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Development of Behavioral Tolerance to Δ^9-THC Without Alteration of Cannabinoid Receptor Binding or mRNA Levels in Whole Brain

MARY E. ABOOD,[1] CHRISTINE SAUSS, FANG FAN, CORI L. TILTON AND BILLY R. MARTIN

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298

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ABOOD, M. E., C. SAUSS, F. FAN, C. L. TILTON AND B. R. MARTIN. *Development of behavioral tolerance to Δ^9-THC without alteration of cannabinoid receptor binding or mRNA levels in whole brain.* PHARMACOL BIOCHEM BEHAV **46**(3) 575–579, 1993.—The effect of repetitive administration of delta-9-tetrahydrocannabinol (Δ^9-THC) in mice on behavioral and biochemical tolerance was determined in this study. Mice were injected twice daily with 10 mg/kg Δ^9-THC for 6.5 days. On day 8, spontaneous activity was assessed or whole-brain homogenates were prepared for the cannabinoid receptor binding and mRNA studies. Although a twenty-sevenfold tolerance to Δ^9-THC was observed in the behavioral assay, there was no significant alteration in receptor binding or mRNA levels.

Delta-9-tetrahydrocannabinol Δ^9-THC Cannabinoid mRNA Receptor

THE principle active ingredient in marijuana, Δ^9-THC, produces a unique syndrome of behavioral effects in animals. At low doses, it produces a mixture of depressant and stimulatory effects, and at high doses, central nervous system depression predominates. This syndrome has been useful as a model for predicting psychotomimetic activity. Cannabinoids that produce a combination of hypoactivity, hypothermia, antinociception, and catalepsy in mice are likely to be psychoactive (11). These effects are most likely mediated by the recently characterized cannabinoid receptor (6,9).

The development of novel synthetic analogs of Δ^9-THC has played a major role in the characterization and cloning of a cannabinoid receptor. The bicyclic compound, CP-55,940, has been radiolabeled to high specific activities, and used in radioligand binding studies in brain tissue homogenates and tissue slices (6). These studies have shown that the receptor has a high affinity for CP-55,940 and is extremely abundant. In rat brain cortical membranes, reported K_d values for CP-55,940 range from 0.13 to 5 nM and B_{max} values on the order of 0.9–1.9 pmol/mg protein (6,15). The cloning of this receptor (12), coupled with receptor binding, provides an excellent opportunity for further characterization of the mechanism of action of the cannabinoids in the central nervous system.

One of the typical means for assessing receptor function is through either desensitization or sensitization studies. Chronic exposure to Δ^9-THC results in development of tolerance to the behavioral effects of Δ^9-THC. In mice, tolerance has been shown to occur to most Δ^9-THC-induced behaviors (4). However, the effect of tolerance development on the cannabinoid receptor has not been reported. Long-term exposure to Δ^9-THC (90 days) apparently does not irreversibly alter the cannabinoid receptor. This conclusion is based upon the fact that 60 days after cessation of the treatment, the receptor affinity and number were the same in both THC- and vehicle-treated animals (15). The effects on receptor binding properties following shorter exposure times and washout periods have not been documented. In addition, the cloning of the cannabinoid receptor affords an opportunity to examine the effect of chronic Δ^9-THC treatment on expression of this mRNA. The question addressed here was whether chronic exposure to Δ^9-THC alters cannabinoid receptor binding properties and/or mRNA levels under conditions where tolerance develops to

[1] Requests for reprints should be addressed to Mary E. Abood, Department of Pharmacology, Box 524 MCV Station, Richmond, VA 23298-0524.

the behavioral effects. The approach was to examine cannabinoid receptor binding and mRNA levels in whole-brain homogenates prepared from mice that had been treated for 7 days with 10 mg/kg Δ^9-THC.

METHOD

Induction of Tolerance

Male ICR mice (25–30 g) were injected intraperitoneally (IP) (with either Δ^9-THC (10 mg/kg) or vehicle (emulphor : ethanol : saline). On days 1–6 they were injected at 7:00 a.m. and 5:00 p.m. On day 7 they received the 7:00 a.m. injection only. On day 8 vehicle- and THC-treated groups received test drug at 7:00 a.m. for pharmacological testing, whereas the animals used in the biochemical studies did not receive an injection on day 8.

Behavioral Evaluations

Mice were acclimated in the laboratory (ambient temperature 20–24°C) overnight. Mice received tail vein injections (0.1 ml/10 g) 5 min prior to being placed into individual Digiscan Animal Activity Monitors (Omnitech Electronics, Inc., Columbus, OH). Spontaneous activity was measured for a 10-min period as the number of interruptions of 16 photocell beams per chamber. The data were analyzed by ALLFIT (5), which generated best-fit sigmoidal curves, determined parallelism, and calculated ED_{50}s and potency ratios.

Membrane Preparation

The method for tissue preparation was similar to that described by Devane et al. (6). The brains from three mice were immersed in 7.5 ml of ice-cold buffer (320 mM sucrose, 2 mM Tris-EDTA, 5 mM $MgCl_2$) and homogenized with a Kontes Potter-Elvehjem glass-Teflon grinding system (Fisher Scientific, Springfield, NJ). The homogenate was centrifuged at 1600 × *g* for 10 min, washed twice (1600 × *g* centrifugation), and the combined supernatant fractions were centrifuged at 39,000 × *g* for 15 min. The P_2 pellet was resuspended in 12.5 ml of buffer A (50 mM Tris-HCl, 2 mM Tris-EDTA, 5 mM $MgCl_2$, pH 7.0), incubated for 10 min at 37°C, then centrifuged at 23,000 × *g* for 10 min. The P_2 membrane was resuspended in 12.5 ml of buffer A and incubated at 30°C for 40 min before centrifugation at 11,000 × *g* for 15 min. The final wash-treated P_2 pellet was resuspended in assay buffer B (50 mM Tris-HCl, 1 mM Tris-EDTA, 3 mM $MgCl_2$, pH 7.4) to a protein concentration of approximately 3 mg/ml. The membrane preparation was quickly frozen in a bath solution of dry ice and 2-methylbutane (Sigma Chemical Co., St. Louis, MO), then stored at −80°C for no more than 2 weeks. Prior to performing a binding assay, an aliquot of frozen membrane was rapidly thawed and protein values determined by the method of Bradford (1) using Coomassie brilliant blue dye (Bio-Rad, Richmond, CA) and BSA standards (fatty acid free, Sigma Chemical Co.) prepared in assay buffer.

Binding Assay

The procedure was essentially that described by Devane et al. (6) with the exception that bound and free drug were separated by filtration rather than centrifugation. Binding was initiated by the addition of 150 μg of protein to test tubes containing [^{3}H]CP-55,940 (79 Ci/mmol), and a sufficient quantity of buffer C (50 mM Tris-HCl, 1 mM Tris-EDTA, 3 mM $MgCl_2$, 5 mg/ml BSA) to bring the total incubation volume to 1 ml. Nonspecific binding was determined by the addition of 1 μM unlabeled CP-55,940. CP-55,940 was prepared by suspension in buffer C from a 1 mg/ml ethanolic stock without evaporation of the ethanol (final concentration of no more than 0.4%).

Following incubation at 30°C for 1 h, binding was terminated by addition of 2 ml of ice-cold buffer D (50 mM Tris-HCl, 1 mg/ml BSA) and vacuum filtration through pretreated filters in a 12-well sampling manifold (Millipore, Bedford, MA). Reaction vessels were washed once with 2 ml of ice-cold buffer D, and the filters washed twice with 4 ml of ice-cold buffer D. Filters were placed into 20-ml plastic scintillation vials (Packard, Downer Grove, IL) with 1 ml of distilled water and 10 ml of Budget-Solve (RPI Corp., Mount Prospect, IL). After shaking for 1 h, the radioactivity present was determined by liquid scintillation spectrometry. All assays were performed in siliconized test tubes. The GF/C glass fiber filters (2.4 cm, Baxter, McGaw Park, IL) were pretreated in a 0.1% solution of pH 7.4 PEI (Sigma Chemical Co.) for at least 6 h. Saturation experiments were conducted with eight concentrations of [^{3}H]CP-55,940 in the range of 250 pM to 10 nM. The B_{max} and K_d values were obtained from Scatchard analysis as determined via the KELL package of binding analysis programs for the Macintosh computer (Biosoft, Milltown, NJ).

RNA Preparation

RNA was prepared from homogenates of whole mice brains via a modification of the acid phenol extraction technique (2). The tissue was homogenized in 4 M guanidinium thiocyanate (GuSCN), 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 50 mM β-mercaptoethanol. The RNA was ex-

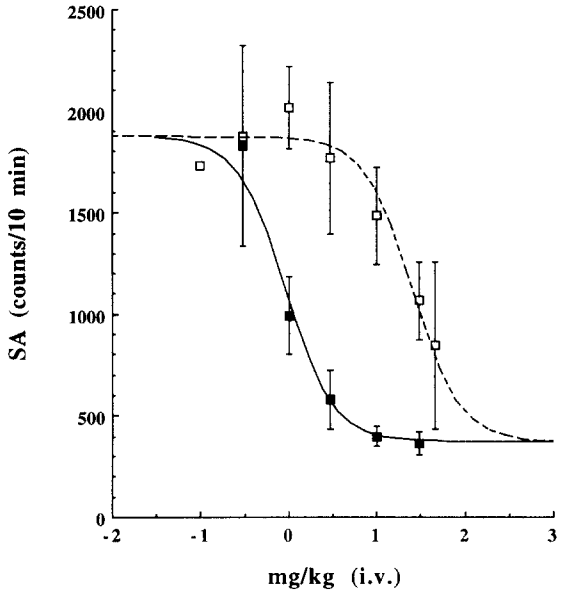

FIG. 1. Effects of Δ^9-THC on spontaneous activity in mice treated repetitively with either vehicle (solid squares) or Δ^9-THC (open squares). Interruptions of the photocell beams (counts/10 min) are plotted vs. the log of the dose in mg/kg (IV). Each point represents the mean ± SEM of three to five experiments, with the exception of one point, which was derived from a single experiment.

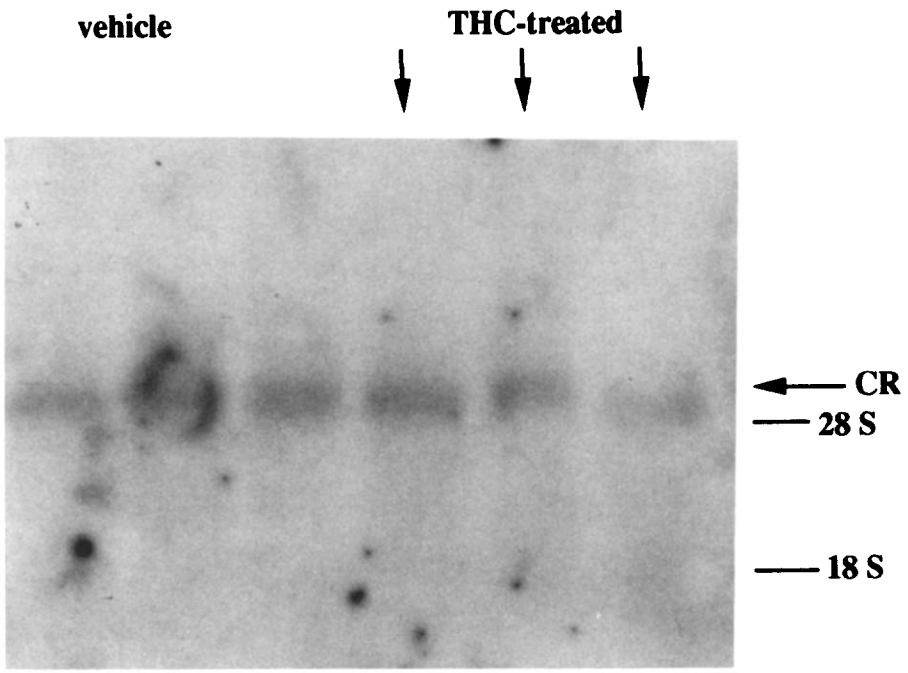

FIG. 2. Northern blot of whole-brain RNA from vehicle- and Δ^9-THC-treated mice (arrows). The cannabinoid receptor message is indicated (CR), along with the positions of 28S and 18S rRNA.

tracted with an equal volume of H_2O-saturated phenol, chloroform with sodium acetate, pH 4.5 (0.25 M final). The RNA was precipitated with an equal volume of isopropanol, the pellet washed with 75% ethanol, and dried. The pellet was resuspended in 1 mM EDTA, pH 7.0.

RNA Gel Electrophoresis and Blotting

Equivalent amounts of RNA (30 μg) were denatured by heating at 70°C for 5 min in loading buffer and loaded onto 1% formaldehyde-agarose horizontal gels. Loading buffer consisted of 50% formamide, 6% formaldehyde, 20 mM boric acid, 10% glycerol, 0.2 mM EDTA, 0.25% bromphenol blue, and 0.25% xylene cyanol. After denaturation, 1 μl of 1 mg/ml ethidium bromide was added to aid visualization of the RNA samples. The gels, which consist of 1% agarose in 20 mM boric acid (pH 8.3), 0.2 mM EDTA, and 3% formaldehyde, were run in a buffer of the same composition. Following electrophoresis, the gels were soaked for 20 min in 0.05 N NaOH, then rinsed in RNase-free water, soaked in 20 × SSC for 45 min, and transferred in 20 × SSC. The gels were transferred to reinforced nitrocellulose by capillary transfer (13) followed by UV irradiation to fix the RNA onto the filter.

Slot Blot Analysis

Total RNA was denatured by heating 2 μg for 5 min at 90°C, and dilutions thereof were dotted onto nylon membranes (Genescreen, NEN DuPont) using a Schleicher and Schuell Minifold II. tRNA was added to more dilute samples to keep the same amount of RNA per slot. The RNA was fixed by UV irradiation.

Hybridization

The hybridization buffer consisted of: 50% formamide; 6 × SSC [20 × SSC is 3 M NaCl, 0.3 M sodium citrate (pH 7.0), 0.1 mg/ml salmon sperm DNA, 50 mM Tris (pH 8.0), and 5 × Denhardt's (50 × Denhardt's is 1 g ficoll, 1 g polyvinylpyrrolidone, 1 g BSA fraction V in 100 ml H_2O)]. Filters were hybridized at 42°C using 10^7 dpm of radiolabeled probe (specific activity > 5 × 10^8 dpm/μg). The filters were washed to a stringency of 0.5 × SSC, 0.1% SDS, 30°C.

Probe Preparation

The probe used in these experiments was a rat cannabinoid receptor cDNA that one of us (Abood) isolated from a λZAP rat brain cDNA library using a probe based on sequences unique to the published cannabinoid receptor (12). Oligonucleotide probes based on bp 1–21 and bp 1410–1422 on the opposite strand were chosen for use in the polymerase chain reaction to generate a 1389-bp probe specific for the cannabinoid receptor. Sequence analysis of the isolate used in these experiments indicated identity with the published sequence. A 1.6-kb insert containing the cannabinoid receptor sequence was radiolabeled by random priming with T7 DNA polymerase for use in the RNA blot analysis experiments.

Quantitation of Blots

The washed blots were exposed to film for 24 h or longer. The resulting autoradiograms were were illuminated with a Northern Light box and digitized with a solid-state video camera coupled to an imaging system designed for quantitative densitometry (MCID System, Imaging Research Inc., Toronto, Canada). To control for differences in loading, a probe prepared from 28S rRNA was used to reprobe the blots, and the quantitated autoradiograms were used for normalization. The results are expressed as relative optical density units.

RESULTS

The objective of the drug treatment regimen was to develop a procedure that would produce a measurable degree of toler-

ance. It is desirable to have a period of time before testing when the animals do not receive drug to minimize the possible confounds of residual drug. To meet these objectives, mice were injected twice daily, once in the morning and once in the evening, for 6 days. On the seventh day of the experiment, the animals received only the morning injection, and on the morning of day 8 they were assessed for development of tolerance. For each dose-response curve, there were six mice per dose. The experiments were repeated a total of five times. The animals showed no weight loss with this treatment paradigm. The potencies of Δ^9-THC in decreasing spontaneous activity in vehicle- and Δ^9-THC-treated animals are presented in Fig. 1. The ED_{50} of Δ^9-THC in the vehicle-treated mice was 0.9 mg/kg, whereas it increased to 27.6 mg/kg in the Δ^9-THC-treated mice. The degree of tolerance was determined to be twenty-sevenfold. It is noteworthy that a maximal effect was obtained for Δ^9-THC in the Δ^9-THC-treated group. Constraining the minimum and maximum effects in both treatment groups during the analysis did not produce a statistically significant difference in the curves.

The identical injection paradigm was used to induce tolerance in the animals that were used for the biochemical studies. For the RNA analysis, three separate groups of six animals each were used to obtain whole-brain RNA. Expression of cannabinoid receptor mRNA was assessed by Northern and slot blot analysis. A representative Northern is shown in Fig. 2. Quantitation of the autoradiograms showed no alterations in cannabinoid receptor mRNA in the chronic Δ^9-THC-treated vs. the chronic vehicle-treated brains (Fig. 3).

Cannabinoid receptor binding properties were also examined following chronic exposure to Δ^9-THC. P_2 membranes were prepared from whole brain, and the cannabinoid analog [^{3}H]CP-55,940 was used as a radioligand in Scatchard analysis. As shown in Table 1, neither the binding capacity nor affinity for [^{3}H]CP-55,940 was altered in the chronic Δ^9-THC-treated mice. The B_{max} for the vehicle groups was 3.4 ± 0.3 pmol/mg protein and the K_d was 3.4 ± 0.7 nM; the B_{max} for the THC groups was 3.0 ± 0.6 pmol/mg and the K_d 4.6 ± 1.6 nM (N = 5).

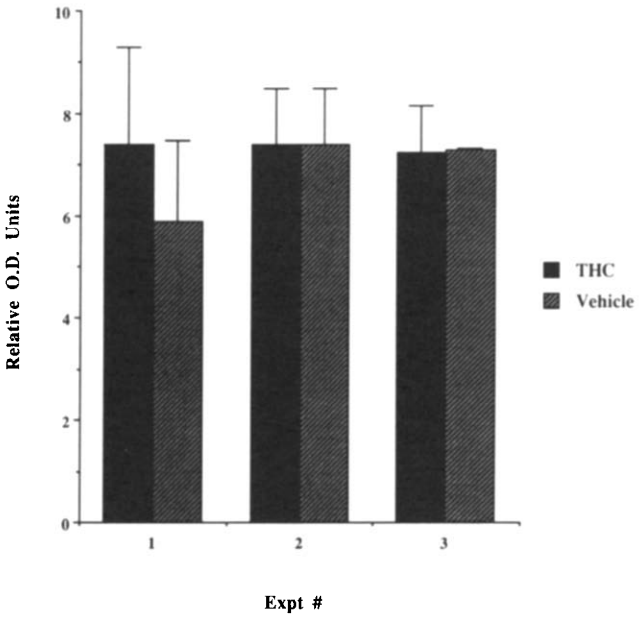

FIG. 3. Slot blot analysis of cannabinoid receptor mRNA levels in Δ^9-THC-treated (solid bars) and vehicle-treated (hatched bars) mice. The data shown are the results of three separate experiments of six mice each. The data were normalized to control for differences in loading using a probe to 28S rRNA. The error bars represent SEM.

TABLE 1

SCATCHARD ANALYSIS OF [^{3}H]CP-55,490 BINDING TO MICE WHOLE-BRAIN MEMBRANES

Treatment	K_d (nM)	B_{max} (pmol/mg)
Δ^9-THC	4.6 ± 1.6	3.0 ± 0.6
Vehicle	3.4 ± 0.7	3.4 ± 0.3

The data are presented as the mean ± SEM (N = 5) for each treatment group. There were no significant differences in either the K_d or B_{max} values between the Δ^9-THC- and vehicle-treated animals.

DISCUSSION

We found no alterations in cannabinoid receptor mRNA or protein levels in mouse whole-brain homogenates following a chronic injection paradigm sufficient to induce twenty-sevenfold tolerance in a behavioral assay. We can conclude that there is no global change in cannabinoid receptor mRNA and receptor binding as measured in whole-brain homogenates. However, the possibility remains that in distinct brain regions receptor mRNA and protein levels are altered, and by measuring whole-brain homogenates these changes would not be apparent. Quantitative in vitro autoradiographic studies have revealed a heterogeneous distribution of brain cannabinoid receptors (9). For example, within the cerebellum, receptor number varies from 0.23 pmol/mg protein in the deep nuclei to 4.2 pmol/mg in the molecular level (8). If repetitive adminstration of Δ^9-THC induced changes in specific areas where cannabinoid receptors are relatively sparse and not in other, receptor-rich areas, these changes would not be observed in whole-brain homogenates.

The protocol used here, a 1-week exposure to 10 mg/ml Δ^9-THC, produces rather robust tolerance to the effects of Δ^9-THC on spontaneous activity. While it would seem that this degree of tolerance should be sufficient to induce biochemical changes in receptor mRNA and protein levels, one cannot rule out the possibility that a more extensive development of tolerance is required for detection of biochemical changes. On the other hand, these data are supported by the previous observation that long-term exposure to Δ^9-THC does not produce irreversible changes in receptor binding properties (15). Nonetheless, behavioral tolerance was observed and biochemical tolerance (as measured by changes in receptor levels) was not.

Another possible explanation for these results is that biochemical tolerance occurs at another level in the cannabinoid receptor transduction system; for instance, the receptors could be uncoupled from their second messenger systems. This has been observed with Δ^9-THC in the N18TG2 neuroblastoma cell line, where exposure to Δ^9-THC results in a loss of cannabinoid-mediated inhibition of adenylate cyclase (7). This type of desensitization could lead to a decrease in the effects of Δ^9-THC, without an alteration in the absolute number of receptors. The cannabinoid receptor is a member of the G-protein-coupled receptor family. In other G-protein-coupled

receptor systems, this desensitization can be manifest as a decrease in the number of high-affinity binding sites (3,14). It is thought to represent alterations in G-protein coupling following prolonged agonist treatment. The high-affinity agonist binding state is the complex between agonist-bound receptor G-protein without guanine nucleotide bound to the G-protein. Under normal circumstances, agonist-bound receptor catalyzes the exchange of GTP for GDP on the G-protein. Following this, the G-protein dissociates into its active subunits and also dissociates from the receptor. The receptor is now in a lower-affinity state. The different affinity states of the receptor can be observed using radiolabeled antagonist and competing with agonist in the presence and absence of guanine nucleotides. As there are no available antagonists for the cannabinoid receptor, this possibility remains to be addressed. It is also possible that high-affinity binding can be retained following prolonged agonist treatment and that the defect resides in the ability of the receptor to stimulate GDP/GTP exchange on the G-protein (10).

In summary, in this preliminary study, we have observed behavioral tolerance to Δ^9-THC without alteration of cannabinoid receptor binding or mRNA levels in whole brain. Future studies will address whether there are biochemical changes in the cannabinoid receptor in specific brain regions following longer exposure to Δ^9-THC.

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REFERENCES

1. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254; 1976.
2. Chomczynski, P.; Sacchi, N. Single step method of RNA isolation by acid guanidium thiocyanate phenol chloroform extraction. Anal. Biochem. 162:156–161; 1987.
3. Collin, E.; Cesselin, F. Neurobiological mechanisms of opioid tolerance and dependence. Clin. Neuropharmacol. 14:465–488; 1991.
4. Compton, D. R.; Dewey, W. L.; Martin, B. R. Cannabis dependence and tolerance production. In: Erickson, C. K.; Javors, M. A.; Morgan, W. W., ed. Addiction potential of abused drugs and drug classes. Binghamton, NY: The Hayworth Press, Inc.: 1990: 129–147.
5. DeLean, A.; Munson, P.; Rodbard, D. Simultaneous analysis of families of sigmoidal curves: Application to bioassay, radioligand assay, and physiological dose–response curves. Am. J. Physiol. 235:E97–E102; 1977.
6. Devane, W. A.; Dysarz, I. F. A.; Johnson, M. R.; Melvin, L. S.; Howlett, A. C. Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. 34:605–613; 1988.
7. Dill, J. A.; Howlett, A. C. Regulation of adenylate cyclase by chronic exposure to cannabimimetic drugs. J. Pharmacol. Exp. Ther. 244:1157–1163; 1988.
8. Herkenham, M.; Lynn, A. B.; Johnson, M. R.; Melvin, L. S.; de Costa, B. R.; Rice, K. C. Characterization and localization of cannabinoid receptors in rat brain: A quantative in vitro autographic study. J. Neurosci. 11:563–583; 1991.
9. Herkenham, M.; Lynn, A. B.; Little, M. D.; Johnson, M. R.; Melvin, L. S.; DeCosta, B. R.; Rice, K. C. Cannabinoid receptor localization in the brain. Proc. Natl. Acad. Sci. USA 87:1932–1936; 1990.
10. Law, P. Y.; Hom, D. S.; Loh, H. H. Effect of chronic d-ala2,d-leu5-enkephalin or pertussis toxin treatment on the high-affinity state of delta opioid receptor in neuroblastoma × glioma NG108-15 hybrid cells. J. Pharmacol. Exp. Ther. 256:710–716; 1991.
11. Martin, B. R.; Compton, D. R.; Thomas, B. F.; Prescott, W. R. J.; Little, P. J.; Razdan, R. K.; Johnson, M. R.; Melvin, L. S.; Mechoulam, R.; Ward, S. J. Behavioral evaluation of cannabinoid analogs for agonistic and antagonistic properties. Pharmacol. Biochem. Behav. 40:471–478; 1991.
12. Matsuda, L. A.; Lolait, S. J.; Brownstein, M. J.; Young, A. C.; Bonner, T. I. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346:561–564; 1990.
13. Sambrook, J.; Fritsch, E. F.; Maniatis, T., Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor: Cold Spring Harbor Press; 1989.
14. Werling, L. L.; McMahon, P. N.; Cox, B. M. Selective changes in μ opioid receptor properties induced by chronic morphine exposure. Proc. Natl. Acad. Sci. USA 86:6393–6397; 1989.
15. Westlake, T. M.; Howlett, A. C.; Ali, S. F.; Paule, M. G.; Scallet, A. C.; Slikker, W., Jr. Chronic exposure to Δ^9-tetrahydrocannabinol fails to irreversibly alter brain cannabinoid receptors. Brain Res. 544:145–149; 1991.