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Cortical GAD₆₇ deficiency results in lower cannabinoid 1 receptor mRNA expression: Implications for schizophrenia

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Abstract

Background—Levels of cannabinoid 1 receptor (CB1R) mRNA and protein, which are expressed most heavily in the cholecystinin class of GABA neurons, are lower in the dorsolateral prefrontal cortex (DLPFC) in schizophrenia, and the magnitude of these differences is strongly correlated with that for glutamic acid decarboxylase (GAD₆₇) mRNA, a synthesizing enzyme for GABA. However, whether this correlation reflects a cause-effect relationship is unknown.

Methods—Using quantitative *in situ* hybridization, we measured CB1R, GAD₆₇, and diacylglycerol lipase alpha (DAGL α ; the synthesizing enzyme for the endocannabinoid 2-arachidonoylglycerol) mRNA levels in the medial prefrontal cortex of genetically-engineered GAD₆₇ heterozygous (GAD₆₇^{+/-}), CB1R heterozygous (CB1R^{+/-}), CB1R knockout (CB1R^{-/-}), and matched wild-type mice.

Results—In GAD₆₇^{+/-} mice, GAD₆₇ and CB1R mRNA levels were significantly reduced by 37% and 16%, respectively, relative to wild-type mice and were significantly correlated across animals ($r=0.61$; $p=0.01$). In contrast, GAD₆₇ mRNA levels were unaltered in CB1R^{+/-} and CB1R^{-/-} mice. Expression of DAGL α mRNA, which is not altered in schizophrenia, was also not altered in any of the genetically-engineered mice.

Conclusions—The findings that reduced GAD₆₇ mRNA expression can induce lower CB1R mRNA expression support the hypothesis that lower cortical levels of CB1Rs in schizophrenia may partially compensate for deficient GAD₆₇-mediated GABA synthesis by reducing endogenous cannabinoid suppression of GABA release.

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Keywords

Cannabis; cholecystokinin; cognition; GABA; in situ hybridization; interneurons; mouse model; working memory

Introduction

Alterations in subpopulations of γ -aminobutyric acid (GABA) neurons appear to contribute to dysfunction of the dorsolateral prefrontal cortex (DLPFC) in schizophrenia. Indeed, lower expression of glutamic acid decarboxylase (GAD_{67}), the enzyme responsible for most GABA synthesis, is consistently found in schizophrenia (1). The affected GABA neurons include basket neurons that express both the cannabinoid 1 receptor (CB1R) and the neuropeptide cholecystokinin (CCK) (2–4). For example, CB1R mRNA and protein levels are lower in the DLPFC of subjects with schizophrenia (5–7), and the magnitude of alterations in CB1R mRNA expression is significantly correlated with that for GAD_{67} mRNA (3,6). These findings suggest that both transcripts are lower in the same population of DLPFC GABA neurons in schizophrenia.

Because CB1R activation suppresses GABA neurotransmission (8), we previously suggested that a lower density of CB1Rs in schizophrenia could be a cell type-specific, homeostatic adaptation to partially compensate for upstream reductions in GAD_{67} -mediated GABA synthesis in CB1R/CCK-containing neurons (5,6). That is, a down-regulation of CB1Rs could reduce the endocannabinoid-mediated block of GABA release from the terminals of CB1R/CCK-containing neurons, thereby enhancing GABA neurotransmission in cells with deficient GABA synthesis (5,6). As a proof-of-concept test of this hypothesis, we assessed GAD_{67} and CB1R mRNA levels in the medial prefrontal cortex of genetically-engineered GAD_{67} heterozygous ($GAD_{67}^{+/-}$), CB1R heterozygous ($CB1R^{+/-}$), CB1R knockout ($CB1R^{-/-}$), and matched wild-type (WT) mice. In addition, to test the specificity of the causal relationship between reduced GAD_{67} mRNA and lower CB1R mRNA levels, we also assessed mRNA levels for diacylglycerol lipase alpha ($DAGL\alpha$), the synthesizing enzyme for 2-arachidonoylglycerol (2-AG) (the principal endocannabinoid in the DLPFC), which are unaltered in schizophrenia (9).

Methods and Materials

Animals and Tissue Processing

Generation of GAD_{67} heterozygous mice— $GAD_{67}^{+/-}$ mice were generated as previously described (10). Exon 2 (the first coding exon) of the *Gad1* gene was flanked by loxP sites using gene targeting in embryonic stem cells. Using FLP recombinase, the Sv-NeoR selectable gene was removed to produce $Gad1^{lox/+}$ mice, which were interbred to generate phenotypically normal $Gad1^{lox/lox}$ mice. $Gad1^{lox/lox}$ mice were bred with Mox2-Cre mice to delete exon 2 in the germ-line ($Gad1^{\Delta/+}$ [$GAD_{67}^{+/-}$]) mice. Interbreeding of $Gad1^{\Delta/+}$ mice generated some mice that did not survive beyond the perinatal period, consistent with previously described *Gad1*-null mice (11). Age, sex, and litter-matched (10 males and 4 females) $GAD_{67}^{+/-}$ and WT mice (n=7 per group) were euthanized at eight weeks of age and brains were removed, frozen, and stored at -80°C .

Generation of CB1R heterozygous and knockout mice— $CB1R^{+/-}$ and $CB1R^{-/-}$ mice were generated as previously described (12). The *Cnr1* gene was mutated in MPI2 embryonic stem cells by replacing the coding region between amino acids 32 and 448 with PGK-neo. Chimeric mice derived from these cells were bred with C57BL/6J animals. Backcrossing of chimeric and heterozygous animals to C57BL/6J mice and interbreeding of

CB1R^{+/-} animals produced CB1R^{-/-} mutants and wild-type mice. Male animals were euthanized at either eight weeks of age (CB1R^{+/-}, CB1R^{-/-}, and WT; n=6 per group) or four weeks of age (CB1R^{-/-} and WT; n=7 per group) and brains were removed, frozen, and stored at -80°C. Fresh frozen brains were provided by Bristol-Myers Squibb.

Coronal sections from all mouse brains were cut on a cryostat at 12 µm, thaw mounted onto SupraFrost slides (Fisher Scientific, Pittsburgh, PA), and stored at -80°C until used.

***In situ* hybridization**

Templates for the synthesis of riboprobes against mouse GAD₆₇, CB1R, and DAGL α mRNA were generated by polymerase chain reaction (see Table). Nucleotide sequencing revealed 100% homology for the amplified template fragments to previously reported sequences. Sense and antisense riboprobes were generated by *in vitro* transcription in the presence of ³⁵S-CTP using T7 or SP6 RNA polymerase, purified, and reduced to approximately 100 bp by alkaline hydrolysis to increase the effectiveness of tissue penetration (13). Standard hybridization procedures were performed as previously described (13). Following hybridization, sections from all mice for a given comparison were exposed to BioMaxMR film (Kodak, Rochester, NY) for 24 hours (GAD₆₇), 48–72 hours (CB1R), or 36 hours (DAGL α). Tissue sections processed by *in situ* hybridization for CB1R mRNA were subsequently coated with NTB2 emulsion (Kodak) using a mechanical dipper (Auto-dip Emulsion Coater, Ted Pella, Redding, CA), exposed at 4°C, then developed using D-19 (Kodak), and counterstained with Cresyl violet. Specificity of the hybridization signal produced by each probe was confirmed by the findings that each antisense probe produced the expected distinctive laminar pattern of expression (4,14,15) and by the absence of labeling with sense probes.

Quantification

Quantification was performed blind to condition and animal number by random coding of slides as previously described (6). Autoradiographic film images of GAD₆₇, CB1R, and DAGL α mRNA were captured using a Microcomputer Imaging Device (MCID) (5.1 µm/pixel resolution) and digitized. All images for slides processed in an experimental run were acquired in the same session under identical room illumination and with the same gain and black levels and flatfield correction. Three sections evenly spaced at ~144 µm intervals containing the medial prefrontal cortex (mPFC; +1.98 to +1.54 bregma (16)), including the cingulate and prelimbic cortices, were selected from each mouse for quantification. For each section, optical density (OD) levels of GAD₆₇, CB1R, and DAGL α mRNA were measured bilaterally from the pial surface to the white matter in the mPFC and expressed as nanocuries per gram of tissue (nCi/g) by reference to carbon-14 standards (ARC Inc., St. Louis, MO) exposed on the same film. All cortical density measures were corrected by subtracting background measured in the white matter.

Quantification of CB1R mRNA at the cellular level was performed as previously described (13) for the 5 pairs of GAD₆₇ WT and heterozygous mice with available emulsion-dipped, Nissl-counterstained sections. Using the MCID software and a Nikon microscope with a motorized stage, sampling boxes (120 × 170 µm) were systematically tiled from the pial surface to the layer 6 - white matter border in both hemispheres of the mPFC. Sampling circles with a fixed diameter of 16 µm (15) were placed over CB1R silver grain clusters, and the number of silver grains per circle was quantified. Background signal, determined for each tissue section by quantifying grains in a 120 × 170 µm sampling box placed in the white matter, was subtracted from each grain cluster before analysis. Examination of individual grain cluster counts revealed 13 clusters that were ≥ 2SD away from the mean,

and these clusters were excluded from analyses as outliers. A total of 119 and 118 CB1R grain clusters were analyzed for GAD₆₇ WT and heterozygous mice, respectively.

Statistics

T tests (GAD₆₇^{+/-} and WT mice) or analysis of variance (CB1R^{+/-}, CB1R^{-/-}, and WT mice) were performed to test the effect of genetic condition on OD measures using mean values across all of the sections from each animal, and two-tailed paired t-tests were used to assess group differences in grain density measures. One-tailed Pearson correlation analysis was performed to test the *a priori* hypothesis that GAD₆₇ mRNA levels positively predict CB1R mRNA levels (6).

Results

Transcript levels in GAD₆₇ heterozygous mice

Mean (\pm SD) GAD₆₇ mRNA levels were significantly 37.2% lower ($t_{12}=7.11$; $p<0.001$) in GAD₆₇^{+/-} mice (664.9 ± 39.0 nCi/g) relative to WT mice (1059.4 ± 141.4 nCi/g) (Figure 1A–C). Mean CB1R mRNA levels were also significantly 15.7% lower ($t_{12}=2.35$; $p=0.036$) in GAD₆₇^{+/-} mice (402.1 ± 56.8 nCi/g) relative to WT mice (477.0 ± 62.4 nCi/g) (Figure 1D–F). Furthermore, GAD₆₇ and CB1R mRNA levels were positively correlated across all mice ($r=0.61$; $p=0.010$; Figure 2). In contrast, DAGL α mRNA levels did not differ ($t_{12}=0.0$; $p=0.999$) between groups (Figure 1G–I).

We next sought to determine whether lower CB1R mRNA levels were specific to mPFC in GAD₆₇^{+/-} mice or were also found in other cortical brain regions, such as the supplementary motor area. First, as expected (17), in WT mice CB1R expression was lower in supplementary motor area (402.5 ± 51.9 nCi/g) than in mPFC (477.0 ± 62.4 nCi/g) and CB1R mRNA levels were highly correlated between these two cortical regions ($r=0.86$, $p<0.001$). Similar to mPFC, mean CB1R mRNA levels were 11.0% lower in GAD₆₇^{+/-} mice (358.3 ± 47.7 nCi/g) relative to WT mice (402.5 ± 51.9 nCi/g), although this difference did not reach statistical significance ($t_{12}=1.66$; $p=0.123$). The reduced strength of the finding in the supplementary motor area relative to mPFC may reflect the lower baseline levels of CB1R expression in motor areas (17); indeed, we previously suggested that reduced CB1R mRNA is likely to be an effective compensatory response to a deficit in GAD67 expression only in regions, like the PFC, with high levels of CB1R expression (6).

Grain counting analyses (Figure 3A) revealed a similar effect of reduced GAD67 expression on cellular CB1R mRNA levels. The mean number of CB1R grains per neuron was 12% lower ($t_{10}=1.79$, $p=0.08$) in GAD₆₇^{+/-} mice (40.8 ± 5.1) relative to WT mice (36.0 ± 3.7) (Figure 3B).

Transcript levels in CB1R heterozygous and knockout mice

Manipulation of the *Cnr1* gene produced the expected gene dose-dependent effect ($F_{1,15}=381.6$; $p<0.001$) on CB1R mRNA expression (Figure 4A–D). Post-hoc analysis demonstrated that mean CB1R mRNA levels were significantly 49.6% lower in CB1R^{+/-} mice (210.9 ± 37.2) compared to WT mice (418.9 ± 21.7) and 94.6% lower in CB1R^{-/-} mice (22.6 ± 2.2). However, neither GAD₆₇ ($F_{1,15}=0.4$; $p=0.653$; Figure 4E–H) nor DAGL α ($F_{1,15}=2.4$; $p=0.126$; Figure 3I–L) mRNA levels were altered in CB1R^{+/-} or CB1R^{-/-} mice relative to WT mice.

To determine if age-related compensations in GAD₆₇ mRNA expression occur during development, we assessed GAD₆₇ mRNA levels in four week old CB1R^{-/-} and WT mice.

In these mice, cortical GAD₆₇ mRNA levels did not differ ($F_{1,12}=0.2$; $p=0.671$) between CB1R^{-/-} (536.7 ± 41.4 nCi/g) and WT mice (549.4 ± 64.7 nCi/g).

Discussion

Because deficits in GAD₆₇ and CB1R mRNA levels are strongly correlated in the PFC in schizophrenia (6), we used genetically-engineered mice to investigate the plausibility of the hypothesis that a deficiency in GAD₆₇ mRNA expression induces a corresponding reduction in CB1R mRNA, versus the alternative hypothesis that lower CB1R mRNA leads to a reduction in GAD₆₇ expression. We found that GAD₆₇^{+/-} mice with a mean 37% decrease in GAD₆₇ mRNA in the mPFC had CB1R mRNA levels that were significantly 16% lower than WT mice, and that GAD₆₇ and CB1R mRNA levels were positively correlated across animals. Together, these data demonstrate that reduced GAD₆₇ mRNA expression in mice is sufficient to produce lower levels of CB1R mRNA in the mPFC. In contrast, GAD₆₇ mRNA levels were not changed in either peripubertal or adult mice with reduced CB1R mRNA expression, demonstrating that reduced CB1R mRNA expression does not affect GAD₆₇ mRNA levels. In concert with our previous finding that alterations in CB1R and GAD₆₇ mRNA expression in schizophrenia are strongly correlated ($r=0.64$; $p=0.001$) (6), these data support the hypothesis that reduced GAD₆₇ mRNA expression may drive lower CB1R mRNA expression in the DLPFC of subjects with schizophrenia, whereas deficient CB1R mRNA expression is unlikely to be a cause of lower GAD₆₇ mRNA in the disorder.

Consistent with this interpretation, it is noteworthy that the relative reductions in GAD₆₇ and CB1R mRNA expression in the mPFC of GAD₆₇^{+/-} mice are similar to those observed in schizophrenia; mean GAD₆₇ and CB1R mRNA levels are significantly ~28–37% (18,19) and 15% (6) lower, respectively, in the DLPFC of subjects with schizophrenia. In addition, the GAD₆₇^{+/-} mice have lower GAD₆₇ expression across the cortical mantle, consistent with the observations that GAD₆₇ mRNA levels are lower to a similar degree in multiple cortical regions in the same subjects with schizophrenia (19). However, GAD₆₇ mRNA expression is reduced from early prenatal life in the GAD₆₇^{+/-} mice and whether this time course matches that of the GAD₆₇ mRNA deficit in schizophrenia is unknown.

The deficit in GAD₆₇ expression in the GAD₆₇^{+/-} mice was only about three-quarters of the 50% predicted reduction for a heterozygote, perhaps reflecting a compensatory increase in transcription from the remaining allele. On the other hand, the deficits in CB1R mRNA expression in the CB1R^{+/-} mice (49.6%) and CB1R^{-/-} mice (94.6%) were nearly exactly those predicted for heterozygotes and knockout animals, respectively. These comparisons suggest that the methods employed were sensitive to detect real differences in gene expression, and the absence of any differences in DAGL α mRNA expression also indicates that the CB1R mRNA deficit observed in the GAD₆₇^{+/-} mice is unlikely to be a false positive finding.

The mechanism through which deficient GAD₆₇ expression results in reduced CB1R expression in the GAD₆₇^{+/-} mice remains to be determined. Lower GAD₆₇ expression could cause alterations in cortical circuitry that produce increased 2-AG levels and subsequently CB1R down-regulation. However, the expression of DAGL α mRNA, which synthesizes 2-AG in pyramidal neurons postsynaptic to CB1R/CCK-containing axon terminals, was not altered in GAD₆₇^{+/-} mice. Alternatively, the effect of decreased GAD₆₇ expression on CB1R expression may occur through a cell autonomous mechanism within CB1R/CCK-containing GABA neurons. Consistent with this idea, DAGL α mRNA expression was not altered in CB1R^{-/-} mice suggesting that alterations in CB1R expression can occur independently of, and without alterations in, other components of the endocannabinoid system. Alternatively, because GAD₆₇ is critical for the development of perisomatic axon

terminals (10), deficient GAD₆₇ expression may result in fewer CB1R-containing axon terminals and thus a reduced need for CB1R mRNA expression.

Together, these findings support the hypothesis that in schizophrenia, a lower density of CB1Rs could be an adaptation that partially compensates for upstream reductions in GAD₆₇-mediated GABA synthesis (5,6) by reducing the 2-AG-mediated block of GABA release from the terminals of CB1R/CCK-containing neurons. By enhancing GABA release specifically from the terminals of those neurons, this homeostatic adaptation could contribute to a partial, albeit insufficient, normalization of neural network activity necessary for working memory function (20). However, although GAD₆₇^{+/-} mice nicely model the magnitude of reduced PFC GAD₆₇ mRNA levels in schizophrenia, this illness is not defined by a single gene heterozygous null mutation and, consequently, other potential pathogenetic processes must be considered. For example, deficits in GABA-related transcripts in schizophrenia may alternatively reflect impaired development of specific classes of GABA neurons due to other upstream pathogenetic sources (15) or perhaps a compensatory downregulation of inhibitory signaling mechanisms in response to deficient excitation in the disorder (21). Furthermore, the extent to which the genetic manipulation of GAD₆₇ expression in mice recapitulates the disease process of schizophrenia requires knowledge of other factors, such as when in development the deficit in GAD₆₇ arises in schizophrenia.

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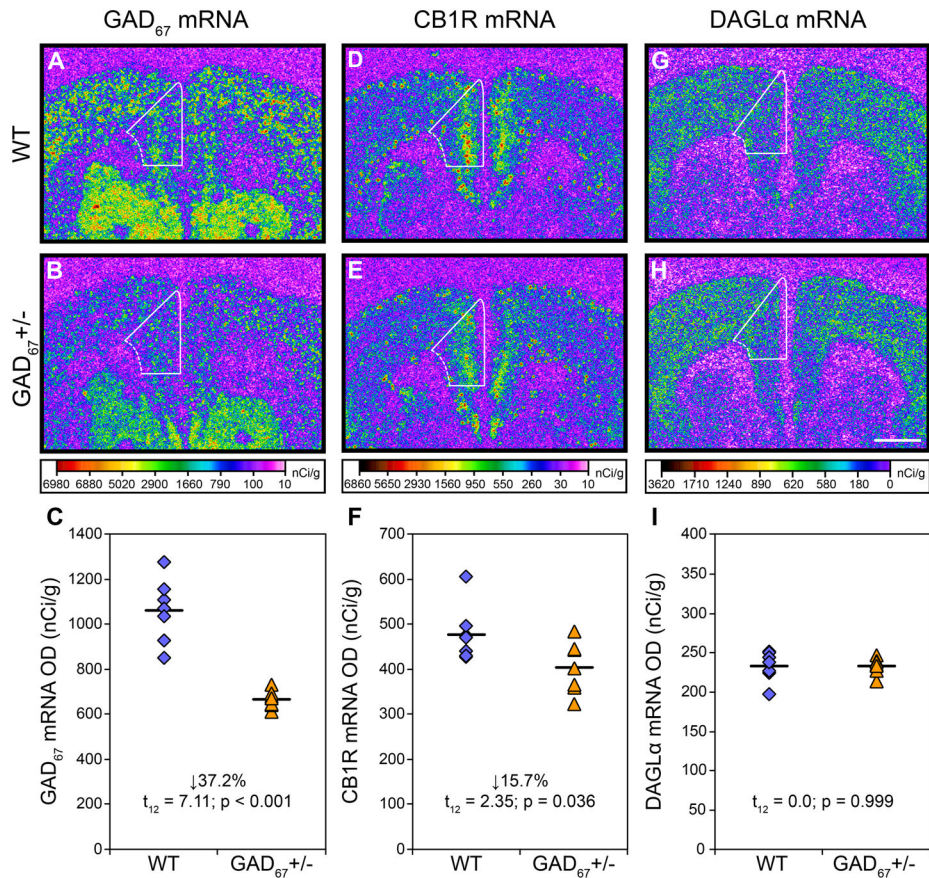


Figure 1.

Transcript levels in the mPFC of adult wild-type and GAD₆₇^{+/-} mice. Representative film autoradiograms illustrating the expression of GAD₆₇ (A, B), CB1R (D, E), and DAGLα (G, H) mRNAs. The density of hybridization signal for each transcript is presented in pseudocolor according to the calibration bars below B, E, and H. Expression of GAD₆₇ and CB1R mRNA in GAD₆₇^{+/-} mice (B, E) appears lower than in wild-type mice (A, D), whereas DAGLα (G, H) does not appear to differ across the two conditions. Note that CB1R mRNA signal is most pronounced in the superficial cortical layers, consistent with the laminar distribution of CCK-containing GABA neurons that heavily express CB1R mRNA and that are the principal CB1R mRNA expressing neuron type in the cortex(4). White contours denote the quantified region of the mPFC. Comparison of cortical GAD₆₇ (C), CB1R (F), and DAGLα (I) mRNA levels by film optical density (OD) in wild-type (diamonds) and GAD₆₇^{+/-} (triangles) mice. Mean values for each genetic condition are indicated by hash marks. Scale bar (1mm) in H applies to all panels.

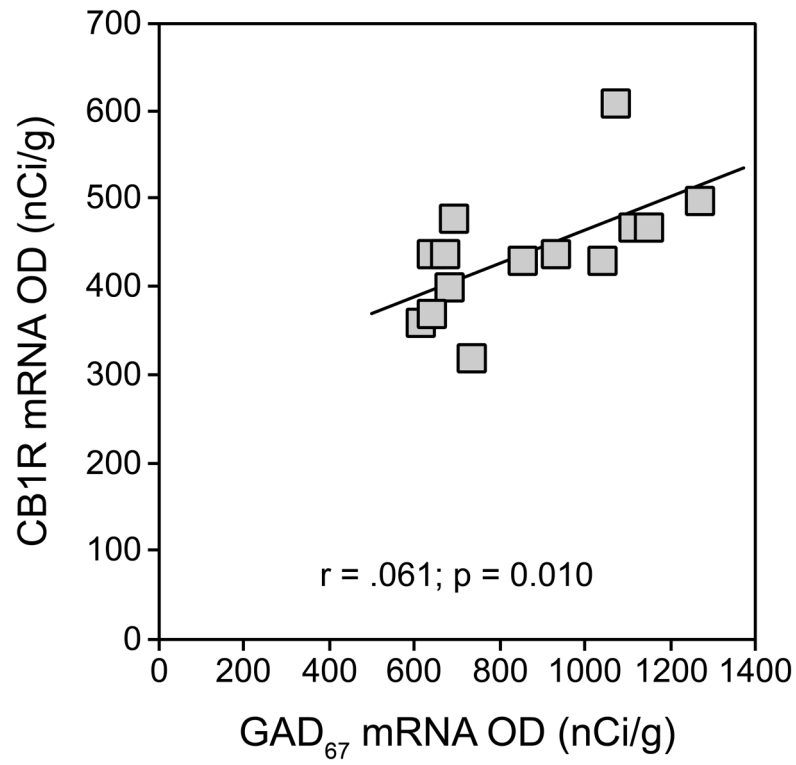


Figure 2. Positive correlation between levels of CB1R and GAD₆₇ mRNAs in adult wild-type and GAD₆₇^{+/-} mice. These findings suggest that changes in CB1R mRNA expression parallel changes in GAD₆₇ mRNA expression.

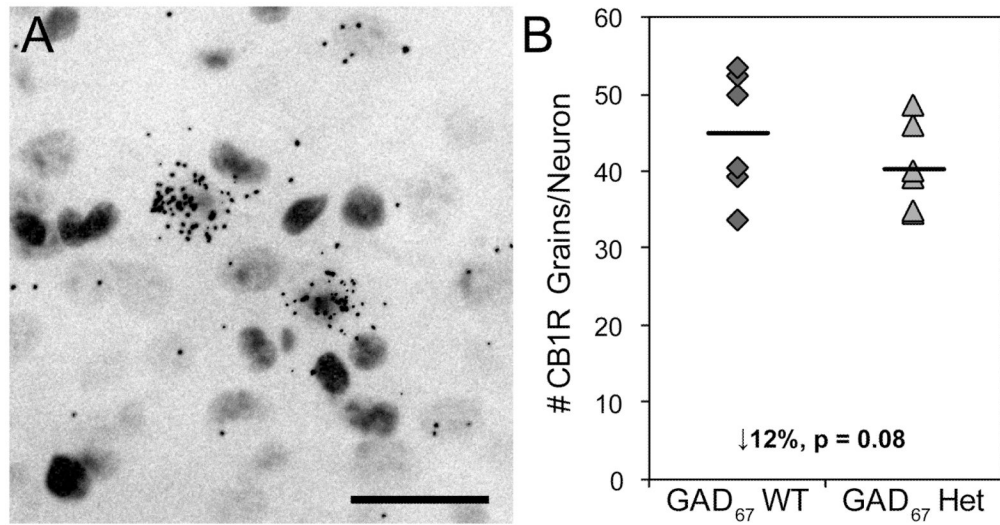


Figure 3. Cellular expression of CB1R mRNA. Representative photomicrograph of Nissl-counterstained, emulsion-exposed tissue section showing silver grains representing CB1R mRNA clustered over a subset of neuronal cell bodies (A). Scale bar = 30 μ m. Expression of CB1R mRNA is lower in GAD₆₇^{+/-} mice relative to wild-type mice (B).

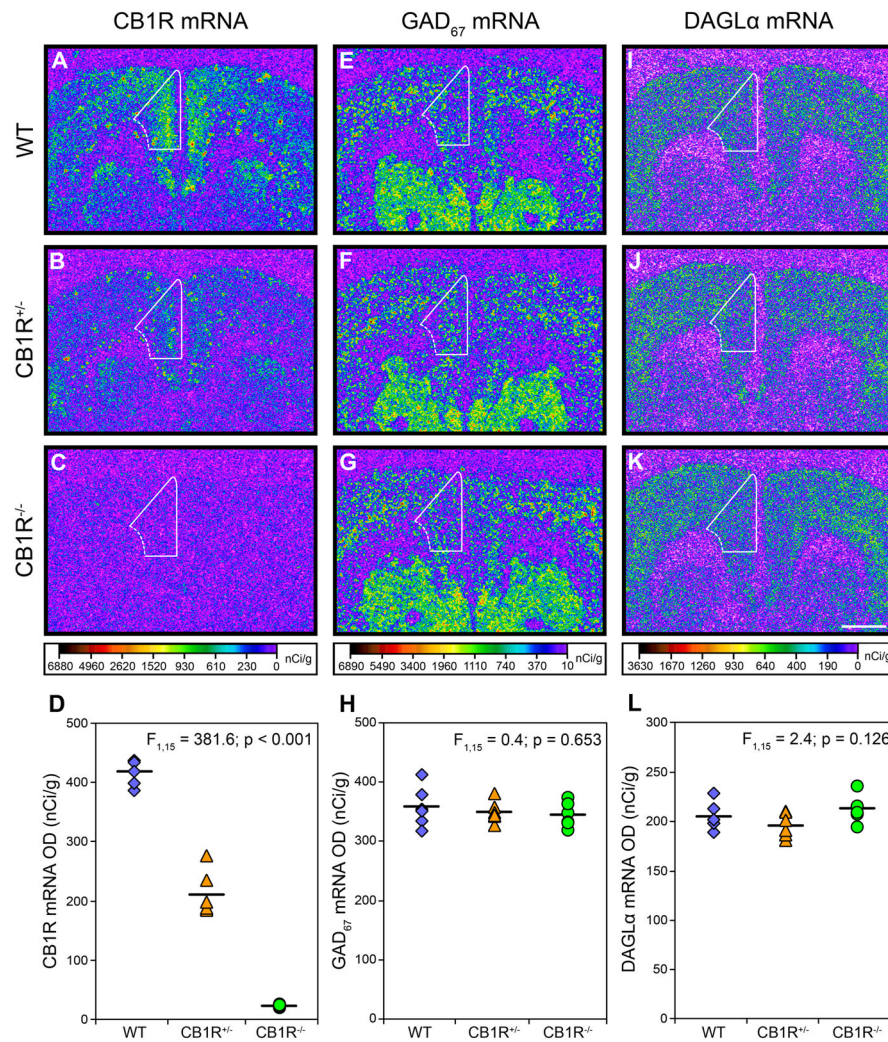


Figure 4.

Transcript levels in the mPFC of adult wild-type, CB1R^{+/-}, and CB1R^{-/-} mice. Representative film autoradiograms illustrating the expression of CB1R (A, B, C), GAD₆₇ (E, F, G), and DAGLα (I, J, K) mRNA. The density of hybridization signal for each transcript is presented in pseudocolor according to the calibration bars below C, G, and K. Expression of CB1R mRNA is markedly reduced in CB1R^{+/-} mice (B) and nearly undetectable in CB1R^{-/-} mice (C) compared to wild-type mice (A). Expression of GAD₆₇ (E, F, G) and DAGLα (I, J, K) mRNA appear unaltered in either genetic condition compared to wild-type mice. White contours denote the quantified region of the mPFC. Comparison of cortical CB1R (D), GAD₆₇ (H), and DAGLα (L) mRNA levels by film optical density (OD) in wild-type (diamonds), CB1R^{+/-} (triangles), and CB1R^{-/-} (circles) mice. Mean values for each condition are indicated by hash marks. Scale bar (1mm) in K applies to all panels.

Table

Template characteristics for riboprobe generation

Gene (mRNA transcript)	Genbank accession number	Target bases of transcript	Template base pair size
<i>Gad1</i> (GAD ₆₇)	Y12257	151–461	311
<i>Cnr1</i> (CB1R)	NM_007726	1130–1454	325
<i>Dagla</i> (DAGL α)	NM_198114	1845–1864	370