Gene expression of opioid and dopamine systems in mouse striatum: effects of CB$_1$ receptors, age and sex

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Abstract

Rationale—Endocannabinoid, opioid, and dopamine systems interact to exhibit cannabinoid receptor neuromodulation of opioid peptides and D$_4$ dopamine receptor gene expression in CB$_1$-cannabinoid-deficient mouse striatum.

Objective—Using CB$_1$-transgenic mice, we examine primary age–sex influences and interactions on opioid and dopamine system members' gene expression in striatum.

Materials and methods—Real-time quantitative polymerase chain reaction was used to analyze gene expression of opioid peptides [preproenkephalin (PPENK); preprodynorphin (PPDYN)], opioid receptors [delta-opioid receptor (δ-OR); mu-opioid receptor (μ-OR)] and dopamine receptor subtypes (D$_1$ through D$_5$) in male/female CB$_1$(+/+)/CB$_1$(−/−) mice striata at two adult ages [young (60–90 days); old (140–300 days)].

Results—(1) Increased PPENK and PPDYN, owing to genotype [CB$_1$(+/+) vs. CB$_1$(−/−)], depended on sex. When genotype-independent, they depended on sex (PPENK) or age (PPDYN). (2) δ-OR was age-dependent (higher in old). (3) μ-OR, owing to genotype, was age-dependent [higher in old CB$_1$(−/−) males]. When genotype-independent, it depended on sex (higher in

females). (4) Female D_1 was genotype-independent and age-dependent, while male D_1 was higher in old over young $CB_1(+/+)$ mice. (5) D_5, owing to genotype, was sex-dependent [higher in young female $CB_1(-/-)$ mice]. (6) D_2, genotype-independent, was higher in old over young male mice. (7) Young female D_3 was higher in $CB_1(-/-)$ over $CB_1(+/+)$ mice. Male D_3 was age-dependent (higher in old mice). (8) D_4, owing to genotype, was sex-dependent [higher in $CB_1(-/-)$ over $CB_1(+/+)$ females]. Genotype-independent D_4 was sex-dependent in young mice (higher in females) and age-dependent in males (higher in old).

Conclusions—Greater striatal expression is genotype-dependent in females (opioid-peptides, D_3, D_4, D_5) and genotype-independent in both females (PPENK, μ-OR, D_4) and old males (PPDYN, δ-OR, D_2, D_3, D_4).

Keywords

Real-time polymerase chain reaction; δ-opioid receptors (DOR); μ-opioid receptors (MOR); κ-opioid receptors (KOR); Enkephalin; Dynorphin; Dopamine receptors; Caudate-putamen; Basal ganglia; C57BL/6 mice

Introduction

The endocannabinoid system in the brain is comprised of CB_1, CB_2, and additional putative cannabinoid receptors that respond to a family of eicosanoid mediators referred to as endocannabinoids, notably anandamide and 2-arachidonoylglycerol. The function of the endocannabinoid system includes the retrograde transmission of endocannabinoids and activation of presynaptic CB_1 receptors to regulate neurotransmitter release (Chevaleyre et al. 2006). CB_1 receptors are highly expressed in the striatum (Herkenham et al. 1991), and they affect directly or indirectly several functions that involve the basal ganglia (i.e., judgment, memory and reward, and the controls for planning and modulation of movement; Gerdeman et al. 2003; van der Stelt and Di Marzo 2003). There is a growing body of evidence that the endogenous cannabinoid system might participate in the motivational and dopamine-releasing effects of several drugs of abuse (Basavarajappa and Hungund 2005; Laviolette and Grace 2006; Parolaro et al. 2005). This may be mediated in part by activation of dopaminergic mesolimbic neurons. For example, stimulation of the CB_1 receptors by the cannabinoid agonist Δ^9-THC caused dopamine release and changes in gene expression in the striatum (Laviolette and Grace 2006). In CB_1 knockout mice [$CB_1(-/-)$], responses to Δ^9-THC are absent (Zimmer et al. 1999), and reinforcement effects of alcohol and opioid drugs are dramatically reduced (Hungund et al. 2003; Ledent et al. 1999). Moreover, CB_1 antagonism by SR141716 (rimonabant) reduced opioid self-administration and conditioned place preference in rodents (Ledent et al. 1999; Martin et al. 2000).

In humans, age and sex differences have been reported when the biological and behavioral effects of drugs of abuse were examined (Greenfield et al. 2003; Randall et al. 1999; von Sydow et al. 2001; Young et al. 2002). These and other studies indicate that the onset and progression to drug addiction and the substances abused differ based on age and sex (Degenhardt et al. 2007). It also suggests the need to identify the neuromodulator mechanisms that are responsible for these differences.

Recent studies in animals indicate that ablation of the CB_1 receptor causes significant differences in the gene expression of the endogenous opioid peptides, preproenkephalin (PPENK) and preprodynorphin (PPDYN), as well as D_4 dopamine receptors in the striatum of $CB_1(-/-)$ mice (Gerald et al. 2006; Steiner et al. 1999) and reduction of D_2 dopamine receptor binding (Houchi et al. 2005). In addition, human genetic variation (polymorphisms) in the cannabinoid, dopamine, and opioid receptor systems are, to varying degrees, linked with neuropathologies including those related to drug addiction (Chakrabarti et al. 2006;

Hoenicka et al. 2007; Hopfer et al. 2006; Saxon et al. 2005; van den Wildenberg et al. 2007). In this present study, the evaluation of gene expression differences in $CB_1(-/-)$ transgenic mice was furthered by including, in our examination, the primary effects and interactions of sex and age on the gene expression of five members of the opioid receptor system and five dopamine receptor subtypes in the striatum.

Materials and methods

Animals

CB_1 transgenic mice studies Zimmer et al. (1999) generated $CB_1(-/-)$ mice by replacing the coding region in the mouse CB_1 receptor gene between amino acids 32 and 448 with a PGK-neo cassette to create chimeric mice which were bred with C57BL/6J animals. In the present study, $CB_1(+/+)$ and $CB_1(-/-)$ mice were produced by inbreeding the $CB_1(+/-)$ mice at North Carolina Central University or the University of North Carolina-Chapel Hill. All animals used in this study were treated in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publications No. 90–23) revised 1996. Animals were anesthetized and decapitated, as approved by institutional Animal Care and Use Committees. Adult mice of both sexes at two different ages [60–90 days (young) and 140–300 days (old)] were used. The number of animals used were males: $CB_1(+/+)$ young (6–8), old (8–10); $CB_1(-/-)$ young (7–8), old (5–8) and females: $CB_1(+/+)$ young (4–5), old (4); $CB_1(-/-)$ young (4–5), old (3).

Reverse transcription and qPCR

Both striata were dissected free hand from each mouse brain and immediately stored at $-80°C$ until extraction of total RNA. Total RNA was isolated from striata of all mice using Trizol Reagent (Invitrogen) stored at $-80°C$. RNA was purified for reverse transcription and quantitative real-time polymerase chain reaction (qPCR) using the RNeasy MinElute Cleanup Kit (Qiagen) and registered a 260/280 nm absorbance ratio of >2.0.

Total purified RNA (1 μg) was reversed transcribed into complementary DNA (cDNA) using a high-capacity cDNA archive kit (Applied Biosystems). Quantitative real-time PCR was performed using a 7500 real-time PCR system (Applied Biosystems) utilizing the TaqMan gene expression assays specific for 18S (human), opioid peptides (mouse preprodynorphin and preproenkephalin), five dopamine receptor subtypes (mouse D_1–D_5), and three opioid receptors (mouse δ-OR, μ-OR, κ-OR). Gene expression was normalized to 18S cDNA. Relative quantitation of gene expression levels was performed using the Delta Delta C_t method ($2^{-\Delta\Delta Ct}$method; Livak and Schmittgen 2001).

Statistics and data analysis

Relative expression data from qPCR for each gene examined was tested for normality using the Anderson–Darling normality test and for homogeneity of variance in group using Levene's test. If data for a particular gene did not comply with the assumption of normality and/or homogeneity of variance, the relative expression ratios for that gene were subjected to a logarithmic transformation. Both transformed and non-transformed relative gene expression ratios were treated as the response in a general linear model (GLM) univariate analysis of variance (ANOVA). The model utilized a 2×2×2 factorial design by which genotype [$CB_1(+/+)$, $CB_1(-/-)$], sex (male, female), and age (young, old) were fixed (i.e., each factor level represented a discrete value). The factors were crossed such that the main effects (genotype, sex, and age), primary interactions (genotype × sex, genotype × age, and sex × age) and the second-order interaction (genotype × sex × age) could be evaluated. A subsequent pairwise comparison of all means was performed using Tukey–Kramer and Bonferroni multiple comparison tests. To further examine the effect of CB_1 ablation, both

transformed and non-transformed data from $CB_1(+/+)$ and $(CB_1(-/-)$ mice of the same age and sex were compared using a two-sample t test. For this t test, equal variance was not assumed. For all data in this study, differences at the p 0.05 level were considered significant. All statistical calculations and analyses were performed using the MINITAB 13.32 statistical software package (Minitab Inc., State College, PA, USA).

Results

The amount of messenger RNA (mRNA) for eight G-protein coupled receptors (GPCRs) were examined in striata from young adult male $CB_1(+/+)$ mice, and the results are presented in Fig. 1 relative to D_1 mRNA levels. The receptors examined included the D_1-like dopamine receptor subtypes for which D_1 was approximately 100-fold greater than D_5. For the D_2-like dopamine receptor subtypes, the D_2 was approximately 100-fold greater than D_3 and 1,000-fold greater than D_4. For the opioid receptors, δ-OR was 200-fold greater than μ-OR and approximately tenfold greater than kappa opioid receptor (κ-OR).

Striatal opioid peptide gene expression

Evaluation of the data, in Fig. 2a, using a GLM ANOVA revealed that PPENK gene expression was dependent on interactions between sex and genotype [$F_{(1,39)}$ =11.29, p= 0.002] and on the main effect of sex [$F_{(1,39)}$=31.94, p< 0.0005]. The apparent interactions between genotype and sex can be attributed to the influence from sex (female) on genotype (CB_1 ablation). Subsequent pairwise comparisons of all means revealed significantly higher gene expression in $CB_1(-/-)$ over $CB_1(+/+)$ female mice (p<0.03) and higher gene expression in females over males independent of age and genotype (p<0.03). There were no main effects due to genotype [$F_{(1,39)}$=2.85, p=0.099] or age [$F_{(1,39)}$= 2.62, p=0.114], no primary interactions between sex and age [$F_{(1,39)}$ =0.10, p=0.751] nor between genotype and age [$F_{(1,39)}$ =1.15, p=0.290], and no second-order interactions among genotype, sex, and age [$F_{(1,39)}$ =0.03, p=0.859]. Although GLM ANOVA did not reveal an effect of genotype in males, a two-sample t test showed that PPENK gene expression in young males was reduced in $CB_1(-/-)$ mice when compared with $CB_1(+/+)$ mice (t =2.79, df=9, p=0.021).

Evaluation of the data in Fig. 2b showed significant differences in PPDYN gene expression due to second-order interactions among genotype, sex, and age [$F_{(1,38)}$=4.4, p= 0.043]; differences due to primary interactions between genotype and sex [$F_{(1,38)}$ =14.59, p<0.0005]; and significant main effects due to age [$F_{(1,38)}$ =33.37, p<0.0005] and genotype [$F_{(1,38)}$=8.82, p=0.005]. There were no effects due to interactions between sex and age [$F_{(1,38)}$=2.70, p= 0.109] nor between genotype and age [$F_{(1,38)}$ =3.45, p=0.071]. Unlike PPENK gene expression, which was higher in female over males, there was no main effect due to sex [$F_{(1,38)}$ =0.29, p=0.594]. Therefore, the significant second-order interactions among genotype, sex, and age were due mainly to the effect of age. The primary interaction between genotype and sex appeared to be mainly due to the effect of genotype, and subsequent pairwise comparisons of all means support this interpretation. Post hoc test revealed significantly higher PPDYN gene expression in old over young male mice regardless of genotype (p<0.002) and that CB_1 ablation increased PPDYN gene expression in female mice regardless of age (p<0.001). When young males were evaluated separately, PPDYN gene expression appeared to be reduced in CB_1 (-/-) mice when compared to their $CB_1(+/+)$ counterparts, and this was confirmed using a two-sample t test (t=3.63, df=9, p=0.006).

Striatal δ-OR and μ-OR gene expression in mice: influence of age and sex

Evaluation of the data from Fig. 3a using a GLM ANOVA revealed significant differences in δ-OR gene expression due to the main effect of age [$F_{(1,40)}$ =14.91, p<0.0005].

Subsequent pairwise comparisons of all means confirmed significantly higher δ-OR gene expression in old over young mice when animals of the same genotype and sex were compared ($p<0.05$). There were no main effects due to genotype [$F_{(1,40)}=0.29$, $p=0.592$] or sex [$F_{(1,40)}=3.74$, $p=0.06$] nor were there primary or second-order interactions between groups: genotype and sex [$F_{(1,40)}=0.25$, $p=0.621$]; genotype and age [$F_{(1,40)}=0.02$, $p=0.878$]; sex and age [$F_{(1,40)}=0.06$, $p=0.800$]; or genotype, sex, and age [$F_{(1,40)}=2.52$, $p=0.121$].

Data from Fig. 3b were evaluated using a GLM ANOVA, and it showed significant differences in μ-OR gene expression due to second-order interactions among genotype, sex, and age [$F_{(1,37)}=9.42$, $p=0.004$] and a significant main effect due to sex [$F_{(1,37)}=29.19$, $p<0.0005$]. Therefore, the interactions among genotype, sex, and age were due mainly to the effects of sex. There were no main effects due to genotype [$F_{(1,37)}=0.05$, $p=0.830$] or age [$F_{(1,37)}=1.05$, $p=0.313$] and no primary interactions between genotype and sex [$F_{(1,37)}=0.07$, $p=0.798$], genotype and age [$F_{(1,37)}=0.00$, $p=0.993$], or sex and age [$F_{(1,37)}=2.04$, $p=0.162$]. Subsequent pairwise comparisons of all means confirmed significantly higher μ-OR gene expression in female over male mice when expression was examined in mice of the same age and genotype ($p<0.002$). When CB_1 (+/+) mice were compared to CB_1(−/−) mice of the same age and sex, using a two-sample t test, a significant increase in μ-OR gene expression was observed in CB_1(−/−) over CB_1(+/+) old male mice ($t=-2.27$, $df=9$, $p=0.05$).

In a preliminary study with a limited number of samples, the influence of CB_1 ablation, sex, and age on κ-OR gene expression was evaluated using a GLM ANOVA. The results revealed significant differences in κ-OR gene expression due to the effect of age [$F_{(1,25)}=5.95$, $p=0.022$], and there were significant interactions between genotype and sex [$F_{(1,25)}=4.25$, $p=0.05$]. No other interaction between groups (genotype and age; sex and age; genotype, sex, and age) were observed. Subsequent pairwise comparisons of all means using Tukey–Kramer and Bonferroni multiple comparison tests revealed significantly higher κ-OR gene expression in old over young male mice ($p=0.043$).

Dopamine D_1-like receptor subtype gene expression: influence of CB_1 ablation, sex, and age

D_1-like receptor (D_1 and D_5) gene expression in striata isolated from CB_1(+/+) and CB_1(−/−) mice were examined in Fig. 4. Evaluation of the data from Fig. 4a revealed significant differences in D_1 dopamine receptor gene expression that were due mainly to the effect of age [$F_{(1,38)}=12.79$, $p=0.001$]. Subsequent pairwise comparisons of all means revealed significantly higher D_1 dopamine receptor gene expression in old over young CB_1(+/+) mice regardless of sex ($p<0.03$) and higher gene expression in old over young female CB_1(−/−) mice ($p<0.03$). There were no significant main effects due to genotype [$F_{(1,38)}=0.95$, $p=0.336$] or sex [$F_{(1,38)}=0.04$, $p=0.834$] nor were there significant interactions between the groups: genotype and sex [$F_{(1,38)}=2.15$, $p=0.151$]; genotype and age [$F_{(1,38)}=0.49$, $p=0.487$]; sex and age [$F_{(1,38)}=1.39$, $p=0.246$]; genotype, sex, and age [$F_{(1,38)}=0.23$, $p=0.636$].

Evaluation of the data from Fig. 4b using a GLM ANOVA revealed significant differences in D_5 dopamine receptor gene expression due to the primary interaction between genotype and sex [$F_{(1,40)}=5.18$, $p=0.028$] and the main effect of sex [$F_{(1,40)}=11.79$, $p=0.001$]. There were no main effects due to genotype [$F_{(1,40)}=0.00$, $p=0.971$] or age [$F_{(1,40)}=0.10$, $p=0.757$], and there were no primary or second-order interactions between genotype and age [$F_{(1,40)}=0.01$, $p=0.905$]; sex and age [$F_{(1,40)}=1.71$, $p=0.199$]; or genotype, sex, and age [$F_{(1,40)}=1.54$, $p=0.221$]. Therefore, the primary interaction between genotype and sex was attributed to the main effect of sex. Subsequent pairwise comparisons revealed significantly higher D_5 dopamine receptor gene expression in female over male young CB_1(−/−) mice

($p<0.03$). A two-sample t test was used to compare the differences between $CB_1(+/+)$ and $CB_1(-/-)$ mice of the same sex and age. The results showed a reduction in receptor gene expression in young male mice ($t=4.32$, $df=10$, $p=0.002$) and an increase in receptor gene expression in young female mice ($t=-3.53$, $df=6$, $p=0.012$) due to CB_1 ablation.

Dopamine D_2-like receptor subtype gene expression: influence of CB_1 ablation, sex and age

Evaluation of the data from Fig. 5a indicate that the combined effects of genotype and sex [$F_{(1,41)}=5.01$, $p=0.031$] appeared to influence the main effect of age [$F_{(1,41)}=7.56$, $p=0.009$] to significantly increase D_2 dopamine receptor gene expression in old over young male mice ($p<0.006$). There were no main effects due to genotype [$F_{(1,41)}=0.54$, $p=0.467$] or sex [$F_{(1,41)}=1.37$, $p=0.248$], and there were no primary or second-order interactions between genotype and age [$F_{(1,41)}=0.57$, $p=0.455$], sex and age [$F_{(1,41)}=1.69$, $p=0.200$], and genotype, sex, and age [$F_{(1,41)}=0.41$, $p=0.527$].

In Fig. 5b, significant differences in D_3 dopamine receptor gene expression were due to the main effect of age [$F_{(1,41)}=9.85$, $p=0.003$], with significantly higher gene expression in old over young male mice ($p<0.01$). There were no main effects due to genotype [$F_{(1,41)}=0.09$, $p=0.763$] or sex [$F_{(1,41)}=0.21$, $p=0.651$] and no significant interactions between genotype and sex [$F_{(1,41)}=0.73$, $p=0.398$]; genotype and age [$F_{(1,41)}=1.21$, $p=0.278$]; sex and age [$F_{(1,41)}=0.71$, $p=0.404$]; or genotype, sex, and age [$F_{(1,41)}=1.11$, $p=0.299$]. A t test analysis comparing $CB_1(+/+)$ to $CB_1(-/-)$ mice of the same sex and age showed higher receptor gene expression in $CB_1(-/-)$ over $CB_1(+/+)$ in young female mice ($t=-3.60$, $df=6$, $p=0.011$).

Differences in D_4 dopamine receptor gene expression, in Fig. 5c, were due to interactions between genotype and sex [$F_{(1,39)}=16.51$, $p<0.0005$] independent from the effect of age and to the main effects of sex [$F_{(1,39)}=24.66$, $p<0.0005$], genotype [$F_{(1,39)}=6.56$, $p=0.014$], and age [$F_{(1,39)}=5.12$, $p=0.029$]. Comparisons of all means indicated D_4 dopamine receptor gene expression was significantly higher in $CB_1(-/-)$ over $CB_1(+/+)$ in both young and old female mice ($p<0.03$). Furthermore, gene expression was higher in female over male young mice ($p<0.03$) and higher in old over young male mice ($p<0.03$) regardless of genotype. There were no primary interaction between genotype and age [$F_{(1,39)}=0.09$, $p=0.768$] or sex and age [$F_{(1,39)}=2.33$, $p=0.135$] and no second-order interactions among genotype, sex, and age [$F_{(1,39)}=0.26$, $p=0.614$].

Discussion

CB_1 receptor ablation in mice produces early mortality and deterioration in cognitive, memory, and motor function which has been characterized as "accelerated aging" (Bilkei-Gorzo et al. 2005; Steiner et al. 1999; Zimmer et al. 1999). Some of these phenotypic deficits involve neostriatal regulation, including increased immobility or catalepsy, hypoactivity, decline in skills learning, deficit social memory, and reduced operant responding to reinforcing stimuli (Bilkei-Gorzo et al. 2005; Holter et al. 2005). The reduced operant response reflects reduced incentive in that $CB_1(-/-)$ mice are characterized by a genotypic reduction in the consumption of sweet foods and alcohol and self-administration of reinforcing substances such as morphine (Cossu et al. 2001; Houchi et al. 2005; Hungund et al. 2003; Ledent et al. 1999; Martin et al. 2000; Sanchis-Segura et al. 2004). This behavioral phenotype has been linked with neurochemical differences in $CB_1(-/-)$ striatal tissue, including differences in the expression of substance P, opioid peptides, and enzymes that synthesize neurotransmitters (Steiner et al. 1999). Furthermore, reinforcing substances, which characteristically are accompanied by increased dopamine in the nucleus accumbens, fail to evoke dopamine accumulation in $CB_1(-/-)$ mice (Hungund et al. 2003; Mascia et al. 1999).

The goal of the present study was to extend our knowledge of the effects of CB_1 receptor ablation on gene expression for members of the opioid (δ-OR, μ-OR, PPENK, and PPDYN) and dopamine (D_1 through D_5 receptor) systems in the striatum. This was accomplished by including in our evaluation the influence of the primary effects and interactions of sex and age on differences produced in CB_1 transgenic mice. The results, presented in Figs. 2, 3, 4, and 5 and summarized in Table 1, showed genotypically greater expression of striatal opioid peptides and D_3, D_4 and D_5 dopamine receptors in $CB_1(-/-)$ compared with $CB_1(+/+)$ mice. It was striking that these genotypic differences were restricted to females, particularly young adult females.

Age-related differences in gene expression

Age-related differences were observed, particularly in males, in the expression of PPDYN and δ-OR in the opioid receptor system and in D_1, D_2, D_3, and D_4 dopamine receptor expression. Genotypic differences in gene expression were age-related in males for opioid peptide and μ-OR expression and in females for D_3 and D_5 dopamine receptors expression. The effect of age and CB_1 receptor ablation on behavior has been extensively investigated in mice with C57BL/6 and CD1 backgrounds (Bilkei-Gorzo et al. 2005). In young mice (6–7 weeks), $CB_1(-/-)$ and $CB_1(+/+)$ mice perform the same in learning and memory paradigms including skill learning, partner recognition, and operant conditioning. In contrast, mature adult (3–5 months) $CB_1(-/-)$ perform poorly, but comparable to older (14–17 months) $CB_1(+/+)$ mice. These studies demonstrate an acceleration of the age-related decrement in cognitive and motor function in $CB_1(-/-)$ mice. One explanation for these age-related differences may be the sensitivity of neurons to neurotoxicity and the protective nature of endocannabinoids exerted through CB_1 receptors (Veldhuis et al. 2003). However, it is not clear whether striatal neurons are as vulnerable to neurotoxicity as some areas of the hippocampus.

Significant age-related differences have been observed in the ability of the endocannabinoid system to modulate the intake of reinforcing substances such as ethanol and palatable food. In C57BL/6 mice that are in the same age range as the young adults used in the present study (6–10 weeks), $CB_1(+/+)$ mice prefer ethanol to a greater extent than do $CB_1(-/-)$ mice or $CB_1(+/+)$ mice treated with the CB_1 antagonist SR141716 (Wang et al. 2003). Greater food intake is also observed in food-restricted juvenile $CB_1(+/+)$ mice compared with $CB_1(-/-)$ mice (Di Marzo et al. 2001) or $CB_1(+/+)$ mice treated with SR141716 (Wang et al. 2003). Juvenile mice have greater ethanol and food intake than old mice (26–48 weeks), and the ethanol and food intake in older adult mice cannot be reduced by SR141716 (Wang et al. 2003). Therefore, age has significant behavioral consequence when CB_1 receptor activity is modified.

Our studies demonstrated several potential neurochemical substrates, in the opioid and dopamine receptor system, which may account for the genotype and age-related differences in motor behaviors and response to reinforcing substances. In previous studies, no differences in anandamide or 2-arachidonoylglycerol content were found in the limbic forebrain of young compared with old mice (Wang et al. 2003). Similarly, no differences were detected in CB_1 receptor density in young compared with old mice. However, these studies do find twofold greater HU210-stimulated GTPγS binding in limbic forebrain in young versus old mice (Wang et al. 2003).

Sexual dimorphic effects of cannabinoids

In our study, PPENK and the μ-OR gene expression was greater in young and old adult females compared with males, with no genotypic increase in PPENK occurring in males but a genotypic increase in μ-OR in old adult males. The D_3 and D_5 receptor expression was

significantly greater in young adult female $CB_1(-/-)$ compared with $CB_1(+/+)$, whereas no such increase was observed with males. In male mice, older adults expressed greater levels of D_2 and D_3 receptors than younger males. One explanation for phenotypic differences in the $CB_1(-/-)$ might be the developmental perturbations of the lack of cannabinoid receptors during critical stages of neurogenesis. Developmental studies (Rodriguez de Fonseca et al. 1993) have previously demonstrated a sexual dimorphic pattern of CB_1 receptors arising in prenatal striata. Prenatal exposure to cannabinoid agonists modifies μ-OR density, and it differs with the sex of the animal (Perez-Rosado et al. 2000; Vela et al. 1998), leading to the possibility of differences established before birth in the $CB_1(-/-)$ genotype that might lead to a sexually dimorphic character when behaviors are tested as adults.

Behaviorally, Tseng and Craft (2001) found that Δ^9-THC produces greater hypoactivity measures in female compared with male rats. The relatively greater hypoloco-motion and catalepsy responses to cannabinoid agonists in females are also observed with 11-OH-THC and CP55940, indicating that the sexual dimorphism is not attributed to pharmacokinetic disposition of Δ^9-THC (Tseng and Craft 2001). The catalepsy response is blocked by SR141716, indicating that CB_1 receptors are responsible (Tseng and Craft 2004). Sexually dimorphic responses in reinforcement behaviors have also been observed. Hungund et al. (2003) report that in a population of young adult C57BL/6 mice (13–15 weeks of age), the female mice consume significantly more alcohol (two-bottle choice test) than males. The ablation of the CB_1 receptor significantly reduces the response in male mice. However, the magnitude of the reduction in females is much greater than males, as consumption of alcohol in both male and female mice does not differ.

Regulation of the dopaminergic and opioid mechanisms in the striatum and nucleus accumbens

The influence of CB_1 receptors on enkephalin-containing neurons in striata has previously been reported. Cannabinoid treatment of rats with the potent agonist CP55940 for 18 days resulted in a robust increment in PPENK mRNA in both the striatum and nucleus accumbens (Manzanares et al. 1998). This may represent a means by which cannabinoid receptors mediate the activation of opioid receptors (Vigano et al. 2005). If our observed increases in PPENK in CB1(-/-) mice are also considered, these studies would suggest that the enkephalin-containing neurons are under tonic regulatory control by endocannabinoids. The converse interaction, the influence of enkephalin on the cannabinoid responses, is not as striking, inasmuch as no differences were observed between PPENK (-/-) and WT C57BL/6J mice in the ability of Δ^9-THC to decrease locomotion or invoke catalepsy (Valverde et al. 2000). This would indicate that enkephalin-containing neurons are not intrinsic to a pathway that mediates these responses to cannabinoid drugs. Nevertheless, some of the withdrawal effects after chronic Δ^9-THC treatment might involve enkephalin-containing neurons in the striatum (Valverde et al. 2000).

The influence of CB_1 receptors on dynorphin-containing neurons in the striatum are beginning to be established. PPDYN(-/-) mice are no different from WT C57BL/6 in the Δ^9-THC-decreased locomotion response attributed to striatal neurons. However, PPDYN(-/-) mice are characterized by the absence of Δ^9-THC-induced conditioned place aversion (Zimmer et al. 2001). This suggests that dynorphin-containing neurons are in the pathway for the Δ^9-THC dysphoric response. Nor-BNI also blocked Δ^9-THC conditioned place aversion, indicating that dynorphin is working through κ-OR (Zimmer et al. 2001). It is not clear whether the striatum is involved in the anxiety and dysphoria due to Δ^9-THC. The aversive response to kappa opioids was reduced in the $CB_1(-/-)$ phenotype, indicating that the aversive responses are a coordinated mechanism rather than one that is linearly downstream from the other (Zimmer et al. 2001). However, limited observations in our present study showed no difference in κ-OR expression due to genotype.

An extensive literature has developed surrounding the interactions between the endocannabinoid and dopaminergic systems in the striatum (Gerdeman et al. 2003; van der Stelt and Di Marzo 2003). CB_1 receptors are expressed in D_1-and D_2-like receptor expressing neurons in the striatum (Hermann et al. 2002), suggesting a degree of cross-talk between these receptor systems. Indeed, D_2-like receptors (D_2, D_3, and D_4) have been shown to be functionally coupled to the endocannabinoid system, as dopamine increased anandamide synthesis and release in dorsal striata, and postsynaptic D_2 receptor stimulation parallels the increase in endocannabinoid in striata (Giuffrida et al. 1999). In situ hybridization showed that chronic treatment with D_2 antagonists up-regulated CB_1 mRNA in rat caudate-putamen (Mailleux and Vanderhaeghen 1993). However, D_1-like (D_1, D_5) receptor activation using the agonist SKF38393 did not exhibit similar effects on the endocannabinoid system (Giuffrida et al. 1999).

Influence of D_4 dopamine receptor activity on behavior

Genetic modification of members of the endocannabinoid, opioid, and dopamine receptor systems have been linked to various neuropathologies with behavioral consequences, including those related to drug addiction, attention deficit hyperactivity disorder (ADHD), and bipolar disease (Saxon et al. 2005; Schmidt et al. 2002; Wong et al. 2000). However, many of these links or associations have been questioned due to conflicts among the published data (Chakrabarti et al. 2006; Helmeste and Tang 2000; Hoenicka et al. 2007; Hopfer et al. 2006; Saxon et al. 2005; van den Wildenberg et al. 2007; Wong et al. 2000). In the present study, large differences in D_4 dopamine receptor gene expression were detected in CB_1 ablated female mice, but gene expression independent of genotype was a function of age or sex. D_4 receptor expression has specifically been linked to neuropathologies, particularly ADHD, whose relationship to genetic modification of the receptor is currently being considered (Helmeste and Tang 2000; Wong et al. 2000). Yet, D_4-receptor-ablated mice exhibited hyperactivity in open field test, supersensitivity to the locomotor-stimulating effects of ethanol, cocaine and methamphetamine, and elevated synthesis of dopamine and its metabolite 3,4-dihydroxyphenylacetic acid in the caudate-putamen, but not in the nucleus accumbens. These D_4-receptor-deficient mice were more adept than wild-type mice at complex motor functions as determined by rotarod performance tests. This has been attributed to the elevated dopamine levels (Rubinstein et al. 1997). Spontaneously hypertensive rats (SHR), considered a suitable rat model for ADHD, are hyperactive in the open field test. In the SHR strain, D_4 dopamine receptor gene expression was reduced in the prefrontal cortex, but not in the striatum (Li et al. 2007). In an ADHD model induced by lateral ventricle 6-hydroxydopamine injection, wild-type mice developed hyperactivity, but D_4-receptor-ablated mice did not (Avale et al. 2004). D_4 receptor antagonists (i.e., PNU-101387G, L-745,870, and U-101,958 but not S-18126) blocked the hyperactivity in this ADHD mouse model (Avale et al. 2004; Zhang et al. 2001, 2002). Despite these observations, the underlying mechanism(s) are still open to speculation.

A possible resolution to this question may reside in further studies involving CB_1 transgenic mice. CB_1-deficient mice lack normal dopamine-mediated suppression of PKA activity, mediated by postsynaptic D_4 receptors, which blocks $GABA_A$ currents in the globus pallidus (Engler et al. 2006; Shin et al. 2003). Selective genes in striata, including those for opioid peptides, are influenced by increased cAMP and PKA activation via a cAMP response element binding protein (CREB)-mediated mechanism. Therefore, modulation of CB_1 receptor activity could influence, either directly or indirectly, CREB-mediated mechanisms that affect gene expression that impacts memory, addiction, depression, and anxiety (Carlezon et al. 2005).

In conclusion, differences in opioid peptide and in D_3, D_4, and D_5 dopamine receptor gene expression, which were greater in females, were observed in striata from CB_1-ablated mice.

Meanwhile, greater differences independent of genotype were observed in the gene expression for PPENK, μ-OR, and D_4 dopamine receptors in females and were also observed in the gene expression for PPDYN, δ-OR, and dopamine receptor subtypes D_2, D_3, and D_4 in old males. These significant genotypic, age-dependent, and sexually dimorphic differences in gene expression may contribute to the phenotypic deficit behaviors associated with these genes. They may also contribute to the various behavioral differences that have been observed with these featured groups.

Acknowledgments

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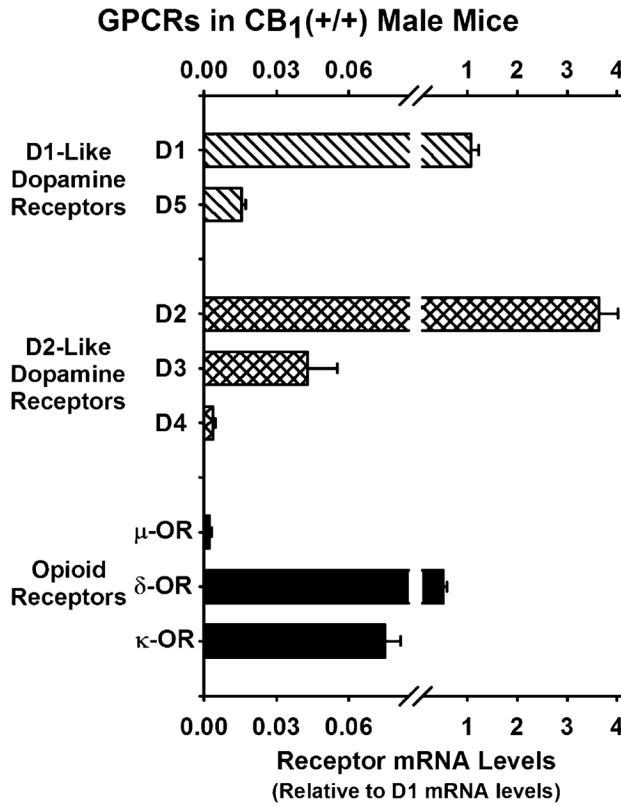
Fig. 1.

G-protein coupled receptor (*GPCR*) mRNA levels in striata of young adult male $CB_1(+/+)$ mice. Relative mRNA levels for GPCRs (D_1-like, D_2-like, and opioid receptors) were determined in total RNA extracts from striata isolated from four to eight young $CB_1(+/+)$ mice brains. Analysis for D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, D_4) dopamine receptors and for opioid receptors (μ-OR, δ-OR, and κ-OR) were performed using RT-qPCR as described in "Materials and methods". Levels of mRNA were quantitated using the Delta Delta C_t method ($2^{-\Delta\Delta Ct}$method; Livak and Schmittgen 2001), and the results are presented relative to D_1 dopamine receptor subtype mRNA levels in the striatum
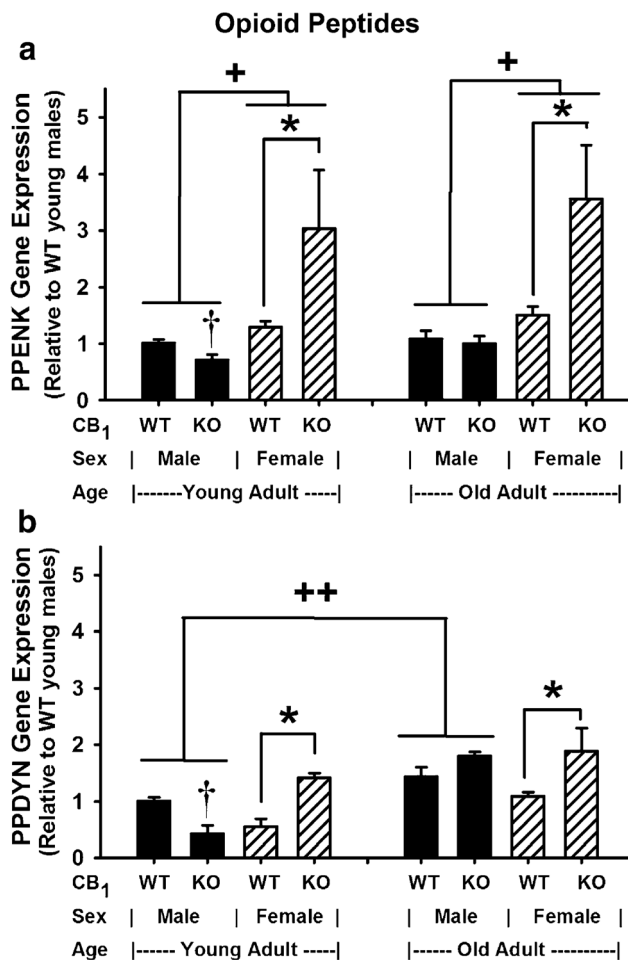
Opioid Peptides

Fig. 2.
Striatal opioid peptide gene expression in mice: The influence of CB_1 ablation, sex, and age. **a** PPENK gene expression, **b** PPDYN gene expression. Gene expression levels were determined in total RNA extracts from brain striata of young and old adult $CB_1(+/+)$ [wild-type (*WT*)] and $CB_1(-/-)$ [mice with CB_1 receptor ablation (KO)] mice (*n*=3–10) using RT-qPCR. All data are presented relative to expression levels in young adult male $CB_1(+/+)$ mice. Data were evaluated using a general linear model (*GLM*) univariate ANOVA. The model utilized a 2×2×2 factorial design where genotype, sex, and age were fixed. Subsequent differences due to genotype, sex, and age were determined using Tukey–Kramer and Bonferroni multiple comparison tests. In mice of the same age and sex, differences due to genotype (WT vs. KO mice) were also determined using a two-sample *t* test. **a** (PPENK gene expression): *differs due to genotype (WT vs. KO), *p*<0.03; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. +differs due to sex, *p*<0.03; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. †WT vs. KO mice of the same age and sex differ with a two-sample *t* test comparison; *p*=0.021. **b** (PPDYN gene expression): *differs due to genotype (WT vs. KO), *p*<0.001; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. ++differs due to age, *p*<0.002; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. †WT vs. KO mice of the same age and sex differ with a two-sample *t* test comparison; *p*=0.006
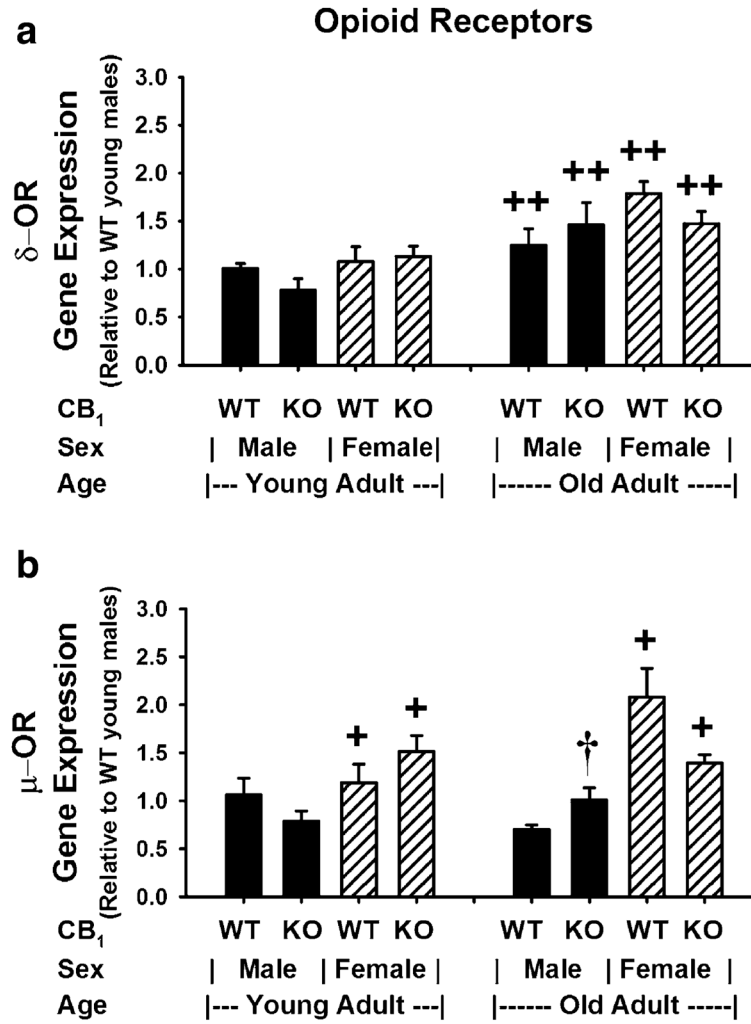
Fig. 3.
Striatal δ-OR and μ-OR in mice: Influence of CB$_1$ ablation, sex, and age. **a** δ-OR (delta opioid receptor) and **b** μ-OR (mu opioid receptor). Gene expression levels were determined in total RNA extracts from brain striata of young and old adult CB$_1$(+/+) and CB$_1$(−/−) mice (n=3–10) and subjected to analysis as described in Fig. 2. **a** (δ-OR gene expression): ++differs due to age, p<0.05; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. **b** (μ-OR gene expression): +differs due to sex, p<0.002; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. †WT vs. KO mice of the same age and sex differ with a two-sample t test comparison; p=0.05
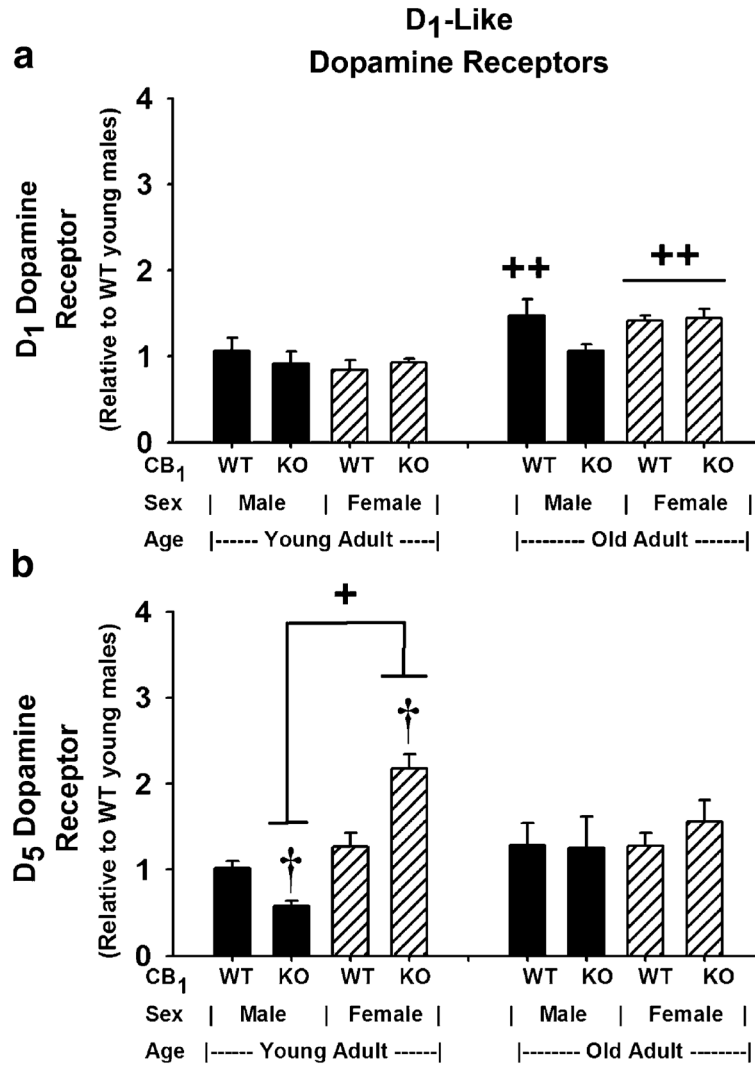
Fig. 4.
Striatal D_1-like dopamine receptor subtype gene expression: Influence of CB_1 ablation, sex, and age. **a** D_1 dopamine receptor and **b** D_5 dopamine receptor. Gene expression levels were determined in total RNA isolated from brain striata of young and old adult $CB_1(+/+)$ and $CB_1(-/-)$ mice ($n=3-10$) and subjected to analysis as described in Fig. 2. **a** (D_1 dopamine receptor gene expression): ++differs due to age, $p<0.03$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. **b** (D_5 dopamine receptor gene expression): +differs due to sex, $p<0.03$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. †WT vs. KO mice of the same age and sex differ with a two-sample t test comparison; p 0.012
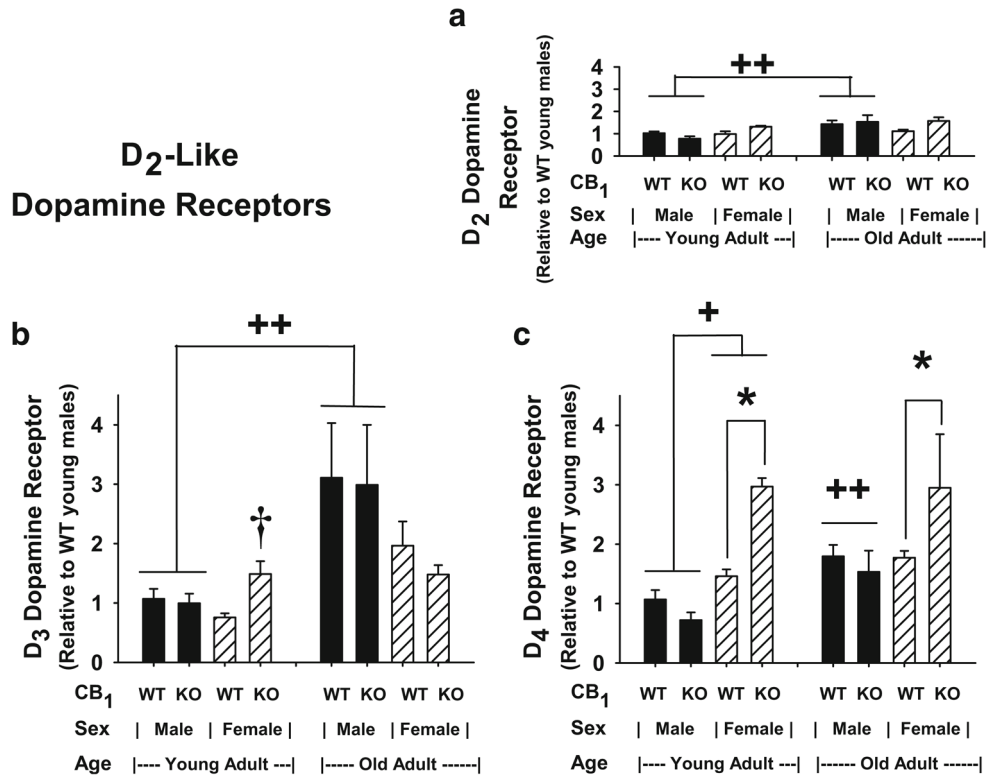
Fig. 5.

Striatal D_2-like dopamine receptor subtype gene expression: Influence of CB_1 ablation, sex, and age. **a** D_2 dopamine receptor, **b** D_3 dopamine receptor, and **c** D_4 dopamine receptor. Gene expression levels were determined in total RNA isolated from brain striata of young and old adult $CB_1(+/+)$ and $CB_1(-/-)$ mice ($n=3$–10) and subjected to analysis as described in Fig. 2. **a** (D_2 dopamine receptor gene expression): ++differs due to age, $p<0.006$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. **b** (D_3 dopamine receptor gene expression): ++differs due to age, $p<0.01$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. †WT vs. KO mice of the same age and sex differ with a two-sample t test comparison; $p=0.011$. **c** (D_4 dopamine receptor gene expression): *differs due to genotype (WT vs. KO), $p<0.03$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. +differs due to sex, $p<0.03$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests. ++differs due to age, $p<0.03$; GLM ANOVA with Tukey–Kramer and Bonferroni multiple comparison tests

Table 1

Relative differences in the expression of selected genes in mouse striatal tissue presented as a function of the following factors—*genotype*: $CB_1(-/-)$ relative to $CB_1(+/+)$ mice; *sex*: female relative to male mice; *age*: old relative to young adult mice

	Genes	Genotype $CB_1(-/-)/CB_1(+/+)$	Sex Female/Male	Age Old/Young
Opioid peptides	PPENK	↑Female[a]	↑Young[b]	–[c]
		↓Male [young]	↑Old[b]	–
	PPDYN	↑Female[a]	–	–
		↓Male [young]	–	↑Male[b]
Opioid receptors	δ-OR	–	–	↑Female[b]
		–	–	↑Male[b]
	μ-OR	–	↑Young[b]	–
		↑Male [old]	↑Old[b]	–
	κ-OR	–	–	–
		–	–	↑Male[b]
D1-like dopamine receptors	D_1 dopamine receptor	–	–	↑Female[b]
		–	–	↑Male [(+/+)]
	D_5 dopamine receptor	↑Female [young]	↑Young [(−/−)]	–
		↓Male [young]	–	–
D2-like dopamine receptors	D_2 dopamine receptor	–	–	–
		–	–	↑Male[b]
	D_3 dopamine receptor	↑Female [young]	–	–
		–	–	↑Male[b]
	D_4 dopamine receptor	↑Female[a]	↑Young[b]	–
		–	–	↑Male[b]

The table is based on data presented in "Results" and in Figs. 2, 3, 4, 5

(+/+), $CB_1(+/+)$: mice with wild-type CB_1 cannabinoid receptors; (−/−), $CB_1(-/-)$: transgenic mice deficient in CB_1 cannabinoid receptors (Zimmer et al. 1999); young: adult mice 60–90 days old; old: adult mice 140–300 days old

PPENK preproenkephalin, *PPDYN* preprodynorphin, *δ-OR* delta opioid receptor, *μ-OR* mu opioid receptor, *κ-OR* kappa opioid receptor

[a] Independent of age

[b] Independent of genotype

[c] No significant difference