The Expression Level of CB₁ and CB₂ Receptors Determines Their Efficacy at Inducing Apoptosis in Astrocytomas

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Abstract

Background: Cannabinoids represent unique compounds for treating tumors, including astrocytomas. Whether CB₁ and CB₂ receptors mediate this therapeutic effect is unclear.

Principal Findings: We generated astrocytoma subclones that express set levels of CB₁ and CB₂, and found that cannabinoids induce apoptosis only in cells expressing low levels of receptors that couple to ERK1/2. In contrast, cannabinoids do not induce apoptosis in cells expressing high levels of receptors because these now also couple to the prosurvival signal AKT. Remarkably, cannabinoids applied at high concentration induce apoptosis in all subclones independently of CB₁, CB₂ and AKT, but still through a mechanism involving ERK1/2.

Significance: The high expression level of CB₁ and CB₂ receptors commonly found in malignant astrocytomas precludes the use of cannabinoids as therapeutics, unless AKT is concomitantly inhibited, or cannabinoids are applied at concentrations that bypass CB₁ and CB₂ receptors, yet still activate ERK1/2.

Introduction

Cannabinoids produce the majority of their biological effects by activating two receptors: CB₁ and CB₂. In healthy brain, CB₁ is expressed by neurons, astrocytes and neural progenitor cells, whereas CB₂ is only expressed by a small population of neurons located in the brain stem [1,2]. Activation of CB₁ and CB₂ receptors regulates fundamental physiological processes, including neurotransmission efficacy, astrocytic function, and the fate and proliferation rate of neural progenitor cells [3,4]. Thus, because these receptors regulate such fundamental physiological processes, extensive effort has been invested toward understanding how cannabinoid compounds activate CB₁ and CB₂ receptors, as well as regulate the signal transduction mechanisms they couple to, with the goal of developing novel therapeutics to treat various neuropathologies [5,6].

One of the most promising therapeutic uses of cannabinoids is linked to their ability to induce apoptosis in tumors, including in astrocytoma subclones grown either in vitro, subcutaneously in immunodeficient mice, or intracranially in rats [7,8]. In fact, these data were so compelling that one clinical trial has already been carried-out, in which patients afflicted with recurrent astrocytomas (grade IV) were treated with cannabinoids stereotactically injected directly into the mass of the malignant tumors [9]. While there are clear results for the use of cannabinoids to treat astrocytomas, the requirement of cannabinoid receptors in mediating this therapeutic effect is still unproven [10]. In fact, evidence suggests that high concentrations of cannabinoids may induce biological effects, including the induction of apoptosis in cells, independently of CB₁ and CB₂ receptors [7,11,12,13]. Thus whether CB₁ and CB₂ receptors, or yet another cannabinoid-like receptor, mediate the therapeutic effects of cannabinoids toward astrocytomas is still unclear.

Other unanswered questions are: Why does CB₁ and CB₂ expression in astrocytomas gradually increase as a function of cancerous transformation? [14,15] Does this increase in receptor expression modify the coupling of CB₁ and CB₂ to signal transduction pathways in astrocytomas? Does this increase in receptor expression modify the efficacy of cannabinoids at inducing apoptosis in astrocytomas? Indeed, while it is known that CB₁ and CB₂ couple to ERK1/2, AKT, JNK, p38 and GSK-3β [8,16,17,18,19], and that both ERK1/2 and AKT mediate the induction of apoptosis by cannabinoids in astrocytomas [8,11,18,20], nothing is known about how changes in CB₁ and CB₂ expression will modify the coupling of these receptors to each kinase, and whether the efficacy with which cannabinoids induce apoptosis is also affected. Answering these questions is important for the following reasons. Most of the studies reporting...
cannabinoid receptors coupling to specific kinases were performed in heterologous expression systems and the expression level of these receptors both surpass those reached by endogenously-expressed receptors and favor promiscuous coupling to effector proteins. One particularly controversial point stems from the ability of cannabinoids to induce apoptosis in astrocytomas when CB1 and CB2 receptors also couple to the pro-survival kinase AKT. Therefore, it is important to increase our understanding of how changes in the expression level of CB1 and CB2 will affect the coupling of these receptors to specific kinases, as well as the efficacy of cannabinoids at killing astrocytomas.

Here we sought to address the following two questions: Are the therapeutic effects of cannabinoids toward astrocytomas truly mediated by CB1 and CB2 receptors? Does the increase in cannabinoid receptor expression known to occur in malignant astrocytomas determine the efficacy of cannabinoids at killing these tumors? To answer these questions, we generated astrocytoma subclones that express low, medium and high levels of either CB1 or CB2, and studied the ability of the cannabinoid agonist CP-55,940 to regulate the activity of various kinases and to induce apoptosis in these tumor cells.

Results

Astrocytoma Subclones Stably Expressing Set Levels of Either CB1 or CB2 Receptors

To determine whether the expression level of CB1 or CB2 receptors dictate cannabinoid-mediated responses in astrocytomas, we sought to use heterologous gene expression technology to drive the expression of these receptors to precise levels in an astrocytoma cell line that normally lacks these receptors. Specifically, we found that the murine delayed brain tumor (DBT) cell line expresses neither CB1 nor CB2 receptors (Table 1). Thus we transfected these cells with IRES constructs containing the gene that encodes either CB1 or CB2 receptors in tandem with the gene encoding eGFP, and used FACS to select for the cells that had stably incorporated the construct (see Experimental Procedures). Using this approach, we obtained 27 CB1-expressing DBT subclones and 49 CB2-expressing DBT subclones. We then randomly selected 24 subclones (twelve CB1 and twelve CB2) and performed qPCR to find out the relative expression level of CB1 and CB2 receptor mRNAs in each single cell subclone (Table S1). Based on these data, we then focused our experiments on three CB1 subclones and three CB2 subclones that expressed relatively low, medium and high levels of receptor mRNA (Table 1). Please note that all CB1-expressing DBT cells were still devoid of CB2 receptor mRNA, and vice versa. Quantification of CB1 and CB2 receptor proteins by radioligand binding showed a correlation between the relative level of mRNA encoding each receptor and the corresponding protein expression level as expressed by the Bmax value (Table 1). Here it is worth noting that the expression level of CB1 and CB2 receptors in the CB1-mid and the CB2-mid subclones, respectively, lie well within the range of their levels when endogenously expressed by various cell lines and tissues [21,22]. Thus, we generated DBT subclones that stably express set levels of either CB1 or CB2 receptors, with expression levels that lie within the range of what is found for cells endogenously expressing these receptors.

Independent of Expression Levels, CB1 and CB2 Receptors Similarly Regulate ERK1/2 Signaling

Because previous studies performed on astrocytoma cell lines have implicated ERK1/2 signaling in the therapeutic actions of cannabinoids [23], we sought to determine whether different expression levels of CB receptors would affect their coupling to this signal transduction pathway. To do so, we treated each subclone with CP-55,940 (1 μM, maximally efficacious concentration [17,24]) and measured ERK1/2 phosphorylation by Western blot analysis. We found that CP-55,940 induced a significant increase in ERK1/2 phosphorylation above vehicle-treated controls in all six subclones (Figure 1a). All these responses were receptor-mediated since they were significantly attenuated by either the CB1 antagonist SR141716A, or the CB2 antagonist SR144528, respectively (Figure 1a). Furthermore, CP-55,940 did not affect ERK1/2 phosphorylation in wild-type DBT cells (Figure S1a). The magnitude and kinetics of CP-55,940-induced ERK1/2 phosphorylation mediated in each subclone were remarkably similar despite a 10-fold difference in receptor expression level between the CB1-low and the CB1-high subclones, and between the CB2-low and the CB2-high subclones (Figure 1b,c). The only differences that we noted between CB1- and CB2-mediated regulation of ERK1/2 phosphorylation were that CP-55,940 induced a slightly slower and sustained increase in ERK1/2 phosphorylation in CB1-expressing subclones (peak at 5 min returning to basal by 20–30 min) compared to CB2-expressing subclones (peak at 5 min returning to basal by 10–20 min) (Figure 1b,c). These results show that CB1 and CB2 receptors heterologously expressed in DBT cells are functional for they regulate ERK1/2 phosphorylation. They also show that the efficacy of this regulation is largely independent of receptor expression levels and receptor subtype.

The Expression Level of CB1 and CB2 Receptors Dictates Their Ability to Regulate AKT, JNK and p38 Signaling

CB1 and CB2 receptors have been shown to regulate a plethora of kinases, including AKT, JNK, p38 and GSK-3β [18,16,17,18,19]. Thus we sought to assess if CB1 and CB2 heterologously expressed in DBT cells also regulate these four kinases. To do so, we treated all DBT subclones with CP-55,940

Table 1. Expression levels of CB1 and CB2 receptors in wild-type and selected DBT subclones.

| Nomenclature subclones | qPCR | Radioligand binding |
|------------------------|------|---------------------|
|                        | CB1  | CB2     | Bmax (cycle threshold) | k_d (pmol/mg) | (nM) |
| Wild type              | no ct| no ct   | no binding             | -             |
| CB1-low                | E3   | 22.4    | 0.2                   | 0.8           |
| CB1-mid                | E7   | 17.7    | 0.7                   | 0.8           |
| CB1-high               | E5   | 14.7    | 1.3                   | 0.7           |
| CB2-low                | 2G5  | no ct   | 24.1                  | 0.3           |
| CB2-mid                | 2H1  | no ct   | 18.0                  | 0.6           |
| CB2-high               | 1D6  | no ct   | 14.0                  | 2.3           |

Wild-type DBT cells and DBT subclones stably expressing cannabinoid receptors were expanded in 10 cm dishes, and either their RNA extracted for qPCR analysis of CB1 and CB2, mRNA levels, or homogenized for radioligand binding analysis of CB1 and CB2 receptor protein levels. mRNA levels are expressed as cycle threshold (ct) value from qPCR performed with Taqman probes. Values represent mean of three independent determinations. Receptor protein level is expressed as Bmax values from radioligand binding experiments performed with [3H]CP-55,940. K_d values were also determined and show consistent low nanomolar affinities. Values represent mean of 3–5 independent determinations. 

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and assessed the phosphorylation state of these kinases using a Luminex multiplexed immunoassay system.

With regard to AKT, three points are noteworthy. First, the CP-55,940-induced increase in AKT phosphorylation correlated with receptor expression levels (Figure 2a). Specifically, CP-55,940 did not significantly increase AKT phosphorylation in the CB2-low subclone, which expresses the lowest level of receptor of all six subclones that we have selected; it induced a small, but significant increase in AKT phosphorylation in CB2-low and CB2-mid, which express slightly more receptors than the CB2-low subclone; and it induced the most pronounced increase in AKT phosphorylation in CB2-mid, CB2-high and CB2-high subclones (see Table 1 for Bmax values). Second, all responses were mediated by cannabinoid receptors since they were antagonized by either SR141716A or SR144528, respectively, and CP-55,940 did not increase AKT in wild type DBT cells (Figures 2a and S1b). Third, there was a clear difference in the kinetics of AKT activation linked to either CB1 or CB2 receptors. Specifically, while the response obtained in the CB2-high subclone peaked at 3 min and returned to the basal level at 5 min, the response measured in the CB2-high clone remained above 250% of basal during the entire 30 min of the incubation period (Figure 2b,c).

With regard to JNK and p38, CP-55,940 induced a small (150–170% of basal) but significant increase in the phosphorylation state of these kinases only in the CB2-high clone that also lasted throughout the 30 min incubation period (Figure S2a,b). With regard to GSK-3β, CP-55,940 did not significantly affect the phosphorylation state of this kinase in any of the subclones throughout the 30 min incubation time period (data not shown).

Taken together, these results suggest that the different expression levels of CB1 and CB2 receptors determine the efficacy with which cannabinoids regulate AKT, JNK and p38 signaling. They also suggest that CB1 and CB2 receptors differentially couple to these kinases.

Only Activation of CB1 Receptors Expressed at Low Levels in DBT Cells Leads to Apoptosis: Involvement of ERK1/2

Several studies suggest that activation of CB1 or CB2 is sufficient to induce apoptosis in astrocytomas [8,14], whereas other studies suggest that only high concentrations of cannabinoids kill astrocytomas and furthermore, independently of cannabinoid receptor activation [7,11,12,13]. We directly tested these possibilities by treating the DBT subclones, as well as wild-type DBT cells, with CP-55,940 at either 1 μM (receptor-mediated) or 10 μM (receptor-independent). To assess for cell viability, we used WST-1, an indicator of mitochondrial activity. We found that 1 μM CP-55,940 induced ~50% cell death in the CB1-low DBT subclone, whereas 10 μM CP-55,940 induced 35–90% cell death in wild type DBT cells and all six subclones (Figure 3a). This result suggests that only the activation of CB1 receptors expressed at low levels in DBT cells leads to significant receptor-dependent killing of these cells. Two additional sets of results support this conclusion. First, SR141171A blocked the 1 μM CP-55,940-induced killing of the CB1-low DBT cells, whereas this antagonist did not affect the 10 μM CP-55,940-induced killing of wild type DBT cells (Figure 3b). Second, CP-55,940 killed CB1-low DBT cells with an EC50 of 0.56 μM, whereas this compound induced the cell death of wild type DBT cells with an EC50 of 5 μM (Figure 3c).

Interestingly, when comparing the effect of 1 μM CP-55,940 on CB1-low DBT cells to the effect of 10 μM CP-55,940 on wild-type DBT, we found that the 10 μM CP-55,940-induced killing of wild-type DBT (receptor-independent) is more rapid than the CB1-mediated cell death (Figure 3d).
We then determined whether the CB₁-dependent and the cannabinoid receptor-independent killing of DBT cells were apoptotic in nature. Specifically, we treated CB₁-low DBT cells with 1 μM CP-55,940 and wild type DBT cells with 10 μM CP-55,940, and assessed for propidium iodide and annexin V staining (to assess for cell permeability and apoptosis, respectively). Figure 4 shows that after five days, cell death by apoptosis was induced in 78% of the wild-type DBT cells treated with 10 μM CP-55,940 compared to 48% of the CB₁-low DBT cells treated with 1 μM CP-55,940. Furthermore, in agreement with the data we have obtained with WST-1, 10 μM CP-55,940 killed more wild-type DBT cells by apoptosis (27%) 3 days after treatment compared to CB₁-low DBT cells treated with 1 μM CP-55,940 (15%) (Figure 4). Together, these data show that both the CB₁-dependent and the cannabinoid receptor-independent killing of DBT cells are due to apoptosis, but that these processes occur with slightly different time courses.

Because we found that 1 μM CP-55,940 increases ERK1/2 signaling in CB₁-low DBT cells without affecting the AKT, JNK, p38 and GSKβ signaling pathways, and one study suggested that ERK1/2 mediates the apoptotic properties of cannabinoids on astrocytomas [8], we then tested if inhibiting ERK signaling would affect the CB₁-mediated and the cannabinoid receptor-independent killing of DBT cells. Thus, we pretreated CB₁-low and wild-type DBT cells with the ERK kinase kinase inhibitor PD98059 before applying either 1 or 10 μM CP-55,940. We found that both responses were partially prevented by this inhibitor (Figure 3b). Thus both the CB₁-dependent and the cannabinoid receptor-independent induced apoptosis of DBT cells rely on ERK signaling.

Together, these results show that cannabinoid concentrations known to activate CB₁ and CB₂ receptors (here 1 μM) induce apoptosis in astrocytomas only when these cells express low levels of CB₁ receptors, and that this response is mediated through ERK signaling. Conversely, high concentrations of cannabinoids known to bypass cannabinoid receptors (here 10 μM) induce apoptosis in all DBT subclones, a mechanism that also depends on ERK signaling.

**CB₁- and CB₂-Induced Apoptosis of DBT Cells Is Uncovered When AKT Is Inhibited**

Because increased AKT signaling promotes cell survival [25], we reasoned that activation of this pathway by CB₁ and CB₂ receptors expressed at mid and high levels might explain why 1 μM CP-55,940 was unable to induce apoptosis in DBT subclones. To test this hypothesis, we treated the six subclones of DBT cells with 1 μM CP-55,940 in combination with Inhibitor X, a cell-permeable inhibitor of AKT [26]. Using the WST-1 assay, we found that while this inhibitor had no effect by itself on cell viability, it uncovered the ability of 1 μM CP-55,940 to kill all the DBT subclones that were normally resistant, with the most pronounced response occurring in CB₂-low DBT cells (Figure 5a). This latter response was due to apoptosis, since 76% of the cells stained positively for annexin V when measured five days after co-application of 1 μM CP-55,940 and Inhibitor X (Figure 5b). These results suggest that the coupling of CB₁ and CB₂ receptors to the AKT pathway (when these receptors are expressed at mid and high levels) eliminates the ability of cannabinoids to induce apoptosis in DBT cells.

**Discussion**

While many laboratories have reported that cannabinoids induce apoptosis in various tumor subtypes, including astrocytomas...
mas, the requirement for CB1 and CB2 in mediating this therapeutic effect has not yet been demonstrated. This point is especially relevant when considering that many of these studies used high concentrations of cannabinoids known to bypass CB1 and CB2 receptor activation. Here we demonstrate that cannabinoid receptors and ERK1/2 indeed mediate this therapeutic effect, but only when these compounds are applied at submicromolar concentrations and the expression level of these receptors remains low. Conversely, high concentrations of cannabinoids kill all astrocytoma subclones independently of CB1, CB2 and AKT, yet still through a mechanisms involving ERK1/2. We also show that increased expression of CB1 and CB2 receptor allows for their coupling to additional kinases, especially AKT, the result of which eliminates the ability of cannabinoids to induce apoptosis even though these receptors still couple to ERK1/2.

One of the most unexpected and interesting findings of our study is that the coupling of CB1 and CB2 receptors to AKT, JNK and p38 is dictated by receptor expression levels, whereas their coupling to ERK1/2 is not. What may be the molecular mechanism underlying a gradual coupling to AKT, JNK and p38, while preserving a steady coupling to ERK1/2? Reports on the molecular mechanisms linking cannabinoid receptors to various kinases suggest that these receptors activate AKT through G-proteins, while activating ERK1/2 through EGF receptor transactivation [27]. Based on these reports, a parsimonious interpretation of our results would be that the rate limiting step controlling the efficacy of cannabinoids at stimulating AKT lies in the cannabinoid receptors themselves, whereas the rate limiting step controlling the efficacy of cannabinoids at stimulating ERK lies in EGF receptors, the expression of which is likely to remain constant in the DBT subclones that we generated. Our results may have broader implications when considering the therapeutic efficacy of cannabinoids toward other neuropathologies. For example, epileptic seizures and cerebral ischemia lead to a 3–4 fold increase in the expression of neuronal CB1 receptors, a response thought to constitute a protective mechanism [28,29],

Figure 3. CP-55,940 kills DBT cells via cannabinoid receptor-dependent and -independent mechanisms. Wild-type (wt) DBT cells and DBT subclones stably expressing cannabinoid receptors were expanded in 24-well plates, incubated with CP-55,940 (CP) at either 1 μM (receptor mediated) or 10 μM (receptor independent), and cell viability was assessed by quantifying WST-1 conversion. (a) Five days after treatment, only CB1-low DBT cells showed significant sensitivity to CP at 1 μM, whereas all subclones were sensitive to CP at 10 μM. Data represent means ±s.e.m. of 5–9 independent experiments in triplicate. (b) Only the pretreatment of CB1-low DBT cells with SR141617A (5 μM) prevented the CP-induced killing, whereas pretreatment with PD 98059 (PD, 10 μM) significantly reduced both the CP 1 μM and CP 10 μM induced toxicity in CB1-low and wild-type, respectively. Data represent means ±s.e.m. of 3–5 independent experiments in triplicate. (c) Dose-response and (d) kinetics of CP-induced killing of wt and CB1-low DBT cells. Data represent means ±s.e.m. of 3–9 independent experiments in triplicate. All results are expressed as % of WST-1 measurements in corresponding cells treated with vehicle (0.1% DMSO) for the indicated time point. In (a), (*) = p<0.05 and (**) = p<0.01 significantly different from the viability in the presence of vehicle only, ANOVA followed by Bonferroni’s post-test. In (b), (**) = p<0.01 significantly different the viability after treatment with either 1 or 10 μM CP, ANOVA followed by Bonferroni’s post-test. In (c), (**) and (#) = p<0.01 significantly different from the viability in the presence of vehicle only; ANOVA followed by Bonferroni’s post-test. In (d), (**) and (#) = p<0.01 significantly different from the viability in the presence of vehicle only at corresponding time point, ANOVA followed by Bonferroni’s post-test. doi:10.1371/journal.pone.0008702.g003
Figure 4. Time course of the apoptotic response induced by CP-55,940 in wild-type and CB₁-low DBT cells. (a,b) Wild-type (wt) DBT cells and (c,d) CB₁-low DBT cells were expanded in 6-well plates, incubated with CP-55,940 (CP) at 10 and 1 μM, respectively, and apoptosis assessed by quantifying propidium iodine (PI) and annexin V fluorescence by FACS after 1, 3, and 5 days. Shown are representative results, with values within each quadrant = mean of 3–5 independent experiments.

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Figure 5. Inhibition of AKT signaling confers sensitivity of cannabinoid receptor-expressing DBT cells to cannabinoid-dependent cytotoxicity. (a) Wild-type (wt) DBT cells and DBT subclones stably expressing cannabinoid receptors were expanded in 24-well plates, incubated with CP-55,940 (CP) at 1 μM (receptor-mediated) without or with Inhibitor X (5 μM added 10 min before CP), and cell viability was assessed after 5 days by quantifying WST-1 conversion. Data represent means ± s.e.m. of 3–9 independent experiments in triplicate. (*) = p < 0.05 and (**) = p < 0.01 significantly different from the viability in the presence of vehicle only. ANOVA followed by Bonferroni’s post-test. (b) wt and CB2-low DBT cells were expanded in 6-well plates, incubated with CP at 1 μM (receptor-mediated) without or with Inhibitor X (5 μM added 10 min before CP), and the apoptotic response assessed after 5 days by propidium iodine (PI) and annexin V staining by FACS after 1, 3, and 5 days. Shown are representative results, with values within each quadrant = mean of 3–5 independent experiments.

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concentrations of cannabinoids will eradicate a significant portion of C6 astrocytomas inoculated into rodent brains without affecting healthy surrounding tissue or inducing overt adverse effects [8]. Since stereotactic injection of chemotherapeutic compounds directly into human brain tumor masses constitutes a routine approach for neurosurgeons, high concentrations of cannabinoids can easily be delivered by this technique [9]. There are two additional advantages to delivering high concentrations of cannabinoids directly into the tumor mass. Malignant transformation of astrocytomas is associated with an increase in cannabinoid receptor expression [14,15], and we show here that this increase in expression precludes the use of low concentrations of cannabinoids known to activate these receptors. Conversely, local injection of high concentrations of cannabinoids will induce apoptosis in all astrocytoma subclones (independently of CB1 and CB2 receptor expression), which constitutes an asset when considering the phenotypic heterogeneity that astrocytomas adopt during malignant transformation [37,38]. Thus, our results suggest that high concentrations of cannabinoids constitute the preferred regimen for neurosurgeons to use when treating malignant astrocytomas with this class of compounds.

One outstanding question that is raised by our study is: what constitutes the molecular target that is engaged by high concentrations of cannabinoids, a target that also leads to ERK activation and induction of apoptosis in astrocytomas? One possibility is another GPCR, several of which have recently been shown to be engaged by high concentrations of cannabinoids, including CP-55,940 [39]. One obvious candidate is GPR55 [40], although this receptor can be ruled out in the context of our studies since we found no evidence for the presence of GPR55 mRNA in DBT cells (as assessed by RT-PCR and qPCR, data not shown). Thus, while the molecular identification of this target remains to be determined, its identification will most certainly raise a lot of interest for research aimed at developing novel therapeutic approaches to treat astrocytomas and other tumors.

In conclusion, our study provides the first evidence for the differential activation of AKT, p38 and JNK by a highly efficacious cannabinoid agonist, CP-55,940, engaging either CB1 or CB2 receptors expressed at different levels. This shift in coupling precludes the use of cannabinoids at submicromolar concentrations as pro-apoptotic therapeutics, unless AKT is coupling precludes the use of cannabinoids at submicromolar concentrations as pro-apoptotic therapeutics, unless AKT is

Materials and Methods

Drugs
Tritium-labeled and unlabeled CP 55,940 ((−)-cis-3-[2-hydroxy-4-[(1,1-dimethylpropyl)phenyl]-trans-4-3-[3-hydroxypropyl]cyclohexanol], SR141716A (5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-[1-piperidyl]-1H-pyrazole-3-carboxamide), and SR141528 (5-(4-chloro-3-methyl phenyl)-1-[4-(methylphenyl)methyl]-N-(1,3,5-trimethoxyboran-2-yl)-pyrazole-3-carboxamide) were provided by the National Institute of Drug Abuse Drug Supply Program (RTI, Research Triangle Park, NC). PD 98059 and Inhibitor X were from CalBiochem (San Diego, CA).

Generation of DBT Subclones Expressing mCB1 and mCB2
mCB1 and mCB2, were PCR-cloned from an existing construct using the following primers, respectively: forward, 5′-gggaatcctgatcttagagggcttcg-3′ and reverse, 5′-gggaatcctcagacacagctctctagagggcttcg-3′; forward, 5′-gggaatcctgagggcttcgagggcttcg-3′ and reverse, 5′-gggaatcctgagggcttcgagggcttcg-3′. Please note that these receptors are not linked to any molecular tag, because such additions often affect the coupling of GPCRs to their signal transduction pathways. Amplicons were digested with EcoR I and BamH I, and ligated into the corresponding multiple cloning site of the pIRE2-EGFP vector (BD Biosciences Clontech, Mountain View, CA). The murine delayed brain tumor (DBT) cell line, which was originally developed using the Schmidt-Ruppin strain of Rous sarcoma virus inoculated into an adult CDF1 mouse brain [41], was expanded at 37°C with 5% CO2 in 10 cm Falcon dishes (BD Biosciences, San Jose, CA) containing 10 ml of culture media: DMEM+GlutaMAXTM-I (Gibco, Carlsbad, CA) supplemented with fetal bovine serum (FBS, 10%), HEPEs (10 mM) and penicillin/streptomycin (100 units/ml and 100 µg/ml, respectively). Once cells reached ~70% confluence, they were transfected with either pIRE2-EGFP-mCB1 or pIRE2-EGFP-mCB2 using SuperFect (QIAGEN, Valencia, CA) according to the manufacturer’s instructions. Three days after transfection, the resulting positive cells (~5% of fluorescent green cells) were isolated using fluorescence assisted cell sorting (FACS, BD FACS Vantage SE) (Figure S4). These enriched cells were put back into culture for an additional 7 days of selection and expanded in media containing G418 (10 µl per 10 cm culture dishes). Cells that survived were isolated again by FACS, and similarly reselected and expanded for three additional rounds to ensure for the stability of each clone. In the final round, we used FACS to isolate single eGFP-positive cells and seeded them into Costar 96-well plates (0.2 ml per well, changing the media every 3–5 days). Three to six weeks later, all single-cell subclones that had expanded were harvested. Please note that both the fluorescence emitted by these single-cell subclones and the receptor expression levels were stable for at least 20 passages, showing that the shuttled plasmid had been stably incorporated in these cells.

qPCR and Radioligand Binding
Cells (10⁶) were expanded in 10 cm dishes using 10 ml culture media. Once they reached ~70% confluence, they were rinsed with PBS and kept for an additional 24 hrs in serum-deprived media, which is composed of DMEM+GlutaMAXTM-I supplemented with fatty acid free bovine serum albumin (0.1%, BSA, Sigma-Aldrich, St. Louis, MO) and insulin-transferin-sodium selenite liquid media (1 x, ITS, Sigma-Aldrich). For qPCR, media was removed and cells washed once with 10 ml PBS, then cells were scraped and total RNA isolated using RNasy extraction kit (QIAGEN) according to the manufacturer’s instructions. The following primer and TaqMan probe sequences were generated using Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA): mCB1 forward, 5′-cacaagcagcgaataacaaca-3′, reverse, 5′-acagctctggtgacatcagct-3′, probe, 5′-gcagcagctcagcagggcgcg-3′; mCB2 forward, 5′-gaacagcagcagcagggcgcg-3′, reverse, 5′-gaacagcagcagcagggcgcg-3′, probe, 5′-cacaagcagcagcagggcgcg-3′. Total RNA from each sample (2.5 µg) was reverse transcribed in the same 10 µl quantitative PCR reaction mix using the Brilliant Single-Step Quantitative RT-PCR Core Reagent Kit (Stratagene, La Jolla, CA). Combined reactions were carried out on a Stratagene Mx3000P Real-Time Detection System using the following protocol: 30 min single-strand synthesis reaction at 45°C; 40-cycle, two-step PCR amplification (15 sec at 95°C followed by 1 min at 56°C). Duplicate reactions with and without reverse transcriptase were run for each sample. For radioligand binding, media was removed and cells washed once with 10 ml PBS. Cells were scraped in 1 ml buffer (50 mM Tris-HCl, 3 mM
Plausible membrane preparations were generated by centrifugation of cell homogenates for 20 min at >10,000 x g. Cell pellets were resuspended in buffer containing BSA (0.1%, fatty acid free) and 0.5 M Hepes containing 0.1% Tween 20. Membranes were then incubated in blocking buffer containing donkey anti-rabbit antibody conjugated to IRDye 800 (1:7500, Rockland Immunochemicals, Inc., Gilbertsville, PA) for 1 hr at 25°C. Membranes were washed 3 times with ice-cold binding buffer containing BSA. Radioactivity on filters was measured using scintillation counter.

Quantifying the Phosphorylation of ERK1/2, AKT, JNK, p38 and GSKβ

Cells were expanded in Costar 24-well plates (10^5 cells per well) in culture media (0.5 ml per well). Once they reached ~70% confluence, they were rinsed with PBS and kept for an additional 24 hrs in serum-deprived media, at which time point drugs or vehicle (DMSO, 0.1%) prepared in 50 μl serum-deprived media were directly added to each well for the corresponding number of minutes. We chose to directly administer the drugs to cell culture media instead of replacing the media to avoid stress-induced increases in basal kinase phosphorylation (Figure S1). To quantify Erk phosphorylation, reactions were stopped by the media and adding NuPAGE® LDS sample buffer (300 μl, Invitrogen, Carlsbad, CA) supplemented with 2-mercaptoethanol (1%). An aliquot of each sample (20 μl) was loaded onto NuPAGE® 4–12% Bis-Tris pre-cast gels (Invitrogen) and electrophoresed according to the manufacturer’s instructions. Gels were electrophoretically transferred to P-nitrocellulose membrane (Millipore, Billerica, MA) and blocked in LiCOR blocking buffer (LiCOR, Lincoln, NE) for 1 hr at 25°C. Membranes were then incubated in blocking buffer containing rabbit anti-phospho-p44/42 antibody (1:1000, Cell Signaling Technology, Inc., Danvers, MA) for 1 hr at 25°C. Membranes were washed 3 times with Tris (50 mM) buffered saline containing 0.1% Tween-20 (TBS-T). Membranes were then incubated in blocking buffer containing donkey anti-rabbit antibody conjugated to IRDye 800 (1:7500, Rockland Immunochemicals, Inc., Gilbertsville, PA) for 1 hr at 25°C. Membranes were washed 3 times with TBS-T. The fluorescence intensity of each band was quantified using the LiCOR Odyssey. To quantify AKT, JNK, p38 and GSKβ phosphorylation, reactions were stopped by addition of NP40 cell lysis buffer (100 μl, Invitrogen). Substrate phosphorylation was quantified using a Biosource Multiplex Bead Immunoassay kit (Invitrogen) according to the manufacturer’s instructions and using a Luminex 100™ instrument (Luminex, Austin, TX). Note that the basal phosphorylation state of these kinases in each subclone did not change significantly when the cells were incubated over the 30 min time-period of the incubation with vehicle only (Figure S1).

Data Analysis

Statistics, B_{max}, k_d, and EC_{50} values were calculated using Prism.

Supporting Information

Figure S1  Kinase activity in wild-type.
Found at: doi:10.1371/journal.pone.0008702.s001 (0.45 MB TIF)

Figure S2  p38 and jnk activity.
Found at: doi:10.1371/journal.pone.0008702.s002 (0.49 MB TIF)

Figure S3  Sequence alignment between mouse CB1 and mouse CB2.
Found at: doi:10.1371/journal.pone.0008702.s003 (0.77 MB TIF)

Figure S4  Scheme outlining the development of stable clones.
Found at: doi:10.1371/journal.pone.0008702.s004 (0.48 MB DOC)

Table S1  qPCR identification of cannabinoid receptor stable subclones.
Found at: doi:10.1371/journal.pone.0008702.s005 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: EC TM NS. Performed the experiments: EC WM. Analyzed the data: EC. Contributed reagents/materials/analysis tools: TM NS. Wrote the paper: EC NS.

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