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DNA Microarray Analysis of Cannabinoid Signaling in Mouse Brain in Vivo

SOPHIE PARMENTIER-BATTEUR, KUNLIN JIN, LIN XIE, XIAO OU MAO, and DAVID A. GREENBERG

Buck Institute for Age Research, Novato, California

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ABSTRACT

To identify novel genes involved in cannabinoid receptor-mediated signaling, we used cDNA microarrays to detect changes in mRNA expression in the forebrains of mice 12 h after they were given a single intraperitoneal dose of the naturally-occurring *Cannabis sativa* alkaloid Δ^9-tetrahydrocannabinol (Δ^9-THC) or the synthetic cannabinoid receptor agonist (*R*)-(+)-2,3-dihydro-5-methyl-3-[(morpholinyl)methyl] pyrrolo[1,2,3-de]-1,4-benzoxazin-yl-1-naphtalenylmethanone mesylate [R(+)-WIN 55,212-2]. Of ~11,000 genes from a mouse brain cDNA library that were probed, 65 showed altered (increased or decreased at least 2-fold) expression after exposure to Δ^9-THC, 41 after exposure to R(+)-WIN 55,212-2, and 20 genes after exposure to both drugs. Genes affected similarly by Δ^9-THC and R(+)-WIN 55,212-2 were considered likely to reflect cannabinoid receptor activation, and expression of the protein products of two such genes not previously implicated in cannabinoid signaling—melanocyte-specific gene-related gene 1 (MRG1) and hexokinase 4 (glucokinase, GK)—was measured by Western blotting and immunohistochemistry. Western blots showed ~2-fold increases in the levels of both proteins in mouse forebrain. Immunohistochemistry revealed preferential localization of MRG1 to cerebral blood vessels and of GK to hypothalamic neurons. These findings suggest that *MRG1* and *GK* are cannabinoid-regulated genes and that they may be involved in the vascular and hypothalamic effects of cannabinoids, respectively.

The discovery of specific cannabinoid receptors and a family of endogenous cannabinoid ligands (endocannabinoids) has led to intensive research on the role of the cannabinoid system in physiology and in pathological conditions. The CB1 receptor subtype is distributed throughout the brain (Devane et al., 1988), whereas the CB2 receptor is located primarily in peripheral tissues (Munro et al., 1993). The brain also produces anandamide, 2-arachidonoylglycerol, and other endogenous cannabinoid ligands of the eicosanoid class (Devane et al., 1992; Stella et al., 1997). Clinically important responses to Δ^9-tetrahydrocannabinol (Δ^9-THC) and synthetic cannabinoids include the attenuation of nausea and vomiting in cancer chemotherapy, stimulation of appetite in wasting syndromes, and reduction in intestinal motility (Robson, 2001). In addition, cannabinoids may have a role in the treatment of neurological disorders, including spasticity associated with multiple sclerosis or spinal cord injury, movement disorders, epilepsy, and pain (Robson, 2001).

Neuronal cannabinoid signaling seems to regulate cell death and survival. Cannabinoids protect the brain from insults such as excitotoxicity (Shen and Thayer, 1998), hypoxia and ischemia (Nagayama et al., 1999; Sinor et al., 2000), and trauma (Panikashvili et al., 2001). Conversely, under some conditions, cannabinoids can induce neuronal apoptosis (Campbell, 2001).

Various signal transduction pathways have been shown to be involved in the action of cannabinoids, which exert most of their known effects through the CB1 receptor. This G protein-coupled receptor signals inhibition of adenylate cyclase and of N-and P/Q-type channels (Pertwee, 1997; Twitchell et al., 1997; Wilson et al., 2001) and activation of mitogen-activated protein kinases (Bouaboula et al., 1995) and protein kinase B (Gomez del Pulgar et al., 2000). CB1 cannabinoid receptors may also signal independently of $G_{i/o}$ proteins through an adaptor protein (neutral sphingomyelinase activation-associated factor, or FAN) that has been implicated in the proapoptotic pathway involving sphingomyelin breakdown and ceramide accumulation (Sanchez et al., 2001). In addition, cannabinoids exert some effects independently of the CB1 and CB2 receptors, such as through gap junctions (Venance et al., 1995), T-type Ca^{2+} channels (Chemin et al., 2001), the vanilloid receptor (Zygmunt et al., 1999) or non-

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ABBREVIATIONS: Δ^9-THC, Δ^9-tetrahydrocannabinol; R(+)-WIN 55,212-2, (*R*)-(+)-2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-yl-1-naphtalenylmethanone mesylate; DMSO, dimethyl sulfoxide; SSC, standard saline citrate; PBS, phosphate-buffered saline; MRG1, melanocyte-specific gene-related gene 1; GK, glucokinase; SR 141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboximide hydrochloride.

CB1/CB2 cannabinoid receptors (Jarai et al., 1999; Breivogel et al., 2001; Hajos et al., 2001).

Microarray analysis is a powerful tool for detecting differential gene expression in neural tissues (Greenberg, 2001), including changes in gene expression associated with the effects of drugs (Thibault et al., 2000). One recent study described changes in the hippocampal expression of 49 of 24,456 arrayed cDNAs after short- (24 h) or long-term (7–21 days) administration of Δ^9-THC to rats (Kittler et al., 2000). To better understand the molecular processes involved in cannabinoid action in vivo, which might suggest mechanisms through which these compounds control cell death and survival decisions, we examined changes in gene expression in the brain after short-term administration to mice of two different cannabinoid receptor agonists—the naturally occurring plant alkaloid Δ^9-THC and the synthetic aminoalkylindole R(+)-WIN 55,212-2. We used large-scale cDNA microarrays that contain probes for ~11,000 known genes and expressed sequence tags selected from a mouse brain cDNA library. Results revealed a total of 65 genes altered by Δ^9-THC exposure, 41 genes altered by R(+)-WIN 55,212-2 exposure, and 20 genes altered by exposure to both drugs. Genes affected similarly by Δ^9-THC and R(+)-WIN 55,212-2 are likely to be involved in cannabinoid receptor-mediated signaling, whereas genes uniquely affected by one or the other drug may reflect nonreceptor effects.

Materials and Methods

Drugs. Drugs were purchased from Sigma-Aldrich (St. Louis, MO). Δ^9-THC was provided dissolved in 100% methanol at 1 mg/ml; methanol was evaporated and Δ^9-THC was redissolved in dimethyl sulfoxide (DMSO) at 5 mg/ml. R(+)-WIN 55,212-2 was also dissolved in DMSO, at 0.5 mg/ml.

Animals and Drug Treatment. Animal experiments were carried out in accordance with National Institutes of Health guidelines and were approved by local committee review. Male CD1 mice (Charles River Laboratories) 2 to 3 months old and weighing 25 to 30 g were housed three per cage and maintained on a 12-h/12-h light/dark cycle, with food and water provided ad libitum, for 1 week before cannabinoid treatment. Mice were given a single 60-μl intraperitoneal injection of R(+)-WIN 55,212-2 (1 mg/kg), Δ^9-THC (10 mg/kg), or DMSO vehicle. Compared with vehicle, both drugs decreased spontaneous locomotion during the first hour after short-term injection, and Δ^9-THC transiently reduced rectal temperature by 1 to 2°C. When mice were killed 12 h later, drug- and vehicle-treated mice were indistinguishable. Whole brains were cut in half, the brainstem and cerebellum were removed, and hemi-forebrains were immediately frozen in dry ice and stored at −80°C until mRNA and protein extracts were prepared.

Microarrays. National Institutes of Mental Health/National Institute of Neurological Disorders and Stroke Brain Molecular Anatomy Project (BMAP) cDNA microarrays, containing 11,200 mouse genes isolated from neural tissues and 32 control genes, were prepared by the Buck Institute's Genomics Core. cDNAs were dissolved at 40 μM in 3× SSC buffer (1× SSC is 140 mM NaCl and 15 mM sodium citrate) and printed on AminoSilane-treated glass microscope slides (CEL Associates, Houston, TX) using a high-performance OmniGrid microarrayer (GeneMachines, San Carlos, CA). Two separate copies of the array were printed per slide. Printed slides were stored in a light-tight box in a bench-top desiccator at room temperature.

Probe Synthesis and Hybridization. Hemi-forebrains from six mice treated with R(+)-WIN 55,212-2, Δ^9-THC, or vehicle were pooled and poly(A) RNA was isolated using Fast Track mRNA isolation kits (Invitrogen, Carlsbad, CA). Fluorescence-labeled probes were prepared by reverse transcription using a superscript II polymerase (Invitrogen) from Cy3-dCTP or Cy5-dGTP and 1 μg of poly(A) RNA. Cy3 was used to label probes from vehicle-treated mice and Cy5 was used to label probes from drug-treated mice. Probes were purified using Millipore columns (Millipore Corporation, Bedford, MA) vacuum-dried, and resuspended in 20 μl of hybridization buffer (10× SSC/50% formamide/0.02% SDS), heated to 90°C for 5 min, cooled to room temperature for 5 min, and applied to slides. The two separate arrays per slide were hybridized with probes from vehicle- and R(+)-WIN 55,212-2-treated or from vehicle- and Δ^9-THC-treated mice. Arrays were separately cover-slipped and hybridized at 60°C for 16 h in a sealed chamber (Corning, Corning, NY), then washed in 0.5× SSC/0.01% SDS at room temperature for 15 min with gentle agitation and in 0.06× SSC/0.01% SDS at room temperature for 5 min. After rapid rinsing in 0.1× SSC, slides were dried by centrifugation at low speed before scanning.

Microarray Scanning, Quantitation, and Statistical Analysis. Microarrays were scanned for Cy3 and Cy5 fluorescence using a ScanArray 3000 microarray scanner (General Scanning, Watertown, MA). QuantArray software (GSI Lunonics, Watertown, MA) was used for quantitation. Spot intensity was corrected by subtracting the immediate background. Background-subtracted element signals were used to calculate Cy3/Cy5 ratios. Each experiment was performed three times and fold-changes in expression were averaged. The selection criteria for differentially expressed genes were a minimum expression ratio of 1.8 on three of three arrays and an average expression ratio of at least 2.0.

Analysis of Protein Expression. For Western blotting, MRG-1, GK, and actin protein expression was assayed in 100-μg protein samples from the hemi-forebrain not used for microarray studies. Samples from six mice treated with R(+)-WIN 55,212-2, Δ^9-THC, or vehicle were pooled, and protein extracts were boiled in reducing SDS sample buffer for 10 min and electrophoresed on a 12% SDS-polyacrylamide gel, then transferred electrophoretically to polyvinylidene difluoridine membranes at 4°C and 200 mA overnight. Membranes were probed with goat polyclonal antibody against the amino terminus of human MRG-1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), sheep polyclonal anti-GK (1:5000; a gift from Dr. M.A. Magnuson, Dept of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN), or monoclonal anti-actin (1:500; Santa Cruz Biotechnology), and then with horseradish peroxidase-conjugated anti-goat (1:5000), anti-sheep (1:5000), or anti-mouse (1:2000) IgG secondary antibody (Santa Cruz Biotechnology), and visualized by chemiluminescence. For immunohistochemistry, sections were treated with 1% H_2O_2 for 15 min (for diaminobenzidine detection) and then placed in blocking buffer containing 5% horse serum and 0.2% Triton X-100 in PBS for 1 h at room temperature. Sections were incubated overnight at 4°C with the following primary antibodies: anti-MRG-1 (as above, 1:100), anti-GK (as above, 1:2000), rat monoclonal

anti-PECAM-1/CD31, (1:50; Cymbus Biotechnology, Chandlers Ford, UK), or mouse monoclonal anti-neuronal nuclear antigen NeuN (1:500; Chemicon, Temecula, CA). Sections were washed in PBS containing 0.1% Tween 20 and immunostaining was completed using the following secondary antibodies: biotinylated anti-goat and anti-sheep IgG (1:200; Vector Laboratories, Burlingame, CA), fluorescein-conjugated anti-goat and anti-sheep IgG (1:200; Vector Laboratories), and rhodamine-conjugated anti-mouse and anti-rat IgG (1:200; Jackson ImmunoResearch, West Grove, PA). The peroxidase reaction was detected with 0.05% diaminobenzidine in PBS and 0.03% H_2O_2. As controls, alternating sections were incubated without primary antibody.

Results

Patterns of Altered Gene Expression Induced by Cannabinoids. Eighty-six genes, representing somewhat less than 1% of the arrayed cDNAs, showed 2-fold or greater changes in expression in the brain after systemic administration of Δ^9-THC or R(+)-WIN 55,212-2. Of these genes, 52% were affected only by Δ^9-THC, 25% were affected only by R(+)-WIN 55,212-2, and 23% were affected by both cannabinoids. More genes were down-regulated (72%) than were up-regulated (28%), although this ratio varied across treatments. Thus, 93% of Δ^9-THC-regulated genes and 65% of genes regulated by both Δ^9-THC and R(+)-WIN 55,212-2, but only 33% of genes regulated by R(+)-WIN 55,212-2, exhibited reduced expression.

Both cannabinoids modified the expression of genes in a broad range of functional categories (Fig. 1). When classified according to these categories, the most prominently regulated class of genes was those involved in cellular signaling, which accounted for 30% of all regulated genes and 45% of genes regulated by both Δ^9-THC and R(+)-WIN 55,212-2. Other functional gene classes that were affected included genes involved in protein processing, cell growth or structure, stress responses, and glial differentiation.

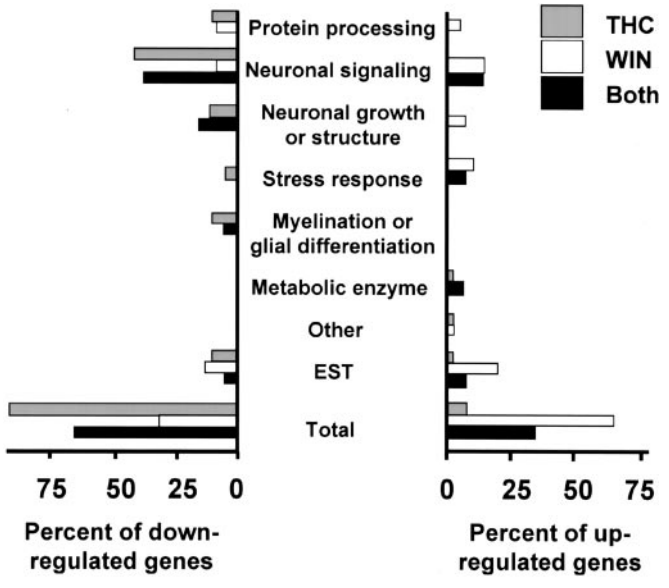

Fig. 1. Functional classes of genes showing cannabinoid-induced changes in expression. Each bar represents the percentage of all genes regulated by THC only, WIN only, or both drugs that belong to a given functional class of genes.

Genes Regulated by Δ^9-THC. Forty-five genes were differentially expressed after Δ^9-THC treatment, but not after treatment with R(+)-WIN 55,212-2 (Table 1). Only two of these genes were up-regulated: aspartyl-tRNA synthase and neuromedin U. Neuromedin U is a peptide that is expressed at high levels in the ventromedial hypothalamus, and its administration suppresses food intake and increases core body temperature (Howard et al., 2000). Because these effects are opposite those produced by Δ^9-THC, enhanced expression of neuromedin U could represent a homeostatic response to the drug. Of the genes that showed Δ^9-THC–induced decreases in expression, ≥3-fold changes were seen for the small inducible cytokine family D member 1 (neurotactin), mouse myelin proteolipid protein, neuron-specific gene family member 1, copine 6, APP-binding protein, and monoglycerate lipase. Neurotactin is a proinflammatory chemokine that is up-regulated in brain microglia in experimental autoimmune encephalomyelitis (Pan et al., 1997), suggesting that its down-regulation by Δ^9-THC could contribute to the drug's anti-inflammatory effect. Copine 6, a calcium-dependent phospholipid-binding protein expressed in hippocampus, is up-regulated by stimuli that evoke long-term potentiation (Nakayama et al., 1998), which is impaired by cannabinoids.

Genes Regulated by R(+)-WIN 55,212-2. Twenty-one genes were regulated only by R(+)-WIN 55,212-2 (Table 2). The greatest up-regulation was observed for the 140-kDa subunit of replication factor C, which promotes cell survival after DNA damage (Pennaneach et al., 2001). R(+)-WIN 55,212-2 also up-regulated Hsp27, which has been implicated in the neuroprotective effect of ischemic preconditioning (Currie et al., 2000), and cyclin-dependent protein kinase 1, which promotes neuronal survival from apoptotic insults (Courtney and Coffey, 1999), whereas the proapoptotic ubiquitin-protein ligase Nedd4 (Anan et al., 1998) was down-regulated.

Genes Regulated by both Δ^9-THC and R(+)-WIN 55,212-2. Twenty genes showed changes in expression associated with both Δ^9-THC and R(+)-WIN 55,212-2; in all cases, the direction of the change induced by both drugs was the same (Table 3). Genes down-regulated in common constituted 31% of those down-regulated by Δ^9-THC and 35% of those down-regulated by R(+)-WIN 55,212-2; for up-regulated genes, the corresponding percentages were 70 and 33%. Because genes regulated by two structurally dissimilar cannabinoids seemed most likely to be affected through a specific, receptor-mediated mechanism, we focused most of our attention on this group of genes. Known genes regulated by both Δ^9-THC and R(+)-WIN 55,212-2 included genes involved in neuronal signaling, neuronal growth and structure, myelination or glial differentiation, and metabolism. Considering the neuroprotective effects of cannabinoids and the proposed role of endogenous cannabinoid signaling in regulating cell death and survival, certain up-regulated genes were of particular interest, including the enzyme GK (Alvarez et al., 2002; Roncero et al., 2000) and the transcription factor MRG1 (Sun et al., 1998; Bhattacharya et al., 1999).

Hexokinase 4, or glucokinase (GK), a member of an enzyme family that catalyzes the first step of glycolysis, is found at high levels in the brain, especially in the hypothalamus (Roncero et al., 2000). Cannabinoid receptors are also abundant in

TABLE 1

Genes specifically modified in mouse brain 12 h after Δ ^{9}-THC administration

Expression levels are ratios of Δ ^{9}-THC-treated versus control samples. Each mRNA sample group was hybridized to three different arrays, and fold-change values are average of the triplicate measurements. Matches of clone sequences from cDNA arrays were based on the closest calculated expect value (*E*).

Gene Name	Symbol	*E*	GenBank Accession No.	Fold Change
Down-regulated genes				
Protein processing				
Ubiquitin specific protease 9, X chromosome	Usp9x	0	NM009481	−2.4
Tumor rejection antigen gp96 homologous to hsp90	Tra1	0	NM011631	−2.2
Acidic ribosomal phosphoprotein	PO	0	BC003833	−2.2
RNA binding protein		$1e^{-118}$	AF130350	−2.2
Neuronal signaling				
Control of post-synaptic Ca^{2+} signaling				
Copine 6	Cpne6	0	NM009947	−3.0
FK506 binding protein 8		$1e^{-180}$	BC003739	−2.6
Control of cell proliferation and differentiation				
Alternative splicing factor	ASF	$1e^{-139}$	X66091	−2.8
Protein tyrosine phosphatase, receptor-type, N	Ptprn	0	NM008985	−2.7
Kruppel-like factor 9	Klf9	0	NM010638	−2.4
Silent mating type information regulation 2	Sir2	0	NM022433	−2.2
Control of signal transduction				
Calcium/calmodulin-dependent protein kinase II, beta	Camk2b	$1e^{-166}$	NM007595	−2.8
Leucine-rich, glioma inactivated 1	Lgi1	0	NM020278	−2.9
A-raf, clone MGC:6964	Araf	$1e^{-63}$	BC004757	−2.4
Protein tyrosine phosphatase	PTPT9	0	D28530	−2.2
Control of transport of synaptic vesicles				
Small GTP-binding protein Rabphillin3C	Rab3C	0	AY026947	−2.5
Kinesin heavy chain member 1A	Kif1a	0	NM008440	−2.0
Neuronal growth/structure				
Neuron specific gene family member 1	Nsg1	$7e^{-98}$	BC008272	−3.1
Neuron specific gene family member 2	Nsg2	$1e^{-106}$	NM008741	−2.4
ADP ribosylation factor-like 2	Arl 2	$1e^{-109}$	BC002131	−2.2
Stress response				
Inflammation				
Small inducible cytokine subfamily D1	Scyd1	$1e^{-138}$	NM00914	−3.6
Transforming growth factor β1-induced transcript 4	Tgfb1i4	0	NM009366–2.2	−2.2
Apoptosis-induced gene				
Apoptosis-associated tyrosine kinase	Aatk	0	NM007377	−2.7
PTEN induced putative kinase 1	PINK1	0	AB053476	−2.2
Tumor-associated genes				
Tumor protein D52, clone MGC:5954	D52	0	BC002036	−2.2
Myelination/glial differentiation				
Mouse myelin proteolipid protein gene, exon 7	PLPM7	0	M37335	−3.3
APP-binding Protein	APPbP	0	AF206720	−3.0
Oligodendrocyte transcription factor 1	Olig1	0	AB038696	−2.8
Stearoyl-CoA A desaturase 2	Scd2	$1e^{-144}$	NM009128	−2.5
Metabolic enzymes				
Arsenite-translocating ATPase	ArsA	0	AF039405	−2.3
Others				
PH domain-containing protein in retina 1	Phr1	$1e^{-51}$	AF100613	−2.3
Monoglyceride lipase	Mgll	$9e^{-77}$	NM011844	−2.1
LDL receptor-related protein 1	Lrp1	0	NM008512	−3.0
Expressed sequence tags				
Highly similar to human protein synthesis initiation factor		$3e^{-45}$	AF104913	−2.5
Highly similar to rat calcium-binding protein		$2e^{-99}$	D14819	−2.3
Highly similar to human zinc finger protein		$1e^{-136}$	XM007221	−2.2
Similar to rat RNA-binding protein SiahBP	SiahBP	$1e^{-145}$	AF165892	−2.2
Up-regulated genes				
Metabolic enzymes				
Aspartyl-tRNA synthetase		$1e^{-112}$	BC008638	+2.5
Others				
Neuromedin	Nmu	$1e^{-150}$	AF203444	+2.3

PTEN, phosphatase and tensin homolog; APP, amyloid βA4 precursor; LDL, low-density lipoprotein.

this region, where they seem to mediate the appetite-stimulating effect of cannabinoids (Di Marzo et al., 2001; Jamshidi and Taylor, 2001). MRG1 is an isoform of the cyclic AMP response element binding protein (CREB)-binding protein CBP-interacting protein 35srj, which is induced by hypoxia-inducible factor-1 (HIF-1) as part of the transcriptional response to hypoxia. To confirm that Δ^9-THC and R(+)-WIN 55,212-2 enhanced GK and MRG1 protein expression, Western blotting was performed. As shown in Fig. 2, expression of GK and MRG1 was increased by 50 to 100% by both Δ^9-THC and R(+)-WIN 55,212-2. Because cannabinoids have a broad range of physiological actions that are likely to be mediated through different cell types and brain regions, the observation that particular proteins are induced by cannabinoid administration provides little information about their functional roles. To begin to address this issue, we investigated the cellular distribution of GK and MRG1 protein expression in brain sections from cannabinoid-treated mice, which were stained with antibodies against GK and MRG1. Fig. 3 shows that MRG1 protein expression was associated with cerebral

TABLE 2

Genes specifically modified in mouse brain 12 h after R(+)-WIN 55,212-2 administration

Expression levels are ratios of R(+)-WIN 55,212-2-treated versus control samples. Each mRNA sample group was hybridized to three different arrays, and fold-change values are average of the triplicate measurements. Matches of clone sequences from cDNA arrays were based on the closest calculated expect value (*E*).

Gene Name	Symbol	*E*	GenBank Accession No.	Fold Change
Down-regulated genes				
Protein processing				
Protein folding				
Chaperonin containing TCP-1β	Ccth	0	AB022160	−2.6
Ubiquitin-protein Ligase				
Neural precursor developmentally down-regulated 4b	Nedd4b	0	NM031881	−2.1
Neuronal signaling				
Synaptic endocytosis				
Protein kinase C and casein kinase substrate in neurons	Pacsin1	$1e^{-110}$	NM011861	−2.1
Control of cell proliferation and differentiation				
Protein tyrosine phosphatase receptor	Ptprn	0	NM008985	−2.1
Expressed sequence tags				
Clone MGC:7904		0	BC004732	−2.2
Highly similar to KIAA0893 protein		$2e^{-96}$	NM014969	−2.0
Clone MGC:7410		0	BC003968	−3.0
Up-regulated genes				
Protein processing				
Protein folding				
Heat shock 27 kDa protein 3	Hspb3	0	NM019960	+2.4
Neuronal signaling				
Control of cell proliferation and differentiation				
Cyclin-dependent protein kinase 1	PFTK1	$4e^{-40}$	NM012395	+2.0
Endothelial differentiation G-protein receptor 3	Edg3	$1e^{-157}$	NM10101	+2.2
Control of signal transduction				
TNF receptor-associated factor 5	Traf5	0	NM011633	+2.2
Neuronal growth/structure				
Netrin 1	Ntn1	0	NM00874	+2.2
Microtubule-associated protein tau	Mapt	$1e^{-105}$	NM010838	+2.6
Stress response				
DNA damage-induced apoptosis				
Myelocytomatosis oncogene	Myc	0	NM010849	+2.1
DNA replication and repair				
Replication factor C, 140 kDa	Recc1	0	NM011258	+2.9
Others				
Down syndrome critical region a, clone MGC:5833		0	BC003740	+2.7

TNF, tumor necrosis factor; TCP-1β, t-complex polypeptide-1β.

TABLE 3

Genes modified in mouse brain 12 h after both Δ^9-THC and R(+)-WIN 55,212-2 administration

Expression levels are ratios of R(+)-WIN 55,212-2- or Δ^9-THC-treated versus control samples. Each mRNA sample group was hybridized to three different arrays, and fold-change values are average of the triplicate measurements. Matches of clone sequences from cDNA arrays were based on the closest calculated expect value (*E*).

Gene Name	Symbol	*E*	GenBank Accession No.	Fold change (WIN)	Fold change (THC)
Down-regulated genes					
Myelination/glial differentiation					
ATP-binding cassette subfamily A, member 2	ABC2	e^{-152}	X75927	−2.1	−3.1
Neuronal signaling					
Control of signal transduction					
Protein phosphatase 2A, α isoform	Ppp2r1a	e^{-114}	NM016891	−2.2	−2.9
Embryonal Fyn-associated substrate	Efyn	e^{-133}	BC005438	−2.1	−2.3
Prostaglandin D synthetase	PGD	0	X89222	−2.1	−2.1
Control of cell proliferation and differentiation					
Survival motor neuron	SMN	e^{-162}	Y12835	−2.1	−2.9
Mortality factor-regulator gene 15	MRG15	e^{-153}	AB042241	−2.2	−2.9
N-myc downstream-regulated gene 4	NDRG4	e^{-108}	BC006595	−2.6	−2.8
Reticulon 3	RTN3	e^{-152}	AF195940	−2.2	−2.6
Neuronal growth/structure					
Microtubule-associated protein 2	Mtap2	e^{-126}	NM008632	−2.2	−3.1
Protease nexin-1	PN-1	e^{-137}	NM009255	−2.1	−2.5
Neuronal protein 25	Np25	e^{-107}	NM019754	−2.2	−2.4
Expressed sequence tags					
Clone MGC:6545		e^{-153}	BC003451	−2.1	−2.3
Up-regulated genes					
Metabolic enzymes					
Glucokinase	GK	e^{-153}	L38990	+2.0	+2.0
Neuronal signaling					
Control of signal transduction					
Gioblastoma-expressed ring finger protein	GERP	e^{-166}	AF281047	+2.4	+2.3
Transcription factor					
Melanocyte-specific gene-related gene 1	MRG1	0	Y15163	+2.3	+2.0
Stress Response					
Apoptosis-induced gene					
P53 apoptosis effector related to Pmp22	Perp	0	NM022032	+2.8	+2.2
Expressed sequence tags					
Clone MGC:12127		0	BC006765	+2.7	+2.2

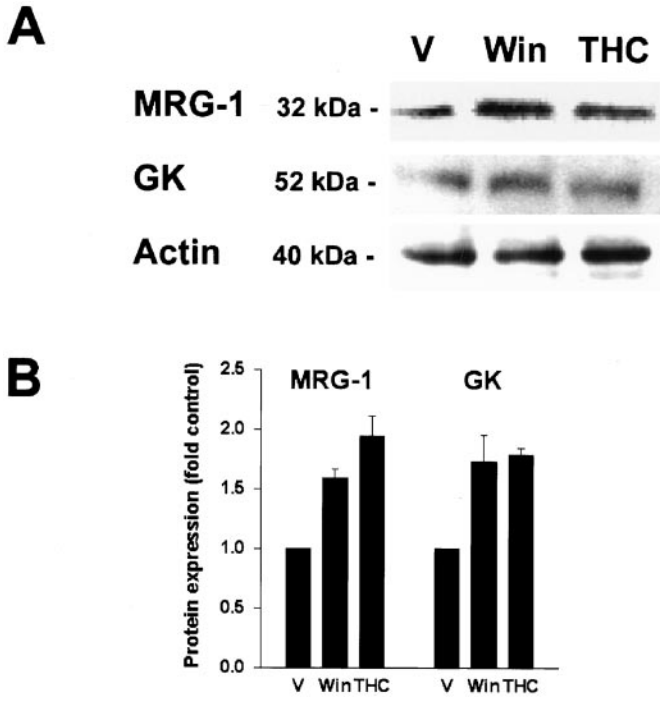

Fig. 2. Western analysis of cannabinoid-induced changes in the expression of MRG1 and GK proteins in rat brain. A, Western blots show MRG1, GK, and actin immunoreactivity after treatment with vehicle (V), R(+)-WIN 55,212-2 (Win), or Δ^9THC (THC). B, bar graphs show the relative optical density of the MRG1 and GK bands under each treatment condition. Values were normalized to the optical density of the actin band and were expressed as a percentage of optical density in vehicle-treated mice. Data shown are means ± S.D. from three experiments.

blood vessels (20–30% of vessels were MRG1-immunopositive), whereas GK protein expression was localized most abundantly to hypothalamic neurons, suggesting that induction of MRG1 and GK may be involved in the vascular (e.g., hypotensive) and hypothalamic (e.g., appetite-stimulating) effects of cannabinoids.

Discussion

The major finding of this study is that the cannabinoids Δ^9-THC and R(+)-WIN 55,212-2 induce distinct but overlapping transcriptional responses in mouse brain, measured 12 h after a single systemic dose of either drug. The overlapping responses are likely to reflect effects of cannabinoid receptor activation. CB1 is the predominant established cannabinoid receptor subtype in the brain and can bind both Δ^9-THC and R(+)-WIN 55,212-2, so transcriptional responses shared by these drugs are likely to be triggered by CB1 activation. Non-CB1/non-CB2 ("CB3") cannabinoid receptors are also thought to exist in brain (Breivogel et al., 2001) but are insensitive to Δ^9-THC. Therefore, although some or all of the genes induced only by R(+)-WIN 55,212-2 in our study might be induced through CB3 receptors, those genes induced by Δ^9-THC alone or by both Δ^9-THC and R(+)-WIN 55,212-2 are presumably not.

In a previously published study of Δ^9-THC -regulated gene expression (Kittler et al., 2000), the genes identified were different from what we observed. There are several explanations for the discrepancy, including differences in species (mouse versus rat), brain region studied (entire forebrain versus hippocampus), time between drug administration and

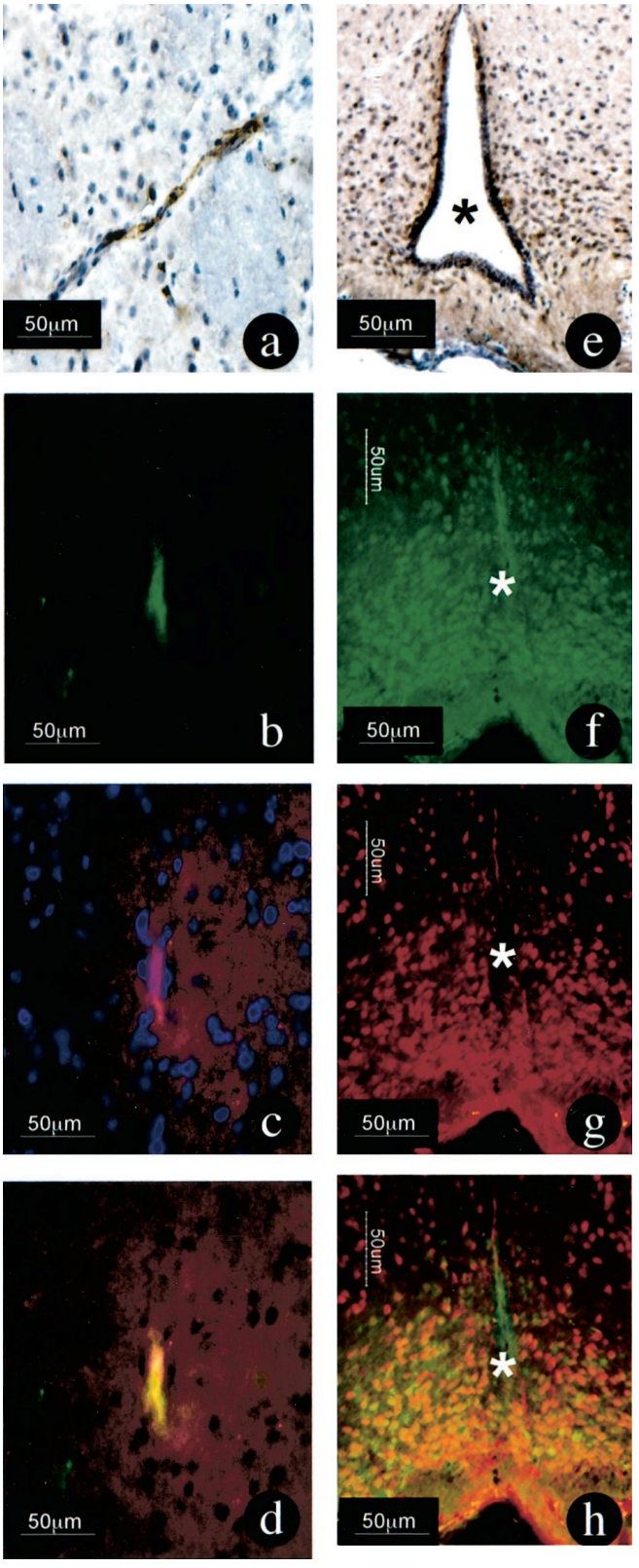

Fig. 3. Immunohistochemical localization of MRG1 (a-d) and GK (e-h) in the brains of cannabinoid-treated mice. MRG1 was localized to blood vessels (a, brown), and MRG1 (b, green) and the endothelial cell marker CD-31 (c, red) were colocalized (d, yellow); nuclei are stained with 4,6-diamidino-2-phenylindole (c, blue). GK was localized preferentially to the hypothalamus (e, brown), and GK (f, green) and the neuronal marker NeuN (g, red) were colocalized (h, yellow). Asterisks in e-h indicate the third ventricle. Data shown are representative fields from three experiments that gave similar results.

sacrifice (12 h versus 24 h to 21 days) and in the genes present on the different arrays (the Kittler study included approximately twice as many genes). We chose our conditions based on an interest in what genes might account for neuroprotective effects of cannabinoids in focal cerebral ischemia from middle cerebral artery occlusion (Nagayama et al., 1999), which affect the forebrain prominently and occur during the first 24 h after onset. In contrast, Kittler et al. were interested in genes involved in tolerance to cannabinoids (Kittler et al., 2000). A microarray study of genes induced by the cannabinoid agonist CP 55,940 in HL-60 promyelocytic cells transfected with the CB2 receptor also showed no overlap with the genes induced in our study (Derocq et al., 2000).

Cannabinoids affect a variety of tissues, which helps to account for the diversity of their actions. Central nervous system effects on motor activity, memory, and pain are related to interaction with neuronal CB1 receptors; hypotension seems to be mediated through vascular CB1 receptors; and CB2 receptors on mono- and polymorphonuclear leukocytes and macrophages have been implicated in immunosuppressive and anti-inflammatory actions. One prominent central effect of cannabinoids is the stimulation of appetite, which may underlie the reported therapeutic value of the cannabinoid drug dronabinol in the AIDS wasting syndrome (Robson, 2001). Intrahypothalamic injection of the endocannabinoid anadamide produces hyperphagia in rats that is blocked by the CB1 antagonist SR141716A, consistent with an effect mediated through CB1 receptors (Jamshidi and Taylor, 2001). SR141716A also reduces food intake in wild-type but not CB1 knockout mice, and the anorexigenic hormone leptin reduces hippocampal levels of two endocannabinoids—anandamide and 2-arachidonoyl glycerol (Di Marzo et al., 2001). Our finding that Δ^9-THC and R(+)-WIN 55,212-2 increase GK protein expression in hypothalamus is of interest in this regard, considering that GK may have an important role in glucose sensing and metabolic regulation in the brain (Roncero et al., 2000; Alvarez et al., 2002).

Cerebrovascular effects of cannabinoids have also been observed, and these seem to be mediated through CB1 receptors on vascular smooth muscle and endothelial cells (Hillard, 2000). Cannabinoids are neuroprotective in conditions, such as cerebral ischemia (Nagayama et al., 1999) and trauma (Panikashvili et al., 2001), in which vascular factors play an important role, and a vascular component to cannabinoid-induced neuroprotection has been proposed (Panikashvili et al., 2001). Our finding that cannabinoids induce different genes in neurons (e.g., *GK*) and in blood vessels (e.g., *MRG1*) suggests that the molecular basis for vascularly and neuronally mediated neuroprotection may be different. More generally, if cannabinoids activate different signaling pathways in different types of cells, therapeutic approaches that target such downstream events may be capable of dissociating desirable from undesirable effects of cannabinoids.

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References

Alvarez E, Roncero I, Chowen JA, Vazquez P, and Blazquez E (2002) Evidence that glucokinase regulatory protein is expressed and interacts with glucokinase in rat brain. *J Neurochem* **80:**45–53.

Anan T, Nagata Y, Koga H, Honda Y, Yabuki N, Miyamoto C, Kuwano A, Matsuda I, Endo F, Saya H, et al. (1998) Human ubiquitin-protein ligase Nedd4: expression, subcellular localization and selective interaction with ubiquitin-conjugating enzymes. *Genes Cells* **3:**751–763.

Bhattacharya S, Michels CL, Leung MK, Arany ZP, Kung AL, and Livingston DM (1999) Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes Dev* **13:**64–75.

Bouaboula M, Poinot-Chazel C, Bourrie B, Canat X, Calandra B, Rinaldi-Carmona M, Le Fur G, and Casellas P (1995) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1. *Biochem J* **312:**637–641.

Breivogel CS, Griffin G, Di Marzo V, and Martin BR (2001) Evidence for a new G protein-coupled cannabinoid receptor in mouse brain. *Mol Pharmacol* **60:**155–163.

Campbell VA (2001) Tetrahydrocannabinol-induced apoptosis of cultured cortical neurones is associated with cytochrome c release and caspase-3 activation. *Neuropharmacology* **40:**702–709.

Chemin J, Monteil A, Perez-Reyes E, Nargeot J, and Lory P (2001) Direct inhibition of T-type calcium channels by the endogenous cannabinoid anandamide. *EMBO (Eur Mol Biol Organ) J* **20:**7033–7040.

Courtney MJ and Coffey ET (1999) The mechanism of Ara-C-induced apoptosis of differentiating cerebellar granule neurons. *Eur J Neurosci* **11:**1073–1084.

Currie RW, Ellison JA, White RF, Feuerstein GZ, Wang X, and Barone FC (2000) Benign focal ischemic preconditioning induces neuronal Hsp70 and prolonged astrogliosis with expression of Hsp27. *Brain Res* **863:**169–181.

Derocq JM, Jbilo O, Bouaboula M, Segui M, Clere C and Casellas P (2000) Genomic and functional changes induced by the activation of the peripheral cannabinoid receptor CB2 in the promyelocytic cells HL-60. Possible involvement of the CB2 receptor in cell differentiation. *J Biol Chem* **275:**15621–15628.

Devane WA, Dysarz FA, 3rd, Johnson MR, Melvin LS and Howlett AC (1988) Determination and characterization of a cannabinoid receptor in rat brain. *Mol Pharmacol* **34:**605–613.

Devane WA, Hanus L, Breuer A, Pertwee RG, Stevenson LA, Griffin G, Gibson D, Mandelbaum A, Etinger A, and Mechoulam R (1992) Isolation and structure of a brain constituent that binds to the cannabinoid receptor. *Science (Wash DC)* **258:**1946–1949.

Di Marzo V, Goparaju SK, Wang L, Liu J, Batkai S, Jarai Z, Fezza F, Miura GI, Palmiter RD, Sugiura T, et al. (2001) Leptin-regulated endocannabinoids are involved in maintaining food intake. *Nature (Lond)* **410:**822–825.

Gomez del Pulgar T, Velasco G, and Guzman M (2000) The CB1 cannabinoid receptor is coupled to the activation of protein kinase B/Akt. *Biochem J* **347:**369–373.

Greenberg SA (2001) DNA microarray gene expression analysis technology and its application to neurological disorders. *Neurology* **57:**755–761.

Hajos N, Ledent C, and Freund TF (2001) Novel cannabinoid-sensitive receptor mediates inhibition of glutamatergic synaptic transmission in the hippocampus. *Neuroscience* **106:**1–4.

Hillard CJ (2000) Endocannabinoids and vascular function. *J Pharmacol Exp Ther* **294:**27–32.

Howard AD, Wang R, Pong SS, Mellin TN, Strack A, Guan XM, Zeng Z, Williams DL Jr, Feighner SD, Nunes CN, et al. (2000) Identification of receptors for neuromedin U and its role in feeding. *Nature (Lond)* **406:**70–74.

Jamshidi N and Taylor DA (2001) Anandamide administration into the ventromedial hypothalamus stimulates appetite in rats. *Br J Pharmacol* **134,** 1151–1154.

Jarai Z, Wagner JA, Varga K, Lake KD, Compton DR, Martin BR, Zimmer AM, Bonner TI, Buckley NE, Mezey E, et al. (1999) Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc Natl Acad Sci USA* **96:**14136–41.

Kittler JT, Grigorenko EV, Clayton C, Zhuang SY, Bundey SC, Trower MM, Wallace D, Hampson R, and Deadwyler S (2000) Large-scale analysis of gene expression changes during acute and chronic exposure to Δ^9-THC in rats. *Physiol Genomics* **3:**175–185.

Munro S, Thomas KL, and Abu-Shaar M (1993) Molecular characterization of a peripheral receptor for cannabinoids. *Nature (Lond)* **365:**61–65.

Nagayama T, Sinor AD, Simon RP, Chen J, Graham S, Jin K, and Greenberg DA (1999) Cannabinoids and neuroprotection from global and focal cerebral ischemia and in vitro. *J Neurosci* **19:**2987–2995.

Nakayama T, Yaoi T, Yasui M, and Kuwajima G (1998) N-copine: a novel two C2-domain-containing protein with neuronal activity-regulated expression. *FEBS Lett* **428:**80–84.

Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, Vath J, Gosselin M, Ma J, Dussault B, et al. (1997) Neurotactin, a membrane-anchored chemokine upregulated in brain inflammation. *Nature (Lond)* **387:**611–617.

Panikashvili D, Simeonidou C, Ben-Shabat S, Hanus L, Breuer A, Mechoulam R, and Shohami E (2001) An endogenous cannabinoid (2-AG) is neuroprotective after brain injury. *Nature (Lond)* **413:**527–531.

Pennaneach V, Salles-Passador I, Munshi A, Brickner H, Regazzoni K, Dick F, Dyson N, Chen TT, Wang JY, Fotedar R, et al. (2001) The large subunit of replication factor C promotes cell survival after DNA damage in an LxCxE motif- and Rb-dependent manner. *Mol Cell* **7:**715–727.

Pertwee RG (1997) Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther* **74:**129–180.

Robson P (2001) Therapeutic aspects of cannabis and cannabinoids. *Br J Psychiatry* **178:**107–115.

Roncero I, Alvarez E, Vazquez P, and Blazquez E (2000) Functional glucokinase isoforms are expressed in rat brain. *J Neurochem* **74:**1848–1857.

Sanchez C, Rueda D, Segui B, Galve-Roperh I, Levade T, and Guzman M (2001) The CB(1) cannabinoid receptor of astrocytes is coupled to sphingomyelin hydrolysis through the adaptor protein fan. *Mol Pharmacol* **59:**955–959.

Shen M and Thayer SA (1998) Cannabinoid receptor agonists protect cultured rat hippocampal neurons from excitotoxicity. *Mol Pharmacol* **54:**459–462.

Sinor AD, Irvin SM, and Greenberg DA (2000) Endocannabinoids protect cerebral cortical neurons from in vitro ischemia in rats. *Neurosci Lett* **278:**157–160.

Stella N, Schweitzer P, and Piomelli D (1997) A second endogenous cannabinoid that modulates long-term potentiation. *Nature (Lond)* **388:**773–778.
Sun HB, Zhu YX, Yin T, Sledge G, and Yang YC (1998) MRG1, the product of a melanocyte-specific gene related gene, is a cytokine-inducible transcription factor with transformation activity. *Proc Natl Acad Sci USA* **95:**13555–60.
Thibault C, Lai C, Wilke N, Duong B, Olive MF, Rahman S, Dong H, Hodge CW, Lockhart DJ, and Miles MF (2000) Expression profiling of neural cells reveals specific patterns of ethanol-responsive gene expression. *Mol Pharmacol* **58:**1593–1600**.**
Twitchell W, Brown S, and Mackie K (1997) Cannabinoids inhibit N- and P/Q-type calcium channels in cultured rat hippocampal neurons. *J Neurophysiol* **78:**43–50.
Venance L, Piomelli D, Glowinski J, and Glaume C (1995) Inhibition by anandamide of gap junctions and intercellular calcium signalling in striatal astrocytes. *Nature (Lond)* **376:**590–594.
Wilson RI, Kunos G, and Nicoll RA (2001) Presynaptic specificity of endocannabinoid signaling in the hippocampus. *Neuron* **31:**453–462.
Zygmunt PM, Petersson J, Andersson DA, Chuang H, Sørgård M, Di Marzo V, Julius D, and Högestätt ED (1999) Vanilloid receptors on sensory nerves mediate the vasodilator action of anandamide. *Nature (Lond)* **400:**452–457.

Address correspondence to: David A. Greenberg, M.D., Ph.D., Buck Institute for Age Research, 8001 Redwood Boulevard, Novato, CA 94945. E-mail: dgreenberg@buckinstitute.org