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Cannabinoid CB1 Receptor Immunoreactivity in the Prefrontal Cortex: Comparison of Schizophrenia and Major Depressive Disorder

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We recently showed that measures of cannabinoid 1 receptor (CB1R) mRNA and protein were significantly reduced in dorsolateral prefrontal cortex (DLPFC) area 9 in schizophrenia subjects relative to matched normal comparison subjects. However, other studies have reported unaltered or higher measures of CB1R levels in schizophrenia. To determine whether these discrepancies reflect differences across brain regions or across subject groups (eg, presence of depression, cannabis exposure, etc), we used immunocytochemical techniques to determine whether lower levels of CB1R immunoreactivity are (1) present in another DLPFC region, area 46, in the same subjects with schizophrenia, (2) present in area 46 in a new cohort of schizophrenia subjects, (3) present in major depressive disorder (MDD) subjects, or (4) attributable to factors other than a diagnosis of schizophrenia, including prior cannabis use. CB1R immunoreactivity levels in area 46 were significantly 19% lower in schizophrenia subjects relative to matched normal comparison subjects, a deficit similar to that observed in area 9 in the same subjects. In a new cohort of subjects, CB1R immunoreactivity levels were significantly 20 and 23% lower in schizophrenia subjects relative to matched comparison and MDD subjects, respectively. The lower levels of CB1R immunoreactivity in schizophrenia subjects were not explained by other factors such as cannabis use, suicide, or pharmacological treatment. In addition, CB1R immunoreactivity levels were not altered in monkeys chronically exposed to haloperidol. Thus, the lower levels of CB1R immunoreactivity may be common in schizophrenia, conserved across DLPFC regions, not present in MDD, and not attributable to other factors, and thus a reflection of the underlying disease process.
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INTRODUCTION

Cannabinoid signaling through the cannabinoid 1 receptor (CB1R) mediates the physiological and psychoactive properties of cannabis (Ameri, 1999), and may have an important function in the pathogenesis and/or pathophysiology of schizophrenia (Murray *et al*, 2007). For example, altered inhibition from γ-aminobutyric acid (GABA) neurons that express CB1Rs and the neuropeptide cholecystokinin (CCK) may contribute to dorsolateral prefrontal cortex (DLPFC) dysfunction in schizophrenia (Lewis and Sweet, 2009); indeed, measures of CB1R mRNA and protein are reported to be significantly lower in DLPFC area 9 in schizophrenia subjects (Eggan *et al*, 2008; Uriguen *et al*, 2009). Furthermore, lower CB1R mRNA expression levels were significantly correlated with those for the mRNAs for both CCK and glutamic acid decarboxylase (GAD_{67}), a synthesizing enzyme for GABA (Eggan *et al*, 2008; Hashimoto *et al*, 2008a), suggesting that all three transcripts are lower in the same population of DLPFC GABA neurons in schizophrenia.

Alterations in these neurons might contribute to DLPFC-mediated cognitive impairments in schizophrenia, such as working memory dysfunction, through the following mechanism (Elvevag and Goldberg, 2000; Weinberger *et al*, 2001; Lewis *et al*, 2005). CB1Rs are heavily localized to the axon terminals of CCK-containing, GABA basket neurons (Bodor *et al*, 2005; Eggan and Lewis, 2007). Activation of CB1Rs inhibits release of GABA from these terminals and strongly suppresses $GABA_A$ receptor-mediated inhibitory postsynaptic currents in pyramidal neurons (Trettel *et al*, 2004; Galarreta *et al*, 2004; Bodor *et al*, 2005). Furthermore, systemic administration of CB1R agonists reduces overall levels of GABA in the prefrontal cortex (Pistis *et al*, 2002). Thus, the lower levels of CB1Rs would be expected to enhance cortical GABA neurotransmission in schizophrenia, providing a compensatory, albeit insufficient, response to the deficit of GAD_{67}-mediated

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GABA synthesis (Eggan *et al*, 2008), which is thought to contribute to working memory impairments in the illness (Lewis *et al*, 2005). Activation of CB1Rs by exogenous cannabinoids would be expected to further impair GABA neurotransmission in an already compromised system and produce the exacerbation of symptoms, including impaired working memory, frequently seen in individuals with schizophrenia who use cannabis (D'Souza *et al*, 2005).

However, in contrast to the findings of lower cortical CB1R levels in schizophrenia (Eggan *et al*, 2008; Uriguen *et al*, 2009), other studies have reported unaltered or higher CB1R levels or binding (Dean *et al*, 2001; Zavitsanou *et al*, 2004; Newell *et al*, 2006; Koethe *et al*, 2007; Deng *et al*, 2007). These discrepancies might reflect differences in the cortical regions examined (eg, DLPFC area 9, anterior cingulate cortex, posterior cingulate cortex, or superior temporal gyrus), or in the presence of comorbid factors, such as depression, which is present in ~50% of schizophrenia subjects (Buckley *et al*, 2009). Interestingly, higher levels of CB1R protein and agonist-stimulated [^{35}S]GTPγS binding are present in DLPFC area 9 of major depressive disorder (MDD) subjects (Hungund *et al*, 2004). Thus, comorbid major depression may confound measures of CB1R levels in schizophrenia subjects. In addition, other factors that may differ across studies, such as the proportion of schizophrenia subjects with cannabis exposure (Linszen *et al*, 1994; Bersani *et al*, 2002), may have an impact on the measures of CB1R levels.

To address these issues and to gain a better understanding of the function CB1Rs might have in the pathophysiology of schizophrenia, we used immunocytochemical techniques in postmortem tissue from 26 schizophrenia subjects, 14 MDD subjects, and monkeys chronically exposed to haloperidol or matched control monkeys (four pairs) to determine whether lower levels of CB1R immunoreactivity are (1) common in schizophrenia, (2) present in a previously unstudied DLPFC region, area 46, that is functionally important for working memory processes, and (3) distinctive to schizophrenia or attributable to other factors, such as depression, suicide, and antipsychotic medications, commonly associated with the illness.

MATERIALS AND METHODS

Human Subject Characteristics

Following informed consent for brain donation from the next-of-kin using procedures approved by the University of Pittsburgh's Committee for Research Involving the Dead and Institutional Review Board for Biomedical Research, brain specimens were obtained from 63 human subjects during autopsies conducted at the Allegheny County Medical Examiner's Office, Pittsburgh, PA. Two cohorts of non-overlapping schizophrenia subjects were used. The first cohort (Table 1; Supplementary Table S1) consisted of 12 matched pairs of schizophrenia and normal comparison subjects used in our earlier study of CB1R mRNA and protein in DLPFC area 9 (Eggan *et al*, 2008), for which tissue containing area 46 was available. The second cohort (Table 2; Supplementary Table S2) comprised 14 matched triads of schizophrenia, normal comparison, and MDD subjects. Only two schizophrenia subjects had a secondary axis I diagnosis of depression (640 in cohort 1, Supplementary Table S1; 422 in cohort 2, Supplementary Table S2). To control experimental variance and to reduce biological variance, each subject with schizophrenia was matched for sex and, as closely as possible for age and postmortem interval (PMI), with one normal comparison subject (cohort 1) or one normal comparison subject and one MDD subject (cohort 2). The mean age, PMI, and tissue storage time did not significantly differ across subject groups in either cohort (Tables 1 and 2). Subject characteristics have been previously reported for subjects in cohort 1 (Eggan *et al*, 2008) and cohort 2 (Volk *et al*, 2002; Cruz *et al*, 2009); for diagnostic evaluations and detailed subject characteristics see Supplementary Materials. As most of these subjects were obtained as part of a fixed brain collection (see below), measures of brain pH could not be obtained; however, brain pH does not predict stability of immunoreactivity (Beneyto *et al*, 2009).

Tissue Preparation and Immunocytochemistry

For each brain, the fresh left hemisphere was cut coronally into 1.0-cm thick blocks, fixed for 48 h in phosphate

Table 1 Summary of Cohort 1 Characteristics

Characteristic	Comparison	Schizophrenia	t-Test[a]	p
Sex, M/F, No.	8/4	8/4		
Race, W/B, No.	8/4	9/3		
Age, mean (SD), years	46.3 (15.7)	45.3 (12.5)	0.57	0.58
PMI, mean (SD), hours	16.5 (5.1)	17.5 (9.2)	−0.59	0.57
ST, mean (SD), months	116.2 (21.4)	119.9 (16.3)	−0.93	0.37

Abbreviations: B, black; F, female; M, male; No., number; PMI; postmortem interval; SD, standard deviation; ST, storage time at −80°C; W, white.
[a]Paired *t*-test, df = 11.

Table 2 Summary of Cohort 2 Characteristics

Characteristic	Comparison	Schizophrenia	MDD	ANOVA F(2, 39)	p
Sex, M/F, No.	9/5	9/5	9/5		
Race, W/B, No.	10/4	10/4	13/1		
Age, mean (SD), years	53.7 (14.4)	52.9 (13.0)	54.0 (13.1)	0.03	0.98
PMI, mean (SD), hours	12.8 (5.8)	13.5 (5.8)	12.2 (5.0)	0.21	0.81
ST, mean (SD), months	182.2 (27.8)	190.9 (25.0)	166.2 (27.5)	3.07	0.06

Abbreviations: B, black; F, female; M, male; No., number; PMI; postmortem interval; SD, standard deviation; ST, storage time at −80°C; W, white.

buffered (0.1 M; pH 7.4) 4% paraformaldehyde at 4°C, cryoprotected by immersion in solutions of increasing sucrose concentration, and then stored at −30°C in a cryoprotectant solution as described earlier (Eggan and Lewis, 2007). Coronal sections were serially cut on a cryostat (40 μm) from tissue blocks containing the DLPFC, and every 10th or 20th section was stained for Nissl substance with thionin to identify the location of area 46 based on cytoarchitectonic criteria (Rajkowska and Goldman-Rakic, 1995; Daviss and Lewis, 1995). For each subject, two sections separated by at least 320 μm were chosen; sections from each pair or triad were matched as closely as possible for rostral-caudal level.

Free-floating tissue sections were processed for CB1R immunoreactivity using the avidin-biotin-peroxidase method (Hsu *et al*, 1981) and 3,3′-diaminobenzidine as described earlier (Eggan and Lewis, 2007; Eggan *et al*, 2008). The specificity of the affinity-purified rabbit anti-CB1R antibody (anti-CB1R-L15; diluted 1 : 4000; kindly provided by Dr Ken Mackie, Indiana University, Bloomington, IN) has been previously demonstrated by multiple lines of evidence including western blot analysis, preadsorption studies, and testing in knockout mice (for details see Eggan and Lewis, 2007). Although CB1Rs are contained in excitatory synapses in the neocortex (Kawamura *et al*, 2006; Katona *et al*, 2006), the antibody used in this study exclusively labels symmetric, inhibitory synapses by electron microscopy in both the monkey DLPFC (Eggan and Lewis, 2007) and rodent hippocampus (Katona *et al*, 1999; Hajos *et al*, 2000), probably because the much lower level of CB1Rs in excitatory terminals is below the threshold of detectability (Katona *et al*, 2006; Eggan and Lewis, 2007). Hence, observed differences between subject groups in CB1R immunoreactivity in this study likely reflect changes in the expression of CB1R protein specifically in inhibitory neurons and axon terminals. Two immunocytochemistry runs were performed for each of the two subject cohorts, with one section from a given subject pair or triad processed together in each run.

Quantification of CB1R Immunoreactivity Levels

The intensity of CB1R immunoreactivity (expressed as relative optical density (ROD)) in DLPFC area 46 was assessed using a Microcomputer Imaging Device system (Imaging Research, London, Ontario, Canada), without the knowledge of diagnosis or subject number by random coding of slide-mounted sections as described earlier (Eggan and Lewis, 2007). Slide-mounted sections were illuminated on a microscope (Leitz Diaplan; Wild Leitz GmbH, Wetzlar, Germany), images were captured at a final magnification of ×74 (4.0 μm/pixel resolution) by a video camera and digitized, and ROD values were measured by drawing contours of the full cortical thickness for all locations where the gray matter was cut perpendicular to the pial surface. Blood vessels in the contours were excluded to minimize any potential effects of differences in vascularization across brains on ROD measures. The mean (±SD) area sampled per subject was 37.4 (8.9) mm^2 for normal comparison subjects and 37.7 (16.0) mm^2 for schizophrenia subjects in cohort 1, and 35.7 (8.8) mm^2 for normal comparison subjects, 37.3 (16.1) mm^2 for schizophrenia subjects, and 34.3 (10.0) mm^2 for MDD subjects in cohort 2.

To assess CB1R immunoreactivity levels across cortical layers, ROD values were measured within three ∼1-mm-wide traverses per section (six traverses per subject) extending from the pial surface to the white matter. The data were divided into 50 bins using Matlab software (The MathWorks, Natick, MA), and the ROD in each layer was determined by dividing the total cortical thickness from the pial surface to white matter into zones of 1–10, 10–30, 30–50, 50–60, 60–80, and 80–100%, which approximate the locations of layers 1, 2-superficial 3 (3s), deep 3 (3d), 4, 5, and 6, respectively (Pierri *et al*, 1999). As the highest density of CB1R-immunoreactive (IR) axons occurs in layer 4 and precisely marks the cytoarchitectonic boundaries between layers 3–4 and 4–5 (Eggan and Lewis, 2007), the bins were aligned so that the peak ROD value of each traverse corresponded to the zone representing the middle of layer 4 for every traverse.

All images for slides processed in an experimental run were acquired in the same session under identical room and microscope illuminations and with the same gain and black levels and flatfield correction. All cortical and laminar gray matter measures were corrected by subtracting background ROD values obtained from the white matter of each subject.

Haloperidol-Exposed Monkeys

To further evaluate the effects of long-term antipsychotic medication exposure on CB1R immunoreactivity levels (Eggan *et al*, 2008), we studied four male macaque monkeys (*Macaca fascicularis*) who had received the antipsychotic, haloperidol decanoate, for 9–12 months and four control animals matched for sex, age, and weight (Akil *et al*, 1999). The mean (±SD) dose of haloperidol decanoate was 16.0 (2.1) mg/kg, was administered by injection every 4 weeks, and yielded trough serum haloperidol concentrations of 4.3 (1.1) ng/ml. Similar concentrations have been associated with a therapeutic response in humans (Volavka *et al*, 1992), and resulted in extrapyramidal symptoms that were effectively controlled with maintenance administration of benztropine mesylate in all treated animals.

Animals were euthanized in pairs and tissue was processed as described earlier (Dorph-Petersen *et al*, 2005; Hashimoto *et al*, 2008a; Sweet *et al*, 2009). Three DLPFC tissue sections from each animal, separated by 800 μm, were processed for CB1R immunoreactivity, as described above, except that the anti-CB1R-L15 antibody was diluted 1 : 5000. Levels of CB1R immunoreactivity were measured without the knowledge of animal or drug condition by random coding of slides. CB1R immunoreactivity levels were quantified from images of the ventral bank of the principal sulcus in DLPFC area 46, as described above.

All monkeys were drug naive before onset of haloperidol exposure. All procedures were conducted in accordance with NIH guidelines and were approved by the University of Pittsburgh's Institutional Animal Care and Use Committee.

Statistical Analyses

To test the effect of diagnosis on CB1R immunoreactivity measures, two analysis of covariance (ANCOVA) models

were performed using background corrected mean ROD values (Eggan *et al*, 2008). For cohort 1, the first ANCOVA model used a paired design with ROD entered as the dependent variable, diagnostic group as the main effect, subject pair as a blocking factor, and tissue storage time as a covariate. Subject pairing reduces biological variance by balancing the diagnostic groups for age, sex, and PMI and minimizes inter-assay experimental variance through the parallel processing of tissue samples from each subject pair. However, because subject pairing does not represent a true statistical paired design, a second ANCOVA model was performed using an unpaired design with ROD entered as the dependent variable, diagnostic group as the main effect, and age, sex, PMI, and storage time as covariates. For cohort 2, the first model used a paired design, as described above, to compare each combination of diagnostic groups. The second model used an unpaired design with the same covariates described above, and with a *post hoc* analysis (least significant difference) to test the between group differences. Sex, age, and PMI did not have a significant effect in either cohort 1 (all $F_{(1,18)}<0.21$; $p>0.656$) or cohort 2 (all $F_{(2,35)}<2.4$; $p>0.134$). Storage time also did not have a significant effect in cohort 2 (paired: all $F_{(1,12)}>3.2$; $p>0.092$; unpaired: $F_{(1,35)}=0.06$; $p=0.808$) or in the unpaired model for cohort 1 ($F_{(1,18)}=0.21$; $p=0.651$), but was observed to have a significant effect in the paired model for cohort 1 ($F_{(1,10)}=9.12$; $p=0.013$). Regression analysis revealed no significant correlation between storage time and CB1R immunoreactivity levels in normal comparison ($r=0.06$; $p=0.851$) or schizophrenia ($r=0.42$; $p=0.170$) subjects, or across all subjects ($r=0.12$; $p=0.579$).

For laminar density measures, a multivariate analysis of variance (MANOVA) was performed, with ROD for each layer entered as the dependent variable, diagnosis as the main effect, and sex, age, PMI, and storage time as covariates. For all significant values, the least significant difference *post hoc* test (with $\alpha=0.05$) was used to assess the differences between subject groups within each layer.

The effect of potential confounding factors on CB1R immunoreactivity levels across all subjects with schizophrenia (both cohorts 1 and 2) or MDD were evaluated using an ANCOVA model, with ROD entered as the dependent variable, each confounding factor as the main effect, and sex, age, PMI, and storage time as covariates. As tissue from each cohort was processed in a different immunocytochemical experiment, immunocytochemical run was entered as a blocking factor; however, immunocytochemical run was never observed to have a significant effect and was excluded in the reported analyses. As the addition of covariates reduces the degrees of freedom and may diminish the ability to detect small differences, we also evaluated the effect of each potential confounding factor using a simple analysis of variance model (ANOVA).

RESULTS

Comparison of CB1R Immunoreactivity Levels in Areas 46 and 9 in Cohort 1

Qualitative examination of tissue sections (Figure 1a) revealed an intensity and pattern of CB1R immunoreactivity in DLPFC area 46 identical to that previously reported in human and monkey area 46 (see Figures 1 and 12A in Eggan and Lewis, 2007) and very similar to that in human area 9 (see Figure 5A in Eggan *et al*, 2008). Specifically, intense CB1R immunoreactivity was primarily observed in axons and axon varicosities; the density of these structures progressively increased across cortical layers 2 and 3, formed a distinct, dense band in layer 4, fell strikingly in layer 5, and intensified again in layer 6 (Figure 1a). As reported earlier, CB1R immunoreactivity was detectable in only a few cell bodies (Eggan and Lewis, 2007).

In cohort 1, mean ($\pm$SD) ROD levels of CB1R immunoreactivity in area 46 were significantly (unpaired: $F_{(1,18)}=6.3$; $p=0.022$; paired: $F_{(1,10)}=17.5$; $p=0.002$) 19.1% lower in schizophrenia subjects (0.156 ± 0.023) relative to matched normal comparison subjects (0.193 ± 0.039) (Figure 2a; Supplementary Figure S1), with CB1R immunoreactivity levels lower in the subject with schizophrenia in 9 of 12 pairs (Supplementary Figure S1).

In area 9 from these subjects, CB1R immunoreactivity levels were also significantly 13.9% lower in subjects with schizophrenia relative to normal comparison subjects (Eggan *et al*, 2008). Significant positive correlations were present between areas 9 and 46 for CB1R immunoreactivity across all subjects ($r=0.73$; $p<0.001$; Figure 2b) and for the within-subject pair percent differences in CB1R immunoreactivity ($r=0.62$, $p=0.032$; Figure 2c).

CB1R Immunoreactivity Levels in Area 46 of Cohort 2

In cohort 2, the overall density of CB1R-IR axons in area 46 appeared to be lower in schizophrenia subjects relative to both matched normal comparison and matched MDD subjects (Figure 1). Quantitative assessments revealed a significant main effect of diagnosis (unpaired: $F_{(2,35)}=3.7$; $p=0.036$) on CB1R immunoreactivity levels (Figure 3). *Post hoc* analysis demonstrated that mean levels of CB1R immunoreactivity in schizophrenia subjects (0.172 ± 0.043) were significantly 20.0% lower than in normal comparison subjects (0.215 ± 0.057; $p=0.027$) and significantly 23.3% lower than in MDD subjects (0.224 ± 0.031; $p=0.022$), but did not differ between MDD and normal comparison subjects ($p=0.747$; Figure 3). Paired analyses also demonstrated that CB1R immunoreactivity levels in area 46 were significantly lower in schizophrenia subjects relative to both matched normal comparison (paired: $F_{(1,12)}=5.4$, $p=0.039$; Supplementary Figure S2A) and matched MDD (paired: $F_{(1,12)}=16.9$; $p=0.001$; Supplementary Figure S2B) subjects and that the schizophrenia subject had lower CB1R immunoreactivity levels relative to the matched normal comparison subject in 9 of the pairs and relative to the matched MDD subject in 13 of the pairs (Supplementary Figure S2A and B). In contrast, CB1R immunoreactivity levels did not significantly differ between MDD and normal comparison subjects (paired: $F_{(1,12)}=0.3$, $p=0.596$; Supplementary Figure S2C).

Laminar Assessment of CB1R Immunoreactivity Levels in Area 46 of Cohort 2

The distinctive laminar pattern of CB1R immunoreactivity in area 46 was conserved across subject groups (Figure 1).

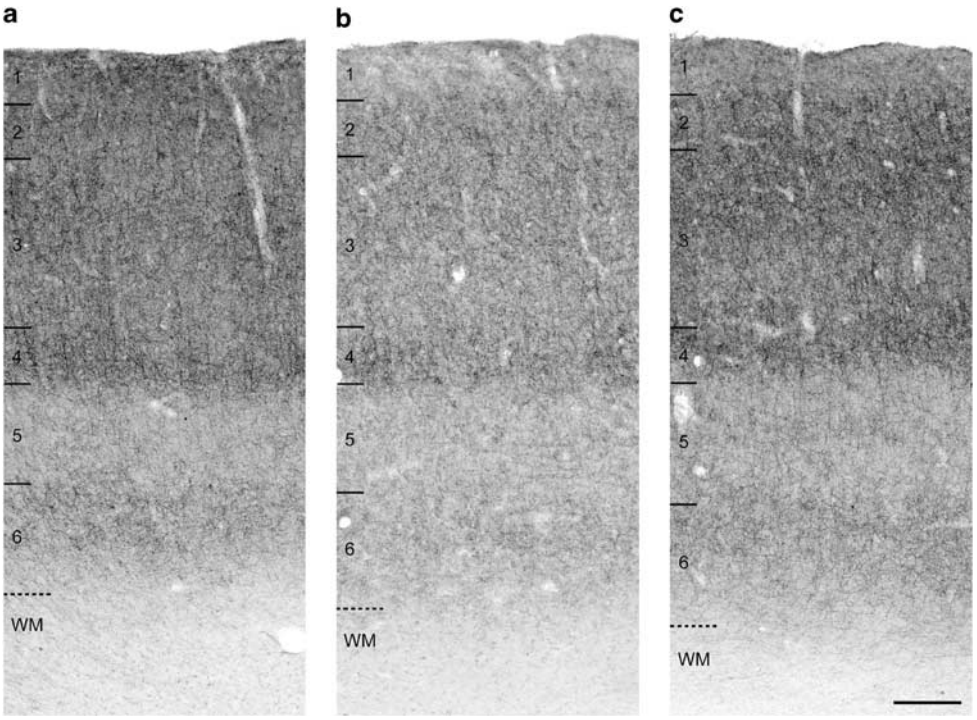

Figure 1 Brightfield photomicrographs demonstrating the density and laminar pattern of CB1R immunoreactivity in DLPFC area 46 of a representative triad of matched normal comparison (a), schizophrenia (b), and major depressive disorder (MDD) subjects (c) (triad 3; Supplementary Table S2). Intense CB1R immunoreactivity was localized to axons and varicosities in all three subjects; however, note the lower density of CB1R-IR axons in the subject with schizophrenia (b). Numbers and hash marks to the left indicate the relative positions of the cortical layers, and the dashed lines denote the layer 6-white matter (WM) border. Scale bar in (c) = 300 μm and applies to all panels.

The levels of CB1R immunoreactivity for the schizophrenia group were lower across all layers relative to both the normal comparison and MDD subject groups, but these differences achieved statistical significance only in layers 1–4 (all $F_{(2,35)} > 3.6$; all $p < 0.036$; Figure 4). *Post hoc* analysis revealed that the schizophrenia group had significantly lower CB1R immunoreactivity in layers 1–4 (all $p < 0.037$) relative to the normal comparison group and in layers 1–4 and layer 6 (all $p < 0.039$) relative to the MDD group (Figure 4).

Confounding Factors and CB1R Immunoreactivity Levels

To assess the effect of potential confounding factors on CB1R protein levels, we compared the levels of CB1R immunoreactivity in area 46 from all 26 subjects with schizophrenia with or without a given confound. ANCOVA analysis demonstrated that mean CB1R immunoreactivity levels in the schizophrenia subjects did not differ as a function sex; diagnosis of schizoaffective disorder; suicide; antidepressant, benzodiazepine, or antipsychotic use at the time of death; diagnosis of substance abuse or dependence at the time of death, or history of cannabis use or abuse (all $F \leqslant 2.8$; all $p \geqslant 0.115$; Figure 5). ANOVA analysis also failed to reveal an effect of each potential confounding variable on CB1R immunoreactivity levels (all $F \leqslant 1.5$; all $p \geqslant 0.228$).

To assess the effect of psychosis or suicide, independent of a diagnosis of schizophrenia, we compared the levels of CB1R immunoreactivity in MDD subjects with or without those factors. The mean levels of CB1R immunoreactivity did not significantly differ in MDD subjects with or without psychotic features (unpaired ANCOVA: $F_{(1,8)} = 2.8$; $p = 0.131$; unpaired ANOVA: $F_{(1,12)} = 0.7$; $p = 0.419$; Supplementary Figure S3A) or who died by suicide or by other means (unpaired ANCOVA: $F_{(1,8)} = 0.8$; $p = 0.403$; unpaired ANOVA: $F_{(1,12)} = 0.6$; $p = 0.468$; Supplementary Figure S3B).

CB1R Immunoreactivity Levels in Haloperidol-Exposed Monkeys

To assess the effect of antipsychotic medications on CB1R protein levels, we compared CB1R immunoreactivity levels in area 46 from monkeys chronically exposed to haloperidol or vehicle. Mean ($\pm$ SD) CB1R immunoreactivity levels in area 46 were 11.5% higher (unpaired: $F_{(1,7)} = 3.9$; $p = 0.095$; paired: $F_{(1,3)} = 10.8$; $p = 0.046$) in haloperidol-exposed monkeys (0.188 ± 0.007) relative to matched control monkeys (0.168 ± 0.018; Figure 6). CB1R immunoreactivity levels were higher in the haloperidol-exposed monkey in each of the four pairs (Figure 6).

DISCUSSION

We recently reported that CB1R mRNA and protein levels were significantly lower in DLPFC area 9 in a cohort of 23 matched pairs of schizophrenia and normal comparison subjects (Eggan *et al*, 2008). In this study, we found that the levels of CB1R immunoreactivity (1) are similarly reduced

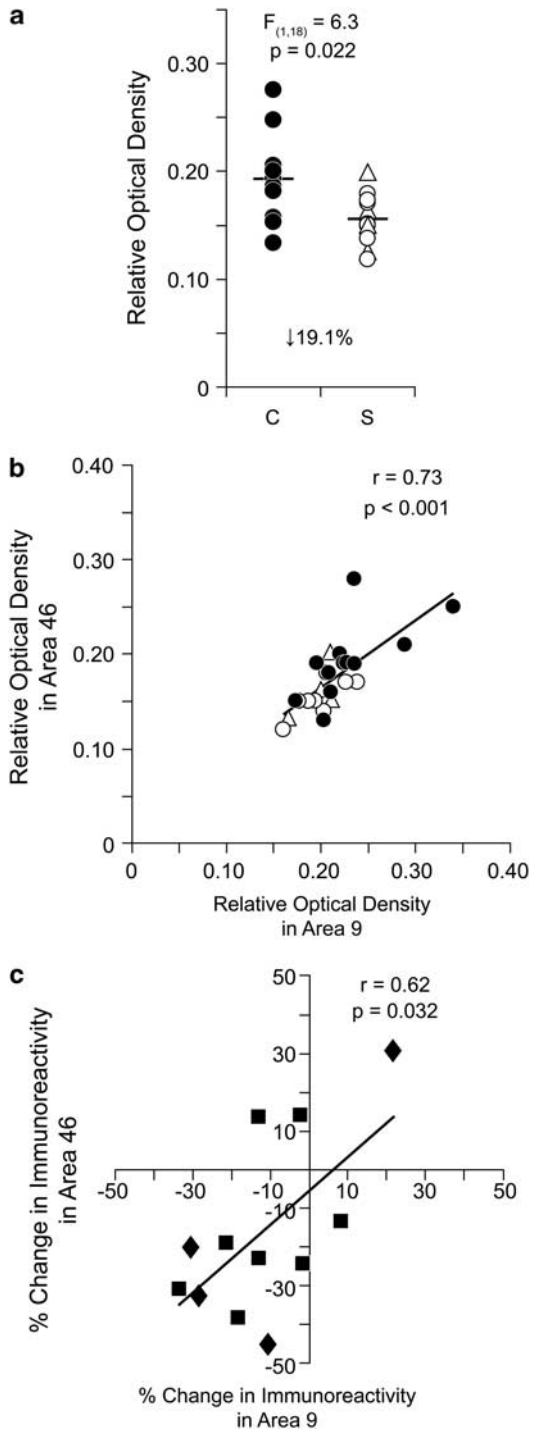

Figure 2 Lower CB1R immunoreactivity in DLPFC area 46 of subjects with schizophrenia from cohort 1. (a) Unpaired analysis of mean cortical ROD levels of CB1R immunoreactivity in normal comparison subjects (C; filled circles) and matched subjects with schizophrenia (S; open circles) or schizoaffective disorder (open triangles). Mean values for each subject group are indicated by horizontal bars. The ROD levels of CB1R immunoreactivity (b) and within-pair percent change in CB1R immunoreactivity levels (c) measured in area 46 correlated with those previously reported in area 9 (Eggan *et al*, 2008) of the same subjects with schizophrenia. (b) Closed circles denote normal comparison subjects and open circles or triangles denote schizophrenia or schizoaffective disorder subjects, respectively. (c) Closed squares denote matched normal comparison and schizophrenia subject pairs and closed diamonds denote normal comparison and schizoaffective disorder subject pairs.

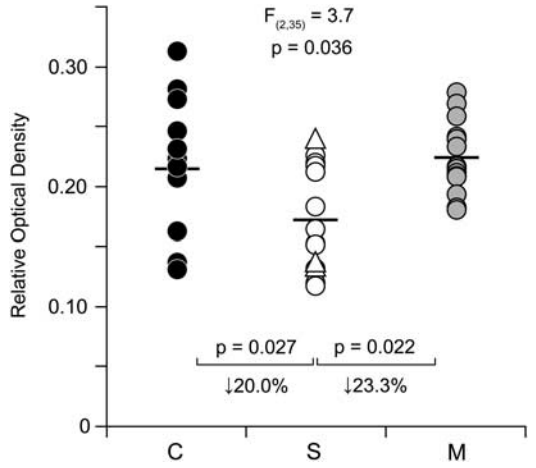

Figure 3 Lower CB1R immunoreactivity in DLPFC area 46 of subjects with schizophrenia from cohort 2. Unpaired analysis of cortical ROD levels of CB1R immunoreactivity in matched normal comparison subjects (C; closed circles), subjects with schizophrenia (S; open circles) or schizoaffective disorder (open triangles), and subjects with major depressive disorder (M; gray circles). Mean values for each subject group are indicated by horizontal bars.

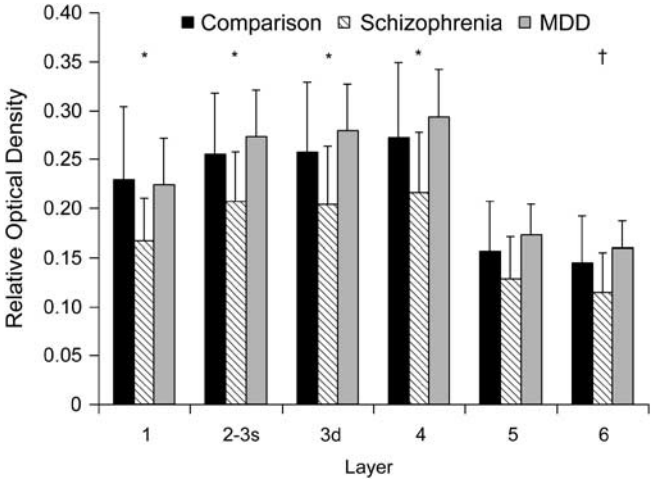

Figure 4 Lower CB1R immunoreactivity levels across cortical layers in DLPFC area 46 of subjects from cohort 2. The distinctive laminar pattern of CB1R immunoreactivity was conserved across subject groups; however, the levels of CB1R immunoreactivity for the schizophrenia group were reduced across all layers relative to the normal comparison and MDD groups. Comparison of mean (±SD) ROD levels of CB1R immunoreactivity in each cortical layer revealed that CB1R immunoreactivity levels were significantly ($p<0.05$) lower in schizophrenia subjects in layers 1–4 relative to both normal comparison and MDD subjects (*), and in layer 6 relative to MDD subjects (†). 3s indicates superficial layer 3; 3d indicates deep layer 3.

in areas 9 and 46 of the same subjects with schizophrenia; (2) are also reduced in area 46 in a new cohort of schizophrenia subjects; (3) are not altered in subjects with MDD, with or without the presence of psychotic features or death by suicide; and (4) are not apparently influenced by antipsychotic exposure or other confounding factors. These data suggest that the lower levels of CB1R protein may be common and conserved across DLPFC regions in

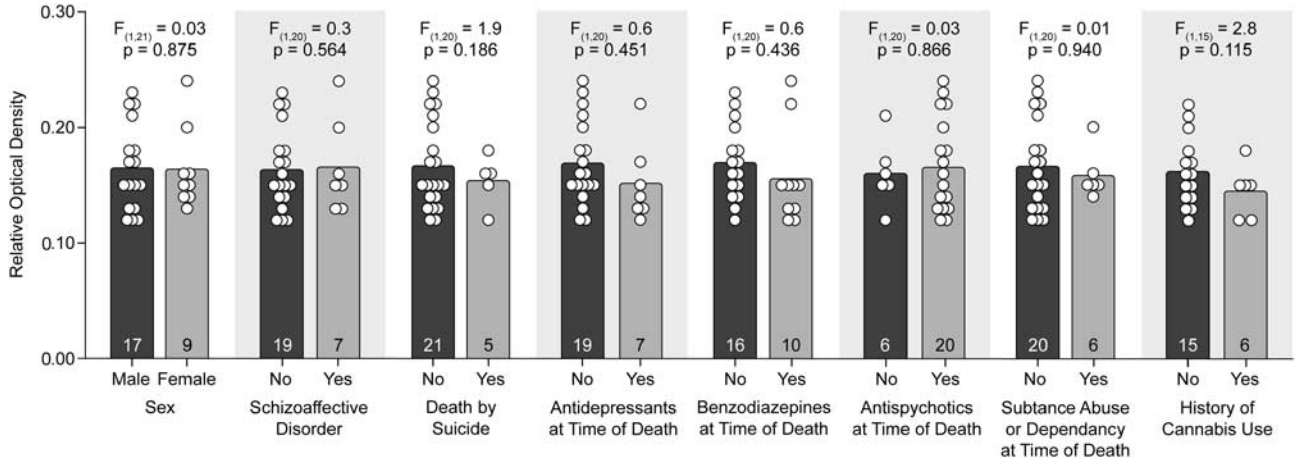

Figure 5 The effects of confounding factors on CB1R immunoreactivity levels in schizophrenia. Mean (bar) and individual subject (circles) ROD levels of CB1R immunoreactivity in the subjects with schizophrenia grouped by potential confounding factors. Neither sex, diagnosis of schizoaffective disorder, suicide, antidepressant medication use at the time of death, benzodiazepine use at the time of death, antipsychotic medication use at the time of death, diagnosis of substance abuse/dependence at the time of death, nor history of cannabis use/abuse significantly affected levels of CB1R immunoreactivity. Numbers in bars indicate the number of subjects with schizophrenia in each category. A history of cannabis use was unknown for five of the schizophrenia subjects in cohort 2; thus those subjects were not included in the analysis.

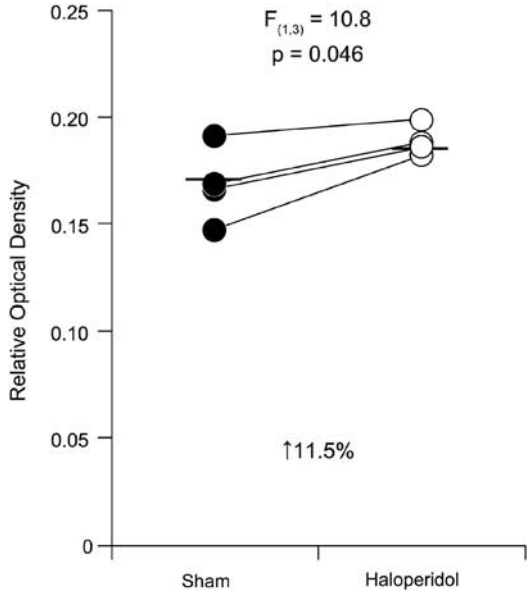

Figure 6 Higher CB1R immunoreactivity in DLPFC area 46 of monkeys chronically exposed to the antipsychotic haloperidol. Paired analysis of mean cortical ROD levels of CB1R immunoreactivity in sham-exposed monkeys (filled circles) and matched monkeys chronically exposed to haloperidol (open circles). Mean values for each subject group are indicated by horizontal bars.

schizophrenia, and show at least some specificity for the disease process of this illness.

Specificity of Lower CB1R Levels to the Disease Process of Schizophrenia

Convergent lines of evidence suggest that lower CB1R protein levels in the DLPFC of subjects with schizophrenia are not due to comorbid depression or suicide, and are not a consequence of psychosis in general. First, in contrast to schizophrenia, no alterations in CB1R immunoreactivity levels were observed in MDD subjects. In another study, CB1R protein levels in MDD subjects, assessed by western blot analysis, were reported to be significantly increased in area 9 of MDD subjects (Hungund *et al*, 2004). Preclinical data demonstrate that CB1R agonists and endocannabinoid enhancers possess antidepressant-like properties (Bortolato *et al*, 2007; Mangieri and Piomelli, 2007) and clinical evidence suggests that the CB1R antagonist rimonabant increases the risk of depression and suicidality (Christensen *et al*, 2007). Thus, although this interpretation is speculative, higher CB1R protein levels in MDD subjects might represent a compensatory, albeit insufficient, response to ameliorate depressive symptoms. Second, the lower levels of CB1R immunoreactivity in schizophrenia were not associated with death by suicide and CB1R immunoreactivity levels did not differ between MDD subjects with or without death by suicide. Third, CB1R immunoreactivity levels were not significantly affected by the presence of psychotic features in some of the MDD subjects. Thus, the lower levels of CB1R protein observed in schizophrenia do not seem to be attributable to depression, suicide, or psychosis, and thus might be specific to the disease process of schizophrenia.

This interpretation is further supported by the finding that lower levels of CB1R immunoreactivity in DLPFC area 46 of subjects with schizophrenia cannot be attributed to pharmacological treatment of the illness; CB1R immunoreactivity levels in schizophrenia subjects did not differ as a function of benzodiazepine, antidepressant, or antipsychotic medication use at the time of death. A recent study reported that CB1R protein levels, assessed by western blot analysis, were significantly lower in the prefrontal cortex of antipsychotic-treated, but not of drug-free, schizophrenia subjects (Uriguen *et al*, 2009); however, several other lines of evidence indicate that prefrontal CB1R levels are not affected by antipsychotic medications. First, CB1R mRNA expression levels were unaltered in the DLPFC of monkeys

chronically exposed to therapeutic serum levels of either typical (haloperidol) or atypical (olanzapine) antipsychotic medications (Eggan *et al*, 2008). Similarly, exposure to a variety of antipsychotic medications did not alter the density of CB1Rs, measured by radioligand binding, in the rat prefrontal cortex (Sundram *et al*, 2005; Wiley *et al*, 2008). Consistent with these observations, the six subjects with schizophrenia in this study (three subjects each from cohorts 1 and 2; Supplementary Tables S1 and S2) who were off antipsychotic medications at the time death all had lower CB1R immunoreactivity levels in area 46 than their matched normal comparison subjects. Second, the mean within-subject pair percent difference in CB1R immunoreactivity was larger in those schizophrenia subjects who were not receiving antipsychotic medications at the time of death (-33.3%; $n=6$) compared with those who were receiving antipsychotic medications at the time of death (-14.9%; $n=20$). Third, although the sample size was small, CB1R immunoreactivity levels showed a trend to being higher in area 46 from monkeys after long-term exposure to haloperidol at doses that mimicked the therapeutic treatment of schizophrenia (Figure 6). Together, these data suggest that exposure to antipsychotic medications does not account for the lower CB1R immunoreactivity levels observed in schizophrenia, and might have actually blunted the disease-related decrease.

The lower levels of CB1R immunoreactivity also do not seem to be driven by other potential confounding factors that are commonly associated with schizophrenia. For example, consistent with our earlier findings for CB1R mRNA (by *in situ* hybridization) and protein (by radioimmunocytochemistry) in area 9 (Eggan *et al*, 2008), CB1R immunoreactivity levels in area 46 of schizophrenia subjects did not differ as a function of sex, a diagnosis of schizoaffective disorder, or a diagnosis of substance abuse/dependence at the time of death (Figure 5).

Although our analysis considered all substances of abuse together (except cannabis that was assessed separately), nicotine and alcohol exhibit distinct interactions with cannabinoid signaling; thus, use of these substances by schizophrenia subjects may represent potential confounds. For example, evidence suggests a function for CB1Rs in modulating nicotine addiction (Fagerstrom and Balfour, 2006) and the high prevalence and extensive use of nicotine by individuals with schizophrenia is well documented. The effect of nicotine is difficult to control for in postmortem studies since, even if schizophrenia and comparison subjects could be matched for pack years of smoking history, individuals with schizophrenia are likely to have had greater nicotine exposure due to their tendency to extract more nicotine per cigarette. However, to our knowledge, only one study has investigated the effect of nicotine exposure on CB1R protein levels (Marco *et al*, 2007). This study in rodents showed that subchronic exposure to nicotine during adolescence produced a significant increase in CB1R immunoreactivity in the hippocampus measured by western blot analysis. Thus, a history of nicotine use in our schizophrenia subjects may have actually blunted the observed reduction in CB1R protein levels. In rodents, chronic ethanol exposure may downregulate CB1Rs in some brain regions (Basavarajappa and Hungund, 2002; Hungund and Basavarajappa, 2004); however, region-specific effects of drugs on CB1R expression are reported (Sundram *et al*, 2005; Wiley *et al*, 2008). Indeed, CB1R mRNA expression is reported to be increased 2.6-fold in the prefrontal cortex of alcohol-exposed rats (Rimondini *et al*, 2002). Consistent with these data, in humans, CB1R ligand-binding levels are higher in both alcoholic subjects and alcoholic suicide victims compared with normal control subjects or non-alcoholic suicide victims, respectively (Hungund *et al*, 2004; Vinod *et al*, 2005). In our study, the mean ($\pm$SD) level of CB1R immunoreactivity was 0.158 ± 0.021 in the subjects with schizophrenia who had a diagnosis of alcohol abuse/dependence at the time of death ($n=6$) (11.2% lower relative to matched comparison subjects) compared with 0.166 ± 0.039 (22.1% lower relative to matched comparison subjects) in the subjects with schizophrenia without a diagnosis of alcohol abuse/dependence ($n=20$). Furthermore, mean ($\pm$SD) CB1R immunoreactivity levels did not differ ($F_{(1,20)}=0.006$; $p=0.940$) between those schizophrenia subjects with or without a diagnosis of alcohol abuse/dependence at the time of death. Thus, a history of alcohol abuse/dependency at the time of death does not seem to account for the observed decreases in CB1R protein in subjects with schizophrenia.

Finally, the findings from this study suggest that cannabis use does not seem to account for lower levels of prefrontal CB1R immunoreactivity in schizophrenia. First, consistent with our earlier findings of CB1R mRNA and protein in area 9 (Eggan *et al*, 2008), CB1R immunoreactivity levels in area 46 of schizophrenia subjects did not differ as a function of a prior history of cannabis use. Second, in cohort 2, both the schizophrenia and matched MDD subject were known to have a history of cannabis use for three subject pairs, and for five of the subject pairs both members did not have a history of cannabis use (Supplementary Table S2). In the five subject pairs without a history of cannabis use, the mean level of CB1R immunoreactivity in area 46 was 23.5% lower in schizophrenia subjects relative to the matched MDD subjects. Similarly, in the three subject pairs with a history of cannabis use, the mean level of CB1R immunoreactivity was 26.9% lower in schizophrenia subjects relative to the matched MDD subjects. Consistent with these observations, measures of CB1Rs do not seem to be permanently altered in the prefrontal cortex of either monkeys or rodents after exposure to CB1R agonists (Westlake *et al*, 1991; Rubino *et al*, 1994; Zhuang *et al*, 1998; Romero *et al*, 1998; Garcia-Gil *et al*, 1999). However, the findings of the effects of CB1R agonist exposure on CB1R levels are mixed and seem to be dependent on the dose administered, length of exposure, and the brain region studied (Romero *et al*, 1995, 1997; Zhuang *et al*, 1998; Sim-Selley, 2003); thus, although an effect of cannabis use cannot be ruled out, the findings are consistent with the idea that earlier cannabis use does not account for lower levels of prefrontal CB1R immunoreactivity in schizophrenia.

In concert, these data suggest that neither depression, suicide, psychosis, antipsychotic medications, other potential confounding factors, nor a prior history of cannabis use seem to account for lower levels of CB1R immunoreactivity in the DLPFC from two independent cohorts of subjects with schizophrenia. Thus, although our analysis of potential confounding factors may suffer from type II error, the

findings all converge on the hypothesis that lower CB1R levels in the DLPFC may reflect the disease process of schizophrenia.

In contrast, CB1R-IR structures in the anterior cingulate cortex were reported to be unaltered in schizophrenia subjects (Koethe *et al*, 2007); importantly, the specificity of the antibody used was not adequately tested and the reported anatomical localization and laminar distribution of CB1R immunoreactivity was inconsistent with those previously demonstrated in rodent, monkey, and human cortex in studies using well-characterized CB1R antibodies (Katona *et al*, 2000; Egertová and Elphick, 2000; Morozov and Freund, 2003; Bodor *et al*, 2005; Eggan and Lewis, 2007; Eggan *et al*, 2008). In addition, increased binding of the CB1R agonist [^{3}H]CP-55940 was reported in the DLPFC (Dean *et al*, 2001) and posterior cingulate cortex (Newell *et al*, 2006) of schizophrenia subjects. However, increased [^{3}H]CP-55940 binding might reflect differences other than the amount of receptor present because CB1Rs contain an allosteric modulation site that, when bound, elicits a conformational change in the receptor increasing the affinity of [^{3}H]CP-55940 for the orthosteric-binding site (Price *et al*, 2005; Horswill *et al*, 2007). Increased binding of the CB1R antagonist [^{3}H]SR141716 was also reported in the anterior cingulate cortex of subjects with schizophrenia (Zavitsanou *et al*, 2004); however, SR141716 has functional effects in CB1R knockout mice, suggesting that it binds receptors other than the CB1R (Breivogel *et al*, 2001; Hajos *et al*, 2001). Importantly, saturation and competition experiments were not performed in any of these studies to determine whether the number of binding sites or the affinity for the ligand were altered in the illness. Thus, the binding of these radioligands may not represent the relative amount of CB1R protein present. However, it is possible that schizophrenia is associated with higher levels of cellular membrane-bound CB1Rs with overall CB1R mRNA and protein levels downregulated due to reduced internalization of CB1Rs. Studies of CB1R binding with specific ligands, and protein and mRNA levels in adjacent tissue sections are necessary to address this possibility; the advent of new CB1R-specific PET ligands (Horti *et al*, 2006; Burns *et al*, 2007; Yasuno *et al*, 2008; Terry *et al*, 2010) could be useful in this regard, as well as allowing for the assessment of CB1R levels *in vivo*.

Pathophysiological Significance of Lower CB1R Protein Levels in Schizophrenia

Although CB1R protein is present in both inhibitory and excitatory cortical axon terminals (Bodor *et al*, 2005; Kawamura *et al*, 2006; Katona *et al*, 2006; Eggan and Lewis, 2007), lower CB1R immunoreactivity levels in schizophrenia likely represent a reduced amount of CB1R protein specifically in the axons and terminals of CCK-containing GABA neurons because (1) CCK-containing axon terminals contain much higher levels of CB1Rs and are more sensitive to the effects of CB1R agonists than are pyramidal cell axon terminals (Marsicano and Lutz, 1999; Ohno-Shosaku *et al*, 2002); (2) CB1R- and CCK-containing structures in macaque monkey DLPFC exhibit similar overall distribution patterns and undergo nearly identical changes in laminar distribution during postnatal development (Oeth and Lewis, 1993; Eggan *et al*, 2010); and (3) the antibody used in this study exclusively labels symmetric, inhibitory synapses (Katona *et al*, 1999, 2001; Eggan and Lewis, 2007). Furthermore, alterations in CB1R mRNA levels in area 9 of schizophrenia subjects strongly correlate with those of GAD_{67} and CCK mRNAs, suggesting that lower CB1R protein levels in the DLPFC in schizophrenia accompany deficient GABA synthesis in CCK neurons (Eggan *et al*, 2008).

The lower levels of CB1R protein in the DLPFC of schizophrenia subjects could be secondary to increased endocannabinoid levels. Indeed, elevated levels of the endocannabinoid anandamide have been reported in the blood and cerebral spinal fluid of schizophrenia patients (Leweke *et al*, 1999; De Marchi *et al*, 2003; Giuffrida *et al*, 2004). However, the principal endocannabinoid for CB1Rs in the cortex and hippocampus seems to be 2-arachidonoylglycerol (2-AG), not anandamide (Kim and Alger, 2004; Makara *et al*, 2005; Hashimotodani *et al*, 2007); however, 2-AG levels cannot be reliably assessed in postmortem tissue and whether 2-AG levels are elevated in the DLPFC of schizophrenia subjects is unknown.

Alternatively, the lower levels of CB1R immunoreactivity in schizophrenia could represent a compensatory downregulation of CB1R protein in response to deficient GABA synthesis in the DLPFC (Eggan *et al*, 2008). Reduced GAD_{67} mRNA levels in the DLPFC are one of the most consistently replicated findings in schizophrenia and are thought to contribute, at least in part, to DLPFC-related working memory deficits in the illness (Lewis *et al*, 2005; Akbarian and Huang, 2006). In the DLPFC, normal GABA-mediated neurotransmission is necessary for working memory function (Sawaguchi *et al*, 1988; Rao *et al*, 2000). In particular, networks of interconnected GABA interneurons are essential for the synchronization of large ensembles of neurons (Connors and Long, 2004), including γ-band oscillations that increase in the DLPFC with working memory load in humans (Howard *et al*, 2003). As the activation of CB1Rs suppress GABA neurotransmission in the DLPFC, a lower density of CB1Rs could, by reducing the endocannabinoid-mediated block of GABA release from the terminals of CB1R/CCK-containing interneurons, compensate for lower levels of GAD_{67}-mediated GABA synthesis in those neurons and contribute to a partial, albeit insufficient, normalization of γ-band power and working memory function.

This interpretation is supported by the finding that, in contrast to the common and conserved lower levels of CB1R immunoreactivity in the DLPFC of schizophrenia subjects, no alterations in CB1R immunoreactivity levels were observed in the DLPFC of MDD subjects in this study. Importantly, other studies report that GAD_{67} mRNA expression levels are unaltered in the DLPFC of MDD subjects (Guidotti *et al*, 2000; Morris and Lewis, 2009). These data suggest that working memory impairments in MDD and schizophrenia may arise from disparate mechanisms. Consistent with this idea, a recent longitudinal study demonstrated that children who developed adult schizophrenia exhibited premorbid developmental impairments including static deficits in cognitive functions such as reasoning and conceptualization and developmental lags in other cognitive functions such as attention and working

memory, whereas children who later developed recurrent depression did not exhibit those premorbid cognitive patterns (Reichenberg *et al*, 2010).

If CB1R downregulation is compensatory for deficient GABA neurotransmission, then reduced levels of CB1R protein may not be restricted to the DLPFC, but may also occur in other cortical regions that exhibit deficient GABA neurotransmission in schizophrenia. For example, overall GAD_{67} mRNA levels, and the magnitude of GAD_{67} mRNA alterations in schizophrenia, are similar across DLPFC area 9, and anterior cingulate, primary motor, and primary visual cortices, suggesting that impaired GABA neurotransmission may broadly contribute to cortical dysfunction in schizophrenia (Hashimoto *et al*, 2008b). However, CB1R immunoreactivity levels are heterogeneous across cortical regions, with regions associated with higher cognitive functions, such as the DLPFC, containing much higher densities of CB1R-containing axons than primary motor or visual cortices (Eggan and Lewis, 2007). These data suggest that the capacity for endocannabinoid-mediated compensatory responses to GABA deficits may differ across cortical regions, with lower levels of CB1R protein being a particularly salient compensation for deficient GABA neurotransmission in the DLPFC.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)