ORIGINAL ARTICLE

Reduced Cortical Cannabinoid 1 Receptor Messenger RNA and Protein Expression in Schizophrenia

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Context: Cannabis use is associated with both impaired cognitive functions, including working memory, and an increased risk of schizophrenia. Schizophrenia is characterized by impairments in working memory that are associated with reduced γ-aminobutyric acid (GABA) neurotransmission in the dorsolateral prefrontal cortex. The cannabinoid 1 receptor (CB1R) is highly expressed in the dorsolateral prefrontal cortex, is contained in the axon terminals of a subpopulation of perisomatic-targeting GABA neurons, and, when activated, suppresses the release of GABA.

Objective: To determine the potential relationship between CB1R signaling and altered GABA neurotransmission in schizophrenia by evaluating CB1R messenger RNA (mRNA) and protein expression in the dorsolateral prefrontal cortex.

Design: In situ hybridization and immunocytochemistry techniques were used to examine the cortical levels of CB1R mRNA and protein, respectively.

Setting: Brain specimens were obtained from autopsies conducted at the Allegheny County Medical Examiner's Office, Pittsburgh, Pennsylvania.

Participants: Postmortem brain specimens from 23 pairs of subjects with schizophrenia and age-, sex-, and postmortem interval–matched comparison subjects, as well as brain specimens from 18 macaque monkeys with long-term exposure to haloperidol, olanzapine, or placebo.

Main Outcome Measures: Optical density measures of CB1R mRNA expression and protein levels and correlations with previously reported glutamic acid decarboxylase 67 and cholecystokinin mRNA measures.

Results: Levels of CB1R mRNA were significantly lower by 14.8% in the subjects with schizophrenia. Similarly, CB1R protein levels, assessed by radioimmunocytochemistry and standard immunocytochemistry, were significantly decreased by 11.6% and 13.9%, respectively. Group differences in CB1R mRNA levels were significantly correlated with those in glutamic acid decarboxylase 67 and cholecystokinin mRNA levels. Expression of CB1R mRNA was not changed in antipsychotic-exposed monkeys, and neither CB1R mRNA levels nor protein levels were affected by potential confounding factors in the subjects with schizophrenia.

Conclusions: This combination of findings suggests the testable hypothesis that reduced CB1R mRNA and protein levels in schizophrenia represent a compensatory mechanism to increase GABA transmission from perisomatic-targeting cholecystokinin interneurons with impaired GABA synthesis.

Arch Gen Psychiatry. 2008;65(7):772-784

A GROWING BODY OF EVIDENCE suggests that alterations in cannabinoid signaling in the brain contribute to the pathophysiological findings of schizophrenia. For instance, markers of endogenous cannabinoids[1-3] and the principal cannabinoid receptor in the brain (cannabinoid 1 receptor [CB1R])[4-6] have been reported to be altered in schizophrenia. Convergent findings from epidemiological studies suggest that cannabis use represents a substantial environmental risk factor for schizophrenia, especially when exposure occurs during adolescence.[7,8] Cannabis use is also associated with a poorer outcome, more frequent hospitalizations, and increased severity of symptoms, especially cognitive impairments, in individuals with schizophrenia.[9-12] Finally, long-term users of cannabis exhibit deficits in cognitive functions, such as working memory,[13] that are impaired in schizophrenia.[14]

Working memory processes depend on the circuitry of the dorsolateral prefrontal cortex (DLPFC), and alterations in this brain region appear to contribute to working memory impairments in schizophrenia.[15] In particular, γ-aminobutyric acid (GABA) neurotransmission within the DLPFC is critical for normal working memory function,[16,17] and reductions in markers of GABA neurotransmission have been consistently identified in the DLPFC of subjects with schizophrenia.[18] Interestingly, the CB1R is highly expressed in the primate DLPFC and

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Table 1. Subject Characteristics

Pair No. and Subject	Case No.	Sex/ Race/ Age, y	PMI, h	RIN	pH	Storage Time, No. of Months at –80°C	Cause of Death	*DSM-IV* Diagnosis	Cannabis Use[a]	Antipsychotic Medication[b]
1[c]										
Comparison	592	M/B/41	22.1	9.0	6.7	120.3	ASCVD	. . .	. . .	. . .
Schizophrenia	533	M/W/40	29.1	8.4	6.8	130.1	Accidental asphyxiation	US	No	Typical
2[c]										
Comparison	567	F/W/46	15.0	8.9	6.7	124.3	Mitral valve prolapse	. . .	. . .	. . .
Schizophrenia	537	F/W/37	14.5	8.6	6.7	129.4	Suicide by hanging	SA	No	None
3[c]										
Comparison	516	M/B/20	14.0	8.4	6.9	131.9	Homicide by gun shot	. . .	. . .	. . .
Schizophrenia	547	M/B/27	16.5	7.4	7.0	128.0	Heat stroke	SA	No	Typical
4[c]										
Comparison	630	M/W/65	21.2	9.0	7.0	114.4	ASCVD	. . .	. . .	. . .
Schizophrenia	566	M/W/63	18.3	8.0	6.8	124.7	ASCVD	US[d]	No	Atypical
5[c]										
Comparison	604	M/W/39	19.3	8.6	7.1	118.0	Hypoplastic coronary artery	. . .	. . .	. . .
Schizophrenia	581	M/W/46	28.1	7.9	7.2	122.5	Accidental combined drug overdose	PS[e,f]	Yes	Typical
6[c]										
Comparison	546	F/W/37	23.5	8.6	6.7	128.3	ASCVD	. . .	. . .	. . .
Schizophrenia	587	F/B/38	17.8	9.0	7.0	121.1	Myocardial hypertrophy	US[d]	Yes	Both
7[c]										
Comparison	551	M/W/61	16.4	8.3	6.6	127.1	Cardiac tamponade	. . .	. . .	. . .
Schizophrenia	625	M/B/49	23.5	7.6	7.3	115.0	ASCVD	DS[g]	No	Typical
8[c]										
Comparison	685	M/W/56	14.5	8.1	6.6	107.4	Hypoplastic coronary artery	. . .	. . .	. . .
Schizophrenia	622	M/W/58	18.9	7.4	6.8	115.2	Right MCA infarction	US	No	None
9[c]										
Comparison	681	M/W/51	11.6	8.9	7.2	108.0	Hypertrophic cardiomyopathy	. . .	. . .	. . .
Schizophrenia	640	M/W/49	5.2	8.4	6.9	113.1	Pulmonary embolism	PS	No	Atypical
10										
Comparison	806	M/W/57	24.0	7.8	6.9	86.5	Pulmonary thromboembolism	. . .	. . .	. . .
Schizophrenia	665	M/B/59	28.1	9.2	6.9	110.6	Intestinal hemorrhage	PS[e]	No	Typical
11										
Comparison	822	M/B/28	25.3	8.5	7.0	83.9	ASCVD	. . .	. . .	. . .
Schizophrenia	787	M/B/27	19.2	8.4	6.7	90.1	Suicide by gunshot	SA[h]	Yes	Typical
12[c]										
Comparison	727	M/B/19	7.0	9.2	7.2	101.0	Trauma	. . .	. . .	. . .
Schizophrenia	829	M/W/25	5.0	9.3	6.8	81.8	Suicide by drug overdose	SA[e,i,j]	Yes	None
13										
Comparison	871	M/W/28	16.5	8.5	7.1	73.3	Trauma	. . .	. . .	. . .
Schizophrenia	878	M/W/33	10.8	8.9	6.7	72.3	Myocardial fibrosis	DS[e]	Yes	Atypical
14[c]										
Comparison	575	F/B/55	11.3	9.6	6.8	123.0	ASCVD	. . .	. . .	. . .
Schizophrenia	517	F/W/48	3.7	9.3	6.7	131.7	Intracerebral hemorrhage	DS[e]	No	Atypical

(continued)

is contained in the axon terminals of a subpopulation of GABA interneurons that express the neuropeptide cholecystokinin (CCK) and that furnish perisomatic inputs to pyramidal neurons.[19,20] Activation of CB1Rs suppresses the release of GABA and reduces inhibitory postsynaptic currents.[21] Thus, CB1Rs play an important role in regulating network activity patterns by controlling proximal inhibitory input to pyramidal neurons.

In concert, the evidence that endocannabinoid signaling is altered in schizophrenia, that cannabis use is a risk factor for schizophrenia, that cannabis use impairs working memory function, and that the CB1R modulates GABA neurotransmission suggests that altered expression of CB1Rs could contribute to the pathophysiological findings of DLPFC dysfunction in schizophrenia. To test this hypothesis, we used in situ hybridization and immunocytochemistry techniques to (1) assess the expression of CB1R messenger RNA (mRNA) and protein in the DLPFC of subjects with schizophrenia; (2) examine the relationship between these measures and markers of GABA neurotransmission in schizophrenia; and (3) determine the effects of potential confounds on the measures of CB1Rs.

METHODS

HUMAN SUBJECTS

Brain specimens from 23 subjects with schizophrenia and 23 healthy comparison subjects were obtained from autopsies conducted at the Allegheny County Medical Examiner's Office, Pittsburgh, Pennsylvania, following consent for brain donation from the next of kin and using procedures approved by the Univer-

Table 1. Subject Characteristics (cont)

Pair No. and Subject	Case No.	Sex/ Race/ Age, y	PMI, h	RIN	pH	Storage Time, No. of Months at –80°C	Cause of Death	*DSM-IV* Diagnosis	Cannabis Use[a]	Antipsychotic Medication[b]
15										
Comparison	700	M/W/42	26.1	8.7	7.0	105.1	ASCVD	. . .	. . .	. . .
Schizophrenia	539	M/W/50	40.5	8.1	7.1	129.2	Suicide by combined drug overdose	SA[k]	No	Atypical
16										
Comparison	988	M/W/82	22.5	8.4	6.2	51.9	Trauma	. . .	. . .	. . .
Schizophrenia	621	M/W/83	16.0	8.7	7.3	115.5	Accidental asphyxiation	US	No	None
17										
Comparison	686	F/W/52	22.6	8.5	7.0	107.1	ASCVD	. . .	. . .	. . .
Schizophrenia	656	F/B/47	20.1	9.2	7.3	111.3	Suicide by gunshot	SA[e]	No	Atypical
18										
Comparison	634	M/W/52	16.2	8.5	7.0	113.8	ASCVD	. . .	. . .	. . .
Schizophrenia	722	M/B/45	9.1	9.2	6.7	101.4	Upper GI tract bleeding	US[i,l]	Yes	Typical
19										
Comparison	852	M/W/54	8.0	9.1	6.8	76.3	Cardiac tamponade	. . .	. . .	. . .
Schizophrenia	781	M/B/52	8.0	7.7	6.7	91.3	Peritonitis	SA[k]	No	Typical
20[c]										
Comparison	987[m]	F/W/65	21.5	9.1	6.8	51.9	ASCVD	. . .	. . .	. . .
Schizophrenia	802	F/W/63	29.0	9.2	6.4	87.1	Right ventricular dysplasia	SA[e,l]	No	Both
21										
Comparison	818	F/W/67	24.0	8.4	7.1	85.0	Anaphylactic reaction	. . .	. . .	. . .
Schizophrenia	917	F/W/71	23.8	7.0	6.8	65.1	ASCVD	US	No	Typical
22										
Comparison	857	M/W/48	16.6	8.9	6.7	75.1	ASCVD	. . .	. . .	. . .
Schizophrenia	930	M/W/47	15.3	8.2	6.2	61.7	ASCVD	DS[j,k]	Yes	Typical
23										
Comparison	739	M/W/40	15.8	8.4	6.9	100.1	ASCVD	. . .	. . .	. . .
Schizophrenia	933	M/W/44	8.3	8.1	5.9	61.1	Myocarditis	DS	No	Atypical

Abbreviations: ASCVD, arteriosclerotic cardiovascular disease; B, black; DS, disorganized schizophrenia; F, female; GI, gastrointestinal; M, male; MCA, middle cerebral artery; PMI, postmortem interval; PS, paranoid schizophrenia; RIN, RNA integrity number; SA, schizoaffective disorder; W, white; US, undifferentiated schizophrenia; ellipses, not applicable.

[a] No comparison subjects had a history of cannabis use.
[b] Prescribed antipsychotic medications at the time of death.
[c] Subject pairs used in immunocytochemistry experiments.
[d] Alcohol abuse, in remission at the time of death.
[e] Alcohol dependence, current at the time of death.
[f] Other substance abuse, current at the time of death.
[g] Alcohol abuse, current at the time of death.
[h] Other substance dependence, current at the time of death.
[i] Cocaine abuse, in remission at the time of death.
[j] Other substance abuse, in remission at the time of death.
[k] Alcohol dependence, in remission at the time of death.
[l] Other substance dependence, in remission at the time of death.
[m] History of posttraumatic stress disorder, in remission for 39 years at the time of death.

sity of Pittsburgh's Committee for Research Involving the Dead and Institutional Review Board for Biomedical Research. Each subject with schizophrenia was matched for sex and, as closely as possible, for age and postmortem interval with 1 comparison subject (**Table 1**) (for details, see the supplemental methods available at http://www.archgenpsychiatry.com). Pairing for these variables was performed to control experimental variance and to reduce biological variance. Subject groups did not differ in mean age, postmortem interval, RNA integrity number (RIN), brain pH, or tissue storage time (**Table 2**).

IN SITU HYBRIDIZATION

For each subject, coronal blocks through the right prefrontal cortex were frozen and stored at –80°C.[22] Cryostat sections from the middle portion of the superior frontal sulcus were collected into tubes containing Trizol (Invitrogen Corp, Carlsbad, California) and were homogenized. Total RNA was subsequently isolated from homogenates and was purified by RNeasy columns (Qiagen Inc, Valencia, California), and the RIN was assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Inc, Santa Clara, California) according to the manufacturer's protocol as previously described.[23] Other sections (20 µm) were thaw mounted on Superfrost slides (VWR Scientific, West Chester, Pennsylvania) and stored at –80°C until processed. Cytoarchitectonic criteria[24] were used to identify the location of DLPFC area 9 in Nissl-stained sections.[22,25] For each subject within a pair, 3 sections separated by at least 320 µm were chosen and sections with the same rostral-caudal level were paired. One pair of sections from each subject pair was processed side by side in an in situ hybridization run.

Templates for the synthesis of riboprobes against human CB1R mRNA were generated by polymerase chain reaction. A 714–base pair (bp) fragment corresponding to bases 435 to 1148 of the human *CNR1* gene (GenBank NM_033181) was amplified with specific primer sets. Nucleotide sequencing revealed 100% homology for the amplified fragment to a previously reported sequence. Sense and antisense riboprobes were gener-

Table 2. Summary of Subject Characteristics

Parameter	Comparison Subjects	Subjects With Schizophrenia	*t* Test Score[a]	*P* Value
Sex, M/F, No.	17/6	17/6		
Race, W/B, No.	18/5	15/8		
Age, mean (SD), y	48.0 (15.5)	47.9 (14.1)	0.16	.88
PMI, mean (SD), h	18.0 (5.5)	17.8 (9.3)	0.22	.83
Brain pH, mean (SD)	6.9 (0.2)	6.8 (0.3)	0.62	.54
RIN, mean (SD)	8.7 (0.4)	8.4 (0.7)	1.84	.08
Storage time, mean (SD), No. of months at –80°C	100.6 (23.5)	104.8 (23.5)	–0.96	.35

Abbreviations: B, black; F, female; M, male; PMI, postmortem interval; RIN, RNA integrity number; W, white.
[a]The *df* is 22.

ated by in vitro transcription, purified, and reduced to approximately 100 bp by alkaline hydrolysis to increase tissue penetration.[23,26,27] Hybridization procedures were performed as previously described (see supplemental methods for details).[26]

RADIOIMMUNOCYTOCHEMISTRY

Tissue sections adjacent to those processed for in situ hybridization were immersed in paraformaldehyde, 4%, diluted in 0.1M phosphate-buffered saline (pH 7.4) for 1 hour, washed in 0.01M phosphate-buffered saline, and incubated in a blocking solution containing Triton X, 0.3% (Sigma-Aldrich, St Louis, Missouri), normal donkey and normal human sera, 4% (Jackson ImmunoResearch Laboratories, Inc, West Grove, Pennsylvania), and bovine serum albumin, 1% (Jackson ImmunoResearch Laboratories, Inc) in phosphate-buffered saline for 1 hour to reduce nonspecific binding. Slides were then placed in humidified boxes, and approximately 300 µL of blocking solution containing an affinity-purified polyclonal rabbit anti-CB1R antibody raised against the last 15 amino acid residues of the C terminus of the rat CB1R (anti-CB1R-L15; diluted 1:5000; kindly provided by Ken Mackie, MD, Indiana University, Bloomington) was pipetted onto each section. The specificity of this antibody has been previously demonstrated by Western blot analysis, preadsorption studies, and testing in knockout animals.[20] Sections were incubated for 48 hours at 4°C, washed, and incubated with approximately 300 µL of secondary antibody solution containing a sulfur 35–labeled donkey anti-rabbit IgG secondary antibody (0.5 µCi/mL; GE Healthcare Bio-Sciences Corp, Piscataway, New Jersey), Triton X, 0.3%, and normal donkey and normal human serum samples, 4%, in phosphate-buffered saline for 2 hours at room temperature. Sections were then washed, dried, and exposed to BioMax MR film (Eastman Kodak, Rochester, New York) for 3 days. Two radioimmunocytochemistry runs were performed, with 1 section from a given pair processed side by side in a single run.

IMMUNOCYTOCHEMISTRY

The fresh left hemisphere of 12 subject pairs (Table 1) was cut into 1.0-cm coronal blocks and immersed in phosphate-buffered (0.1M; pH 7.4) paraformaldehyde, 4%, for 48 hours at 4°C, cryoprotected, and stored at –30°C.[20] Coronal tissue blocks containing DLPFC area 9 were serially sectioned at 40 µm on a cryostat. For each subject pair, 2 sections separated by at least 400 µm were chosen as described earlier.

Free-floating tissue sections were processed for CB1R immunoreactivity using a previously described protocol[20] except that tissue sections were pretreated with hydrogen peroxide, 1%, for 15 minutes to remove endogenous peroxidase activity; the anti-CB1R-L15 antibody was diluted at 1:6000. Four immunocytochemistry runs were performed, with 1 section from a given pair processed side by side in a single run.

ANTIPSYCHOTIC-TREATED MONKEYS

To evaluate the effects of long-term exposure to antipsychotic medications on CB1R mRNA expression levels, 3 groups (n=6 per group) of young adult, male, macaque monkeys (*Macaca fascicularis*) were exposed for 17 to 27 months to oral haloperidol, olanzapine, or placebo at doses that produced trough plasma levels (approximately 1.5 ng/mL for haloperidol and approximately 15 ng/mL for olanzapine) in the therapeutic range for the treatment of schizophrenia.[23,28] Animals were euthanized in triads and tissue was processed as described previously.[23,28] For each triad, 2 sections separated by 224 µm from each animal were processed for in situ hybridization as described earlier. All housing and experimental procedures were conducted in accordance with US Department of Agriculture and National Institutes of Health guidelines and with approval of the University of Pittsburgh's Institutional Animal Care and Use Committee.

QUANTIFICATION OF CB1R MRNA, RADIOIMMUNOREACTIVITY, AND IMMUNOREACTIVITY

Levels of CB1R mRNA expression and radioimmunoreactivity were quantified using a Microcomputer Imaging Device system (Imaging Research Inc, London, Ontario, Canada) without knowledge of diagnosis or subject number by random coding of film autoradiograms.[23,26,27] Optical density (OD) was measured in the gray matter of DLPFC area 9 and expressed as nanocuries per gram of tissue by reference to radioactive carbon 14 standards (American Radiolabeled Chemicals, St Louis) exposed on the same film. In measurements of CB1R mRNA expression, the mean (SD) total area sampled per subject was 385 (140) mm^2 for comparison subjects and 354 (112) mm^2 for subjects with schizophrenia. The mean (SD) total area sampled per subject for radioimmunoreactivity levels was 70 (35) mm^2 for comparison subjects and 75 (32) mm^2 for subjects with schizophrenia.

To determine differences in CB1R mRNA expression across lamina, OD was measured in approximately 1-mm-wide cortical traverses extending from the pial surface to the white matter. Three cortical traverses per section (9 traverses per subject) were placed in locations where the tissue section was cut perpendicular to the pial surface as determined by the presence of pyramidal neurons with vertically oriented apical dendrites in adjacent Nissl-stained sections. Within each traverse, the OD in each layer was determined by dividing the total cortical thickness from the pial surface to white matter into zones of 1% to 10%, 10% to 30%, 30% to 50%, 50% to 60%, 60% to 80%, and 80% to 100% approximating layers 1, 2 to superficial 3, deep 3, 4, 5, and 6, respectively.[29]

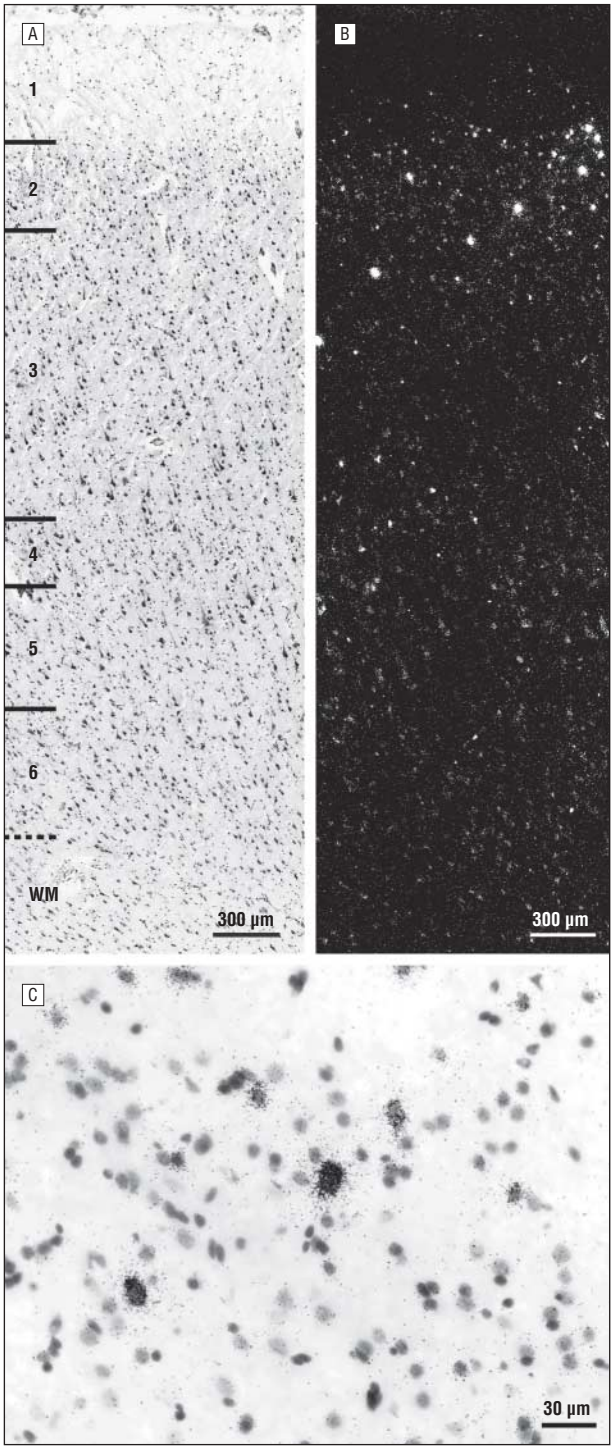

Figure 1. Distribution of silver grain clusters representing cannabinoid 1 receptor messenger RNA–positive neurons. A, Brightfield photomicrograph of a representative traverse from a Nissl-stained section of a comparison subject. Numbers and hash marks indicate the relative positions of the cortical layers; dashed line, the layer 6–white matter (WM) border. B, Darkfield photomicrograph of an adjacent emulsion-dipped section illustrating silver grain accumulation over neuronal nuclei of cannabinoid 1 receptor messenger RNA–positive neurons. Note that the density of cannabinoid 1 receptor messenger RNA–positive neurons appears greatest in layers 2 through superficial 3 and that cells in these layers express very high levels of cannabinoid 1 receptor messenger RNA. C, Representative high-power brightfield photomicrograph illustrating silver grain accumulation around neuronal nuclei.

Immunoreactivity of CB1R in DLPFC area 9 of subject pairs was assessed using the Microcomputer Imaging Device system and expressed as relative OD. Slide-mounted sections were illuminated on a microscope (Leitz Diaplan; Wild Leitz GmbH, Wetzlar, Germany) and images were captured at a final magnification of ×74 by a video camera and digitized. Relative OD values of CB1R immunoreactivity were measured within 3 cortical traverses per section (6 traverses per subject) as described earlier. The mean (SD) total area sampled per subject was 15 (1) mm^2 for comparison subjects and 15 (1) mm^2 for subjects with schizophrenia.

The OD of CB1R mRNA expression in the antipsychotic-exposed monkeys was assessed in contours encompassing the gray matter between the cingulate and principal sulci, which includes DLPFC areas 9 and 46. The mean (SD) total area sampled per animal was 49 (8) mm^2 for placebo, 49 (9) mm^2 for haloperidol, and 47 (10) mm^2 for olanzapine.

For slides processed in an experimental run, all of the images were acquired in the same session under the identical room and lightbox or microscope illumination as well as with the same gain and black levels and flatfield correction. All of the cortical gray matter OD values were corrected by subtracting background OD values obtained from the white matter of each subject.

STATISTICAL ANALYSES

Analysis of covariance (ANCOVA) models were performed to test the effect of diagnosis on each OD measure using mean values across all of the sections from each subject.[23,26,27] In the first ANCOVA model, OD was entered as the dependent variable, diagnostic group as the main effect, and subject pair as a blocking factor. In analyses of mRNA, the OD, pH, RIN, and tissue storage time were entered as covariates because pH and RIN reflect mRNA quantity[30] and integrity[31] and because storage time may affect mRNA preservation. A second unpaired ANCOVA model was performed to validate the first model using diagnostic group as the main effect and sex, age, postmortem interval, pH, RIN, and storage time as covariates. Similar paired and unpaired analyses of radioimmunoreactivity and immunoreactivity OD values were conducted with the same covariates except RIN. Tissue storage time never had a significant effect and was excluded in the reported analyses. The results for diagnostic group effect from both paired and unpaired ANCOVA models for each of the 3 dependent variables are reported.

The influences of potential confounding variables on the OD values in subjects with schizophrenia were assessed with ANCOVA models using each confounding variable as the main effect and sex, age, postmortem interval, pH, and RIN (in analyses of mRNA OD values) as covariates. A 1-way analysis of variance model with OD as the dependent variable and treatment group as the main effect was used to compare CB1R mRNA expression levels in the DLPFC of antipsychotic-exposed monkeys.

RESULTS

ANALYSIS OF CB1R MRNA EXPRESSION

The specificity of the riboprobe for CB1R mRNA was confirmed by several observations. First, in emulsion-dipped tissue sections, dense silver grain clusters were present over Nissl-stained neuronal nuclei of medium size, presumably inhibitory neurons, whereas very low levels of silver grains appeared over large, presumably pyramidal, neuronal nuclei as previously reported in rodent cortex (**Figure 1**).[32] Silver grain clusters were not present over glial cells identified by small, intensely Nissl-

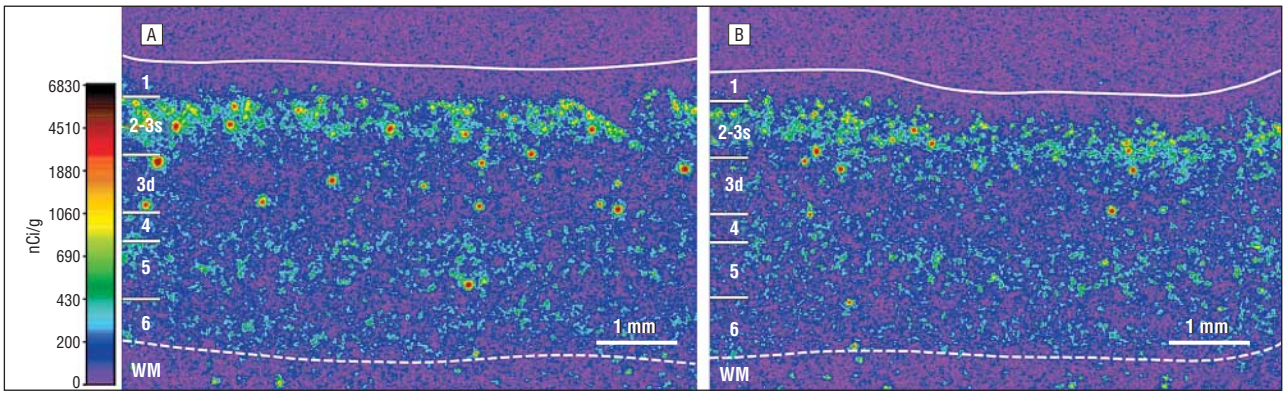

Figure 2. Representative film autoradiograms illustrating the expression of cannabinoid 1 receptor messenger RNA in dorsolateral prefrontal cortex area 9 of a comparison subject (A) and a matched subject with schizophrenia (B) (pair 5 in Table 1). The density of hybridization signal is presented in pseudocolor according to the calibration bar. Cannabinoid 1 receptor messenger RNA was expressed across layers 2 through 6, with the highest expression in layers 2 and superficial 3. Expression levels of cannabinoid 1 receptor messenger RNA in the subject with schizophrenia (B) appear lower than those in the matched comparison subject (A). Solid lines indicate the pial surface; dashed lines, the gray matter–white matter (WM) border; numbers and hash marks, the relative positions of the cortical layers; 3s, superficial layer 3; and 3d, deep layer 3.

stained nuclei. Second, the density of CB1R mRNA–positive neurons was highest in layers 2 to superficial 3, lowest in deep layer 3, intermediate in layers 4, 5, and 6 (Figure 1), and not detectable in layer 1; this laminar distribution pattern matched that previously reported for CB1R mRNA–expressing cells and CB1R-immunoreactive cell bodies in monkey and human DLPFC.[20,33,34] Third, specificity was confirmed by an absence of signal above background in tissue sections hybridized with the sense riboprobe for CB1R mRNA (data not shown).

Expression of CB1R mRNA was qualitatively reduced in DLPFC area 9 of subjects with schizophrenia compared with matched comparison subjects (**Figure 2**). Indeed, quantitative film OD measures of the entire cortical gray matter revealed that the subject with schizophrenia had lower OD measures in 20 of the 23 pairs and that the mean (SD) level of CB1R mRNA expression was significantly lower by 14.8% (paired: $F_{1,20}=8.1$; $P=.01$; unpaired: $F_{1,39}=9.2$; $P=.004$) in subjects with schizophrenia (105.4 [24.3] nCi/g) compared with the matched comparison subjects (123.8 [17.2] nCi/g) (**Figure 3**A).

The pattern of OD values for CB1R mRNA across cortical layers was similar between the schizophrenia and comparison groups; however, the OD values for the schizophrenia group were lower in all of the layers compared with the comparison group (Figure 3B). Expression of CB1R mRNA was significantly lower in the schizophrenia group by 15.9% (paired: $F_{1,20}=7.7$; $P=.01$; unpaired: $F_{1,39}=8.1$; $P=.007$) in layers 2 to superficial 3, by 15.5% (paired: $F_{1,20}=9.3$; $P=.006$; unpaired: $F_{1,39}=6.8$; $P=.01$) in layer 5, and by 17.7% (paired: $F_{1,20}=6.1$; $P=.02$; unpaired: $F_{1,39}=6.2$; $P=.02$) in layer 6 (Figure 3C).

ANALYSIS OF CB1R PROTEIN LEVELS

Qualitative examination of film autoradiograms revealed a laminar pattern of CB1R radioimmunoreactivity identical to that of CB1R-immunoreactive axons previously reported in human DLPFC.[20] The density of CB1R radioimmunoreactivity progressively increased across layers 2 and 3, formed a distinct band in layer 4, fell sharply in layer 5, and rose again in layer 6 (**Figure 4**A).

Radioimmunoreactivity of CB1R was qualitatively reduced in subjects with schizophrenia relative to matched comparison subjects (Figure 4A and B). Indeed, quantitative film OD measures of the entire cortical gray matter revealed that the subject with schizophrenia had lower OD measures in 19 of 23 pairs and that the mean (SD) level of CB1R radioimmunoreactivity was 11.6% lower (paired: $F_{1,21}=12.1$; $P=.002$; unpaired: $F_{1,40}=2.0$; $P=.16$) in subjects with schizophrenia (192.4 [57.4] nCi/g) relative to matched comparison subjects (217.6 [70.1] nCi/g) (Figure 4C). The within-pair percentage change in CB1R radioimmunoreactivity in the subjects with schizophrenia strongly correlated with the within-pair percentage change in CB1R mRNA expression ($r=0.67$; $P<.001$) (Figure 4D).

To confirm the observed decrease in CB1R protein levels by radioimmunocytochemistry and to assess the potential effect of laterality, we performed standard immunocytochemistry techniques in the fixed left hemisphere of 12 subject pairs. In both comparison subjects and subjects with schizophrenia, intense CB1R immunoreactivity was observed primarily in axons and boutons as previously described.[20]

The overall density of CB1R-immunoreactive axons was qualitatively reduced in the subjects with schizophrenia compared with matched comparison subjects (**Figure 5**). Indeed, quantitative OD measures of the entire cortical gray matter revealed that the subject with schizophrenia had lower OD measures in 10 of 12 pairs and that the mean (SD) level of CB1R immunoreactivity was significantly lower by 13.9% (paired: $F_{1,10}=7.4$; $P=.02$; unpaired: $F_{1,18}=4.6$; $P=.045$) in subjects with schizophrenia (0.230 [0.045]) than in comparison subjects (0.198 [0.023]) (**Figure 6**A).

The pattern of OD values for CB1R immunoreactivity across cortical layers was similar between the schizophrenia and comparison groups; however, the OD values for the schizophrenia group were lower in all of the layers relative to the comparison group (Figure 6B). Immunoreactivity of CB1R was significantly lower in the schizophrenia group by 15.2% (paired: $F_{1,10}=8.4$; $P=.02$; unpaired: $F_{1,18}=5.9$; $P=.03$) in deep layer 3, by 16.9%

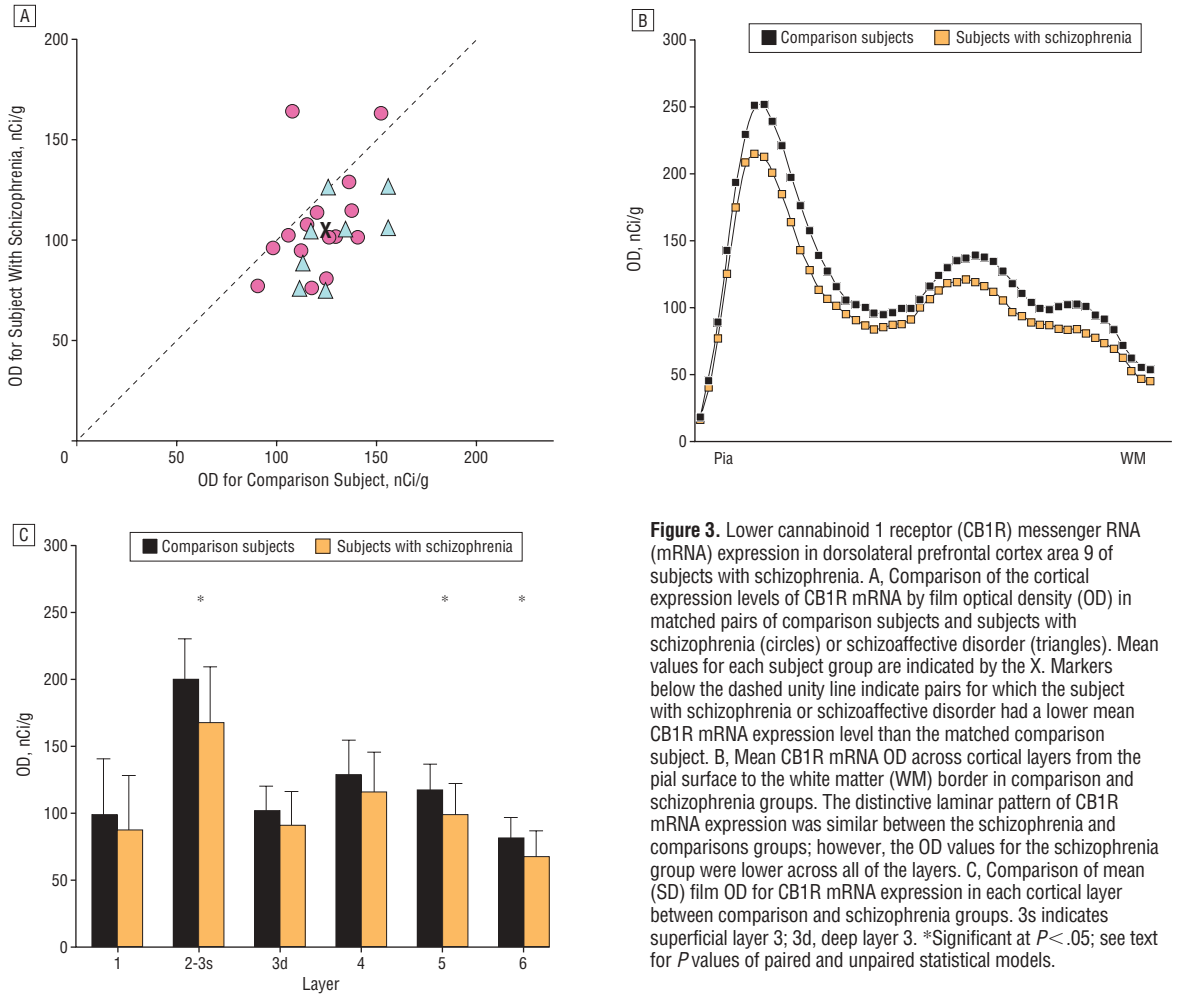

Figure 3. Lower cannabinoid 1 receptor (CB1R) messenger RNA (mRNA) expression in dorsolateral prefrontal cortex area 9 of subjects with schizophrenia. A, Comparison of the cortical expression levels of CB1R mRNA by film optical density (OD) in matched pairs of comparison subjects and subjects with schizophrenia (circles) or schizoaffective disorder (triangles). Mean values for each subject group are indicated by the X. Markers below the dashed unity line indicate pairs for which the subject with schizophrenia or schizoaffective disorder had a lower mean CB1R mRNA expression level than the matched comparison subject. B, Mean CB1R mRNA OD across cortical layers from the pial surface to the white matter (WM) border in comparison and schizophrenia groups. The distinctive laminar pattern of CB1R mRNA expression was similar between the schizophrenia and comparisons groups; however, the OD values for the schizophrenia group were lower across all of the layers. C, Comparison of mean (SD) film OD for CB1R mRNA expression in each cortical layer between comparison and schizophrenia groups. 3s indicates superficial layer 3; 3d, deep layer 3. *Significant at $P<.05$; see text for P values of paired and unpaired statistical models.

(paired: $F_{1,10}=7.7$; $P=.02$; unpaired: $F_{1,18}=6.4$; $P=.02$) in layer 4, and by 17.5% (paired: $F_{1,10}=12.4$; $P=.006$; unpaired: $F_{1,18}=5.0$; $P=.04$) in layer 6 (Figure 6C).

CORRELATION OF CB1R MRNA EXPRESSION WITH OTHER GABA-RELATED TRANSCRIPTS

We previously reported that glutamic acid decarboxylase 67 (GAD_{67}) and CCK mRNA levels were significantly reduced in DLPFC area 9 of the same subjects used in this study.[23] In addition, the within-pair percentage changes in GAD_{67} and CCK mRNA levels in the subjects with schizophrenia were significantly correlated ($r=0.81$; $P<.001$), suggesting that CCK-containing neurons exhibit a deficit in GABA synthesis.[23] Given that CB1R mRNA is preferentially expressed by CCK interneurons in the neocortex, the observed differences in CB1R mRNA were hypothesized to be associated with differences in GAD_{67} and CCK mRNA levels; consistent with this prediction, the within-pair percentage difference in CB1R mRNA expression was significantly correlated with the within-pair percentage differences in GAD_{67} mRNA ($r=0.64$; $P=.001$) and CCK mRNA ($r=0.68$; $P<.001$) levels (**Figure 7**).

ANALYSIS OF POTENTIAL CONFOUNDING FACTORS

The mean OD value in the subjects with schizophrenia did not differ as a function of sex, diagnosis of schizoaffective disorder, suicide, antidepressant medication use at the time of death, use of benzodiazepines or sodium valproate at the time of death, antipsychotic medication use at the time of death, diagnosis of substance abuse or dependence at the time of death, or history of cannabis use or abuse in measures of CB1R mRNA (all $F\leq 2.7$; all $P\geq .12$) (**Figure 8**A), radioimmunoreactivity (all $F\leq 3.3$; all $P\geq .09$) (Figure 8B), or immunoreactivity (all $F\leq 2.4$; all $P\geq .18$) (data not shown).

To further test the potential effect of antipsychotic medications on the expression of CB1R mRNA, we evaluated film OD values in monkeys with long-term exposure to haloperidol, olanzapine, or placebo (**Figure 9**). The laminar distribution of CB1R mRNA expression in all of the 3 groups matched the pattern observed in humans (Figure 9A-C). The mean (SD) OD of CB1R mRNA did not differ ($F_{2,15}=1.26$; $P=.31$) between the haloperidol (184.6 [13.3] nCi/g), olanzapine (199.1 [16.0] nCi/g), and placebo (192.1 [18.2] nCi/g) groups (Figure 9D).

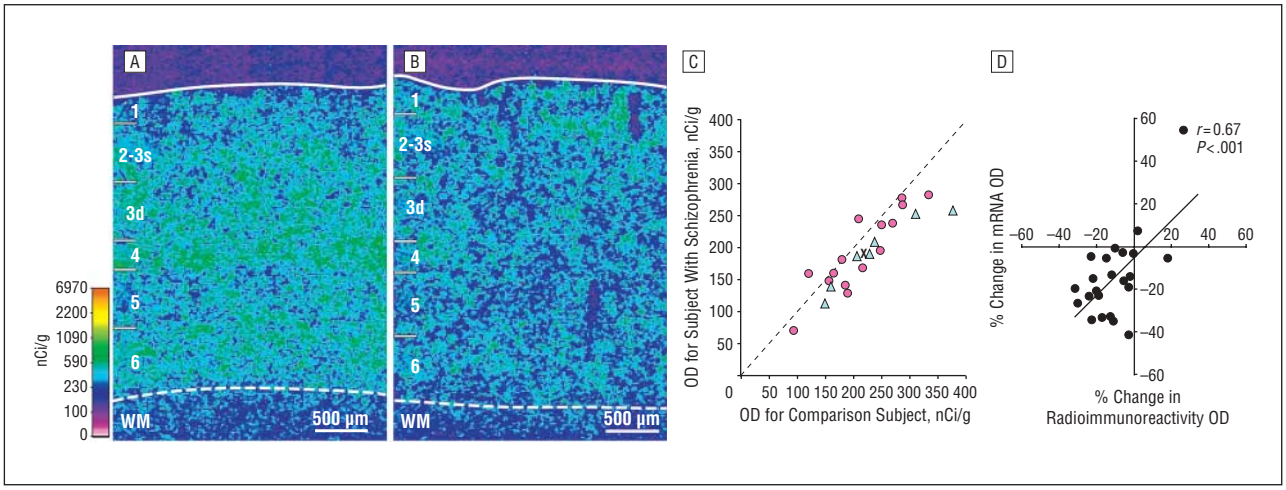

Figure 4. Representative film autoradiograms illustrating the expression of cannabinoid 1 receptor (CB1R) radioimmunoreactivity in dorsolateral prefrontal cortex area 9 of a comparison subject (A) and a matched subject with schizophrenia (B) (pair 8 in Table 1). The density of radioimmunoreactivity signal is presented in pseudocolor according to the calibration bar. The CB1R radioimmunoreactivity was expressed across all of the layers, with the highest expression in layer 4. The level of CB1R radioimmunoreactivity in the subject with schizophrenia (B) appeared lower than that in the matched comparison subject (A). Solid lines indicate the pial surface; dashed lines, the gray matter–white matter (WM) border; numbers and hash marks, the relative positions of the cortical layers; 3s, superficial layer 3; and 3d, deep layer 3. C, Comparison of the cortical levels of CB1R radioimmunoreactivity by film optical density (OD) in matched pairs of comparison subjects and subjects with schizophrenia (circles) or schizoaffective disorder (triangles). Mean values for each subject group are indicated by the X. Markers below the dashed unity line indicate pairs for which the subject with schizophrenia or schizoaffective disorder had a lower mean CB1R radioimmunoreactivity than the matched comparison subject. D, The within-pair percentage change in CB1R radioimmunoreactivity strongly correlated with the within-pair percentage difference in CB1R messenger RNA (mRNA) expression.

COMMENT

We found that (1) levels of CB1R mRNA and protein were significantly reduced and highly correlated in the DLPFC of subjects with schizophrenia; (2) the observed differences in CB1R mRNA expression were significantly correlated with those in GAD_{67} and CCK mRNA in the same subject pairs, suggesting that downregulation of CB1R in schizophrenia may be a compensatory response to impaired GABA neurotransmission in CCK-containing neurons; and (3) the reductions in CB1R mRNA and protein could not be explained by potential confounding factors.

Several lines of evidence indicate that the reductions in CB1R mRNA and protein levels in schizophrenia are not a consequence of factors frequently associated with the illness. First, CB1R mRNA expression was not altered in the DLPFC of monkeys with long-term exposure to typical or atypical antipsychotics in a manner that mimics the clinical treatment of schizophrenia (Figure 9). Consistent with these observations, the 4 subjects with schizophrenia who were not receiving antipsychotic medications at the time of death (Table 1) all had lower CB1R mRNA and protein levels than their matched comparison subjects. In addition, mean CB1R mRNA and protein levels did not differ between the subjects with schizophrenia who were receiving or had stopped receiving antipsychotic medication at the time of death (Figure 8). Second, neither a diagnosis of substance abuse or dependence nor a history of cannabis use accounted for the group differences in CB1R mRNA or protein levels (Figure 8). In fact, mean CB1R mRNA and protein levels in those subjects with schizophrenia with a substance use disorder or a history of cannabis use were actually higher than those in subjects who did not meet these criteria, suggesting that these factors might have blunted the decreases in CB1R mRNA and protein levels in schizophrenia. Consistent with these observations, substances of abuse do not affect CB1R mRNA expression or CB1R binding in rodent neocortex (with the exception of cocaine, which only affected CB1R mRNA levels[35,36]), and long-term exposure to CB1R agonists either does not alter CB1R mRNA expression[37-39] or increases its expression in cortical structures.[40] In addition, monkeys with long-term exposure to Δ9-tetrahydrocannabinol or marijuana smoke did not exhibit alterations in CB1R density in the prefrontal cortex.[41] However, not all animal studies have produced similar results,[42] so an effect of prior cannabis exposure cannot be definitively excluded. Third, the lower CB1R mRNA and protein levels in schizophrenia were not associated with the use of antidepressant medication, benzodiazepines, or valproate at the time of death, death by suicide, or a diagnosis of schizoaffective disorder (Figure 8). Finally, measures of RNA quality and quantity (RIN and pH) were in the ranges associated with excellent RNA preservation[31] in all of the subjects (Table 2), and the effect of diagnosis on CB1R measures remained significant when the effects of these variables were controlled statistically. Furthermore, the expression of other transcripts was not altered in these same subjects with schizophrenia,[23,26,27] confirming that the observed reductions in CB1R mRNA levels are not attributable to a general loss of mRNA integrity in the subjects with schizophrenia.

In contrast to our findings of reduced CB1R mRNA and protein levels, increased binding of the CB1R agonist [^{3}H]CP-55940 was reported in the DLPFC[4] and posterior cingulate cortex[6] of subjects with schizophrenia. Increased [^{3}H]CP-55940 binding might reflect the presence of an allosteric modulation site on CB1Rs that, when bound, elicits a conformational change in the receptor,

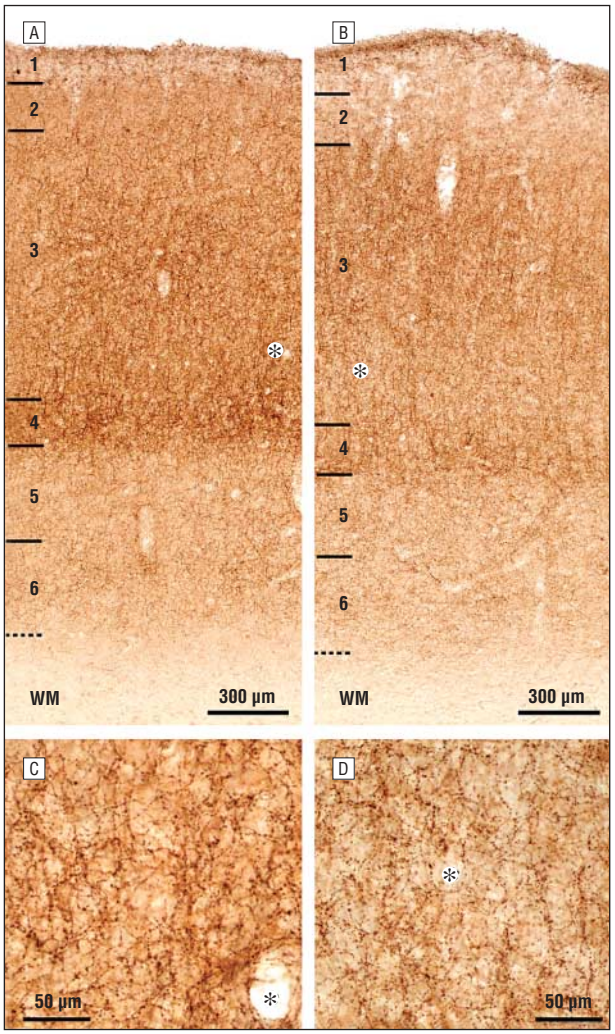

Figure 5. Brightfield photomicrographs demonstrating the density and laminar pattern of cannabinoid 1 receptor immunoreactivity in dorsolateral prefrontal cortex area 9 of a comparison subject (A and C) and a matched subject with schizophrenia (B and D) (pair 4 in Table 1). Numbers and hash marks indicate the relative positions of the cortical layers; dashed lines, the layer 6–white matter (WM) border. Intense cannabinoid 1 receptor immunoreactivity was observed primarily in axons and boutons in the comparison subject (C) and the subject with schizophrenia (D). The density of cannabinoid 1 receptor–immunoreactive axons and varicosities appeared to be decreased in the subject with schizophrenia (B and D) relative to the matched comparison subject (A and C). Asterisks indicate the same blood vessel in A and C as well as the same blood vessel in B and D.

increasing the affinity of [^{3}H]CP-55940 for the orthosteric binding site.[43] Thus, [^{3}H]CP-55940 binding can reflect differences other than the amount of receptor present. Increased binding of the CB1R antagonist [^{3}H]SR141716 was also reported in the anterior cingulate cortex of subjects with schizophrenia.[5] Functional effects of SR141716 have been found in CB1R knockout mice, suggesting that it binds receptors other than the CB1R.[44,45] Importantly, no studies reporting increased CB1R binding in schizophrenia conducted saturation and competition experiments to determine whether Kd as opposed to Bmax was altered in schizophrenia. Finally, the laminar patterns of radioligand binding in these studies, when reported, are not consistent with those previously reported in human, monkey, or rat neocortex[46-48] and do not match the laminar distribution of CB1R-immunoreactive axons in the same regions of monkey and human cortex[20]; these findings suggest that the binding of these radioligands does not represent the relative amount of CB1R protein present.

In the neocortex, CB1Rs are heavily localized to inhibitory axon terminals of the subpopulation of GABA basket neurons that contain CCK.[19,20] Asymmetric, excitatory, CB1R-immunoreactive synapses have also been observed,[49,50] and CB1R agonists modulate glutamate release, consistent with a presynaptic localization of CB1Rs in pyramidal cell axon terminals.[51] However, CB1R mRNA levels are much higher in GABA neurons than in pyramidal cells,[32] the density of CB1Rs is more than 20-fold higher in inhibitory terminals than in excitatory terminals, and the concentration of CB1R agonist necessary for 50% suppression of glutamate release is approximately 30 times higher than that necessary to suppress GABA release.[52] These data indicate that CCK neuron axon terminals contain much higher levels of CB1Rs than pyramidal cell axon terminals and are more sensitive to the effects of CB1R agonists. Importantly, the antibody used in this study exclusively labels symmetric, inhibitory synapses by electron microscopy,[20] probably because the level of CB1Rs in excitatory terminals is below the threshold of detectability. Thus, the observed reductions in CB1R protein levels in subjects with schizophrenia are likely to reflect lower CB1R levels specifically in inhibitory neurons and axon terminals rather than in pyramidal neurons and axons. Furthermore, the strong correlations between the differences in CB1R mRNA and GAD_{67} and CCK mRNA support the interpretation that the observed deficits in CB1R levels reflect changes in GABA neurons and not pyramidal cells.

Disturbances in inhibitory neurotransmission appear to play a prominent role in the dysfunction of the DLPFC of subjects with schizophrenia[18] as demonstrated by the consistent finding of an approximately 25% to 35% reduction in the expression of GAD_{67} mRNA across layers 2 through 5.[22,53-55] Parvalbumin-containing interneurons appear to account for the decreased GAD_{67} mRNA expression in layers 3 and 4,[26] and the results of the present study suggest that CB1R- and CCK-containing neurons might contribute to the GAD_{67} mRNA deficit in layers 2 through superficial 3. In the primate DLPFC, the highest densities of both CB1R- and CCK-positive neurons are found in these layers, and both CB1R- and CCK-positive axon terminals densely innervate layer 4.[20,56] In addition, these 2 proteins are colocalized in terminals that furnish perisomatic inputs to pyramidal neurons.[19,32,57] Thus, our findings of reduced CB1R mRNA in layers 2 through superficial 3, reduced CB1R immunoreactivity in layer 4, and correlated changes in CB1R, CCK, and GAD_{67} mRNA in schizophrenia converge on the interpretation that GABA neurotransmission is altered in the subset of CB1R- and CCK-containing GABA neurons that project from the superficial to middle cortical layers.

How might these disturbances be related to the working memory impairments associated with DLPFC dysfunction in schizophrenia? In the human DLPFC, the power of gamma band oscillations (30-80 Hz) increases directly with working memory load,[58] and impaired working memory performance in individuals with schizophrenia is

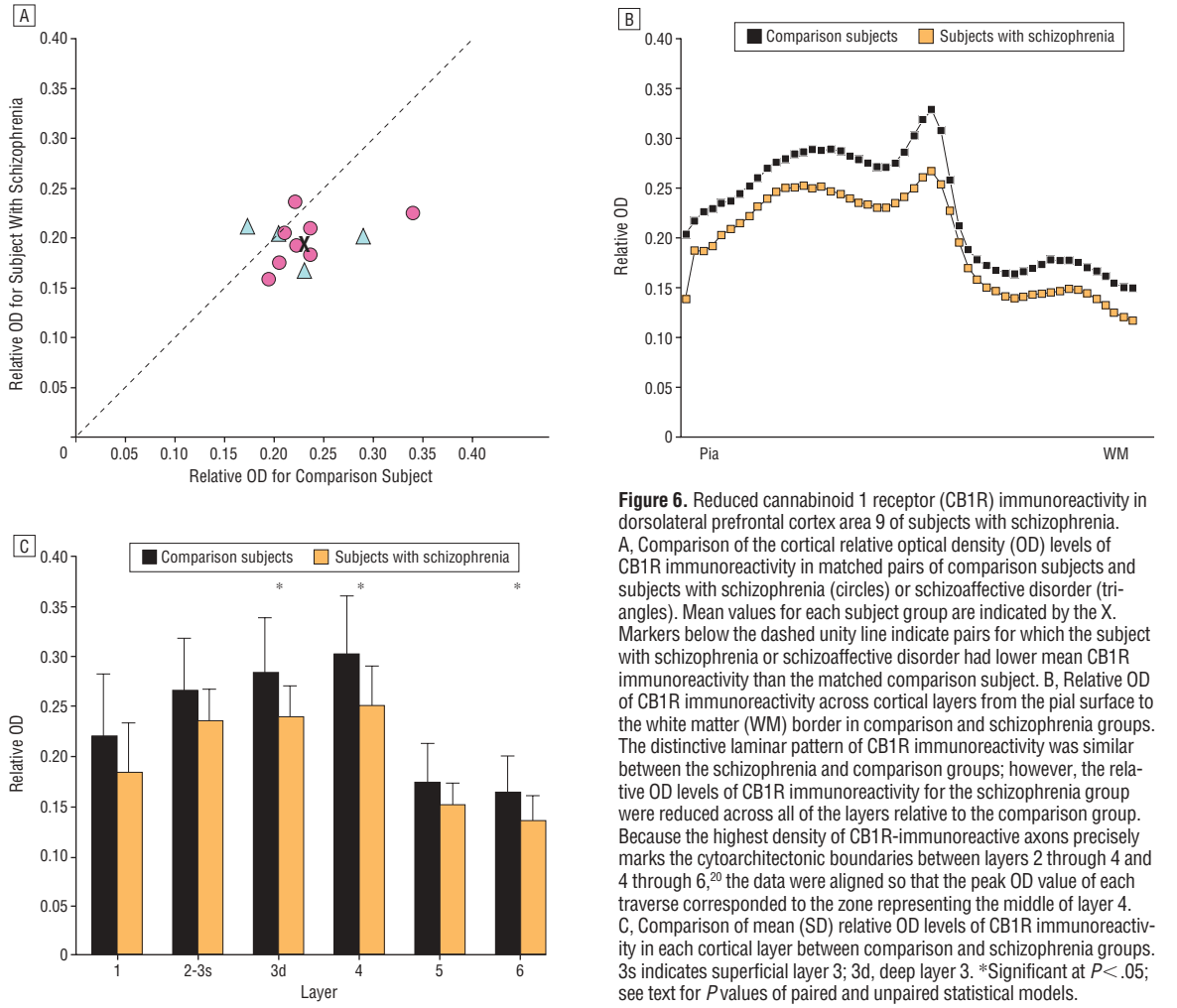

Figure 6. Reduced cannabinoid 1 receptor (CB1R) immunoreactivity in dorsolateral prefrontal cortex area 9 of subjects with schizophrenia. A, Comparison of the cortical relative optical density (OD) levels of CB1R immunoreactivity in matched pairs of comparison subjects and subjects with schizophrenia (circles) or schizoaffective disorder (triangles). Mean values for each subject group are indicated by the X. Markers below the dashed unity line indicate pairs for which the subject with schizophrenia or schizoaffective disorder had lower mean CB1R immunoreactivity than the matched comparison subject. B, Relative OD of CB1R immunoreactivity across cortical layers from the pial surface to the white matter (WM) border in comparison and schizophrenia groups. The distinctive laminar pattern of CB1R immunoreactivity was similar between the schizophrenia and comparison groups; however, the relative OD levels of CB1R immunoreactivity for the schizophrenia group were reduced across all of the layers relative to the comparison group. Because the highest density of CB1R-immunoreactive axons precisely marks the cytoarchitectonic boundaries between layers 2 through 4 and 4 through 6,[20] the data were aligned so that the peak OD value of each traverse corresponded to the zone representing the middle of layer 4. C, Comparison of mean (SD) relative OD levels of CB1R immunoreactivity in each cortical layer between comparison and schizophrenia groups. 3s indicates superficial layer 3; 3d, deep layer 3. *Significant at $P<.05$; see text for P values of paired and unpaired statistical models.

associated with reduced frontal lobe gamma band power.[59] Neurotransmission of GABA in the DLPFC is essential for both working memory performance[16,17] and oscillatory activity.[60] Consistent with the anatomical localization of CB1Rs to CCK-containing neuron axon terminals, activation of CB1Rs inhibits GABA release from these terminals and strongly suppresses $GABA_A$ receptor–mediated inhibitory postsynaptic currents in pyramidal neurons.[19,57,61] Indeed, the acute activation of CB1Rs with exogenous cannabinoids decreases the power of gamma oscillations in the rodent hippocampus, entorhinal cortex, and prefrontal cortex, presumably by disrupting the synchronous firing of pyramidal neurons.[62-64] Thus, the disruption of gamma oscillations by CB1R activation in the DLPFC may explain the impairments in working memory performance in both humans and animals following systemic administration of cannabinoids[65-67]; however, this interpretation remains to be tested in primates.

Our findings might represent a downregulation of CB1Rs in response to elevated levels of endocannabinoids in schizophrenia (eg, increased cerebrospinal fluid and blood levels of anandamide[1-3]). However, whether the DLPFC contributes to reported elevated levels of anandamide is

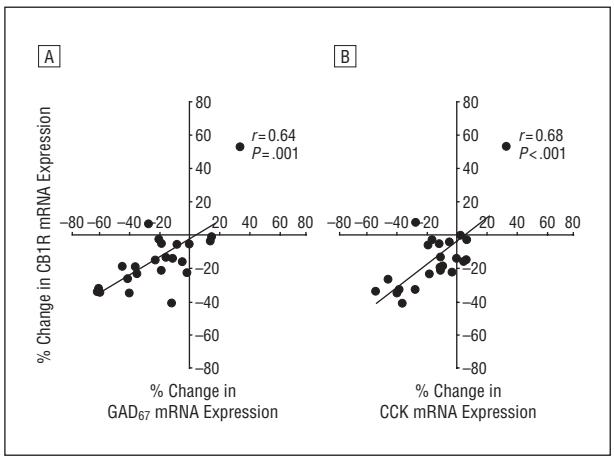

Figure 7. Correlation analyses between the differences in cannabinoid 1 receptor (CB1R) messenger RNA (mRNA) and glutamic acid decarboxylase 67 (GAD_{67}) (A) or cholecystokinin (CCK) (B) mRNA expression across subject pairs. The within-pair percentage difference in CB1R mRNA expression is plotted against those for GAD_{67} (A) or CCK (B) mRNA expression[23] for the 23 subject pairs. Changes in CB1R mRNA expression significantly correlated with changes in GAD_{67} and CCK mRNA expression across the 23 subject pairs.

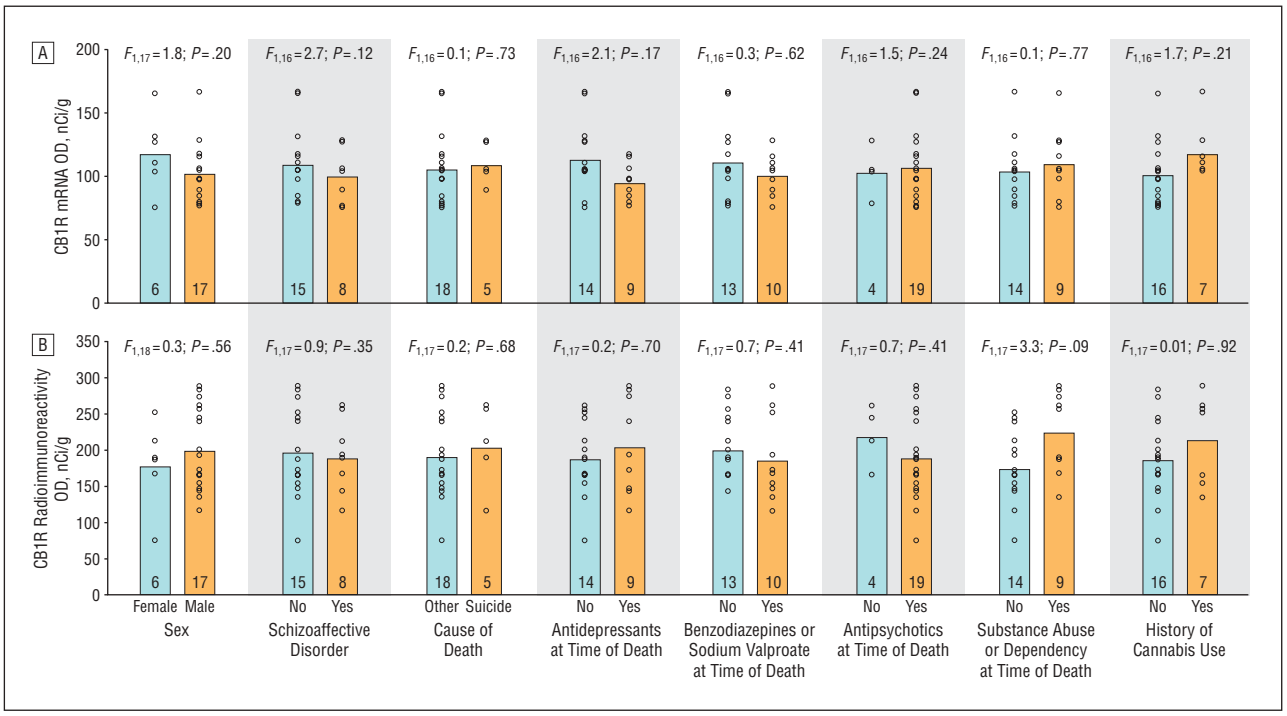

Figure 8. The effects of confounding factors on cannabinoid 1 receptor (CB1R) messenger RNA (mRNA) expression and radioimmunoreactivity in subjects with schizophrenia. Mean (bar) and individual (circle) optical density (OD) values for CB1R mRNA expression (A) or radioimmunoreactivity (B) are shown for the subjects with schizophrenia grouped by potential confounding factors. Sex, diagnosis of schizoaffective disorder, suicide, antidepressant medication use at the time of death, use of benzodiazepines or sodium valproate at the time of death, antipsychotic medication use at the time of death, diagnosis of substance abuse or dependence at the time of death, and history of cannabis use or abuse did not significantly affect CB1R mRNA expression (A) or radioimmunoreactivity (B). Numbers in bars indicate the number of subjects with schizophrenia in each category.

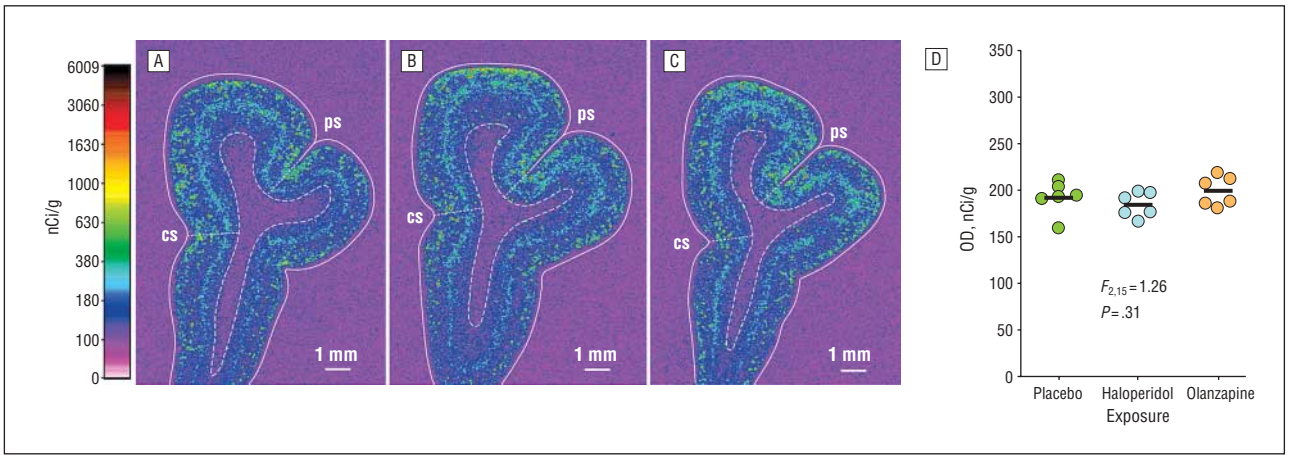

Figure 9. Representative film autoradiograms illustrating the expression of cannabinoid 1 receptor messenger RNA in the dorsolateral prefrontal cortex of placebo-exposed (A), haloperidol-exposed (B), and olanzapine-exposed (C) monkeys used to mimic the clinical treatment of individuals with schizophrenia. The density of hybridization signal is presented in pseudocolor according to the calibration bar. Cannabinoid 1 receptor messenger RNA expression was assessed between the cingulate sulcus (cs) and the principal sulcus (ps). Solid lines indicate the pial surface; dashed lines, the gray matter–white matter border. D, Comparison of cannabinoid 1 receptor messenger RNA expression levels by film optical density (OD) in the dorsolateral prefrontal cortex of placebo-exposed, haloperidol-exposed, and olanzapine-exposed monkeys. Hash bars indicate group means.

unknown. Furthermore, 2-arachidonoyl-glycerol, not anandamide, appears to be the principal endocannabinoid for CB1Rs in cortical and hippocampal GABA neurons.[68-70] Alternatively, we suggest that the downregulation of CB1R mRNA and protein in schizophrenia may represent a compensatory response to a deficit of GABA synthesis in CCK-containing neurons. That is, a lower density of CB1Rs could, by reducing the endocannabinoid-mediated suppression of GABA release from the perisomatic terminals of CB1R- and CCK-containing interneurons, contribute to a partial normalization of gamma band power and working memory function. Downregulation of CCK expression may also represent a compensatory response that, by reducing CCK_B receptor–mediated enhancement of 2-arachidonoyl-glycerol synthesis, decreases endocannabinoid-mediated suppression of GABA release from CB1R- and CCK-containing axon terminals.[71] This interpretation implies that cannabis use in vulnerable individuals would counteract

these compensatory responses, providing a potential mechanism linking cannabis exposure with an increased risk for the cognitive impairments of schizophrenia.

This interpretation also suggests possible novel molecular targets for treating the cognitive deficits in schizophrenia. For instance, CB1R antagonists would be predicted to augment the intrinsic compensatory downregulation of CB1R expression, further limit the endocannabinoid-mediated suppression of GABA release from CB1R- and CCK-containing terminals, and enhance the ability of CCK basket neurons to synchronize pyramidal neurons in gamma oscillations. In addition, at least in the hippocampus, $GABA_A$ receptors containing the $\alpha 2$ subunit are selectively located on pyramidal cell bodies postsynaptic to CB1R- and CCK-containing terminals.[72] Thus, positive allosteric modulators of the benzodiazepine binding site with selectivity for $GABA_A$ receptors containing the $\alpha 2$ subunit would be predicted to increase the efficacy of GABA released from CB1R- and CCK-containing terminals and might be synergistic with the proposed effects of such agents at augmenting the input from parvalbumin-containing chandelier neurons to the axon initial segment of pyramidal neurons.[73,74] Together, such agents might enhance the synchronization of pyramidal neuron activity by restoring normal levels of perisomatic GABA input to pyramidal neurons.

Submitted for Publication: October 9, 2007; final revision received March 17, 2008; accepted March 19, 2008.
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Author Contributions: Dr Lewis had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. *Study concept and design:* Eggan and Lewis. *Acquisition of data:* Eggan and Lewis. *Analysis and interpretation of data:* Eggan, Hashimoto, and Lewis. *Drafting of the manuscript:* Eggan and Lewis. *Critical revision of the manuscript for important intellectual content:* Eggan, Hashimoto, and Lewis. *Statistical analysis:* Eggan and Hashimoto. *Obtained funding:* Lewis. *Administrative, technical, and material support:* Eggan and Lewis. *Study supervision:* Hashimoto and Lewis.
Financial Disclosure: Dr Lewis currently receives research support from the Bristol-Myers Squibb Foundation, Merck and Co, Inc, and Pfizer Inc and in 2006 to 2008 served as a consultant to Bristol-Myers Squibb, Hoffman-La Roche Inc, Merck and Co, Inc, Neurogen Corp, Pfizer Inc, Sepracor Inc, and Wyeth.
Funding/Support: This work was supported by grants MH045156 and MH043784 from the National Institutes of Health and by the Andrew Mellon Predoctoral Fellowship (Dr Eggan) and Scottish Rite Fellowship (Dr Eggan).
Additional Information: Supplemental methods are available online at http://www.archgenpsychiatry.com.
Additional Contributions: Ken Mackie, MD, kindly donated the CB1R antibody, Mary Brady, BS, assisted with the graphics, Holly Bazmi, MS, assisted in generating templates for the CB1R riboprobes, and Jim Kosakowski developed the Matlab program.

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