Large-scale analysis of gene expression changes during acute and chronic exposure to $\Delta^9$-THC in rats

JOSEF T. KITTLER,¹ ELENA V. GRIGORENKO,⁴ CHRIS CLAYTON,² SHOU-YUAN ZHUANG,⁴ SARAH C. BUNDEY,³ MICHAEL M. TROWER,² DON WALLACE,² ROBERT HAMPSON,⁴ AND SAM DEADWYLER⁴

¹University of Bath, BA2 7GY Bath, United Kingdom; ²Glaxo Wellcome, SG1 2NY Stevenage; ³Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157

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Kittler, Josef T., Elena V. Grigorenko, Chris Clayton, Shou-Yuan Zhuang, Sarah C. Bundey, Michael M. Trower, Don Wallace, Robert Hampson, and Sam Deadwyler. Large-scale analysis of gene expression changes during acute and chronic exposure to $\Delta^9$-THC in rats. Physiol Genomics 3: 175–185, 2000.—Large-scale cDNA microarrays were employed to assess transient changes in gene expression levels following acute and chronic exposure to cannabinoids in rats. A total of 24,456 cDNA clones were randomly selected from a rat brain cDNA library, amplified by PCR, and arrayed at high density to investigate differential gene expression following acute (24 h), intermediate (7 days), and chronic (21 days) exposure to $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), the psychoactive ingredient of marijuana. Hippocampal mRNA probes labeled with $^{33}$P obtained from both vehicle and $\Delta^9$-THC-treated animals were hybridized with identical cDNA microarrays. Results revealed a total of 49 different genes altered by $\Delta^9$-THC exposure; of these, 28 were identified, 10 had homologies to expressed sequence tags (ESTs), and 11 had no homology to known sequences in the GenBank database. Chronic or acute cannabinoid receptor activation altered expression of several genes (i.e., prostaglandin D synthase, calmodulin) involved in biochemical cascades of cannabinoid synthesis or cannabinoid effector systems. Other genes (i.e., neural cell adhesion molecule (NCAM), myelin basic protein), whose relation to cannabinoid exposure (10). These findings suggest that other molecular changes in hippocampus may be affected in a similar manner during chronic exposure to cannabinoid drugs. However, to date there has been no systematic study of the molecular changes underlying both acute and chronic exposure to $\Delta^9$-THC. Recently developed large-scale cDNA microarray technology allows comparison of expression profiles of thousands of genes simultaneously probed with labeled cDNA derived from mRNA pools of differential gene analysis; hippocampus; $\Delta^9$-tetrahydrocannabinol

ILlicit USE OF MARIJUANA (Cannabis sativa) is a major drug problem in both the adolescent and adult popula-

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Address for reprint requests and other correspondence: S. A. Deadwyler, Dept. of Physiology and Pharmacology, Wake Forest Univ. School of Medicine, Winston-Salem, NC 27157 (E-mail: sdeadwyl @wfubmc.edu).
“control” vs. “differentially treated” groups of animals or cells (15, 21, 25, 37, 56). We employed such large-scale cDNA microarray profiling to assess effects of chronic cannabinoid exposure on a total of 24,456 gene clones in rat hippocampus. From this pool, a subset of differentially expressed genes were identified by directly comparing the hybridization patterns of RNA-derived probes from Δ9-THC- and vehicle-injected animals. Results revealed alterations in gene expression patterns in 28 different genes occurring at different stages of chronic Δ9-THC exposure.

METHODS

Animals and drug treatment. Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing 240–300 g at the time of Δ9-THC treatment were housed two per cage and maintained on a 12-h light/dark cycle with ad libitum food and water provided. All animals were given 1 wk home cage adaptation before initiation of Δ9-THC or vehicle injections. Rats were injected daily with a single dose of 9-THC (10 mg·kg−1·day−1 ip) or vehicle (Pluronic F-68; Sigma; St. Louis, MO) and killed at 24 h, 7 days, or 21 days, 2 days after receiving the last injection. Δ9-THC was provided by National Institute on Drug Abuse. Δ9-THC was dissolved in 100% ethanol at a concentration of 50 mg/ml, then 150 mg of Δ9-THC was mixed with 240 mg Pluronic F-68 and dissolved in 15 ml pure ethanol and 6 ml saline to form a suspension. The ethanol was later evaporated under a stream of nitrogen gas, and the Δ9-THC/Pluronic mixture was suspended in saline (10 mg/ml) for intraperitoneal injection. Vehicle-injected animals received the same Pluronic/saline solution without Δ9-THC. Rats were killed by rapid decapitation, and the hippocampus was dissected and immediately frozen in dry ice and stored at −80°C until preparation of RNA.

RNA extraction and preparation. Total RNA was isolated from rat hippocampus by the single-step procedure of Chomczynski and Sacchi (18) using Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer’s instructions. mRNA was purified from total RNA using the Oligotex mRNA kit (Qiagen, Valencia, CA), then the concentration of mRNA was determined and the integrity of mRNA samples was assessed on denaturing gels containing 1% agarose, 2.7 M formaldehyde, and 0.1 M MOPS buffer, pH 7.4.

cDNA library and array construction. Poly(A) RNAs were isolated from the brain of male Sprague Dawley rats using Oligotex mRNA kits (Qiagen). This material was used as a template for cDNA library construction using an oligo-dT primed first-strand cDNA synthesis kit (Life Technologies, Gaithersburg, MD). The cDNAs were directionally ligated into the pSport 1 plasmid and electroporated into E. coli (Stratagene, La Jolla, CA) and vector-specific primers in 50 μl containing 0.2 mM dNTPs and 1× Promega PCR buffer. The PCR conditions were as follows: 95°C for 1 min; then 94°C for 30 s, 50°C for 1 min, and 72°C for 2 min, for 30 cycles; and 72°C for 5 min as a final elongation step. Agarose gel electrophoresis revealed the average cDNA insert size to be 1.5 kb with 80% success rate for PCR amplification. The PCR products were grided in duplicate on positively charged 10 × 12 cm nylon filters (Boehringer Mannheim, Indianapolis, IN) using an automated 384-pin gridding device (Genetix Q-bot). Each 10 × 12 cm cDNA array contained 12,228 different clones in duplicate; hence, each probe was assessed on two arrays comprising a total of 24,456 different cDNA clones (Fig. 1). The filters were then denatured (0.5 M NaOH, 100 mM NaCl) for 2 min, neutralized (1 M Tris, pH 7.2; 100 mM NaCl) for 2 min, and ultraviolet (UV) cross-linked in Stratalinker (Stratagene).

To determine the variability of PCR and robotic arraying of PCR products, a series of hybridizations was performed on the arrays using a T7 promoter oligonucleotide probe internal to the PCR products. There was <5% variation between arrays in the amount of template DNA spotted at a particular coordinate (Fig. 2A) in the arrays used to compare the hybridization profiles of labeled probes from either Δ9-THC- or vehicle-injected animals (Fig. 2B). Whereas the T7 promoter oligonucleotide probe labeled all 45,912 spots on the two arrays, clones derived from hippocampal tissue hybridized to an average of only 11,225 of these spots, including duplicates or roughly 23% of the total number of spots in the grid. The fact that hippocampal probes hybridized to only this many locations on the array was expected, since the RNA used to produce the cDNA probes most likely did not contain all the genes represented in the whole rat brain cDNA library. This was verified by the fact that cDNA probes derived from cerebellar RNA in the same animals produced a different hybridization pattern and a different percentage of hybridized loci from the same rat whole brain library (Grigorenko et al., unpublished observations).

Complex probe preparation and hybridization. Radiolabeled probes were prepared using 4 μl of [32P]dCTP (1–3,000 Ci/ml; Amersham, Piscataway, NJ) and 1 μg of poly(A) RNA or 20 μg of total RNA by anchor oligo-dT primed first-strand synthesis using Superscript II reverse transcriptase (Life Technologies). Probes were purified using Sephadex G-50 spin columns according to the manufacturer’s instructions (Pharmacia Biotech, Piscataway, NJ). Quality and length of complex probes were checked by polyacrylamide gel electrophoresis on a 6% urea-Tris borate-EDTA gel (0.045 M Tris borate, 0.001 M EDTA, pH 8.0) buffered polyacrylamide gel. cDNA arrays were prehybridized in 15 ml DIG EasyHyb (Boehringer Mannheim) at 45°C for 30 min in a rotisserie oven. Ten micrograms of human COT1 DNA (Life Technologies) and 435 μl EasyHyb were added to each probe prior to denaturation at 100°C for 5 min. The probe solution was quenched at 45°C for 90 min and hybridized to gridded cDNA arrays for 72 h at 45°C in a total volume of 10 ml EasyHyb. After hybridization, three washes of 20 min duration at high stringency in SSC consisting of 3 M NaCl, 0.5 M sodium citrate, pH 7.2, and 0.1% SDS, diluted to 1:200 in H2O, were performed at 68°C. Filters were exposed to a phosphor storage screen for 72 h before image capture on a phosphorimaging device (Storm scanner; Molecular Dynamics, Sunnyvale, CA).

Image analysis. Image analysis of cDNA array spot hybridizations were performed using a proprietary, PC (computer)-based, differential gene expression analysis program (DGENT) developed by Glaxo Wellcome (Stevenage, UK). Intensity values of identically located cDNA amplicon spots were compared and normalized statistically using median spot intensity of each filter for any differences in cDNA probe activity between filters. The selection criteria for differentially expressed clones were 1) a minimum 1.5-fold difference in intensity for a particular cDNA spot comparison and 2)
cDNA TECHNOLOGY:

STUDY OF DIFFERENTIAL GENE EXPRESSION IN THC-TOLERANT RATS

1. Grid preparation

- cDNA clones from rat brain cDNA library were selected and arrayed into 384-well plates.
- PCR of each robotically picked out clone.
- Arrayed PCR products derived from cDNA clones.
- Robotic gridding of PCR products onto nylon membranes to produce a library grid.
- 12,428 PCR products in duplicates.

2. Construction of complex cDNA probe and hybridization

VEHICLE

- mRNA
- Generate complex probes
- Radiolabel first strand of cDNA from different brain regions
- [32P]-labeled cDNA

THC

- mRNA
- Each probe is hybridized to identical grids of the same rat brain cDNA library
- [32P]-labeled cDNA

Hybridized grids are analyzed for differences in intensity and exposed to phosphorimaging screens.

Fig. 1. Schematic of cDNA microarray technology for analysis of gene expression in rat hippocampus following Δ⁹-tetrahydrocannabinol (Δ⁹-THC) exposure. The diagram shows the method of arraying and assessing gene expression differences in vehicle-injected and Δ⁹-THC-treated animals.

consistency of intensity between duplicate cDNA clones in the same array (49). Only candidate clones that met these two criteria were assessed by further sequencing and tests using RNA dot-blot analyses.

Sequencing. Bacterial colonies containing the cDNAs of interest were picked from the 384-well plate arrayed master cDNA library and grown in 384-well plates in LBA (ampicillin, 100 µg/ml) overnight. The cDNA inserts were amplified by PCR and products visualized by agarose gel electrophoresis. These inserts were sequenced using T7 and SP6 fluorescently labeled primers on an ABI model 377 DNA sequencer. The resulting sequences were compared with the GenBank database using the Blast program.

RNA dot blots. Dot blots were produced with 1 µg mRNA/dot using the Minifold system II (Scheicher and Schuell, Keene, NH), cross-linked with UV light in a Stratalinker (Stratagene, Palo Alto, CA). Prior to the candidate clone hybridization, RNA dot blots were quantitated for the amount of poly(A) RNA using [32P]-end-labeled oligo-d(T)₁₆. Differences in mRNA loading were taken into account in the calculation of candidate clone hybridization intensity. Probes from cDNA clones were prepared by random priming PCR product from cDNA inserts of interest using a Decaprime II kit (Ambion, Austin, TX) in the presence of [32P]dCTP. Hybridization was carried out according to Sambrook et al. (51). Dot blots were exposed to phosphor imaging screens, and images were captured with the imager’s Tina 2.0 software (Fuji Systems). The gene expression level was normalized to the level of mRNA used in the dot blots. Changes in relative levels of gene expression were determined as a ratio to their respective vehicle controls as follows [where V is vehicle treated, ODT is oligo-d(T)₁₆, and THC is Δ⁹-THC]: for up-regulated gene, (THC/ODT)/(V/ODT); for downregulated gene, (V/ODT)/(THC/ODT). This calculation of gene expression controlled for differences in loaded mRNA (49).

In situ hybridization. Brains were dissected rapidly from a new series of control and treated adult rats (n = 3 per group) and snap-frozen in isopentane precooled at −65°C in dry ice. Tissue blocks are stored at −80°C for up to 4 wk. Cryosections of 20 µm were cut at −20°C, collected on Superfrost Plus slides (Fisher), and stored desiccated at −80°C.

Fixation and all pretreatment steps were carried out in baked Coplin jars. Sections were fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min at room temperature.
then immersed in freshly prepared 0.25% acetic anhydride in 0.1 M tetraethanolamine/0.9% NaCl for 10 min, rinsed in SSC diluted to 1:10, and then dehydrated through a series of graded ethanol solutions (50, 70, 95, and 100%). Each section was overlaid with 100 µl of hybridization buffer, which contained 50% formamide, 0.6 M NaCl, 1 mM EDTA, 1× Denhardt’s solution, 10% dextran sulfate, 100 µg/ml salmon sperm DNA, 250 µg/ml yeast tRNA, 10 mM Tris-HCl (pH 7.4), 1.2 mg/ml heparin, 0.1 M dithiothreitol, and 750,000 cpm of 35S-labeled oligonucleotide probe. The oligonucleotide probe was radiolabeled at the 3'-end with α-35S-dATP using terminal deoxyribonucleotidyl transferase. The sections were incubated at 37°C overnight in a moist chamber, washed three times at room temperature, and then dehydrated in a series of ethanol washes. Slides were washed two times in SSC diluted to 1:10 at 45°C for 20 min, then two times in SSC diluted to 1:40 at room temperature for 20 min. The specificity of the signal was assessed in the preliminary experiments where the sections were incubated with a mix of radiolabeled/unlabeled probe in a ratio 1:50. Slide-mounted tissue sections were exposed directly to X-ray film (Hyperfilm-3H, Amersham). Both the 14C standards and experimental slides were exposed to the same film. The slides were exposed together with 14C standards to calculate the density values from slides with brain paste standards in correspondence with values of 14C standards. The quantification of optical density on X-ray films was analyzed using Power Macintosh-assisted NIH imaging analysis software (version 1.65).

RESULTS

Detection of differentially expressed clones. cDNA arrays were used to identify the degree of altered gene expression in hippocampus following acute and chronic exposure to Δ9-THC. Hybridization patterns of hippocampal mRNA from Δ9-THC exposed animals were compared with the patterns derived from vehicle-injected control animals (see Fig. 1). The similarity of the overall hybridization patterns for probes from vehicle- and Δ9-THC-injected animals is quite apparent in Fig. 2, since the majority of the 5,605 genes expressed in hippocampus (25% of 24,456) were unchanged by acute or chronic Δ9-THC exposure (Fig. 2B).

For each duration of Δ9-THC exposure (24 h, 7 days, and 21 days), two different arrays containing 12,228 clones in duplicate were constructed and compared for each animal. The resolution of the technique only allowed genes with a ≥1.5-fold difference in labeled 33P spot intensity to be considered significantly altered (see Table 1) and automatically excluded genes which did not show this degree of change in expression level (58).

Figure 3 shows two examples of differentially expressed genes identified on separate cDNA microarrays. The spot that corresponded to the cDNA clone for prostaglandin D synthase was more intense in the array from the acute, single injected Δ9-THC (24 h) animal than the animal injected with vehicle (Fig. 3, top). In the same animal, expression of myelin basic protein was downregulated as indicated by the decrease in spot intensity relative to the vehicle-injected animal.

Comparative analyses of the hybridization patterns following different stages of Δ9-THC exposure revealed a total of 180 detections of differential spot intensity across all durations of Δ9-THC exposure (see METHODS). Sequence analyses revealed 159 clones representing known genes, 10 clones with high homology to sequences in the expressed sequence tag database (EST) and 11 without homology to any sequences in GenBank. Since a nonnormalized rat whole brain cDNA library was employed, it was possible to have multiple detections of the same clones, indicated by the numbers in parentheses in Table 1. Taking into account this redundancy, we found a net total 28 genes in hippocampus exhibited altered expression at various time points following acute or chronic exposure to Δ9-THC. Table 1 shows the GenBank accession numbers of the 28 genes (and one EST) together with the closest homology match using an expected value (E) cutoff of ≤ 1.0 e-20 (36).

http://physiolgenomics.physiology.org
Identified genes whose expression was altered by Δ⁹-THC exposure were grouped by the following classification scheme: metabolism, cell adhesion/cytoskeletal proteins, myelination/glial differentiation, signal transduction proteins and proteins involved in folding and/or proteolytic processes. Clones that could not be placed in the above scheme were designated as “other” in Table 1.

### Table 1. List of genes differentially expressed in rat hippocampus following 24 h, 7 days, and 21 days of Δ⁹-THC exposure

<table>
<thead>
<tr>
<th>Clone/Cellular Function</th>
<th>GenBank Accession Number</th>
<th>24 h</th>
<th>7 days</th>
<th>21 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fructose-bisphosphate aldolase</td>
<td>M12919</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>M17701</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>S79304</td>
<td>↑(15)</td>
<td>↑(7)</td>
<td>↑(43)</td>
</tr>
<tr>
<td>α-Enolase</td>
<td>X53279</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipid glutathione peroxidase</td>
<td>X82679</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell adhesion/structural</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCAM</td>
<td>X06564</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC1 protein</td>
<td>U27562</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Myelination/glial differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelin-associated protein</td>
<td>X96368</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myelin proteolipid protein</td>
<td>M11185</td>
<td>↑</td>
<td>↑(2)</td>
<td></td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>M15060</td>
<td>↓</td>
<td>↓(2)</td>
<td>↓(6)</td>
</tr>
<tr>
<td>Brain lipid binding protein</td>
<td>U02096</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptors/transporters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin AT₁ receptor</td>
<td>S66402</td>
<td>↓</td>
<td>↑</td>
<td>↑(12)</td>
</tr>
<tr>
<td>Signal transduction/receptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proteosomal ATPase</td>
<td>D83521</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calmodulin</td>
<td>E02315</td>
<td>↓(2)</td>
<td>↑(2)</td>
<td></td>
</tr>
<tr>
<td>Calreticulin</td>
<td>D78308</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostaglandin D synthase</td>
<td>J04488</td>
<td>↑(11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-3-3 Protein γ-subunit</td>
<td>D17447</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GDP dissociation inhibitor</td>
<td>L07925</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKU β-subunit</td>
<td>AB004885</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein folding</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HSP70</td>
<td>X70065</td>
<td>↓(2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme</td>
<td>AF031141</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyubiquitin</td>
<td>D17296</td>
<td>↓(2)</td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>Chaperonin containing TCP-1</td>
<td>Z31553</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12S and 16S rRNA</td>
<td>J01438</td>
<td>↓</td>
<td>↓(11)</td>
<td>↓(7)</td>
</tr>
<tr>
<td>Acidic 82-kDa protein</td>
<td>U15552</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>D38380</td>
<td>↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elongation factor</td>
<td>X63961</td>
<td>↑(5)</td>
<td>↑(2)</td>
<td>↑(2)</td>
</tr>
<tr>
<td>T cell receptor β-locus</td>
<td>AE000663</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>EST</td>
<td>AA048564</td>
<td>↑</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Expression level is measured as ratio of Δ⁹-tetrahydrocannabinol (Δ⁹-THC)-treated vs. control samples for upregulated (↑) genes and control vs. Δ⁹-THC treated samples for downregulated (↓) genes. Two array measurements were made per animal for each clone, for two different animals at each time point. Multiple detections of the same gene are in parentheses. Matches of clone sequences from cDNA arrays were based on the calculated expect value ($E$), where $E \leq 1.0 \times 10^{-20}$ was considered as the criteria for sequence homology in the identified clones. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NCAM, neural cell adhesion molecule; HSP70, heat-shock protein-70; and PKU, protein kinase U.

Fig. 3. Enlargement of the microarray array images at two different locations on the membrane and hybridized with complex cDNA probe from a vehicle-injected (Vehicle) and a Δ⁹-THC-injected (THC) (24 h) animal. The circled spots represent prostaglandin D synthase (top, 1) and myelin basic protein (bottom, 2) cDNAs. Images do not reflect normalization for DNA loading by T7 promoter oligonucleotide (Fig. 2A), which is performed in the analysis by DGENT.
A single high (10 mg/kg) acute dose of Δ⁹-THC initiated changes in expression within 24 h in 15 of the 28 altered genes which were dispersed across the different classifications shown in Table 1. Six genes had transiently altered expression levels after the single dose of Δ⁹-THC, four were upregulated [neural cell adhesion molecule (NCAM), prostaglandin D synthase, 14-3-3 protein, and ubiquitin-conjugating enzyme], and two were downregulated (GDP dissociation inhibitor protein and transferrin). Changes in expression of these six genes returned to vehicle-injected control levels 7 days after chronic Δ⁹-THC exposure (see below). The acute changes likely reflect direct physiological consequences of the large dose (10 mg/kg) of Δ⁹-THC, which produces severe catalepsy, hypothermia, and other symptoms in rats for the initial 3–5 days of repeated injections (20). Expression levels of four other genes; SC1 protein, cytochrome oxidase, myelin proteolipid protein, and T cell receptor protein (Table 1), were also upregulated after the single injection of Δ⁹-THC but, unlike the above genes, remained significantly elevated throughout the entire 21 days of Δ⁹-THC exposure. Another gene, elongation factor, remained upregulated for 7 days but returned to control levels after 21 days of exposure. Finally, expression levels of the calmodulin and polyubiquitin transcripts changed in a biphasic manner across the duration of the chronic treatment period as evidenced by downregulation after a single injection, reversing to upregulation at 7 days and in the case of calmodulin was again downregulated after 21 days (Table 1). The overall pattern of gene expression changes after 7 days of Δ⁹-THC exposure was different from that following a single acute injection. As indicated above 8 of 15 genes whose expression was altered at 24 h remained altered at 7 days of Δ⁹-THC exposure. In addition, transcripts for α-enolase, and myelin associated protein (upregulated) as well as phospholipid gluthionine peroxidase, myelin basic protein, angiotensin AT₁ receptor, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and acidic 82-kDa protein (downregulated), were all affected after 7 days.

By day 21 of Δ⁹-THC exposure, the expression levels of 16 of 28 genes either remained altered or became changed from their prior status at 7 days. At this time, seven genes were upregulated and nine were downregulated (Table 1) relative to vehicle-injected animals. Five of the nine downregulated genes [fructose-bisphosphate aldolase, brain lipid binding protein, pro- teosomal ATPase, calreticulin, and heat shock protein-70 (HSP70)] were evident only after 21 days of exposure (Table 1), whereas expression levels of genes encoding myelin basic protein and mitochondrial 12–16S RNA, downregulated at 7 days, were also downregulated at 21 days (see Table 1). The upregulation in expression of calmodulin and elongation factor observed at 7 days was reversed to downregulation at 21 days of Δ⁹-THC exposure (Table 1). The EST clone 407167 was upregulated at 21 days.

Verification of primary large-scale array gene analysis by RNA dot-blot analysis. To validate the specificity and magnitude of the Δ⁹-THC-induced gene expression changes shown in Table 1, dot blots of mRNA isolated from a different series of rats subjected to the same acute and chronic Δ⁹-THC treatments, were employed to validate the expression patterns of 15 of the 28 genes identified in the primary screen. Table 2 shows the mean change (±SE) in expression levels for the selected genes examined in groups of three rats, with each group exposed to Δ⁹-THC for one of the three different durations (24 h, 7 days and 21 days) relative to equal numbers of vehicle-injected controls.

The RNA dot-blot analyses confirmed the changes in transcriptional regulation that occurred at various stages of Δ⁹-THC exposure indicated in the primary screen (Fig. 4). In agreement with reports from other

### Table 2. DGE in rat hippocampus monitored by primary and secondary microarray screens and RNA dot blot analyses

<table>
<thead>
<tr>
<th>Clone</th>
<th>DGE Confirmed</th>
<th>Primary Screening, fold difference</th>
<th>24 h</th>
<th>7 day</th>
<th>21 day</th>
<th>RNA Dot Blot, fold difference</th>
<th>24 h</th>
<th>7 day</th>
<th>21 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin D synthase</td>
<td>Y</td>
<td>1.8↑</td>
<td>1.6±0.3*↑</td>
<td>1.2±0.2↑</td>
<td>1.5±0.2↑</td>
<td>1.0±0.3↑</td>
<td>1.6±0.2↑</td>
<td>1.2±0.3↑</td>
<td>1.2±0.3↑</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Y</td>
<td>1.8↑</td>
<td>1.5±0.2↑</td>
<td>1.5±0.1↓</td>
<td>1.2±0.3↓</td>
<td>1.0±0.1↓</td>
<td>1.4±0.1↑</td>
<td>1.9±0.2*↑</td>
<td>0.9±0.2↑</td>
</tr>
<tr>
<td>NCAM</td>
<td>Y</td>
<td>3.2↑</td>
<td>1.4±0.1↑</td>
<td>1.9±0.2*↑</td>
<td>0.9±0.2↑</td>
<td>1.4±0.2↑</td>
<td>1.0±0.3↑</td>
<td>1.2±0.3↑</td>
<td>1.2±0.3↑</td>
</tr>
<tr>
<td>Sodium channel</td>
<td>N</td>
<td>1.9↑</td>
<td>1.2±0.1↑</td>
<td>1.3±0.2↓</td>
<td>1.4±0.2↓</td>
<td>1.2±0.1↓</td>
<td>1.5±0.2↑</td>
<td>1.5±0.2↓</td>
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<tr>
<td>α-Enolase</td>
<td>Y</td>
<td>2.3↑</td>
<td>2.5↑</td>
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<tr>
<td>GST peroxidase</td>
<td>N</td>
<td>1.8↓</td>
<td>1.0±0.2↑</td>
<td>1.0±0.1↑</td>
<td>1.2±0.3↑</td>
<td>1.0±0.2↑</td>
<td>1.4±0.2↑</td>
<td>1.7±0.2*↑</td>
<td>1.7±0.2*↑</td>
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<tr>
<td>PKU β-subunit</td>
<td>Y</td>
<td>2.5↑</td>
<td>1.4±0.3↑</td>
<td>1.2±0.4↑</td>
<td>1.7±0.2*↑</td>
<td>1.7±0.2*↑</td>
<td>1.4±0.3↑</td>
<td>1.4±0.3↑</td>
<td>1.4±0.3↑</td>
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<tr>
<td>Peptide-histidine transporter</td>
<td>Y</td>
<td>4.0↑</td>
<td>1.5±0.1↑</td>
<td>1.6±0.2↓</td>
<td>1.7±0.3*↑</td>
<td>1.7±0.3*↑</td>
<td>1.5±0.1↑</td>
<td>1.6±0.2↓</td>
<td>1.7±0.3*↑</td>
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<tr>
<td>HSP70</td>
<td>Y</td>
<td>2.3↑</td>
<td>1.1±0.05↑</td>
<td>0.6±0.3↑</td>
<td>2.0±0.5*↑</td>
<td>2.0±0.5*↑</td>
<td>1.2±0.2↑</td>
<td>1.5±0.1*↑</td>
<td>1.7±0.2*↑</td>
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<tr>
<td>Proteosomal ATPase</td>
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<td>8.6↑</td>
<td>1.0±0.1↑</td>
<td>1.5±0.2↑</td>
<td>1.7±0.2*↑</td>
<td>1.7±0.2*↑</td>
<td>1.3±0.2↑</td>
<td>1.7±0.2*↑</td>
<td>1.7±0.2*↑</td>
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<tr>
<td>Brain specific lipid binding protein</td>
<td>Y</td>
<td>2.5↑</td>
<td>1.0±0.3↑</td>
<td>1.2±0.2↑</td>
<td>1.7±0.2*↑</td>
<td>1.7±0.2*↑</td>
<td>1.3±0.2↑</td>
<td>1.2±0.2↑</td>
<td>1.7±0.2*↑</td>
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<tr>
<td>Angiotensin A₁ receptor</td>
<td>Y/N</td>
<td>1.6↑</td>
<td>1.3±0.4↑</td>
<td>1.5±0.1*↑</td>
<td>1.6±0.2*↑</td>
<td>1.3±0.4↑</td>
<td>1.2±0.2↑</td>
<td>1.5±0.1*↑</td>
<td>1.6±0.2*↑</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>Y/N</td>
<td>2.0↑</td>
<td>1.3±0.2↑</td>
<td>2.0±0.4*↑</td>
<td>2.7±0.5*↑</td>
<td>2.7±0.5*↑</td>
<td>1.3±0.2↑</td>
<td>2.0±0.4*↑</td>
<td>2.7±0.5*↑</td>
</tr>
<tr>
<td>SC1</td>
<td>Y/N</td>
<td>1.6↑</td>
<td>1.5±0.1↑</td>
<td>1.8±0.15↑</td>
<td>1.4±0.3↑</td>
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<td>1.5±0.1↑</td>
<td>1.8±0.15↑</td>
<td>1.4±0.3↑</td>
</tr>
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</table>

Expression levels in RNA dot blot analyses were normalized to levels of labeled oligo(d)T primer for each blot (see METHODS) and expressed as a mean fold change from corresponding vehicle-injected controls. Values for primary screens were determined from microarray scan software (Fig. 2). Values are means ± SE; n = 3. *P < 0.01, †P < 0.001, t-test. DGE, differential gene expression.

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investigators (38, 49), the magnitude of the differences in expression levels assessed by RNA dot-blot analyses were in general lower relative to that reported in the primary screen. There was considerable agreement, however, in the effect of duration of drug exposure on the direction (up or downregulation) and relative magnitude of altered gene expression detected by both methods (Table 2).

Expression levels of prostaglandin D synthase, NCAM, and SC1 verified the increase after a single acute dose of $\Delta^9$-THC reported by the primary screen. Of these, prostaglandin D synthase, found to be upregulated 1.8-fold by the primary screen (Table 1), showed a mean change of $1.6 \pm 0.3$ (fold change) relative to vehicle-injected animals ($n = 3$ per group) by dot-blot analysis ($t = 4.32, P < 0.01$, Table 2). Also in agreement with the primary screen, this increase in prostaglandin D synthase expression level was not maintained after 7 days of $\Delta^9$-THC exposure (Table 2, 7–21 days). Similarly, NCAM expression was found by dot-blot analysis to be significantly upregulated (mean change from control = $1.6 \pm 0.2, P < 0.001$) after a single $\Delta^9$-THC injection but to a lesser degree than in the primary screen (3.2-fold, Table 2); however, like prostaglandin D synthase, expression levels also returned to vehicle control levels after 7 days as in the primary screen. The expression pattern for $\alpha$-enolase was found to be upregulated in the primary screen at 7 days but not at 21 days which was also confirmed in the RNA dot-blot assay (mean fold change at 7 days = $1.9 \pm 0.2, P < 0.001$). Finally, in contrast to primary screen results, the increased expression of the sodium channel subunit 1 detected at 7 days (1.9-fold) was not confirmed by subsequent RNA dot-blot analyses (mean difference = $1.0 \pm 0.1, P < 0.5$).

Maintained increases in expression levels of SC1 (hevin) were observed in the primary screen during the entire time course of $\Delta^9$-THC exposure (Table 1), but dot-blot analyses verified significant differences only for days 7 and 21 (mean change = $2.0 \pm 0.5$ and $2.7 \pm 0.4$, respectively, $P < 0.01$ in Table 2). However, changes in expression levels of peptide/histidine transporter and the PKU $\beta$-subunit identified in the primary screen as significantly altered at 21 days of $\Delta^9$-THC exposure (Table 2) were confirmed by RNA dot-blot analysis (mean change = $2.5 \pm 0.4, P < 0.01$; and $1.7 \pm 0.2, P < 0.01$, respectively).

In the primary screen, brain-specific lipid binding protein and myelin basic protein were found to be significantly downregulated at 7 and 21 days of $\Delta^9$-THC exposure (2.5- and 2.0- fold difference, respectively, Table 2). The changes in direction of expression of these two genes were confirmed by RNA dot-blot analyses, but again the magnitudes of the changes were somewhat lower (see Table 2). HSP70 and proteosomal ATPase, found to be downregulated at 21 days by primary screen (2.3- and 8.6-fold), were also confirmed by RNA dot-blot analysis (mean change = $1.7 \pm 0.3$ and $2.0 \pm 0.5, P < 0.01$, respectively). However, downregulation of phospholipid glutathione peroxidase expression, detected in the primary screen (1.8-fold) after 7 days exposure to $\Delta^9$-THC, was not confirmed by RNA dot-blot analysis (mean difference = $1.3 \pm 0.2$, not significant, Table 2). The strongest validation of primary screen results was the detection by both methods of the biphasic alteration in expression of calmodulin. This transcript was significantly downregulated at 24 h (1.6-fold, mean change = $1.5 \pm 0.1, P < 0.01$), upregulated at 7 days (2.2-fold, mean change = $1.8 \pm 0.1, P < 0.01$), and downregulated at 21 days (1.5-fold, mean change = $1.4 \pm 0.3, P < 0.01$) in both assays conducted on different set of animals (Table 2). Finally, the upregulation in the primary screen of a novel gene (Novel 5, 2.1-fold) at 21 days was not verified by RNA dot-blot analysis (mean change = $1.5 \pm 0.3$, not significant, not shown).

In situ hybridization depiction of transient upregulation of NCAM expression. Neural cell adhesion molecules (NCAM) represent a family of alternatively spliced cell surface proteins whose expression is increased during development (53). In normal adult hippocampus immunohistochemical and in situ hybridization studies show widespread expression of several NCAM isoforms in the dentate gyrus and throughout Ammon’s horn (52, 53) with decreased expression in other brain regions. Increased expression of NCAM was detected and verified after a single dose of $\Delta^9$-THC (10 mg/kg) in the cDNA microarray screen and RNA dot-blot analyses (Table 1 and 2). In situ hybridization techniques were therefore implemented to reveal those regions of hippocampus in which the changes in NCAM expression occurred following acute (24 h) $\Delta^9$-THC injections. Since the primary and secondary screens showed the change to be transient, returning to vehicle levels at 7 days, in situ histochemistry was also performed on tissue from animals after 7 days of exposure.

Figure 5 shows the distribution of NCAM in the dorsal hippocampus of rats treated with $\Delta^9$-THC at 24 h and 7 days. It is clear that label was abundant in the hippocampal region as revealed by increased density in the pyramidal cell layers in Ammon’s horn and granule cell layer in the dentate gyrus as previously
reported (52). Semiquantitative analyses of the density of label (see METHODS) showed a significant increase (45%, \( P < 0.01 \)) in density of the hybridization signal for NCAM-positive cells in the dentate gyrus and pyramidal cell layers (42%, \( P < 0.01 \)) 24 h after a single acute injection of \( \Delta^9 \)-THC compared with vehicle-treated control animals (Fig. 5B). The increase at 24 h was significantly reduced if the cannabinoid CB1 receptor antagonist SR171416A (19) was injected 5 min prior to \( \Delta^9 \)-THC injection (12%, \( P = 0.3 \), Fig. 5). Consistent with results from the primary and secondary (dot blot) screens, after 7 days of chronic \( \Delta^9 \)-THC exposure the in situ label increase in NCAM expression was no longer apparent (3% decrease, \( P = 0.15 \)). There were no significant changes in the density of NCAM in situ label in somatosensory cortex in any of the animals that exhibited altered expression of NCAM in the hippocampus (Fig. 5B).

**DISCUSSION**

cDNA microarray technology was employed to examine alterations in gene expression in hippocampus that occurred as the result of both acute (24 h), intermediate (7 days), and long-term (21 days) exposure to \( \Delta^9 \)-THC. The large-scale cDNA microarray analysis revealed 28 known genes, 10 previously sequenced ESTs, and 11 potential novel gene sequences that were altered at one or more of the above time points of chronic \( \Delta^9 \)-THC exposure. Distinct patterns of differential transcript expression over the three stages of drug exposure were revealed in the primary screen (Tables 1 and 2). Verification of the findings agreed with other reports (34, 49) regarding comparison with mRNA dot blots that gave reduced levels of change, but a high degree of concordance (70%) with the results from the large-scale primary screens. Utilizing two animals per group and assessments at three different time points during \( \Delta^9 \)-THC exposure, we found that the primary large-scale screen showed no differences in expression levels of 5,556 (22% of total probed) genes expressed in hippocampus (Figs. 1 and 2). Therefore, the exceedingly small number of altered genes (\( n = 28 \), Table 1), identified by the primary and largely confirmed by the secondary (dot blot) screen (Tables 1 and 2) indicate it is unlikely that the changes reported here resulted from chance variations in expression levels of the identified clones. However, because proteins levels corresponding to the above mRNA changes were not monitored, the functional significance of the change in expression levels cannot be fully ascertained.

The altered expression level of several genes after a single injection of \( \Delta^9 \)-THC (Tables 1 and 2) could be produced by at least two (if not more) different cannabinoid receptor-mediated cellular changes: 1) a decrease in the level of cAMP via receptor-coupled inhibition of adenyl cyclase, which could eventually alter mRNA transcription through cAMP response element-binding protein (CREB) or other gene pathways (12, 28, 32, 54); or 2) the high dose (10 mg/kg) of \( \Delta^9 \)-THC may have acted via cannabinoid receptor-produced physiological changes such as severe catalepsy and hypothermia (11, 14, 19, 26, 34) to initiate expression of multiple transcripts, including immediate early genes (39, 43). Furthermore, genes identified (Table 1) and confirmed (Table 2) as differentially expressed after acute injection can be subdivided again into those affected only after a single injection, returning to vehicle-injected control levels after 7 or 21 days (i.e., NCAM, prostaglandin D synthase, transferrin), and those whose expression levels remained dysregulated over the entire 21 days of \( \Delta^9 \)-THC exposure (calmodulin, SC1). The transient increase in expression levels of NCAM only after a single acute dose of \( \Delta^9 \)-THC is interesting, since it has been suggested that changes in
cAMP levels lead to a downregulation of cell adhesion molecules involved in synaptic remodeling (41). Up-regulation of NCAM expression has been traditionally associated with the maturation and differentiation of neurons in the developing rat hippocampus (52). However, recently, a transient increase in NCAM expression has been shown in certain learning paradigms (47). In addition, altered induction of long-term potentiation (LTP) in hippocampal slices by endoneuraminidase N, an enzyme which removes polysialic acid from NCAM (6, 45), has been reported. This is consistent with reports of inhibition of LTP by cannabinoids in hippocampal slice preparations (44) and enhancement of LTP in mice lacking CB1 receptors (8), if one views the upregulation of NCAM as a consequence of the suppression of cannabinoid suppression of LTP-related mechanisms. To date there has been no report of the sensitivity of NCAM to cannabinoid receptor activation or the fact that such changes are transient in nature and decrease with chronic exposure to Δ9-THC. Our data suggests that acute Δ9-THC treatment may alter NCAM levels in the hippocampus acutely as a consequence of LTP suppression, but following continued suppression of LTP by chronic Δ9-THC treatment, NCAM expression is no longer affected.

The fact that the expression of calmodulin was bidirectionally altered at 24 h, 7 days, and 21 days may reflect similar phasic changes in cAMP levels following development of cannabinoid tolerance (34). In contrast, the basis for the 21 day elevated level of SC1 (hevin), a newly cloned gene for a protein, which binds to cytokine receptors, proteases, and matrix proteins (9), throughout the 21 days of Δ9-THC exposure is not well understood; however, the fact that its expression is changed so significantly suggests a possible convergence with cannabinoid receptor-mediated cellular changes.

Both the primary screen and RNA dot-blot analyses showed significantly elevated prostaglandin D synthase expression after a single injection of Δ9-THC. Cannabinoids stimulate prostaglandin D synthase activity (50) and the production of prostaglandins (33), which is a likely result of release of arachidonic acid as has been shown in adult brain slices (50), neuroblastoma cells (33), and in cultured hippocampal neurons (31). Prostaglandin D2, the end product of this enzyme’s reaction with arachidonic acid, is abundantly expressed in brain and has recently been shown to induce sleep in rats (57). The above findings suggest that acute cannabinoid exposure can act directly to transiently increase expression of an enzyme present in one of the synthesis pathways for endogenous cannabinoids (22).

By day 7 of drug exposure, animals do not exhibit catalepsy to the high (10 mg/kg) dose of Δ9-THC and do display normal locomotor behavior; however, they remain significantly impaired in cognitive function (3, 20). Hippocampal CB1 receptor-stimulated GTPγS binding is maximally depressed at day 7 and coincides with peak elevation of CB1 receptor mRNA expression levels (11, 58). The increased expression of calmodulin (see above) at day 7 (Table 2) could therefore reflect increased levels of Ca2+/calmodulin-stimulated adenyl cyclase (55) following CB1 receptor desensitization (54).

Unlike the above upregulated genes, myelin basic protein mRNA was significantly downregulated at 7 and 21 days. This protein has been shown to inhibit high-affinity cannabinoid binding in brain (48). Curiously, the expression of myelin proteolipid protein, which is functionally related to myelin basic protein, was significantly elevated at the same time points (days 7 and 21, Tables 1 and 2). Whether the simultaneous downregulation of HSP70, which induces conformational changes in myelin basic protein (5), was related to these changes is not known.

Previous reports show that both behavioral tolerance as well as CB1 receptor downregulation and desensitization reach a steady state at 21 days of Δ9-THC exposure (11, 54), at which point expression levels of the CB1 receptor are not different from vehicle control animals (58). Table 2 shows that an additional seven transcripts, not affected during the first 7 days of drug exposure, were significantly changed after an additional 14 days of Δ9-THC exposure. The significant increase in expression of angiotensin AT1 receptor mRNA after 21 days of exposure (Table 2) suggests an increase in receptor number (29) in this transmitter system perhaps produced by an increase in arterial pressure in chronically exposed animals (16). The up-regulation of the PKU β-subunit at 21 days could likewise reflect its role in nuclear translocation and phosphorylation of other transcription factors in response to chronic exposure to Δ9-THC.

In this study, large-scale cDNA microarray analysis successfully identified genes within rat hippocampus susceptible to acute, intermediate, and long-term cannabinoid exposure. A number of transcripts involved in important cellular functions (e.g., NCAM, SC1, PKU β-subunit, HSP70) had altered expression levels at some or all time points during the extended course of Δ9-THC treatment. Detection by differential gene expression criteria at these different time points (Table 1 and 2) provided information about the complexity of cellular and molecular changes affected by both acute and chronic exposure to cannabinoids. Specifically, the technique revealed changes in several cellular processes not previously associated with the development of tolerance to cannabinoids (17, 19). Such changes in expression levels incorporate a wide variety of signaling mechanisms within hippocampal cells, which were differentially susceptible to chronic cannabinoid treatment. The significance of many of these changes with respect to cannabinoid system function has yet to be determined. However, it is unlikely that such profound and widespread alterations in gene regulation revealed through cDNA microarray technology would not have an impact on hippocampal function (19, 30).

REFERENCES

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ACUTE AND CHRONIC EXPOSURE TO Δ9-THC IN RATS


