in the disinhibition of AC activity and an increase in cAMP levels and PKA activity. Cell culture studies have demonstrated this phenomenon as chronic cannabinoid exposure produced an increase in AC activity [14]. Decreases in $CB_1$ receptor signaling (desensitization and downregulation) have also been demonstrated in animal studies to produce an effect on downstream effector activity in brain. More specifically, chronic treatment with $\Delta^9$-THC produced an up-regulation of the cAMP/Protein Kinase A (PKA) cascade in several rat brain regions [15]. In addition, Selley et al. [16] reported that chronic treatment with $\Delta^9$-THC resulted in a reduction in $CB_1$ receptor-mediated inhibition of forskolin-stimulated AC activity in mouse cerebellum. Evidence for alterations in the cAMP/PKA cascade during $\Delta^9$-THC tolerance also comes from studies in which the PKA inhibitor KT-5720 reversed antinociceptive tolerance in mice treated chronically with $\Delta^9$-THC [17]. Thus, some evidence exists for the involvement of PKA in the expression of $\Delta^9$-THC tolerance. However, the direct effect of chronic treatment with $\Delta^9$-THC on PKA activity in brain and spinal cord has not been investigated in $\Delta^9$-THC-tolerant mice.

In the present study, we attempted to relate the level of $\Delta^9$-THC antinociceptive tolerance expressed at the behavioral level to alterations in PKA activity at the cellular level in mouse striatum, hippocampus, cerebellum, cortex and lumbar spinal cord (LSC). These areas were chosen because they possess neurons that contain high levels of $CB_1$ receptors which previous studies have shown undergo desensitization and downregulation following chronic treatment with $\Delta^9$-THC [11–13,18]. Thus, the uncoupling of the $CB_1$ receptor from $G_{i/o}$ proteins produced by chronic $\Delta^9$-THC treatment should result in an increase in AC activity that leads to an increase in cAMP levels and PKA activity. To conduct this investigation we developed models in which two different levels of antinociceptive tolerance were induced in mice treated chronically with 10 mg/kg $\Delta^9$-THC or 80 mg/kg $\Delta^9$-THC for 6.5 days. Basal and total cytosolic and particulate PKA activity were measured in homogenates from the aforementioned anatomical regions taken from drug-naïve mice, as well as in non-tolerant and $\Delta^9$-THC-tolerant mice from each treatment group. Overall, this study represents the first investigation into the effect of different levels of $\Delta^9$-THC antinociceptive tolerance on PKA activity in mouse brain and spinal cord.

## 2. Materials and methods

### 2.1. Materials

$\Delta^9$-THC was obtained from the National Institute on Drug Abuse, Bethesda, MD and was dissolved in a 1:1:18 solution of ethanol, emulphor and saline. 8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS) was purchased from Axxora (San Diego, CA). Protein Kinase Inhibitor-(6-22)-amide ($Thr^6$-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-$Ile^{22}$-$NH_2$) was purchased from Sigma (St. Louis, MO). All other reagent grade chemicals were purchased from Sigma.

### 2.2. Methods of handling mice

Male Swiss Webster mice (Harlan Laboratories, Indianapolis, IN, USA) weighing 25–30 g were housed six to a cage in animal care quarters maintained at 22 ± 2 °C on a 12 h light–dark cycle. Food and water were available ad libitum. The mice were brought to the test room (22 ± 2 °C, 12 h light–dark cycle), marked for identification and allowed 24 h to recover from transport and handling. The Institutional Animal Care and Use Committee (IACUC) at the Virginia Commonwealth University approved all procedures. IACUC procedures comply with the European Communities Council Directive of 24 November 1986 (86/609/EEC) to minimize pain and discomfort.

### 2.3. Intracerebroventricular injections

Intracerebroventricular (i.c.v.) injections were performed as described by Pedigo et al. [19]. Mice were anaesthetized with ether and a transverse incision was made in the scalp. A free-hand 5 μl injection of Sp-8-Br-cAMPS (10 nmol) was made into the lateral ventricle. This laboratory has more than 25 years of experience injecting drugs i.c.v. in mice in the published literature. We have repeatedly conducted dye studies over the years to confirm accurate placement of the needle within the lateral ventricle. Thus, we state with high confidence (at least 98% accuracy) that we are correctly placing the needle. In addition, we have conducted dye studies to insure even distribution of drug throughout the brain.

### 2.4. Intrathecal injections

Intrathecal (i.t.) injections were performed in mice following the protocol of Hylden and Wilcox [20]. Una-

nesthetized mice were injected with 5 μl of Sp-8-Br-cAMPS (10 nmol) between the L5 and L6 area of the spinal cord with a 30-gauge needle.

### 2.5. Activation of PKA by Sp-8-Br-cAMPS

8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-Br-cAMPS) is a cAMP analogue that is a potent membrane-permeant activator of PKA [21]. In contrast to cAMP, Sp-8-Br-cAMPS is resistant to degradation by mammalian cyclic nucleotide phosphodiesterases. Experiments were conducted in which Sp-8-Br-cAMPS was added in vitro to homogenates of thalamus, striatum, hippocampus, cortex and LSC taken from drug-naïve mice to demonstrate the ability of Sp-8-Br-cAMPS to activate PKA in these regions. Additionally, experiments were conducted in drug-naïve mice in which Sp-8-Br-cAMPS was administered i.t. or i.c.v. In these studies, Sp-8-Br-cAMPS was utilized for the development of a positive control to demonstrate the biochemical assay used in this study was sensitive enough to detect an increase in mouse neuronal PKA activity following the dissection and processing of the anatomical regions utilized in this study.

### 2.6. Chronic treatment with $\Delta^9$-THC

Mice were rendered tolerant to $\Delta^9$-THC over 7 days. The mice received intraperitoneal (i.p.) injections of $\Delta^9$-THC (10 mg/kg or 80 mg/kg) twice per day at 12 h intervals (approximately 8 a.m. and 8 p.m.) for 6 days. On the seventh day, the mice received the morning injection only. Thus, each mouse received a total of 13 injections. Control animals were given vehicle (ethanol, emulphor, and saline 1:1:18) at the same times. Twenty-four hours following the final injection, the mice were challenged with a single i.p. dose of $\Delta^9$-THC. The mice were then tested using the tail-flick test to assess for antinociceptive tolerance.

### 2.7. The tail-flick test

The warm-water tail-flick apparatus used to assess for antinociception in the mice was maintained at 56 ± 0.1 °C. Base-line (control) latency values were determined first. Only mice with a control reaction time from 2 to 4 s were used in these studies. The average baseline tail-flick latency for these experiments was 3.1 s. Mice were then administered $\Delta^9$-THC i.p. and test latency values were determined at 30 min post-injection. A 10 s maximum cut-off time was imposed to prevent tissue damage. Immediately upon testing the animals were euthanized to minimize any type of distress, according to IACUC guidelines. Antinociception was quantified according to the method of Harris and Pierson [22] as the percentage of maximum possible effect (percent MPE) which was calculated as: percent MPE = $[(\text{test} - \text{control})(10 - \text{control})^{-1}] \times 100$. Percent MPE was calculated for each mouse using at least six mice per dose. Dose–response curves were generated for calculation of $ED_{50}$ values and 95% confidence limits. These values were calculated using least squares linear regression analysis followed by calculation of 95% confidence limits by Bliss [23]. Tests for parallelism were conducted before calculation of potency ratio values and 95% confidence limits by the method of Colquhoun [24]. A potency ratio value greater than one, with the lower 95% confidence limit greater than one, was considered a significant difference in potency.

### 2.8. Determination of PKA activity in cytosolic and particulate fractions of mouse brain and spinal cord

PKA activity was determined in both particulate and cytosolic fractions obtained from cortex, cerebellum, striatum, hippocampus, and LSC. Mice received injections (i.p.) of vehicle, 10 mg/kg $\Delta^9$-THC or 80 mg/kg $\Delta^9$-THC twice per day for 6.5 days. Twenty-four hours after receiving the final injection, mice were decapitated and these regions were removed by dissection from each mouse and stored at −80 °C. Accurate dissection of each region was verified using boundaries defined by Paxinos and Franklin [25]. Tissue was homogenized in buffer that contained 25 mM Tris–HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 20 mM β-glycerol phosphate, 50 mM sodium pyrophosphate, 1 mM *para*-nitrophenylphosphate and one Complete Protease Inhibitor tablet (Roche Pharmaceuticals, Indianapolis, IN). The homogenate was centrifuged at 100,000 × *g* for 60 min at 4 °C. The supernatant was saved and used as the cytosolic fraction. The pellet was resuspended in homogenization buffer and recentrifuged at 100,000 × *g* for 60 min at 4 °C. The pellet was then resuspended again in homogenization buffer and was used as the particulate fraction. The protein content of each fraction was determined using the method of Bradford [26]. Preliminary studies were conducted to determine optimal protein concentrations to be used where a linear relationship existed between protein concentration and PKA activity. Aliquots from each fraction were then used to measure PKA activity using the SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System (Promega Corp., Madison, WI). The assay measures the transfer of the γ-phosphate of [γ-$^{32}$P]ATP by PKA to a biotinylated peptide substrate called kemptide (LRRASLG) that is highly specific for PKA. PKA activity was determined in duplicate in a final volume of 25 μl containing 200 mM Tris–HCl (pH 7.4), 100 mM $MgCl_2$, 0.5 mg/ml BSA, 100 μM kemptide, and 0.5 μCi [γ-$^{32}$P]ATP (3000 Ci/mmol prepared in 100 μM ATP). PKA activity was determined in the presence (total PKA activity) and absence (basal PKA activity) of cAMP. Total PKA activity was maximal in each region at 1–100 μM cAMP. As a result, 5 μM cAMP was used to determine total PKA activity. In order to confirm kinase activity measured in the assay was catalyzed by PKA,

enzyme activity was measured in the presence of 10 μM PKI-(6-22)-amide which inhibited all phosphate incorporation measured in each assay. PKI-(6-22)-amide is an selective and potent inhibitor of PKA [27]. Reactions were carried out at 30 °C for specific times and were terminated using 7.5 M guanidine hydrochloride. Aliquots (10 μl) were spotted in duplicate on a SAM$^{2®}$ Biotin Capture Membrane. This membrane is coated with strepavidin which allowed the $^{32}$P-labeled biotinylated kemptide substrate to be recovered from the reaction mix. Membranes were washed step-wise in 2 M NaCl, 2 M NaCl in 1% $H_3PO_4$, dd$H_2O$ and then air-dried. The $^{32}$P contained in each membrane was then quantified by liquid scintillation spectrometry. Background counts, calculated for each sample from reactions that did not contain kemptide, were subtracted. Data are expressed as picomoles of [$^{32}$P]phosphate transferred to kemptide substrate per minute per microgram of protein.

### 2.9. Data analysis of PKA activity

Data are reported as mean ± S.E.M. of at least six separate experiments that were each performed in duplicate. Statistical significance of the data was determined by analysis of variance followed by the nonpaired two-tailed Student's *t*-test, using JMP (SAS Institute, Cary, NY).

## 3. Results

### 3.1. Induction of antinociceptive tolerance to $\Delta^9$-THC

Antinociceptive tolerance was observed following chronic treatment for 6.5 days with 10 and 80 mg/kg $\Delta^9$-THC. The dose–response curves for $\Delta^9$-THC in $\Delta^9$-THC-tolerant mice and mice not tolerant to $\Delta^9$-THC in the tail-flick test are shown in Fig. 1. In addition, the $ED_{50}$ values and potency ratios for $\Delta^9$-THC in the non-tolerant versus $\Delta^9$-THC-tolerant mice are shown in Table 1. Chronic treatment with 10 mg/kg $\Delta^9$-THC resulted in a 13-fold reduction in the potency of $\Delta^9$-THC to produce antinociception, while a 49-fold reduction in the potency of $\Delta^9$-THC to produce antinociception was observed in mice treated chronically with 80 mg/kg $\Delta^9$-THC.

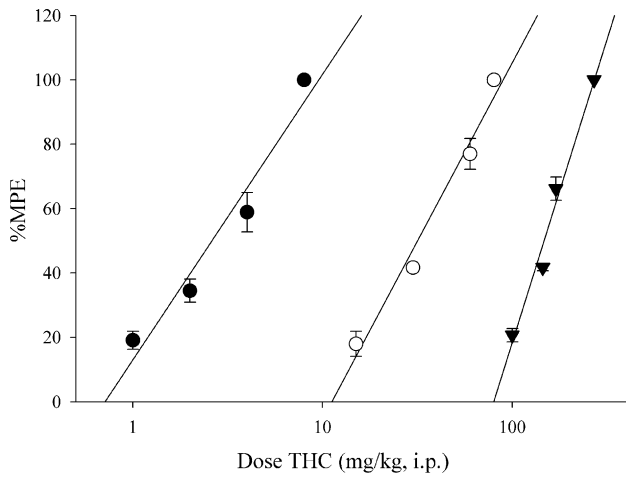


Fig. 1. Mice received injections (i.p.) of vehicle, 10 mg/kg $\Delta^9$-THC, or 80 mg/kg $\Delta^9$-THC twice per day for 6.5 days. Twenty-four hours after receiving the final injection, mice were challenged with various doses of $\Delta^9$-THC and tested for the production of antinociception 30 min later using the tail-flick test. The treatment groups consisted of vehicle (●), 10 mg/kg $\Delta^9$-THC (○), 80 mg/kg $\Delta^9$-THC (▼). Vehicle was ethanol, emulphor and saline (1:1:18).

Table 1
Induction of tolerance to the antinociceptive effects of $\Delta^9$-THC

| Group | $ED_{50}$ (mg/kg) (95% C.L.) | Potency ratio (95% C.L.) |
|---|---|---|
| Vehicle | 2.6 (2.3–2.9) | – |
| 10 mg/kg THC | 33.9 (27.2–42.1)[a] | 12.9 (10.1–16.4)[a] |
| 80 mg/kg THC | 137.3 (130.0–144.9)[a] | 49.0 (45.7–52.5)[a] |

Mice received injections (i.p.) of vehicle, 10 mg/kg $\Delta^9$-THC, or 80 mg/kg $\Delta^9$-THC twice per day for 6.5 days. Twenty-four hours after receiving the final injection, mice were challenged with various doses of $\Delta^9$-THC i.p. and tested for the production of antinociception 30 min later using the tail-flick test. $ED_{50}$ values and potency ratios were calculated as described in Section 2.

[a] Denotes significantly different compared to vehicle.

### 3.2. Regional differences in levels of PKA activity

The direct measurement of cytosolic and particulate basal and total PKA activity in homogenates from mouse hippocampus, cortex, cerebellum, striatum and LSC revealed regional differences in levels of PKA activity. As seen in Table 2, both basal and total PKA activity were significantly lower (∼3–6-fold) in particulate fractions compared to cytosolic fractions from each region taken from drug naïve mice. In addition, differences in both basal and total cytosolic and particulate PKA activity were seen between the regions used in this study. More specifically, it is apparent that basal cytosolic PKA activity is greater in cerebellum and hippocampus compared to striatum, LSC and cortex. In contrast, total cytosolic PKA activity is greater in striatum and cortex compared to cerebellum, LSC and hippocampus. Additionally, total particulate PKA activity is greater in striatum and cortex compared to hippocampus, LSC and cerebellum. Finally, in this study basal and total cytosolic and particulate PKA activity were measured in the presence of the PKA inhibitor PKI-(6-22)-amide. The presence of PKI-(6-22)-amide resulted in complete inhibition of both particulate and cytosolic enzyme activity in each assay which provided confirmation that the phosphate incorporation measured in these assays was catalyzed by PKA.

### 3.3. Activation of PKA by Sp-8-Br-cAMPS in vitro

Experiments were conducted in which 8-Bromoadenosine-3′,5′-cyclic monophosphorothioate, Sp-isomer (Sp-8-

Table 2
Effect of chronic treatment with Delta-9-THC on Protein Kinase A activity in various mouse brain regions and lumbar spinal cord

| Region | Treatment | Cytosolic | | Cytosolic | | Particulate | | Particulate | |
|---|---|---|---|---|---|---|---|---|---|
| | | Basal activity | Percent change | Total activity | Percent change | Basal activity | Percent change | Total activity | Percent change |
| Striatum | Naïve | 58.1 ± 6.4 | – | 326.2 ± 3.2 | – | 14.2 ± 1.2 | – | 90.1 ± 3.4 | – |
| Striatum | Vehicle | 57.1 ± 1.3 | – | 310.3 ± 11.2 | – | 14.3 ± 2.1 | – | 95.2 ± 1.4 | – |
| Striatum | 10 mg/kg THC | 52.4 ± 2.1 | −8 | 313.2 ± 4.1 | 1 | 14.2 ± 1.3 | −1 | 97.2 ± 3.2 | 2 |
| Striatum | 80 mg/kg THC | 50.2 ± 4.3 | −13 | 309.2 ± 7.8 | −1 | 11.1 ± 1.4 | −23 | 97.1 ± 2.3 | 3 |
| Hippo | Naïve | 65.2 ± 1.2 | – | 167.3 ± 4.8 | – | 11.3 ± 1.4 | – | 43.1 ± 2.3 | – |
| Hippo | Vehicle | 68.1 ± 2.1 | – | 161.3 ± 6.2 | – | 10.3 ± 1.2 | – | 38.3 ± 4.1 | – |
| Hippo | 10 mg/kg THC | 74.1 ± 5.3 | 9 | 171.2 ± 8.1 | 6 | 9.3 ± 0.3 | −10 | 35.4 ± 1.1 | −8 |
| Hippo | 80 mg/kg THC | 63.2 ± 3.1 | −7 | 161.4 ± 8.2 | 1 | 10.1 ± 3.2 | −2 | 38.4 ± 1.1 | 1 |
| LSC | Naïve | 43.1 ± 2.1 | – | 154.1 ± 4.3 | – | 8.3 ± 0.9 | – | 28.1 ± 2.1 | – |
| LSC | Vehicle | 55.2 ± 1.3 | – | 145.3 ± 4.2 | – | 7.2 ± 1.2 | – | 25.2 ± 3.2 | – |
| LSC | 10 mg/kg THC | 58.2 ± 2.3 | 6 | 158.1 ± 5.1 | 9 | 8.1 ± 2.1 | 12 | 20.1 ± 0.8 | −20 |
| LSC | 80 mg/kg THC | 63.1 ± 3.1 | 13 | 150.2 ± 5.3 | 4 | 9.3 ± 1.3 | 23 | 21.3 ± 2.1 | −15 |
| Cerebellum | Naïve | 86.3 ± 2.1 | – | 139.3 ± 4.3 | – | 14.4 ± 2.4 | – | 29.3 ± 2.3 | – |
| Cerebellum | Vehicle | 78.1 ± 1.1 | – | 129.3 ± 2.3 | – | 14.2 ± 2.2 | – | 30.1 ± 1.3 | – |
| Cerebellum | 10 mg/kg THC | 81.2 ± 7.1 | 4 | 135.3 ± 6.3 | 5 | 16.3 ± 1.4 | 13 | 34.1 ± 2.2 | 12 |
| Cerebellum | 80 mg/kg THC | 87.2 ± 3.2 | 10 | 138.3 ± 3.3 | 7 | 18.2 ± 2.3 | 22 | 34.3 ± 2.1 | 13 |
| Cortex | Naïve | 49.3 ± 3.2 | – | 213.2 ± 4.1 | – | 16.3 ± 1.3 | – | 77.2 ± 2.1 | – |
| Cortex | Vehicle | 48.3 ± 4.2 | – | 206.4 ± 5.3 | – | 16.1 ± 1.3 | – | 81.2 ± 5.2 | – |
| Cortex | 10 mg/kg THC | 49.3 ± 3.2 | 2 | 219.1 ± 13.1 | 6 | 15.3 ± 2.1 | −5 | 80.1 ± 5.4 | −2 |
| Cortex | 80 mg/kg THC | 45.3 ± 2.1 | −6 | 210.3 ± 10.2 | −2 | 12.3 ± 1.2 | −24 | 73.1 ± 2.1 | −10 |

Mice were administered vehicle or $\Delta^9$-THC (10 mg/kg or 80 mg/kg, i.p.) twice per day for 6.5 days. Twenty-four hours after the last injection, mice were sacrificed and brain regions and LSC were removed. Particulate and cytosolic fractions were assayed for PKA activity. Basal activity represents Protein Kinase A activity measured in the absence of exogenous cAMP addition to the reaction mixture. Total activity represents Protein Kinase A activity measured in the presence of exogenously added cAMP (5 μM) to the reaction mixture. Units for enzyme activity are in pmol/μg/min. Data for each condition in each region are expressed as mean values ± S.E.M. from assays run in six different mice. Vehicle was ethanol, emulphor, and saline (1:1:18). Tissue was diluted 30-fold in homogenization buffer when processed. Hippo is hippocampus.

Br-cAMPS) was added in vitro to homogenates of thalamus, striatum, hippocampus, cortex, and LSC taken from drug naïve mice. Sp-8-Br-cAMPS is a cAMP analogue that is a potent membrane-permeant activator of PKA [21]. In contrast to cAMP, Sp-8-Br-cAMPS is resistant to degradation by mammalian cyclic nucleotide phosphodiesterases. As seen in Fig. 2, the addition of 10 μM Sp-8-Br-cAMPS to homogenates of thalamus, striatum, hippocampus, cortex, and LSC taken from drug naïve mice resulted in a significant increase in cytosolic PKA activity in each region. This increase in PKA activity was similar in magnitude to that produced by 5 μM cAMP. This demonstrated that Sp-8-Br-cAMPS can produce activation of PKA in each region.

### *3.4. Activation of PKA by Sp-8-Br-cAMPS administered i.c.v. and i.t.*

The i.c.v. administration of 10 nmol Sp-8-Br-cAMPS resulted in region-specific increases in basal cytosolic PKA activity in drug-naïve mice. As seen in Fig. 3, mice administered Sp-8-Br-cAMPS exhibited a significant increase in basal cytosolic PKA activity in thalamus, cortex, and hippocampus. In addition, the i.t. administration of 10 nmol Sp-8-Br-cAMPS resulted in an increase in LSC basal cytosolic PKA activity (Fig. 4). These results confirmed that the assay used in this study was sensitive enough to detect an increase in mouse neuronal PKA activity following the dissection and processing of each anatomical region used in this study for the assay of PKA activity.

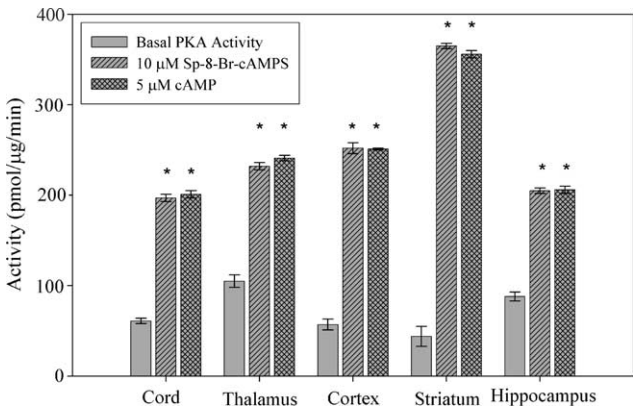


Fig. 2. In vitro effect of Sp-8-Br-cAMPS on cytosolic Protein Kinase A (PKA) activity in mouse brain regions and spinal cord. Naïve mice were decapitated and brain regions and lumbar spinal cords were removed. Cytosolic fractions from each region were assayed for PKA activity as described in Section 2. Basal PKA activity was measured in the absence of exogenous cAMP or Sp-8-Br-cAMPS addition to the reaction mixture. PKA activity was also measured following the addition of 10 μM Sp-8-Br-cAMPS or 5 μM cAMP to homogenates from each region. Data for each condition in each region are expressed as mean values ± S.E.M. from assays run in six different mice. $^{*}p < 0.01$ using Student's *t*-test. Vehicle was saline.

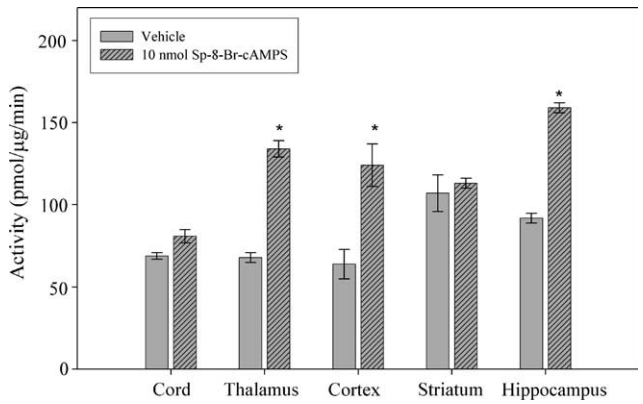


Fig. 3. Effect of Sp-8-Br-cAMPS on cytosolic Protein Kinase A (PKA) activity in mouse brain regions and spinal cord. Naïve mice were administered vehicle or 10 nmol Sp-8-Br-cAMPS i.c.v. Thirty minutes later, mice were decapitated and brain regions and lumbar spinal cords were removed. Cytosolic fractions from each region were assayed for PKA activity as described in Section 2. PKA activity was measured in the absence of exogenous cAMP addition to the reaction mixture. Data for each condition in each region are expressed as mean values ± S.E.M. from assays run in six different mice. $^{*}p < 0.01$ using Student's *t*-test. Vehicle was saline. Tissue was diluted 10-fold for processing.

### 3.5. Effect of chronic treatment with $\Delta^9$-THC on cytosolic and particulate PKA activity in mouse brain regions

The effect of chronic treatment with either 10 mg/kg $\Delta^9$-THC or 80 mg/kg $\Delta^9$-THC on PKA activity was assayed in particulate and cytosolic fractions of homogenates from mouse striatum, hippocampus, cerebellum, LSC and cortex. As seen in Table 2, basal and total cytosolic and particulate PKA activity were not altered in any region from mice treated chronically with either dose of $\Delta^9$-THC.

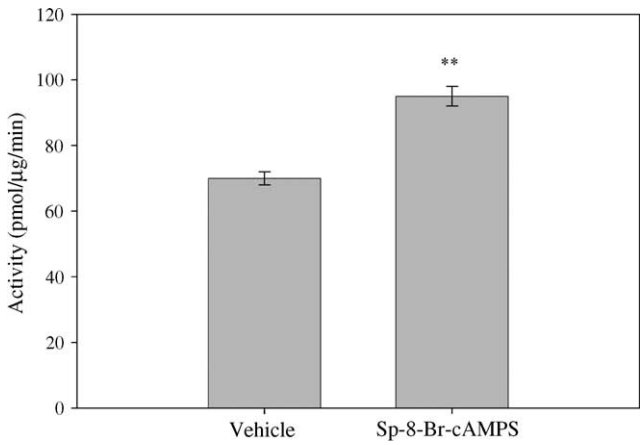


Fig. 4. Effect of Sp-8-Br-cAMPS on cytosolic Protein Kinase A (PKA) activity in mouse lumbar spinal cord. Naïve mice were administered vehicle or 10 nmol Sp-8-Br-cAMPS i.t. Thirty minutes later, mice were decapitated and lumbar spinal cords were removed. Cytosolic fractions from each LSC were assayed for PKA activity as described in Section 2. PKA activity was measured in the absence of exogenous cAMP addition to the reaction mixture. Data for each condition in each region are expressed as mean values ± S.E.M. from assays run in six different mice. $^{**}p < 0.05$ using Student's *t*-test. Vehicle was saline. Tissue was diluted 10-fold for processing.

## 4. Discussion

The present study represents the first investigation of the effect of different levels of $\Delta^9$-THC antinociceptive tolerance on PKA activity in mouse brain and LSC. Results from this study demonstrate that chronic treatment with either 10 mg/kg $\Delta^9$-THC or 80 mg/kg $\Delta^9$-THC for 6.5 days produced significant antinociceptive tolerance in mice. More specifically, mice treated chronically with 10 mg/kg $\Delta^9$-THC expressed a 13-fold level of antinociceptive tolerance, while mice treated chronically with 80 mg/kg $\Delta^9$-THC expressed a 49-fold level of antinociceptive tolerance. However, chronic exposure to either dose of $\Delta^9$-THC did not produce an increase in basal or total cytosolic and particulate PKA activity in mouse cerebellum, hippocampus, striatum, LSC or cortex.

The observation in the present study that chronic treatment with $\Delta^9$-THC did not increase PKA activity in mouse brain and LSC was supported by the development of a positive control which demonstrated the biochemical assay used in this study was sensitive enough to detect an increase in mouse neuronal PKA activity following the dissection and processing of the anatomical regions utilized in this study for the assay of PKA activity. Sp-8-Br-cAMPS was used to develop this positive control. Sp-8-Br-cAMPS is a cAMP analogue and is a potent, membrane-permeant activator of PKA that is resistant to degradation by cyclic nucleotide phosphodiesterases [21]. The cAMP mimetic effect of Sp-8-Br-cAMPS was evident following its addition in vitro to homogenates of mouse thalamus, striatum, hippocampus, cortex, and LSC as Sp-8-Br-cAMPS induced activation of PKA in each region. More importantly, the i.c.v. administration of Sp-8-Br-cAMPS in drug-naïve mice produced significant increases in basal cytosolic PKA activity in thalamus, cortex and hippocampus, while the i.t. administration of Sp-8-Br-cAMPS in drug-naïve mice produced an increase in LSC basal cytosolic PKA activity.

The anatomical regions (striatum, hippocampus, cortex, cerebellum, LSC) that were studied in this investigation are all regions that possess $CB_1$ receptors. In a prior investigation, Rubino et al. [15] demonstrated that chronic $\Delta^9$-THC administration (15 mg/kg i.p.) for 6.5 days resulted in an increase in PKA activity in rat cerebellum, striatum and cortex. In the current investigation, mice treated chronically with a similar dose of $\Delta^9$-THC (10 mg/kg $\Delta^9$-THC i.p.) for 6.5 days expressed a 13-fold level of $\Delta^9$-THC antinociceptive tolerance. However, no changes in PKA activity were observed in striatum, hippocampus, cortex, cerebellum or LSC of these mice. This finding led us to postulate that a higher level of $\Delta^9$-THC antinociceptive tolerance may be required in mice in order for changes in

PKA activity to occur. As a result, a second study was conducted in which mice treated chronically with a higher dose of $\Delta^9$-THC (80 mg/kg i.p.) for 6.5 days expressed a 49-fold level of $\Delta^9$-THC antinociceptive tolerance. This study revealed that PKA activity was not increased in these regions in mice that expressed a very high level of antinociceptive tolerance.

The lack of an effect of chronic $\Delta^9$-THC treatment on PKA activity in mouse brain and LSC raises some important question regarding the role of PKA in the development and maintenance of $\Delta^9$-THC antinociceptive tolerance in mice. First, based on this study it appears that the level of antinociceptive tolerance is not critical to the expression of alterations in PKA activity in mouse brain and spinal cord. Instead, the duration of $\Delta^9$-THC antinociceptive tolerance may be more critical to the expression of this phenomenon in mice. Support for this idea comes from studies by Bass and Martin [28] which demonstrated $\Delta^9$-THC antinociceptive tolerance was maximal in mice following 6.5 days of dosing with $\Delta^9$-THC (10 mg/kg s.c.). Furthermore, tolerance to the antinociceptive effects of $\Delta^9$-THC did not disappear in these mice until 11.5 days following cessation of drug treatment. This finding raises the possibility that there are multiple phases of tolerance which include tolerance development and tolerance maintenance. Different biochemical events may be involved in each phase of tolerance. The desensitization and downregulation of the $CB_1$ receptor that occurs with chronic $\Delta^9$-THC treatment may contribute to tolerance development. Once tolerance is established and reaches its maximal level other biochemical events may contribute to the maintenance of tolerance, such as increased PKA activity. This would explain why Fan et al. [29] observed a reduction in $CB_1$ receptor density in mouse cerebellum following chronic treatment for 6.5 days with CP-55,940, while no desensitization of adenylate cyclase activity was observed. In contrast, Selley et al. [16] observed desensitization of adenylate cyclase in mouse cerebellum following chronic treatment with $\Delta^9$-THC for 15 days. Thus, it is possible that species differences exist between rats and mice regarding the development of tolerance to $\Delta^9$-THC. More specifically, it is possible that tolerance develops more quickly in rats compared to mice which would explain why Rubino et al. [15] observed a significant reduction in $CB_1$ receptor density in striatum, cerebellum and cortex along with higher PKA activity in these regions after $\Delta^9$-THC administration for 6.5 days. In contrast, a longer duration of $\Delta^9$-THC treatment (>6.5 days) may be required in mice before an increase in PKA activity is observed in mouse brain or LSC.

In conducting this investigation, cytosolic and particulate PKA activity were quantified in an attempt to determine if one particular isoform of PKA was involved in $\Delta^9$-THC antinociceptive tolerance expression. Depending on the regulatory subunit present, PKA is localized in either the cell cytosol or it is tightly associated with cellular membranes [30]. More specifically, PKA composed of RI regulatory subunits is localized in the cell cytosol, while PKA composed of RII regulatory subunits is associated with cellular membranes. At present, a conclusion cannot be drawn regarding the involvement of either cytosolic or particulate PKA in the expression of $\Delta^9$-THC antinociceptive tolerance due to the lack of an effect that chronic treatment with $\Delta^9$-THC had on cytosolic and particulate PKA activity in the regions investigated in this study. This study also determined if chronic treatment with $\Delta^9$-THC produced a change in either basal or total PKA activity in mouse brain and spinal cord. These measures were taken because cAMP regulates the activity of PKA. More specifically, PKA exists as an inactive holoenzyme composed of two regulatory and two catalytic subunits in the absence of cAMP [31]. The binding of two molecules of cAMP to each regulatory subunit causes the regulatory subunits to dissociate from the catalytic subunits which are then free to phosphorylate protein substrates. Basal PKA activity reflects PKA activity measured in the absence of exogenously supplied cAMP to the reaction mixture. This measure is indicative of the basal level of free PKA catalytic subunits in each region. In contrast, total PKA activity refers to PKA activity measured when exogenous cAMP is supplied to the reaction mixture. This measure reflects the total amount of PKA that is available to be activated in each region. Previous studies have demonstrated that chronic treatment with $\Delta^9$-THC produces an increase in total PKA activity in rat brain [15]. In addition, studies have shown that basal PKA activity is increased with chronic morphine treatment [32]. However, this study demonstrated that basal and total PKA activity were not changed in any region in this study following chronic treatment with either dose of $\Delta^9$-THC.

We expected that chronic $\Delta^9$-THC treatment would alter PKA activity in the striatum, hippocampus, cerebellum, LSC and cortex. However, it is possible that analgesic tolerance could be occurring in other mouse brain regions that possess $CB_1$ receptors. such as the periaqueductal gray. Future studies will investigate whether PKA activity is altered in other anatomical regions. It could also be argued that the tail-flick test used in this study reflects adaptations at the spinal level and not at the supraspinal level. As a result, the level of analgesic tolerance in supraspinal regions determined by the tail-flick test may not be accurate. More specifically, the level of analgesic tolerance in the supraspinal regions may be less compared to the level of analgesic tolerance in the LSC which would affect the level of PKA activity in supraspinal regions. However, while research has shown that the tail-flick test predominantly is a spinal reflex, studies have shown that it is subject to control by supraspinal structures such as descending bulbospinal antinociceptive systems [33]. Moreover, no change in PKA activity was observed in LSC even at a very high level of $\Delta^9$-THC antinociceptive tolerance. The absence of an effect of chronic treatment

with $\Delta^9$-THC on PKA activity in LSC was supported by the increase in LSC basal cytosolic PKA activity produced by Sp-8-Br-cAMPS (i.t.) which demonstrated that the assay used in this study was sensitive enough to detect an increase in LSC PKA activity.

In summary, the present study demonstrated that PKA activity is not increased in mouse brain and LSC following chronic treatment with $\Delta^9$-THC for 6.5 days. More importantly, PKA activity was not increased in mice that expressed a very high level of $\Delta^9$-THC antinociceptive tolerance. Future studies will investigate the effects of $\Delta^9$-THC antinociceptive tolerance for longer durations on PKA activity in mouse brain and spinal cord. Ultimately, it is our hope that these studies utilizing in vivo models of $\Delta^9$-THC antinociception will contribute to a better understanding of the phenomenon of $\Delta^9$-THC antinociceptive tolerance and the role that PKA plays in this process.

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