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Beta-arrestin-2 regulates cannabinoid CB₁ receptor signaling and adaptation in a CNS region-dependent manner

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Abstract

Background—Cannabinoid CB₁ receptors (CB₁R) mediate the effects of Δ⁹-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 CB₁R effects and receptor regulation is unclear.

Methods—CB₁R signaling and behaviors (antinociception, temperature, 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 (SPM) and region-of-interest analyses.

Results—β-arrestin2 deletion increased CB₁R-mediated G-protein activity in subregions of the cortex, but did not affect CB₁R 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 CB₁R 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 to wild-type mice. Enhanced tolerance to THC-induced catalepsy was observed in βarr2-KO mice.

Conclusions—β-arrestin2 regulation of CB₁R signaling following acute and repeated THC administration was region-specific, and results suggest that multiple, overlapping mechanisms regulate CB₁Rs. 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 CB₁R interactions with β-arrestin2 might produce improved cannabinoid analgesics with reduced motor suppression.

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Keywords

Cannabinoid receptors; beta-arrestin; GPCR; tolerance; statistical parametric mapping

Introduction

CB₁ receptors (CB₁R) are widely distributed in the CNS (1) and mediate the central effects of Δ^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 cyclase, 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. Co-expression 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 co-distributed with β -arrestin2 in certain CNS regions (12, 14), suggesting that β -arrestin2 might regulate CB₁R signaling in the CNS.

As there are no pharmacological β -arrestin inhibitors, β -arrestin2 knockout (β arr2-KO) mice (15) provide a model to study its role in regulating GPCRs *in vivo* (16). Acute administration of THC to β arr2-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]GTP γ S 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]GTP γ S binding in brains from vehicle and THC-treated β arr2-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 β arr2-KO mice. We demonstrate region-specific regulation of CB₁Rs by β -arrestin2 that parallel changes in THC-mediated behavior and tolerance.

Methods and Materials

Detailed Methods are provided in the Supplement.

Mice

Male WT and β arr2-KO mice (littermates, 4 months) (15) were injected intraperitoneally with THC (10 mg/kg) or vehicle (1:1:18 ethanol:cremaphor:0.9% 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, i.p.) every 40 minutes, with responses assessed 30 minutes after each injection. Studies followed the NIH *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-minutes (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 %MPE = $100 \times [(\text{experimental response latency} - \text{basal response latency}) / (\text{maximal possible response} - \text{basal response latency})]$. Non-linear regression analysis was calculated using GraphPad Prism.

[^{35}S]GTP γ S and [^3H]SR141716A binding

Whole spinal cord was collected (see Supplement), tissue was homogenized and agonist-stimulated [^{35}S]GTP γ S binding was conducted as published (10). Concentration-effect curves were generated using 0.01–3 μM CP55,940 or 0.03–10 μM WIN55,212-2. Percent stimulation = $[(\text{agonist-stimulated} - \text{basal}) / \text{basal}] \times 100\%$. Curves were fit using non-linear regression in GraphPad Prism. [^3H]SR141716A binding was performed as published (21) using [^3H]SR141716A (0.1–2.5 nM) and non-specific binding was measured with 5 μM SR141716A. Data were fit to a one-site model in GraphPad Prism. Statistical comparisons used Student-Newman Keuls post-hoc test.

[^{35}S]GTP γ S and [^3H]CP55,940 autoradiography

Agonist-stimulated [^{35}S]GTP γ S autoradiography was conducted on duplicate serial sections as published (7, 18). Basal and CP55,940-stimulated [^{35}S]GTP γ S 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). [^3H]CP55,940 autoradiography was modified from (1, 22). Total binding was assessed with 3nM [^3H]CP55,940 and non-specific binding was measured using 10 μM CP55,940. Image reconstructions, SPM and region-of-interest (ROI) analysis were conducted as published (18, 23). ROI measurements were made on original unprocessed images, averaged across hemispheres, and analyzed by two-way ANOVA (significance $p < 0.05$) and Student-Newman Keuls post-hoc comparisons.

Results

THC-induced responses in β arr2-KO mice

THC-mediated antinociception was assessed in vehicle- or THC-treated WT and β arr2-KO mice by cumulative dosing of THC. The basal latencies in WT and β arr2-KO mice subchronically treated with vehicle were 1.650 ± 0.176 and 1.625 ± 0.251 , respectively. Basal latencies for WT and β arr2-KO mice treated with THC were 1.438 ± 0.092 and 1.788 ± 0.210 , respectively. Cumulative dosing of THC produced a greater degree of antinociception in β arr2-KO mice subchronically treated with vehicle, compared to their WT littermates

(Figure 1A: for genotype: $F_{1,78}=8.95$, $p=0.0037$; for dose: $F_{5,78}=92.00$, $p<0.0001$; for interaction: $F_{5,78}=5.11$, $p=0.0004$). This difference was due to a difference in potency (ED_{50} value) between WT and β arr2-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 β arr2-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 β arr2-KO mice (Figure 1B: genotype: $F_{1,78}=2.53$, $p=0.1159$; dose: $F_{5,78}=65.19$, $p<0.0001$; interaction: $F_{5,78}=0.71$, $p=0.6193$). Subchronic THC treatment reduced the time that both WT and β arr2-KO mice spent immobile compared to vehicle-treated mice given the same acute dose of THC, indicating that both WT and β arr2-KO mice become tolerant to THC-mediated catalepsy (WT, treatment: $F_{1,84}=18.13$, $p<0.0001$; dose: $F_{5,84}=84.00$, $p<0.0001$; interaction: $F_{5,84}=2.49$, $p=0.0376$; β arr2-KO, treatment: $F_{1,72}=53.27$, $p<0.0001$; dose: $F_{5,72}=54.97$, $p<0.0001$; interaction: $F_{5,72}=4.91$, $p=0.0006$). Comparison of the THC-induced shift in the ED_{50} for each genotype revealed that, in contrast to antinociception, β arr2-KO mice displayed a greater degree of tolerance to THC-induced catalepsy (4.76-fold shift) than WT mice (2.12-fold shift) ($F_{1,70}=7.873$; $p<0.01$ sum of least squares f test) (Table 1).

THC-induced hypothermia was also assessed in WT and β arr2-KO mice. Vehicle-treated β arr2-KO mice displayed greater decreases in body temperature across the THC dosing regimen, compared to vehicle-treated WT mice (Figure 1C: genotype: $F_{1,78}=8.15$, $p=0.0055$; dose: $F_{5,78}=41.96$, $p<0.0001$; interaction: $F_{5,78}=0.16$, $p=0.9775$). Subchronic THC treatment induced significant tolerance to THC-mediated hypothermia in both genotypes (WT, treatment: $F_{1,84}=51.15$, $p<0.0001$; dose: $F_{5,84}=32.38$, $p<0.0001$; interaction: $F_{5,84}=13.26$, $p<0.0001$; β arr2-KO, treatment: $F_{1,72}=65.02$, $p<0.0001$; dose: $F_{5,72}=14.76$, $p<0.0001$; interaction: $F_{5,72}=6.94$, $p<0.0001$). 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 β arr2-KO mice (genotype: $F_{1,78}=0.18$, $p=0.6765$). Collectively these studies suggest region-specific CB_1R 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_1R desensitization in spinal cord

The finding that THC-mediated antinociception is enhanced, while development of tolerance is reduced in β arr2-KO mice suggests that β -arrestin2 might negatively regulate CB_1R s in the spinal cord because CB_1R s in this region contribute to tail-flick antinociception (27). Therefore, cannabinoid-stimulated [^{35}S]GTP γ S binding was assessed in spinal cords from WT and β arr2-KO mice. Basal [^{35}S]GTP γ S binding did not significantly differ between genotypes or treatments (WT/vehicle = 215 ± 11 , WT/THC = 208 ± 18 , β arr2-KO/vehicle = 221 ± 20 , and β arr2-KO/THC = 198 ± 15 nCi/g). Residual THC would stimulate [^{35}S]GTP γ S binding above basal levels in vehicle-treated mice, thus THC washout was sufficient. CP55,940-mediated G-protein activity was first compared between vehicle-treated WT and β arr2-KO mice and showed no differences between genotypes (Figure 2A, Table 2). Subchronic treatment with THC significantly reduced CP55,940-stimulated [^{35}S]GTP γ S binding in WT mice, with an approximately 38% decrease in receptor-mediated activity (Figure 2A, Table 2, $p<0.001$). CB_1R desensitization was attenuated in β arr2-KO mice, where the E_{max} value for CP55,940-stimulated [^{35}S]GTP γ S binding did not significantly

differ from its vehicle control, but differed from THC-treated WT mice (Table 2, $p < 0.001$). EC_{50} values for CP55,940-stimulated [35 S]GTP γ S binding did not significantly differ between treatment groups (Table 2), showing sufficient washout of THC. A significant interaction was found between drug and genotype [$F_{1,24} = 5.68$, $p < 0.05$] in CP55,940-stimulated [35 S]GTP γ S binding, suggesting that β -arrestin2 contributes to CB $_1$ R desensitization in the spinal cord following THC treatment. Similar results were found using WIN55,212-2 (Table 2). These studies suggest that β -arrestin2-mediated CB $_1$ R desensitization might contribute to tolerance to THC-mediated antinociception, because both CB $_1$ R desensitization in the spinal cord and tolerance to the tail-flick test were significantly attenuated in β arr2-KO mice.

β -arrestin2 regulates CB $_1$ R downregulation in spinal cord

β -arrestin2 facilitates endocytosis for many GPCRs (11). Internalized receptors can be degraded and the resulting downregulation could contribute to antinociceptive tolerance. To determine the role of β -arrestin2 in CB $_1$ R downregulation, [3 H]SR141716A binding was performed in spinal cord. No differences were found in [3 H]SR141716A binding between genotypes in vehicle-treated mice (Figure 2B, Table 2), showing that alterations in acute behavior were not due to a change in CB $_1$ R density in β arr2-KO mice. In contrast, subchronic THC treatment significantly reduced [3 H]SR141716A binding in WT mouse spinal cord ($B_{max} = 59.4\%$ of vehicle, $p < 0.01$) an effect that was significantly attenuated in β arr2-KO mice ($B_{max} = 82.3\%$ of vehicle). The B_{max} value for THC-treated β arr2-KO mice did not differ from its vehicle control, but was significantly different from THC-treated WT mice ($p < 0.05$). No differences in [3 H]SR141716A K_D values were found between groups (Figure 2B), indicating adequate washout of THC. These results indicate that β -arrestin2 is also involved in CB $_1$ R downregulation in the spinal cord, which could contribute to attenuation of THC-mediated antinociceptive tolerance.

β -arrestin2 regulates CB $_1$ R-mediated G-protein activity in a brain region-specific manner

THC-induced catalepsy (25), hypothermia (26) and antinociception (24) involve different populations of CB $_1$ R in the brain. Therefore, CB $_1$ R-mediated G-protein activity was assessed throughout the brains of vehicle- and THC-treated β arr2-KO and WT mice using CP55,940-stimulated [35 S]GTP γ S autoradiography with SPM and ROI analyses. CP55,940-stimulated [35 S]GTP γ S binding was widely distributed in brains from mice of both genotypes, with the highest levels in the basal ganglia, hippocampus, cerebellum and cortex, as previously reported (Figure 3) (18). SPM and ROI analyses of basal [35 S]GTP γ S binding showed no significant differences based on genotype or drug treatment in any region (Table S1 in the Supplement). CP55,940-stimulated [35 S]GTP γ S binding was first assessed in vehicle-treated mice of both genotypes. CP55,940-stimulated [35 S]GTP γ S binding appeared higher in cortical regions of β arr2-KO compared to WT mice (Figure 3) and SPM corroborated this observation. SPM revealed that CP55,940-stimulated [35 S]GTP γ S binding was significantly ($p < 0.05$) greater in the cortex and caudal hippocampus of β arr2-KO compared to WT mice (Figure 4). This was confirmed by ROI analysis of individual sections, which showed significantly greater CP55,940-stimulated [35 S]GTP γ S binding in the piriform, auditory and visual cortices of β arr2-KO compared to WT mice (Figure 5, Table 3). Intra-regional effects were found within the hippocampus, where CB $_1$ R-mediated G-protein activity was significantly higher in β arr2-KO compared to WT mice only in the caudal hippocampus (Figure 4, Table 3). A non-significant trend toward greater CP55,940-stimulated [35 S]GTP γ S binding in β arr2-KO mice was observed in the cingulate cortex ($p = 0.051$), somatosensory cortex ($p = 0.078$) and amygdala ($p = 0.058$) (Table 3). These results indicate region-specific regulation of acute CB $_1$ R signaling that was apparent in β arr2-KO mice.

To investigate the role of β -arrestin2 in CB₁R regulation after repeated THC, CP55,940-stimulated [³⁵S]GTP γ S binding was compared between vehicle- and THC-treated WT or β arr2-KO mice. SPM showed regions in which THC-induced decreases in CP55,940-stimulated [³⁵S]GTP γ S binding were significant ($p < 0.05$) (Figure 6). SPM and ROI analysis also revealed differences between β arr2-KO and WT mice in both the regional extent and magnitude of CB₁R desensitization. Genotype-specific attenuation in desensitization was seen in some regions in β arr2-KO mice, similar to spinal cord. In the cerebellum, SPM showed that CP55,940-stimulated [³⁵S]GTP γ S binding was significantly reduced in brains from THC-treated WT mice (Figure 6), and ROI analysis confirmed that CB₁R-mediated G-protein activity in THC-treated WT mice was reduced to $71 \pm 7\%$ of levels in vehicle-controls (Figure 5, Table 3). In contrast, no significant difference in CP55,940-stimulated [³⁵S]GTP γ S binding in the cerebellum was observed between THC- and vehicle-treated β arr2-KO mice using SPM or ROI analysis (Figure 5, Table 3). A significant interaction of drug treatment \times genotype was found [$F_{1,28} = 4.51$, $p < 0.05$], suggesting that β -arrestin2 regulates CB₁R desensitization in the cerebellum following this THC treatment paradigm. SPM revealed intra-regional differences in the PAG (Figure 6). Similar significant decreases in CP55,940-stimulated [³⁵S]GTP γ S binding were detected in the rostral PAG (~Bregma -3.40) from THC-treated β arr2-KO and WT mice. In contrast, CP55,940-stimulated [³⁵S]GTP γ S binding in the caudal PAG was not significantly affected by THC treatment in β arr2-KO mice (~Bregma -4.60), suggesting that CB₁R desensitization was attenuated in β arr2-KO mice. This was confirmed by ROI analysis, which showed that THC treatment significantly reduced CP55,940-stimulated [³⁵S]GTP γ S binding in the caudal PAG of WT mice ($48 \pm 5\%$ of vehicle), whereas activity was not significantly different between vehicle- and THC-treated β arr2-KO mice ($84 \pm 6\%$ of vehicle) (Table 3, Figure 5). In contrast, ROI analysis revealed a similar magnitude of desensitization in the rostral PAG of WT ($55 \pm 6\%$ of vehicle) and β arr2-KO ($51 \pm 5\%$ of vehicle) mice.

SPM identified several regions in which CB₁R desensitization appeared similar in β arr2-KO and WT mice (Figure 6). In the amygdala, ROI analysis showed that THC treatment reduced cannabinoid-stimulated [³⁵S]GTP γ S binding to $59 \pm 5\%$ and $65 \pm 8\%$ of vehicle control in β arr2-KO and WT mice, respectively (Figure 5, Table 3). Significant reductions in CP55,940-stimulated [³⁵S]GTP γ S binding were also found in the lateral entorhinal cortex and rostral hippocampus, and did not significantly differ between genotypes (Figure 5, Table 3).

CP55,940-stimulated [³⁵S]GTP γ S binding was also similar between genotypes in the caudate-putamen and nucleus accumbens, where CB₁R-mediated G-protein activity did not differ between THC- and vehicle-treated groups of either genotype (Figures 5–6, Table 3). Different results were observed in the globus pallidus and substantia nigra when comparing genotypes. CP55,940-stimulated [³⁵S]GTP γ S binding did not differ between THC- and vehicle-treated WT mice in these regions, showing a lack of CB₁R desensitization. However, CP55,940-stimulated [³⁵S]GTP γ S binding was significantly reduced in the substantia nigra (~85% of vehicle) and globus pallidus (~79% of vehicle) of THC-treated compared to vehicle-treated β arr2-KO mice (Figures 5–6, Table 3). Therefore, deletion of β -arrestin2 appeared to enhance CB₁R desensitization in the globus pallidus and substantia nigra, without altering acute CB₁R-mediated G-protein activity in vehicle-treated mice.

SPM analysis revealed enhanced desensitization in several other regions of β arr2-KO compared to WT mice (Figure 6). In the hypothalamus, CP55,940-stimulated [³⁵S]GTP γ S binding in THC-treated mice was $57 \pm 8\%$ and $79 \pm 16\%$ of vehicle in β arr2-KO and WT mice, respectively (Figure 5, Table 3) and occurred in the absence of genotype-dependent differences in vehicle-treated mice (Table 3). Subchronic THC also induced CB₁R desensitization in the piriform, auditory and visual cortices of β arr2-KO, but not WT, mice

(Figure 5, Table 3). SPM revealed intra-regional differences in desensitization within the hippocampus, wherein desensitization was significantly greater in magnitude in the caudal hippocampus of β arr2-KO mice (Figures 6). ROI analysis showed a significantly greater reduction in CP55,940-stimulated [35 S]GTP γ S binding in the caudal hippocampus in β arr2-KO mice ($58.6\pm 4\%$ of vehicle), compared to WT mice ($72.6\pm 4\%$ of vehicle) (Figure 5, Table 3).

Increases in the apparent efficacy of CP55,940-stimulated [35 S]GTP γ S binding in some regions of β arr2-KO brains appeared to be associated with the enhancement or unmasking of CB $_1$ R desensitization in these same regions following THC administration. The relative magnitude of desensitization in β arr2-KO mice when normalized to WT animals was positively correlated with the relative efficacy of CP55,940-stimulated [35 S]GTP γ S binding in β arr2-KO mice when normalized to WT animals ($p=0.0011$, $R^2=0.60$; Figure 7, Table 5). For example, a 1.5-fold greater relative efficacy of CP55,940-stimulated [35 S]GTP γ S binding in β arr2-KO mice compared to WT mice corresponded to an approximately 2-fold greater magnitude of desensitization in β arr2-KO relative to WT mice.

β -arrestin2 is involved in CB $_1$ R downregulation in the cerebellum

The role of β -arrestin2 in CB $_1$ R downregulation was assessed using [3 H]CP55,940 autoradiography. [3 H]CP55,940 binding did not differ between genotypes in vehicle-treated mice (Table 4), suggesting that genotype-dependent differences in CP55,940-stimulated [35 S]GTP γ S binding were not due to differences in CB $_1$ R levels. [3 H]CP55,940 binding was significantly decreased in the cerebellum of WT mice following THC treatment ($55\pm 10\%$ of vehicle), whereas [3 H]CP55,940 binding was similar in both treatment groups in β arr2-KO mice. [3 H]CP55,940 binding did not significantly differ between THC- and vehicle-treated mice of either genotype in any other region examined (Table 4). A significant interaction of drug treatment \times genotype was found in the cerebellum [$F_{1,28}=4.277$, $p<0.05$], suggesting that β -arrestin2 is involved in CB $_1$ R downregulation in the cerebellum.

Discussion

This study shows that β -arrestin2 regulates CB $_1$ R-mediated G-protein activity in a CNS region-specific manner and that these differences are reflected in THC-mediated responses observed in β arr2-KO mice. Genotype-specific differences were observed after acute THC, as β arr2-KO mice displayed enhanced THC-mediated hypothermia, antinociception and receptor-mediated G-protein activity in the forebrain compared to their WT littermates. Following repeated THC administration, β -arrestin2 deletion attenuated tolerance to THC-mediated antinociception and CB $_1$ R desensitization and/or downregulation in the cerebellum, caudal PAG, and spinal cord. These findings are consistent with the prediction that β -arrestin2 regulates CB $_1$ R adaptations that occur following subchronic THC administration. CB $_1$ R desensitization was similar between genotypes in other regions, indicating that other mechanisms might mediate desensitization or compensate for β -arrestin2 deletion. Interestingly, CB $_1$ R desensitization was enhanced in a subset of regions, some of which also showed genotype-specific differences in acute signaling.

THC-mediated hypothermia and antinociception were enhanced in β arr2-KO compared to WT mice, whereas catalepsy was similar between genotypes. These results agree with Breivogel et al (17), who showed that THC-mediated antinociception and hypothermia were enhanced by β -arrestin2 deletion. Antinociception and hypothermia produced by the high efficacy agonist CP55,940 did not differ between WT and β arr2-KO mice (17), indicating that effects of β -arrestin2 on CB $_1$ R are ligand-dependent. Microinjection studies showed that the preoptic anterior hypothalamus is involved in cannabinoid-mediated hypothermia (26), but cannabinoid-stimulated G-protein activity did not significantly differ between

genotypes in the preoptic area or hypothalamus. Similarly, CB₁R in the spinal cord and PAG mediate cannabinoid-induced antinociception (24, 27, 28), but acute cannabinoid-stimulated G-protein activity did not differ between genotypes in these areas despite an enhancement in THC-induced antinociception. Physiological responses result from the interplay of various brain regions, so it is possible that enhanced CB₁R signaling in the forebrain contributes indirectly to the observed enhancements in antinociception and hypothermia. Alternatively, THC might indirectly produce these responses via modulation of other receptor systems that are regulated directly by β -arrestin2. In either case, our results suggest that β -arrestin2 dampens THC-induced antinociception and hypothermia. Interestingly, morphine-mediated antinociception and hypothermia were also enhanced in β arr2-KO mice (15, 19, 29), supporting the idea that β -arrestin2 negatively regulates receptor signaling in these functional circuits. However, enhanced antinociceptive responses in β arr2-KO mice are not due to non-specific dysregulation in these systems, as other opioid agonists, such as fentanyl or methadone, produced similar responses in β arr2-KO and WT mice (30, 31).

A possible explanation for the finding that CB₁R-mediated G-protein activity was enhanced in some regions of β arr2-KO mice is that CB₁R constitutive activity or high endocannabinoid tone might induce β -arrestin2-mediated desensitization/internalization. In fact, previous studies showed that CB₁R undergo constitutive endocytosis with substantial localization in intracellular vesicles (32), although there is disagreement on the role of CB₁R activation in this process (33, 34). If deletion of β -arrestin2 increases the population of functional CB₁R localized to the plasma membrane, as suggested by enhanced CP55,940-stimulated [³⁵S]GTP γ S binding in regions such as cortex and caudal hippocampus, then more receptors might be available for agonist-stimulated activation of G-proteins (35), thereby increasing signaling efficacy and in vivo potency of the agonist. Alternatively, CB₁R in these regions might exhibit desensitization to the acute cumulative-dosing THC exposure in WT mice, although we previously found no significant effect of acute THC treatment on CB₁R-mediated G-protein activation (7).

Deletion of β -arrestin2 attenuated THC-induced desensitization of CB₁R-mediated G-protein activity in the cerebellum, caudal PAG, and spinal cord, a similar finding to reports in cell models (8, 13). Tolerance to THC-mediated antinociception was also attenuated in β arr2-KO mice, which likely can be attributed to the reduced CB₁R desensitization/downregulation observed in the spinal cord and caudal PAG following THC treatment, as these regions mediate cannabinoid-induced antinociception (24, 28). Findings in cerebellum are consistent with the expression of β -arrestin2 and CB₁R mRNA in this region as demonstrated by the Allen brain atlas (<http://mouse.brain-map.org/brain/Arrb2.html>) (36). These results suggest that the development of cannabinoids that minimize CB₁R interactions with β -arrestin2 might enhance antinociception while reducing the development of tolerance, thereby producing more effective analgesics.

Previous studies found that CB₁R in the basal ganglia appear resistant to desensitization compared with other regions (5, 21, 37). Similarly, CB₁R in the caudate-putamen, globus pallidus and substantia nigra from WT mice were not desensitized following subchronic THC treatment in this study. Surprisingly, however, CB₁R desensitization was observed in the globus pallidus and substantia nigra of β arr2-KO mice. While expression of β -arrestin2 mRNA in the striatum is low (36), our results suggest a functional interaction with CB₁R. β -arrestin1 mRNA expression is high in this region (36), thus β -arrestin1 could contribute to enhanced desensitization. The globus pallidus has been implicated in THC-induced catalepsy (25), which might explain the observation that tolerance to THC-induced catalepsy was enhanced in β arr2-KO compared to WT mice. This finding suggests that β -arrestin2 inhibits desensitization in these striatal output nuclei and thereby contributes to the reduced

CB₁R adaptations usually reported in these regions. Thus, β -arrestin2 would promote cataleptic side effects of THC by inhibiting the development of tolerance.

The finding that desensitization was unaffected or enhanced in certain regions of β arr2-KO mice indicates that non- β -arrestin2-mediated CB₁R regulation contributes to these adaptations. β -arrestin1 exhibits significant homology to β -arrestin2 (12) and has a widespread CNS localization that overlaps with CB₁Rs (12, 38). Deletion of β -arrestin1 enhanced isoproterenol-mediated cardiac responses (39) and decreased apomorphine-induced climbing in the absence of baseline differences in locomotion (11). These findings suggest that β -arrestin1 both negatively and positively regulates receptor function. Several kinases regulate CB₁R desensitization and tolerance and might contribute to desensitization observed in β arr2-KO mice. cAMP-dependent protein kinase (PKA) appears to contribute to desensitization (40) and PKA inhibitors reverse tolerance to THC-induced antinociception, catalepsy, and hypoactivity (41, 42). Protein kinase C (PKC) phosphorylates CB₁R in cell models (43). CB₁R adaptation and tolerance also involve ERK in a region- and effect-specific manner (44, 45). Deletion of G-protein-associated sorting protein1 (GASPI1), which targets receptors to lysosomes for degradation, attenuates CB₁R downregulation and tolerance to cannabinoid-mediated antinociception (46). Together, these results suggest that additional mechanisms can regulate CB₁R activity, either in a parallel or overlapping manner with β -arrestin2.

In summary, the present studies demonstrate multiple effects of β -arrestin2 deletion on CB₁R function, and confirm its role in attenuating acute THC-mediated antinociception and hypothermia. Moreover, results demonstrate both positive and negative modulatory effects of β -arrestin2 on THC tolerance, such that tolerance to antinociception was reduced whereas tolerance to catalepsy was enhanced in β arr2-KO mice. These findings corresponded with reduced CB₁R desensitization in the caudal PAG and spinal cord, and enhanced CB₁R desensitization in striatal output nuclei, respectively. These findings suggest that developing cannabinoid agonists that minimize interactions between CB₁R and β -arrestin2 might be therapeutically beneficial, as data suggest that these agonists might enhance antinociception but attenuate antinociceptive tolerance. The side effect profile might also improve because deletion of β -arrestin2 enhanced tolerance to THC-induced cataleptic effects, suggesting that motor side effects might be minimized. These findings demonstrate that interactions between CB₁Rs and β -arrestin2 should be considered in future drug development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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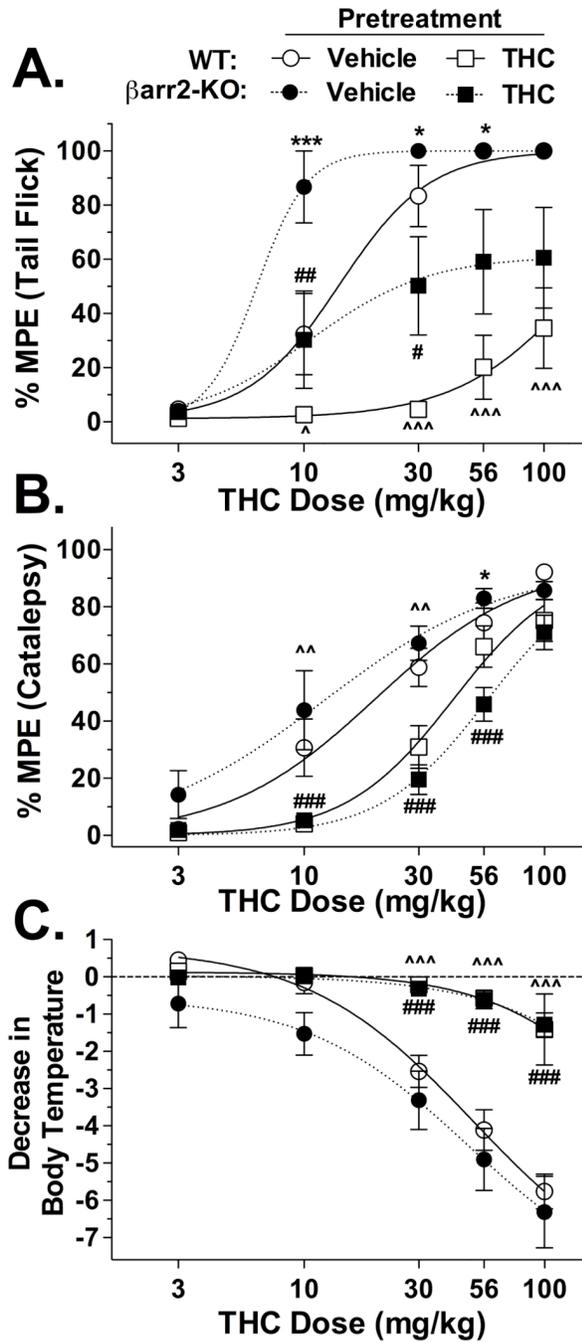


Figure 1. Cumulative THC dose-response curves in WT and β arr2-KO mice subchronically treated with either vehicle or THC (10 mg/kg twice daily, i.p.). **A.** Following vehicle treatment, β arr2-KO mice display enhanced responses to THC compared to WT mice in the tail-flick antinociceptive test (for interaction of dose and genotype: $F_{5,78} = 5.11$, $p = 0.0004$, $***p < 0.001$ Bonferroni post-hoc analysis). Following THC pretreatment, β arr2-KO mice remain more responsive than WT mice (for genotype: $F_{1,78} = 12.31$, $p = 0.0008$, $*p < 0.05$ Bonferroni post-hoc analysis). Both genotypes display tolerance following THC pretreatment (vehicle vs. THC: WT: for interaction of dose and pretreatment: $F_{5,84} = 11.25$, $p < 0.0001$, $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.001$ Bonferroni post-hoc analysis. β arr2-KO: for pretreatment:

$F_{1,72} = 22.78, p < 0.0001, \#p < 0.05, \##p < 0.01$ Bonferroni post-hoc analysis.) **B.** Following vehicle treatment, β arr2-KO mice display equivalent hypoactivity in response to THC compared to WT mice as assessed by the ring test for catalepsy (for genotype: $F_{1,78} = 2.53, p = 0.1159$). Both genotypes displayed tolerance following the THC pretreatment (vehicle vs. THC: WT: for interaction of dose and pretreatment: $F_{5,84} = 2.49, p = 0.0376, \wedge p < 0.01$ Bonferroni post-hoc analysis. β arr2-KO: for interaction of dose and pretreatment: $F_{5,72} = 4.91, p = 0.0006, \###p < 0.001$ Bonferroni post-hoc analysis). Following THC pretreatment, β arr2-KO mice become more tolerant to the hypolocomotor effects of THC than WT mice (for genotype: $F_{1,78} = 3.97, p = 0.0497, \#p < 0.05$ Bonferroni post-hoc analysis). **C.** Following vehicle treatment, β arr2-KO mice display greater hypothermia in response to THC compared to WT mice as determined by changes in rectal body temperature (for genotype: $F_{1,78} = 8.15, p = 0.0055$). Following THC pretreatment, WT and β arr2-KO mice did not differ in their response profiles (for genotype: $F_{1,78} = 0.09, p = 0.9941$). Both genotypes displayed tolerance following the THC pretreatment (vehicle vs. THC: WT: for interaction of dose and pretreatment: $F_{5,84} = 13.26, p < 0.0001, \wedge\wedge p < 0.001$ Bonferroni post-hoc analysis. β arr2-KO: for interaction of dose and pretreatment: $F_{5,72} = 6.94, p < 0.0001, \###p < 0.001$ Bonferroni post-hoc analysis). Data are presented as the mean \pm S.E.M. ($n = 8$ WT, $n = 7$ β arr2-KO). The log values of the indicated doses are graphed on the abscissa and nonlinear regression curves are provided.

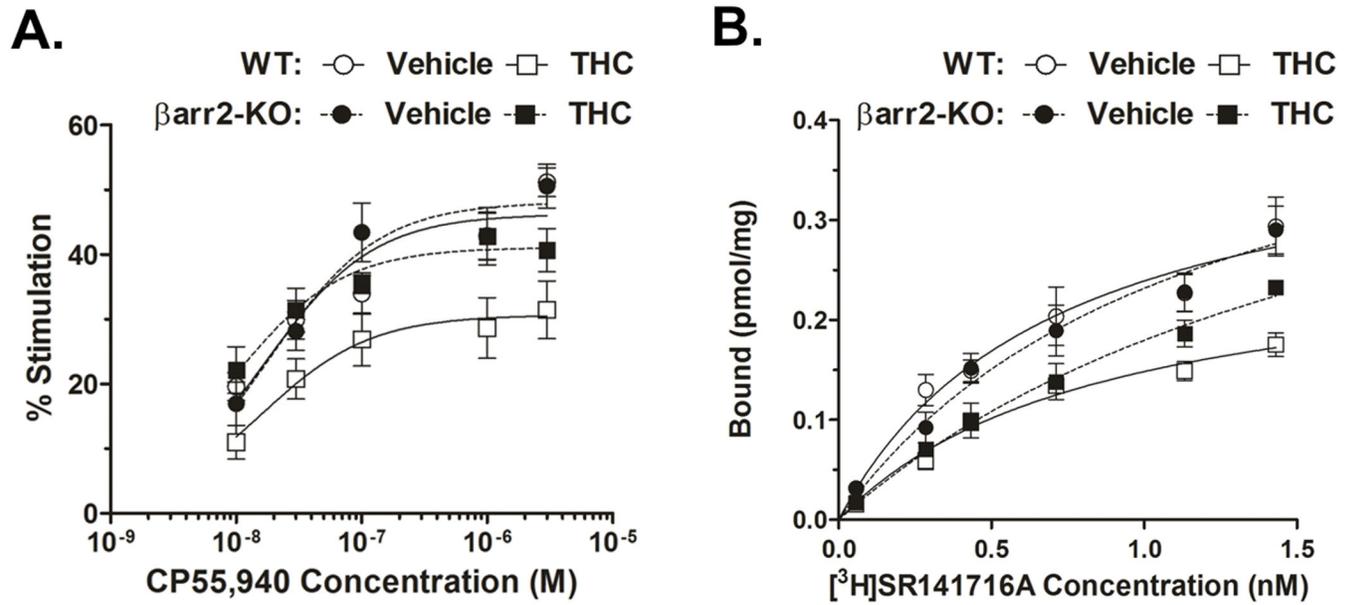


Figure 2. Agonist-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ concentration-effect curves using CP55,940 and CB_1R binding using $[^3\text{H}]\text{SR141716A}$ in WT and β arr2-KO mice subchronically treated with either vehicle or THC (10 mg/kg twice daily, i.p.). **A.** Desensitization of CB_1R -mediated G-protein activation was attenuated in spinal cords of β arr2-KO mice following subchronic treatment with THC. **B.** Subchronic administration of THC produced downregulation of CB_1Rs in spinal cords of WT animals, but was attenuated in β arr2-KO mice. EC_{50} values for CP55,940- or WIN55,212-2-stimulated $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding did not significantly differ between treatment groups.

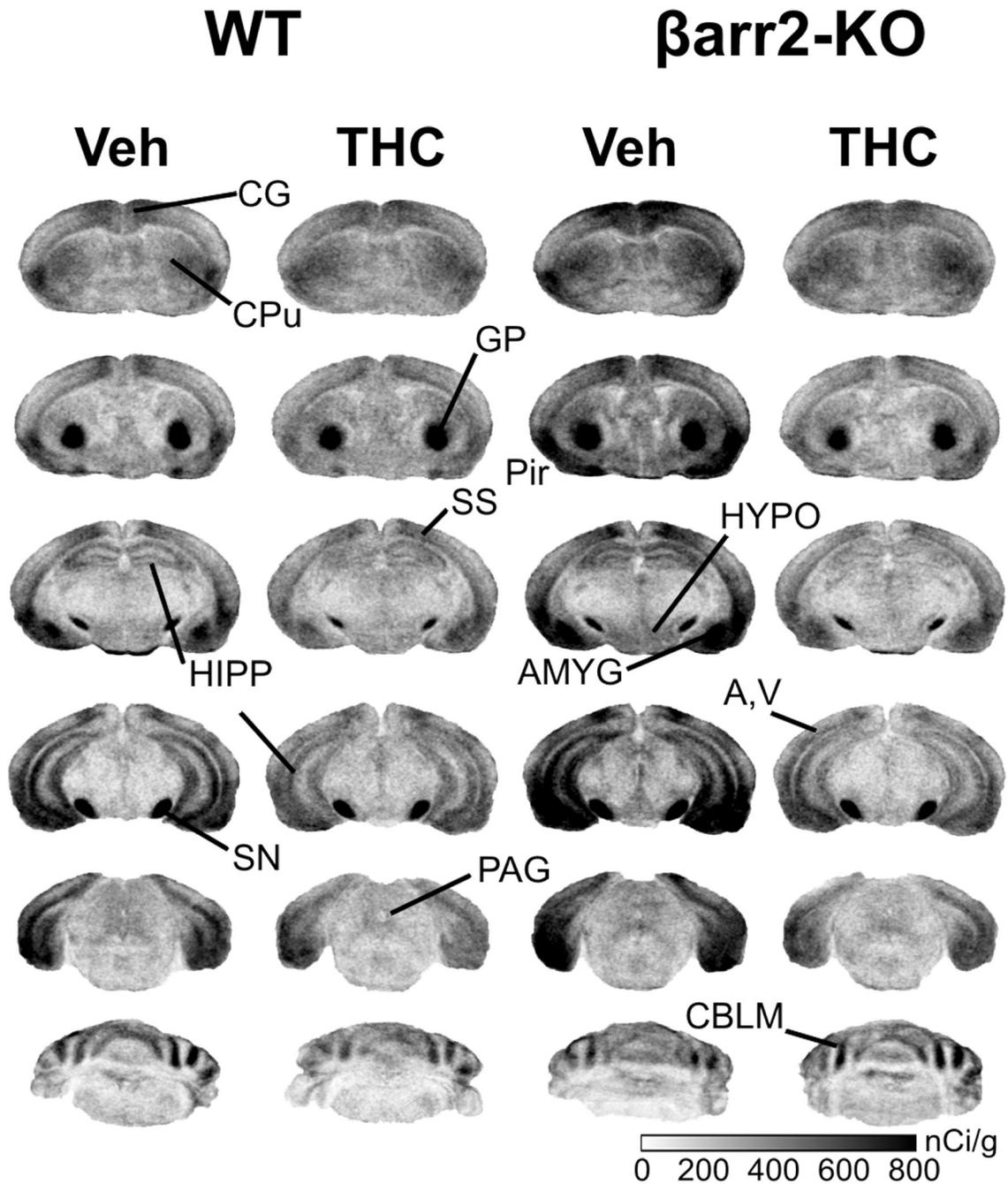


Figure 3. Net CP55,940-stimulated [³⁵S]GTPγS binding reconstructions derived from the image average of all subjects for vehicle- and THC-treated mice of each genotype (*n* = 8). Original autoradiographic images are shown in grayscale and correspond to the scale (bottom right). *AMYG*, amygdala; *A,V*, auditory & visual cortex; *CBLM*, cerebellum; *Cg*, cingulate cortex; *CPu*, caudate-putamen; *GP*, globus pallidus; *HIPP*, hippocampus; *HYP*, hypothalamus; *PAG*, periaqueductal gray; *Pir*, piriform cortex; *SS*, somatosensory cortex; *SN*, substantia nigra.

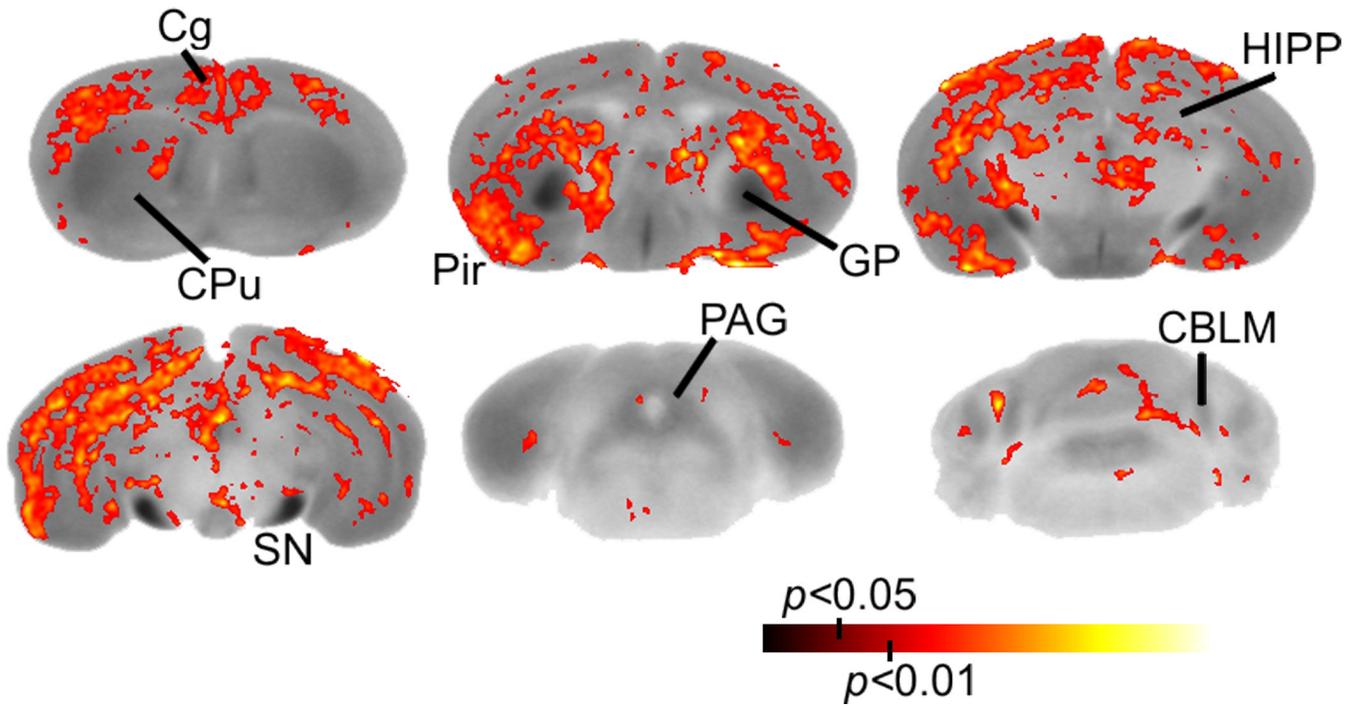


Figure 4. Genotype-specific differences in CP55,940-stimulated [³⁵S]GTP γ S binding were distributed primarily in the cortex and caudal hippocampus. Statistical parametric maps (significance is shown in red to yellow color and corresponds to the significance scale bar) show regions where significantly greater CP55,940-stimulated [³⁵S]GTP γ S binding was found in β arr2-KO compared to WT mice following vehicle treatment ($n = 8$ per group). *Cg*, cingulate cortex; *CPu*, caudate-putamen; *GP*, globus pallidus; *Pir*, piriform cortex; *HIPP*, hippocampus; *SN*, substantia nigra; *PAG*, periaqueductal gray; *CBLM*, cerebellum.

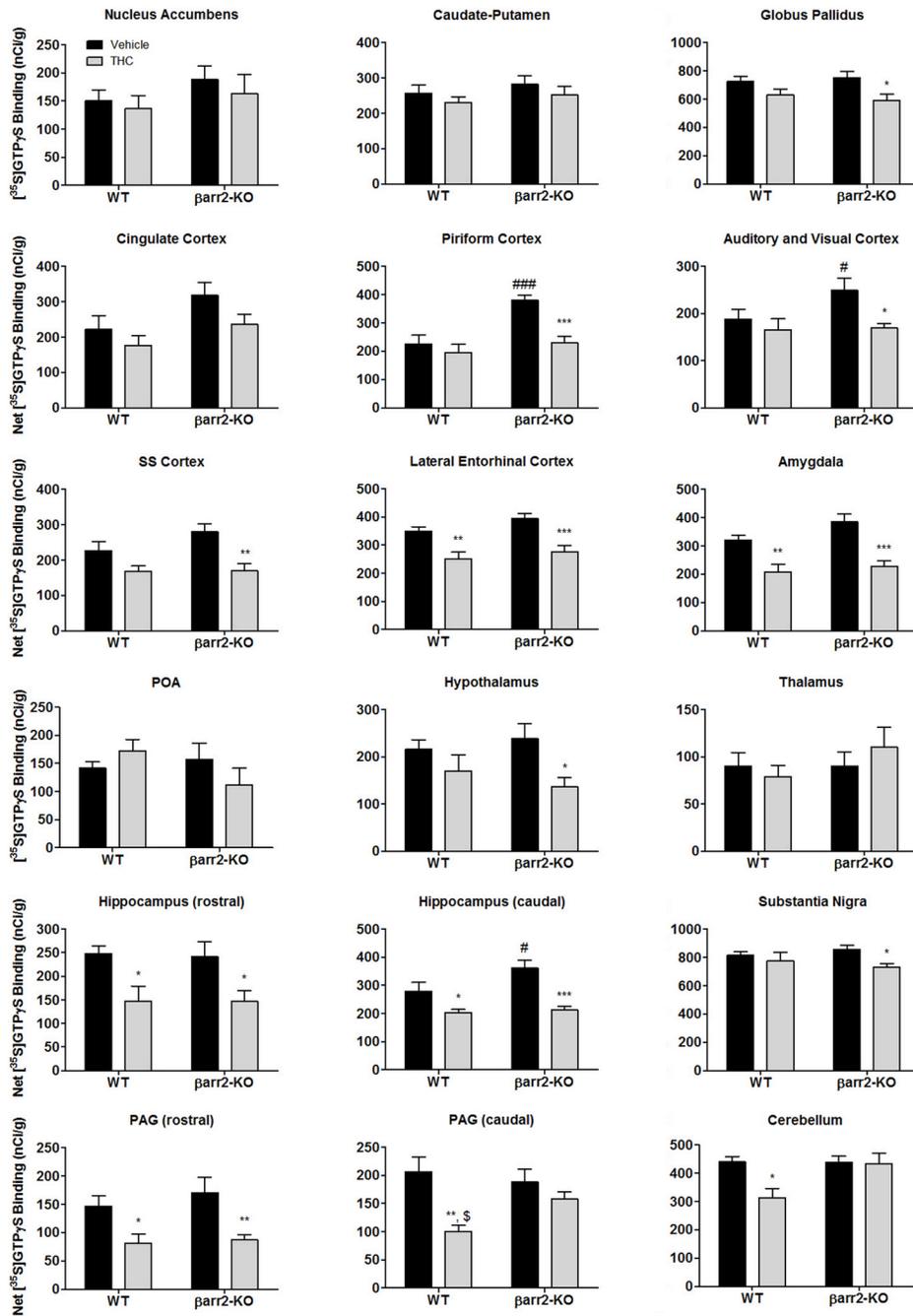


Figure 5. Net CP55,940-stimulated [³⁵S]GTPγS binding (mean ± SEM) in sampled brain regions (*n* = 8 per group) of WT and βarr2-KO mice following 6.5 day subchronic administration of either vehicle or 10 pmg/kg THC twice daily. ****p* < 0.001, ***p* < 0.01, **p* < 0.05 versus respective vehicle; ###*p* < 0.001, #*p* < 0.05 versus WT vehicle; § versus KO THC (Two way ANOVA, Student-Newman Keuls Post-hoc). *PAG*, periaqueductal gray; *POA*, preoptic area; *SS*, somatosensory cortex.

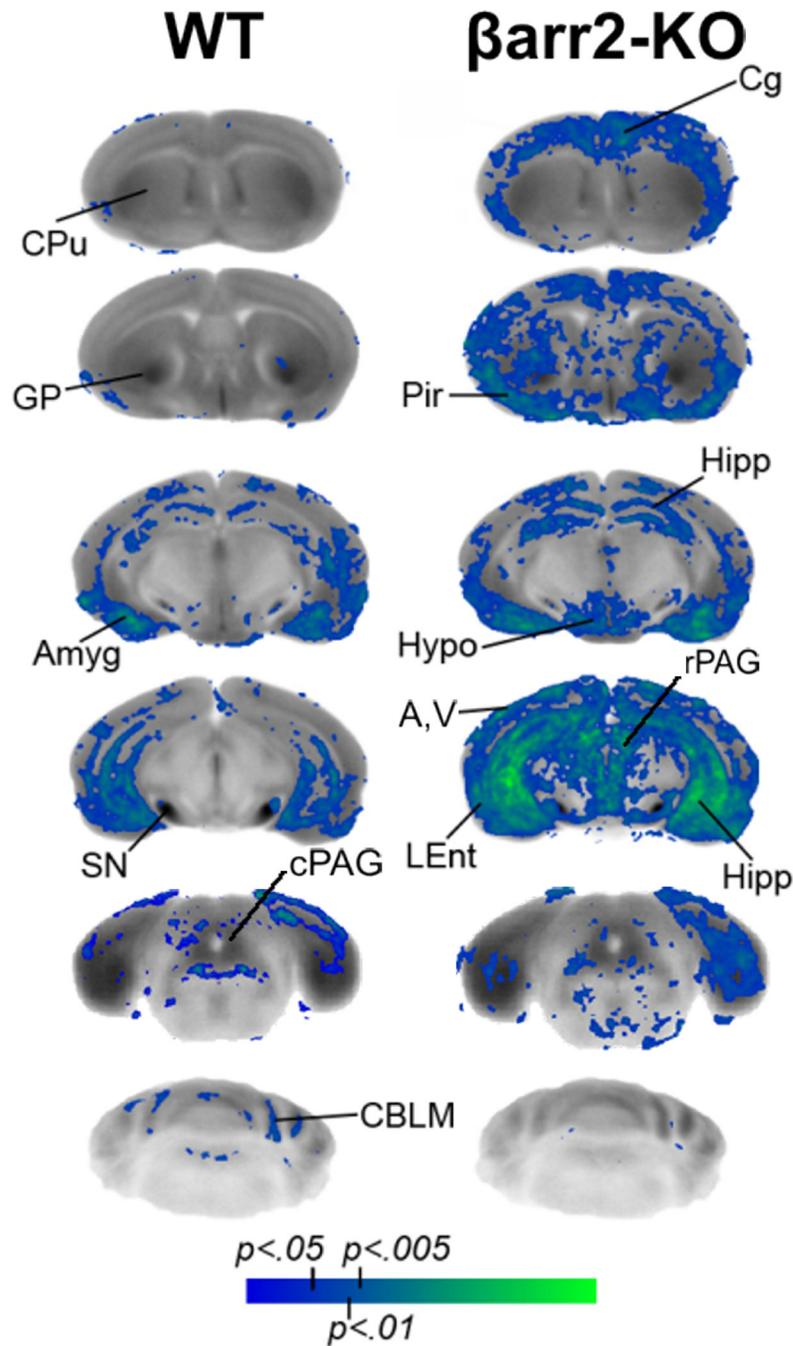


Figure 6.

SPM analysis revealed both region- and genotype-specific differences in desensitization of CB₁R-mediated G-protein activation in the reconstructed mouse brain of WT and β arr2-KO mice following 6.5 day subchronic administration of either vehicle or 10 mg/kg THC ($n = 8$ per group) twice daily. Brain regions within each genotype demonstrating significant desensitization ($p < 0.05$, 2-way ANOVA, $n = 8$) are colored in blue/green and correspond to the significance scale (bottom). *AMYG*, amygdala; *A,V*, auditory & visual cortex; *CBLM*, cerebellum; *Cg*, cingulate cortex; *CPu*, caudate-putamen; *GP*, globus pallidus; *HIPP*, hippocampus; *Hypo*, hypothalamus; *LEnt*, lateral entorhinal cortex; *PAG*, periaqueductal

gray; *Pir*, piriform cortex; *cPAG*, caudal periaqueductal gray; *rPAG*, rostral periaqueductal gray; *SN*, substantia nigra; *SS*, somatosensory cortex.

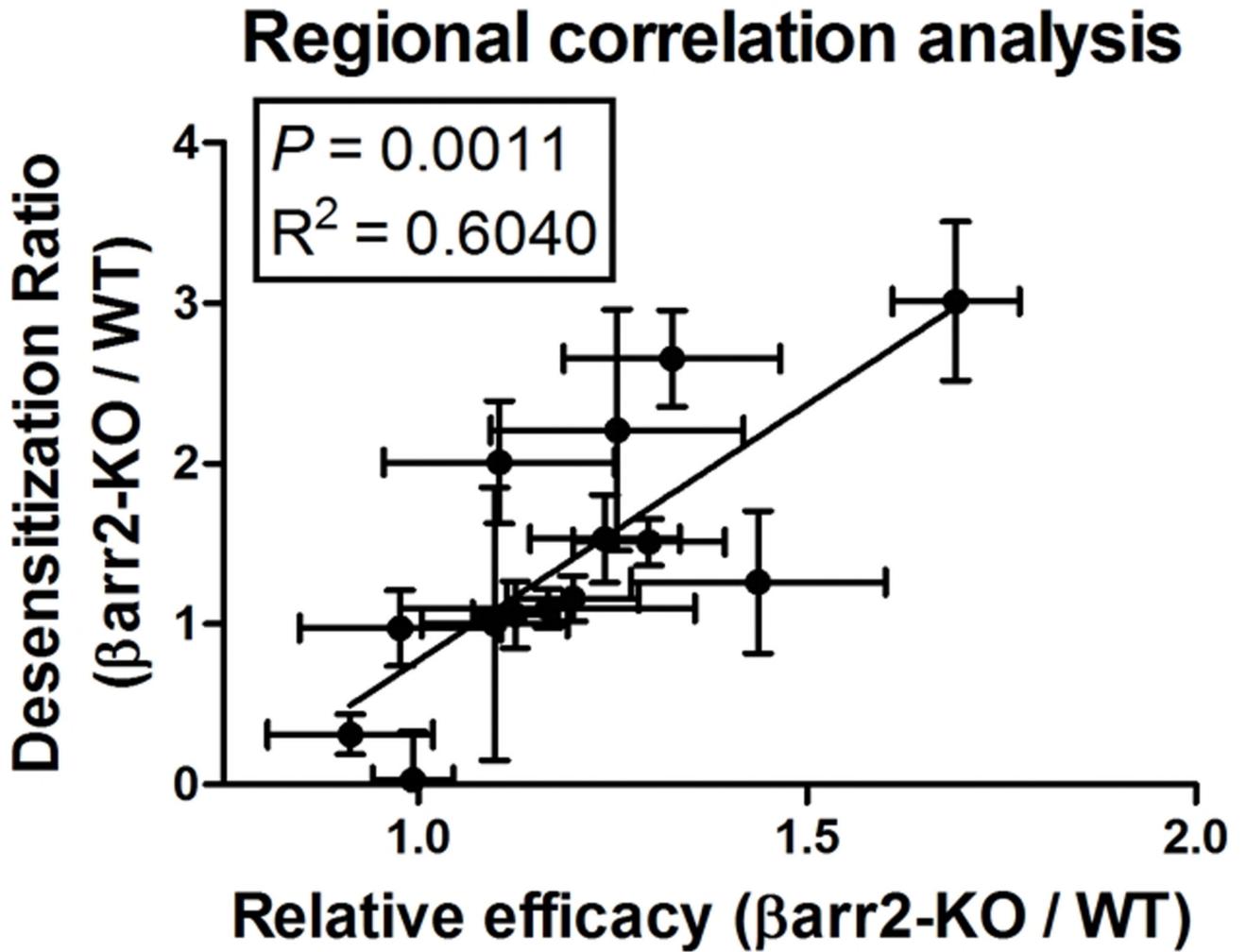


Figure 7. Relative efficacy of CP55,940-stimulated [³⁵S]GTPγS binding is positively correlated with the relative magnitude of CB₁R desensitization in βarr2-KO mice following subchronic THC administration. Each point in the graph represents a brain area (see Table 5) corresponding to its mean Desensitization Ratio and mean Relative efficacy. The Desensitization Ratio was calculated by normalizing the magnitudes of desensitization in βarr2-KO mice to the mean magnitude of desensitization in WT mice. Relative efficacy was calculated by normalizing the magnitudes of CP55,940-stimulated [³⁵S]GTPγS binding in vehicle treated βarr2-KO mice by the mean magnitude of CP55,940-stimulated [³⁵S]GTPγS binding in vehicle treated WT mice. Calculated ratios in the graph represent the mean ± SEM (*n* = 6–8). Linear regression analysis was performed in Graphpad Prism 5.

Table 1

ED₅₀ values (mg/kg) (\pm 95% confidence intervals) for THC cumulative dosing curves obtained in WT and β arr2-KO mice for tail-flick, catalepsy and hypothermia assessments

Behavioral Assessment	Genotype	Vehicle ED ₅₀ (95% CI)	THC ED ₅₀ (95% CI)	Fold Shift in ED ₅₀
Antinociception	WT	13.85 (9.30–20.61)	126.9 (81.50–197.4)	8.45**
	β arr2-KO	6.37 (4.65–8.73)	10.71 (2.60–44.10)	1.68
Catalepsy	WT	19.79 (11.56–33.88)	41.94 (29.22–60.18)	2.12**
	β arr2-KO	12.03 (6.04–23.96)	57.32 (39.82–82.53)	4.76*
Hypothermia	WT	50.24 (17.22–146.50)	NC	--
	β arr2-KO	47.97 (14.06–163.60)	NC	--

(WT: vehicle vs. THC and KO: vehicle vs. THC, * $p < 0.05$, ** $p < 0.01$; sum of squares f test; $n = 8$ /group) (NC, not converged)

Table 2

Agonist-stimulated [³⁵S]GTPγS (top rows) and [³H]SR141716A (bottom rows) binding in spinal cords from WT and βarr2-KO mice following subchronic treatment with vehicle or THC. E_{max} values (expressed as % stimulation [(agonist-stimulated - basal)/basal × 100%]) and EC₅₀ (nM) values were calculated with Prism using a non-linear fit. B_{max} (pmol/mg) and K_D (nM) values were calculated using a one-site hyperbola function.

Group	EC ₅₀ (nM)	E _{max} (%Stim.)	% Vehicle
CP55,940			
WT Vehicle	38 ± 17	45.04 ± 1.91	100 ± 4.25
WT THC	17 ± 11	27.88 ± 3.64 ^{***, ###}	61.89 ± 8.08
βarr2-KO Vehicle	24 ± 7	46.41 ± 2.13	100 ± 4.60
βarr2-KO THC	11 ± 3	41.10 ± 1.80	88.56 ± 3.88
WIN55,212-2			
WT Vehicle	85 ± 21	75.46 ± 6.82	100 ± 9.04
WT THC	198 ± 37	49.39 ± 4.41 [*]	65.45 ± 5.84
βarr2-KO Vehicle	338 ± 187	80.87 ± 11.55	100 ± 14.28
βarr2-KO THC	220 ± 69	72.53 ± 5.99	89.67 ± 7.41
Group	K _D (nM)	B _{max} (pmol/mg)	% Vehicle
[³H]SR141716A			
WT Vehicle	1.03 ± 0.24	0.47 ± 0.04	100 ± 9.07
WT THC	0.91 ± 0.11	0.28 ± 0.02 ^{**} , #	59.40 ± 4.12
βarr2-KO Vehicle	1.43 ± 0.27	0.54 ± 0.04	100 ± 6.67
βarr2-KO THC	1.51 ± 0.34	0.44 ± 0.07	82.30 ± 12.21

Values represent the mean ± SEM (n = 6–7 per group).

* p < 0.05,

** p < 0.01,

*** p < 0.001, versus respective vehicle control group;

p < 0.001 versus βarr2-KO THC (2-way ANOVA, Student-Newman Keuls Post-hoc).

Table 3

Net CP55,940-stimulated [³⁵S]GTPγS binding in brain sections from WT and βarr2-KO mice following 6.5 day administration of either vehicle or THC (*n* = 8 per group). [³⁵S]GTPγS binding is expressed as net agonist-stimulated [³⁵S]GTPγS binding in nCi/g.

Region	Vehicle		THC	
	WT	βarr2-KO	WT	βarr2-KO
Nucleus Accumbens	150 ± 20	189 ± 24	136 ± 24	163 ± 33
Caudate-Putamen	256 ± 23	282 ± 24	230 ± 17	252 ± 25
Globus Pallidus	726 ± 36	751 ± 47	631 ± 41	593 ± 43*
Cingulate Cortex	221 ± 39	318 ± 36	176 ± 29	235 ± 29
Piriform Cortex	225 ± 32	380 ± 18###	195 ± 29	229 ± 25***
Auditory & Visual Cortices	188 ± 21	249 ± 26#	165 ± 23	169 ± 9*
Somatosensory Cortex	226 ± 25	281 ± 22	168 ± 15	169 ± 20**
Lateral Entorhinal Cortex	350 ± 16	393 ± 19	252 ± 23**	277 ± 23***
Amygdala	321 ± 17	385 ± 27	209 ± 26**	228 ± 19***
Hippocampus (rostral)	248 ± 17	242 ± 32	147 ± 32*	146 ± 23*
Hippocampus (caudal)	279 ± 31	362 ± 27#	203 ± 12*	212 ± 14***
Preoptic area	141 ± 12	157 ± 29	172 ± 20	111 ± 30
Hypothalamus	216 ± 21	238 ± 32	169 ± 35	136 ± 20*
Thalamus	90 ± 15	90 ± 15	79 ± 12	110 ± 21
Substantia Nigra	814 ± 26	856 ± 30	776 ± 58	733 ± 24*
PAG (rostral)	146 ± 19	171 ± 28	81 ± 16*	88 ± 9**
PAG (caudal)	207 ± 26	189 ± 22	100 ± 12**,\$	158 ± 12
Cerebellum	440 ± 19	437 ± 23	314 ± 32*	434 ± 38

Values represent mean ± SEM.

* *p* < 0.05,

** *p* < 0.01,

*** *p* < 0.001 versus respective vehicle;

p < 0.05,

p < 0.001 versus WT vehicle;

\$ *p* < 0.05 versus KO THC (*n* = 8 per group, two way ANOVA, Student-Newman Keuls Post-hoc).

Table 4

[³H]CP55,940 binding in brain sections from WT and β arr2-KO mice treated for 6.5 days with either vehicle or THC ($n = 8$ per group). [³H]CP55,940 binding is expressed as nCi/mg.

Region	Vehicle		THC	
	WT	β arr2-KO	WT	β arr2-KO
Caudate-Putamen	2.39 ± 0.36	2.10 ± 0.43	1.76 ± 0.46	2.05 ± 0.38
Globus Pallidus	5.51 ± 0.86	5.29 ± 0.89	4.90 ± 1.11	4.88 ± 0.72
Cingulate Cortex	1.63 ± 0.29	1.41 ± 0.22	1.22 ± 0.24	1.14 ± 0.13
Somatosensory Cortex	1.57 ± 0.27	1.57 ± 0.25	1.23 ± 0.22	1.23 ± 0.16
Amygdala	1.77 ± 0.40	1.72 ± 0.24	1.20 ± 0.30	1.64 ± 0.22
Hippocampus	1.66 ± 0.29	1.48 ± 0.21	1.24 ± 0.28	1.43 ± 0.19
Hypothalamus	1.75 ± 0.42	1.67 ± 0.23	1.25 ± 0.35	1.27 ± 0.29
Thalamus	1.12 ± 0.16	1.17 ± 0.18	1.17 ± 0.26	1.24 ± 0.12
Substantia Nigra	5.66 ± 0.80	5.52 ± 0.82	5.28 ± 1.09	5.15 ± 1.07
PAG	1.76 ± 0.37	1.47 ± 0.21	1.21 ± 0.27	1.51 ± 0.30
Cerebellum	3.18 ± 0.46	2.63 ± 0.40	1.74 ± 0.32*	2.80 ± 0.36

[³H]CP55,940 binding values represent the mean ± SEM. $p < 0.05$ versus respective vehicle (Two way ANOVA, Student-Newman Keuls Post-hoc).

Table 5

Relative Efficacy and Desensitization Ratio values for each brain region from WT and β arr2-KO mice treated for 6.5 days with either vehicle or THC ($n = 6-8$ per group). Calculation of values is explained in the legend of Figure 7.

Relative Efficacy and Desensitization Ratio values represent the mean \pm SEM ($n = 6-8$).

Brain Region	Relative Efficacy	Desensitization Ratio
Nucleus Accumbens	1.26 \pm 0.16	2.21 \pm 0.75
Caudate-Putamen	1.10 \pm 0.09	1.00 \pm 0.85
Cingulate Cortex	1.44 \pm 0.16	1.26 \pm 0.44
Piriform Cortex	1.69 \pm 0.08	3.01 \pm 0.49
Auditory & Visual Cortices	1.33 \pm 0.14	2.65 \pm 0.30
Somatosensory Cortex	1.24 \pm 0.10	1.53 \pm 0.27
Lateral Entorhinal Cortex	1.12 \pm 0.05	1.06 \pm 0.21
Amygdala	1.20 \pm 0.08	1.16 \pm 0.14
Hippocampus (rostral)	0.98 \pm 0.13	0.97 \pm 0.24
Hippocampus (caudal)	1.30 \pm 0.10	1.51 \pm 0.14
Hypothalamus	1.10 \pm 0.15	2.01 \pm 0.38
PAG (rostral)	1.17 \pm 0.19	1.10 \pm 0.12
PAG (caudal)	0.91 \pm 0.11	0.31 \pm 0.12
Cerebellum	0.99 \pm 0.05	0.03 \pm 0.30

PAG, periaqueductal gray