β-Arrestin2 Regulates Cannabinoid CB₁ Receptor Signaling and Adaptation in a Central Nervous System Region–Dependent Manner

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Background: Cannabinoid CB_1 receptors (CB_1Rs) mediate the effects of $\triangle^9$-tetrahydrocannabinol (THC), the psychoactive component in marijuana. Repeated THC administration produces tolerance and dependence, which limit therapeutic development. Moreover, THC produces motor and psychoactive side effects. β-arrestin2 mediates receptor desensitization, internalization, and signaling, but its role in these CB_1R effects and receptor regulation is unclear.

Methods: CB_1R signaling and behaviors (antinociception, hypothermia, catalepsy) were assessed in β-arrestin2-knockout (βarr2-KO) and wild-type mice after THC administration. Cannabinoid-stimulated [³⁵S]GTPγS and [³H]ligand autoradiography were assessed by statistical parametric mapping and region-of-interest analysis.

Results: β-arrestin2 deletion increased CB_1R-mediated G-protein activity in subregions of the cortex but did not affect CB_1R binding, in vehicle-treated mice. βarr2-KO mice exhibited enhanced acute THC-mediated antinociception and hypothermia, with no difference in catalepsy. After repeated THC administration, βarr2-KO mice showed reduced CB_1R desensitization and/or downregulation in cerebellum, caudal periaqueductal gray, and spinal cord and attenuated tolerance to THC-mediated antinociception. In contrast, greater desensitization was found in hypothalamus, cortex, globus pallidus, and substantia nigra of βarr2-KO compared with wild-type mice. Enhanced tolerance to THC-induced catalepsy was observed in βarr2-KO mice.

Conclusions: β-arrestin2 regulation of CB_1R signaling following acute and repeated THC administration was region-specific, and results suggest that multiple, overlapping mechanisms regulate CB_1Rs. The observations that βarr2-KO mice display enhanced antinociceptive responses to acute THC and decreased tolerance to the antinociceptive effects of the drug, yet enhanced tolerance to catalepsy, suggest that development of cannabinoid drugs that minimize CB_1R interactions with β-arrestin2 might produce improved cannabinoid analgesics with reduced motor suppression.

CB₁ receptors (CB₁Rs) are widely distributed in the central nervous system (CNS) (1) and mediate the central effects of $\triangle^9$-tetrahydrocannabinol (THC) and cannabinoids (2). The endocannabinoid system is implicated in numerous physiological processes and is a potential therapeutic target for disorders including neurodegenerative and neuropsychiatric diseases and chronic pain (3). However, therapeutic use is limited by psychoactive and motor side effects. Moreover, repeated cannabinoid treatment produces tolerance to cannabinoid-mediated in vivo effects (4). Region-specific CB₁R desensitization and downregulation occur in conjunction with tolerance (5), but the molecular mechanisms that underlie CB₁R adaptations and tolerance are not well defined.

CB₁Rs primarily activate $G_{i/o}$-proteins, which regulate adenylyl cyclases, ion channels, and kinases (6). Persistent cannabinoid exposure induces CB₁R uncoupling from G-proteins (desensitization) (7), with subsequent receptor internalization (8) and degradation (downregulation) (9,10). One mechanism for these adaptations occurs by G-protein-coupled receptor (GPCR) kinase (GRK)-mediated phosphorylation of activated receptors and subsequent β-arrestin binding (11). β-arrestin2 is one of two arrestin isoforms in the brain (12), and findings in cell models support a role for β-arrestin2 in CB₁R adaptations. Coexpression of GRK3 and β-arrestin2 was required for rapid desensitization of CB₁R-mediated potassium currents following exposure to WIN55,212-2 in *Xenopus oocytes* (8). Similarly, expression of dominant negative β-arrestin2 attenuated desensitization of WIN55,212-2-mediated inhibition of glutamatergic neurotransmission in hippocampal neurons (13). Immunohistochemical studies show that CB₁Rs are codistributed with β-arrestin2 in certain CNS regions (12,14), suggesting that β-arrestin2 might regulate CB₁R signaling in the CNS.

Because there are no pharmacological β-arrestin inhibitors, β-arrestin2 knockout (βarr2-KO) mice (15) provide a model to study its role in regulating GPCRs in vivo (16). Acute administration of THC to βarr2-KO mice revealed enhanced sensitivity to its antinociceptive and hypothermic effects (17). However, direct evidence for the role of β-arrestin2 in CB₁R adaptations and tolerance following repeated THC is lacking.

We adapted statistical parametric mapping (SPM) to analyze [³⁵S]GTPγS autoradiography (18). SPM has the advantage of assessing changes in G-protein activation in an unbiased and anatomically inclusive manner. We applied SPM to examine the role of β-arrestin2 in CB₁R regulation by comparing cannabinoid-stimulated [³⁵S]GTPγS binding in brains from vehicle and THC-treated βarr2-KO and wild-type (WT) littermates. Combining this approach with behavioral assessment following THC administration allowed us to compare CB₁R signaling with behavioral responses observed in the βarr2-KO mice. We demonstrate region-specific regulation of

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CB$_1$Rs by β-arrestin2 that parallel changes in THC-mediated behavior and tolerance.

Methods and Materials

Detailed Methods are provided in Supplement 1.

Mice

Male WT and βarr2-KO mice (littermates, 4 months) (15) were injected intraperitoneally with THC (10 mg/kg) or vehicle (1:1:18 ethanol:cremaphor: .9% saline) twice daily for 6.5 days (subchronic treatment). Twenty-four hours after the final injection, mice were challenged with increasing doses of THC (3, 7, 20, 26, and 44 mg/kg, intraperitoneally) every 40 min, with responses assessed 30 min after each injection. Studies followed the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*.

Behavior

Antinociception was assessed using the warm-water (52°C) tail-immersion assay (19). Duplicate measurements determined baseline responses, but mice were assessed only once following each injection to minimize tissue damage. A trained observer assessed immobility by determining the time mice spent motionless on a metal ring-stand over 5 min (20). Mice were gently restrained, and body temperature was measured using a rectal probe thermometer (15). Mice were sacrificed by decapitation 24 hours after testing. The spinal cord and brain were extracted, frozen, and stored at −80°C. For antinociception and catalepsy, data are presented as the percentage of the maximum possible effect (%MPE) = 100% × [(experimental response latency – basal response latency)/(maximal possible response – basal response latency)]. Nonlinear regression analysis was calculated using GraphPad Prism software (La Jolla, California).

[^{35}S]GTPγS and [^{3}H]SR141716A Binding

Whole spinal cord was collected (Supplement 1), tissue was homogenized, and agonist-stimulated [^{35}S]GTPγS binding was conducted as published (10). Concentration-effect curves were generated using .01–3 μmol/L CP55,940 or .03–10 μmol/L WIN55,212-2. Percent stimulation = [(agonist-stimulated – basal)/basal] × 100%. Curves were fit using nonlinear regression in GraphPad Prism. [^{3}H]SR141716A binding was performed as published (21) using [^{3}H]SR141716A (.1–2.5 nmol/L), and nonspecific binding was measured with 5 μmol/L SR141716A. Data were fit to a one-site model in GraphPad Prism. Statistical comparisons used Student-Newman-Keuls post hoc test.

[^{35}S]GTPγS and [^{3}H]CP55,940 Autoradiography

Agonist-stimulated [^{35}S]GTPγS autoradiography was conducted on duplicate serial sections as published (7,18). Basal and CP55,940-stimulated [^{35}S]GTPγS binding were conducted in adjacent sections. CP55,940 is a high-efficacy agonist for G-protein activation but does not activate non-CB$_1$ sites in brain sections (18). Net stimulation (nCi/g) = (agonist-stimulated – basal). [^{3}H]CP55,940 autoradiography was modified from Herkenham *et al.* (1) and Moise *et al.* (22). Total binding was assessed with 3 nmol/L [^{3}H]CP55,940 and nonspecific binding was measured using 10 μmol/L CP55,940. Image reconstructions, SPM, and region-of-interest (ROI) analysis were conducted as published (18,23). The ROI measurements were made on original unprocessed images, averaged across hemispheres, and analyzed by two-way analysis of variance (significance $p < .05$) and Student-Newman-Keuls post hoc comparisons.

Results

THC-Induced Responses in βarr2-KO Mice

THC-mediated antinociception was assessed in vehicle- or THC-treated WT and βarr2-KO mice by cumulative dosing of THC. The basal latencies in WT and βarr2-KO mice subchronically treated with vehicle were 1.650 ± .176 and 1.625 ± .251, respectively. Basal latencies for WT and βarr2-KO mice treated with THC were 1.438 ± .092 and 1.788 ± .210, respectively. Cumulative dosing of THC produced a greater degree of antinociception in βarr2-KO mice subchronically treated with vehicle, compared with their WT littermates [Figure 1A; for genotype: $F(1,78) = 8.95$, $p = .0037$; for dose: $F(5,78) = 92.00$, $p < .0001$; for interaction: $F(5,78) = 5.11$, $p = .0004$]. This difference was due to a difference in potency (ED$_{50}$ value) between WT and βarr2-KO mice (Table 1). To determine the degree of antinociceptive tolerance, the ED$_{50}$ of THC and 95% confidence intervals were calculated using nonlinear regression analysis of each curve. Comparison between genotypes revealed that subchronic THC treatment shifted the antinociceptive dose-response curve to the right to a much greater extent in WT (8.45-fold shift) compared to βarr2-KO mice (1.68-fold shift; Table 1).

Cannabinoids decrease rodent motility measured as time spent in a cataleptic state. Cumulative dosing of THC induced a similar degree of catalepsy in vehicle-treated WT and βarr2-KO mice [Figure 1B; genotype: $F(1,78) = 2.53$, $p = .1159$; dose: $F(5,78) = 65.19$, $p < .0001$; interaction: $F(5,78) = .71$, $p = .6193$]. Subchronic THC treatment reduced the time that both WT and βarr2-KO mice spent immobile compared with vehicle-treated mice given the same acute dose of THC, indicating that both WT and βarr2-KO mice become tolerant to THC-mediated catalepsy [WT, treatment: $F(1,84) = 18.13$, $p < .0001$; dose: $F(5,84) = 84.00$, $p < .0001$; interaction: $F(5,84) = 2.49$, $p = .0376$; βarr2-KO, treatment: $F(1,72) = 53.27$, $p < .0001$; dose: $F(5,72) = 54.97$, $p < .0001$; interaction: $F(5,72) = 4.91$, $p = .0006$]. Comparison of the THC-induced shift in the ED$_{50}$ for each genotype revealed that, in contrast to antinociception, βarr2-KO mice displayed a greater degree of tolerance to THC-induced catalepsy (4.76-fold shift) than WT mice [2.12-fold shift; ($F(1,70) = 7.873$; $p < .01$ sum of least squares F test; Table 1].

THC-induced hypothermia was also assessed in WT and βarr2-KO mice. Vehicle-treated βarr2-KO mice displayed greater decreases in body temperature across the THC dosing regimen, compared with vehicle-treated WT mice [Figure 1C; genotype: $F(1,78) = 8.15$, $p = .0055$; dose: $F(5,78) = 41.96$, $p < .0001$; interaction: $F(5,78) = .16$, $p = .9775$]. Subchronic THC treatment induced significant tolerance to THC-mediated hypothermia in both genotypes [WT, treatment: $F(1,84) = 51.15$, $p < .0001$; dose: $F(5,84) = 32.38$, $p < .0001$; interaction: $F(5,84) = 13.26$, $p < .0001$; βarr2-KO, treatment: $F(1,72) = 65.02$, $p < .0001$; dose: $F(5,72) = 14.76$, $p < .0001$; interaction: $F(5,72) = 6.94$, $p < .0001$]. The data for THC groups did not converge, therefore ED$_{50}$ values could not be calculated, and comparison of the degree of tolerance was not possible. However, statistical analysis of the two curves revealed no significant difference between THC-pretreated WT and βarr2-KO mice [genotype: $F(1,78) = .18$, $p = .6765$]. Collectively these studies suggest region-specific CB$_1$R regulation by β-arrestin2, because THC-induced antinociception, catalepsy, and hypothermia are mediated by different neuronal populations in the CNS (24–26).

β-Arrestin2 Regulates CB$_1$R Desensitization in Spinal Cord

The finding that THC-mediated antinociception is enhanced, whereas development of tolerance is reduced, in βarr2-KO mice suggests that β-arrestin2 might negatively regulate CB$_1$Rs in the spinal cord because CB$_1$Rs in this region contribute to tail-flick