Cannabinoids suppress inflammatory and neuropathic pain by targeting α3 glycine receptors

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Certain types of nonpsychoactive cannabinoids can potentiate glycine receptors (GlyRs), an important target for nociceptive regulation at the spinal level. However, little is known about the potential and mechanism of glycineergic cannabinoids for chronic pain treatment. We report that systemic and intrathecal administration of cannabidiol (CBD), a major nonpsychoactive component of marijuana, and its modified derivatives significantly suppress chronic inflammatory and neuropathic pain without causing apparent analgesic tolerance in rodents. The cannabinoids significantly potentiate glycine currents in dorsal horn neurons in rat spinal cord slices. The analgesic potency of 11 structurally similar cannabinoids is positively correlated with cannabinoid potentiation of the α3 GlyRs. In contrast, the cannabinoid analgesia is neither correlated with their binding affinity for CB1 and CB2 receptors nor with their psychoactive side effects. NMR analysis reveals a direct interaction between CBD and S296 in the third transmembrane domain of purified α3 GlyR. The cannabinoid-induced analgesic effect is absent in mice lacking the α3 GlyRs. Our findings suggest that the α3 GlyRs mediate glycineergic cannabinoid-induced suppression of chronic pain. These cannabinoids may represent a novel class of therapeutic agents for the treatment of chronic pain and other diseases involving GlyR dysfunction.

Chronic pain, particularly neuropathic pain, is a major clinical problem that is difficult to treat (Zhuo, 2007). Despite an intensive search for new analgesics in the last several decades, the need for novel therapeutic strategies remains unmet because virtually every blockbuster drug for the treatment of chronic pain produces aversive side effects. Marijuana has been used to treat chronic pain for thousands of years (Burns and Ineck, 2006; Murray et al., 2011). However, there is a need to improve the efficacy and tolerability of these agents in treating components of marijuana, respectively (Howlett et al., 2002; Costa, 2007). There is strong evidence suggesting that nonpsychoactive cannabinoids can also alleviate chronic inflammatory and neuropathic pain in animals (Costa et al., 2007; Izzo et al., 2009). Several recent clinical studies have demonstrated that combination of THC and CBD can be an effective therapeutic option for patients with neuropathic pain and other types of chronic pain (Nurmikko et al., 2007; Turcotte et al., 2010; Lynch and Campbell, 2011). However, there is a need to improve the efficacy and tolerability of these agents in treating

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chronic pain. One primary obstacle to development of these agents is the uncertainty about the molecular targets for cannabinoid-induced analgesic effects. For instance, the role of spinal CB1 receptors (CB1Rs) in the pain process is debatable. Some studies suggest that activation of CB1Rs in the spinal dorsal horn can facilitate pain (Pernía-Andrade et al., 2009; Zhang et al., 2010; Zeilhofer et al., 2012). Notably, THC-induced analgesia in the tail flick reflex, a test for nociceptive pain threshold, remains intact in mice devoid of CB1 receptors (CB1−/−; Zimmer et al., 1999; Howlett et al., 2002).

Recent studies have shown that glycine receptors (GlyRs) are an important target for cannabinoids in the central nervous system. For instance, several synthetic and phytocannabinoids, including THC and CBD, can potentiate glycine currents (I_Gly) in native neurons isolated from the ventral tegmental area, amygdala, hippocampus, and spinal cord and in various heterologous cells expressing recombinant GlyRs (Hejazi et al., 2006; Yang et al., 2008; Ahrens et al., 2009a,b; Demir et al., 2009; Foadi et al., 2010; Xiong et al., 2011, 2012; Yevenes and Zeilhofer, 2011a,b). GlyRs are thought to play an important role in the antinociceptive process (Harvey et al., 2004, 2009; Zeilhofer, 2005; Lynch and Callister, 2006; Pernía-Andrade et al., 2009; Zeilhofer et al., 2012). There are four isoforms of the α subunits (α1–4) and a single isoform of the β subunit. The adult form of GlyRs are composed of α and β subunits in a pentameric assembly (Lynch, 2004). The role of the α3 subunit in modulating inflammatory pain has been the focus of many discussions. The α3-containing GlyRs are abundantly located in the lamina II of the spinal dorsal horn, an area known for integrating nociceptive information. Experimental evidence suggests that prostaglandin E2 (PGE2), a critical mediator of central and peripheral pain sensitization, selectively inhibits the α3 GlyR function (Ahmadi et al., 2002; Harvey et al., 2004, 2009). Such disinhibition of the α3 GlyRs is found to contribute to the mechanism of chronic inflammatory pain induced by the intraplantar injection of CFA (Harvey et al., 2004, 2009).

Our recent study suggests that cannabinoid potentiation of GlyRs can produce a potent analgesic effect in mice (Xiong et al., 2011). The idea was mainly based on the results obtained in the tail flick test, a measure of transient nociception which only resembles the normal physiological state (Grossman et al., 1982). It is important to determine whether allosteric facilitation of GlyRs by cannabinoids contributes to the treatment of pathological or chronic pain states. Here, we demonstrate that glycinegic cannabinoids suppress inflammatory and neuropathic pain without significantly causing major psychoactive side effect and analgesic tolerance. The suppression of pathological pain by glycinegic cannabinoids is mediated through an α3 GlyR–dependent mechanism. We also provide mechanistic details of drug–receptor interaction and strategies for future studies to develop a new generation of glycinegic cannabinoid-based agents for the treatment of chronic pain and other diseases involving GlyR dysfunction.

RESULTS

Glycinegic cannabinoids suppress persistent inflammatory pain in both mice and rats

Previous studies have shown that both CBD and dehydroxyl-CBD (DH-CBD) potentiated I_Gly in HEK 293 cells expressing
the α1 and α3 subunits (Ahrens et al., 2009a; Xiong et al., 2011). In view of this, we first examined the effect of DH-CBD and CBD on inflammatory pain induced by intraplantar injection of 20 µl CFA (1:4 in saline) into one hind paw of the mice. CFA induced prolonged hypersensitivity to thermal pain in mice, reflected by a significant decrease in paw withdrawal latency (PWL) upon exposure to heat stimulus (Fig. 1 A, P < 0.001). The magnitude of CFA-induced persistent pain reached maximum 2 h after injection and persisted throughout the observation period in mice. i.p. (50 mg/kg) and intrathecal (i.t.; 50 µg) administration of DH-CBD caused a significant increase in the PWL (Fig. 1 B). The increase in the PWL developed and peaked within the 1st h, and persisted for 2 h. DH-CBD–induced analgesia was fully replicated at multiple times within the same day or next day (Fig. 1 C). This suggests that there is no apparent tolerance with DH-CBD–induced analgesia. In HEK 293 cells expressing the α3 GlyRs, DH-CBD was more efficacious than CBD in potentiating I_{Gly} (Fig. 1 D). For instance, the maximal magnitude of DH-CBD (1 µM)–induced potentiation was 989 ± 171%, whereas the magnitude of CBD-induced potentiation was 491 ± 101% (Fig. 1 E). These values are significantly different (P < 0.01). Both DH-CBD and CBD given i.p. or i.t. increased PWL in a dose–dependent manner (Fig. 1 F). Consistent with the observation of an in vitro study that DH-CBD was more efficacious than CBD in potentiating I_{Gly}, DH-CBD was more potent than CBD in alleviating heat pain hypersensitivity (Fig. 1 F).

Next, we examined whether or not DH-CBD can potentiate I_{Gly} in rat spinal dorsal horn neurons and whether or not i.t. application of DH-CBD can attenuate inflammatory thermal and mechanical pain hypersensitivity in rats. DH-CBD at concentrations from 1 to 20 µM increased the amplitudes of I_{Gly} of lamina II neurons, produced by puff application of 30 µM glycine in rat spinal cord slices (Fig. 2, A and B). This potentiation appeared dependent on the concentrations of DH-CBD and required sustained application of DH-CBD for at least 6 min to reach a peak. The DH-CBD–induced potentiation of I_{Gly} was maximal at the lowest concentrations of glycine. DH-CBD did not significantly alter the amplitude of current activated by glycine at concentrations equal to or higher than 300 µM (unpublished data). The specific GlyR antagonist strychnine completely abolished I_{Gly} and the potentiation of DH-CBD (Fig. 2 A), suggesting that the native GlyRs are the target that mediates the DH-CBD–induced potentiation.

Consistent with the observation in the previous paragraph, the ipsilateral PWLs were significantly decreased from the preinflammation baselines in rats at days 1–3 after receiving an intraplantar injection of CFA into the left hind paw (Fig. 2 C, P < 0.01). We then injected rats i.t. with DH-CBD (10 µg/15 µl, 50 µg/15 µl, or 100 µg/15 µl) or vehicle, and PWL was measured again at 30–60 min after injection. DH-CBD dose–dependently increased the ipsilateral PWL from preinjection values (Fig. 2 C, 50 µg: P < 0.01, 100 µg: P < 0.001) and also increased the contralateral PWLs from the preinflammation baselines at 50 µg (P < 0.01) and 100 µg (P < 0.01) doses (Fig. 2 D). The ipsilateral paw withdrawal thresholds (PWTs) to punctuate mechanical stimuli were significantly decreased from preinflammation baselines in rat at day 1–3 after intraplantar injection of CFA (Fig. 2 E, P < 0.001). The ipsilateral PWTs were significantly increased at 30–60 min after i.t. injection of DH-CBD at 50 µg (P < 0.01) and 100 µg (P < 0.01) doses, but not at a 10 µg dose (Fig. 2 E, P = 0.075). The contralateral PWTs were not significantly changed (Fig. 2 F).

DH-CBD suppress chronic neuropathic pain
Peripheral nerve injury can cause clinically relevant chronic neuropathic pain (Guan et al., 2008). The fifth lumbar spinal nerve injury (SNL) produced long-lasting mechanical and thermal hypersensitivity on the ipsilateral hind paw in rats (Fig. 3, A and C). The PWTs to the application of calibrated von Frey filaments to the plantar side of the hind paw ipsilateral to the nerve injury significantly decreased from the preinjury baseline values, a behavioral indication...
DH-CBD rescues PGE₂-induced inhibition of α₃ GlyR activity and i.t. PGE₂-induced persistent pain

PGE₂ is one of the major proinflammatory substances that promote nociceptive processing in the spinal cord upon various noxious stimuli (Vanegas and Schaible, 2001; Zeilhofer et al., 2012). There is also evidence to suggest that the PGE₂ signaling pathway critically contributes to pain hypersensitivity after peripheral nerve injury (Marchand et al., 2005; Patapoutian et al., 2009). A previous study has suggested that elevated PGE₂ promotes nociceptive action through inhibiting spinal α₃ GlyRs function (Harvey et al., 2004). We proposed that the spinal α₃ GlyR is the target for the cannabinoid-induced analgesic effect. A legitimate question remains as to whether or not cannabinoids can modulate the α₃ GlyRs upon activation of PGE₂ receptors (EP₂Rs). To address this question, we coexpressed the α₃ GlyRs with EP₂Rs in HEK 293 cells. In these cells, preincubation with 10 µM PGE₂ significantly reduced the amplitude of current activated by 200 µM (EC₂₀) glycine (Fig. 4, A and B). In contrast, PGE₂ did not significantly alter I₃₈, in cells coexpressing EP₂Rs and α₁ GlyRs. These findings are consistent with the observations described in a previous study (Harvey et al., 2004). The PGE₂ inhibition of the α₃ GlyRs developed slowly and reached the maximal 5–8 min after application of PGE₂ (Fig. 4 B). In the same cells with continuous incubation of PGE₂, DH-CBD at 1 µM significantly potentiated I₃₈ (Fig. 4, C and D, P < 0.001). i.t. application of PGE₂ (0.2 nmol per mouse) induced persistent thermal pain hypersensitivity (Fig. 4 E). Consistent with a previous study (Harvey et al., 2004, α₃ GlyR knockout (α₃⁻/⁻) mice showed a complete lack of pain sensitization compared with WT littermates (Fig. 4 E, P < 0.001), suggesting an involvement of the α₃ GlyRs in spinal PGE₂-dependent pain signaling pathway. DH-CBD completely reversed the reduction in PWL induced by i.t. PGE₂ in mice (Fig. 4 E, P < 0.001).

Didesoxy-CBD (DD-CBD) inhibits DH-CBD–induced potentiation of I₃₈ and analgesic effect

Consistent with our recent observations (Xiong et al., 2011, 2012), chemically modified CBD with removal of both hydroxyl and oxygen groups (DD-CBD) did not significantly alter the amplitude of I₃₈ in HEK 293 cells expressing the α₃ GlyRs (Fig. 5 A). In these cells, DD-CBD inhibited the DH-CBD (1 µM)–induced potentiation on I₃₈ in a concentration-dependent manner (Fig. 5 A and B). DD-CBD at 3 µM shifted in a parallel manner the concentration-response curve of DH-CBD–induced potentiation of the α₃ GlyRs to the right (Fig. 5 C). We next examined whether or not DD-CBD can inhibit DH-CBD–induced analgesic effect in persistent pain. Although systemic or i.t. application of DD-CBD (50 mg/kg i.p. or 10 µg i.t.) alone did not significantly alter the PWL in CFA-treated mice (Fig. 5 D), DD-CBD when applied either i.t. or i.p. antagonized the DH-CBD (50 mg/kg i.p.)–induced analgesic effect in CFA-induced pain hypersensitivity in mice (Fig. 5, E and F). This DD-CBD–induced antagonism appeared to be dose dependent and reached the maximal magnitude (complete inhibition of DH-CBD–induced analgesia) when applied at 10 mg/kg i.p. or 10 µg i.t. (P < 0.001).
measuring the following functional indexes: the binding affinity of the cannabinoids bound for both CB1 receptors (in purified brain membrane) and CB2 receptors (in purified CB2 receptor protein from transformed Escherichia coli bacteria cells), the potentiation of IGly induced by 1 µM cannabinoids and the EC50 values of cannabinoid potentiation of IGly in HEK 293 cells expressing the α3 GlyRs, and cannabinoid-induced analgesic effect in CFA-induced inflammatory pain in mice. Although these cannabinoids share a high degree of structural similarity, they differed significantly in their binding affinity for CB1/CB2 receptors, their potencies in potentiating IGly, and their inhibition of pain hypersensitivity in mice (Fig. S1). Except for inducing an analgesic effect, some of these cannabinoids, such as THC and HU210, substantially reduced body temperature, locomotor activity, and balance and coordination skills in mice (Fig. 7). There was a strong correlation between the cannabinoid-induced potentiation of IGly and cannabinoid-induced analgesic effect in chronic inflammatory pain in mice (Fig. 7 A, 1 µM cannabinoid-induced potentiation vs. i.p. 50 mg/kg cannabinoid-induced percentage changes of PWL, P < 0.001). There was also a strong correlation between the EC50 values of cannabinoid potentiation of IGly and cannabinoid-induced percentage changes of PWL (r2 = 0.662, P < 0.05). In contrast, the magnitudes of cannabinoid-induced analgesia in chronic pain were not significantly correlated with the binding affinity of cannabinoids

The α3 GlyR is essential for the DH-CBD-induced analgesic effect in chronic pain

To date, there is no antagonist highly selective for a specific GlyR subunit. The α3 GlyR subunit has been shown to be valuable for exploring the α3 GlyR-mediated behaviors (Harvey et al., 2004, 2009; Hösl et al., 2006; Manzke et al., 2010; Xiong et al., 2011). To study the role of the α3 subunit in CBD and DH-CBD–induced analgesia, we conducted the following experiments using the α3−/− mice. We observed that both CBD and DH-CBD–induced analgesic effects in CFA-induced pain hypersensitivity were significantly reduced in mice lacking the α3 subunits as compared with the WT littermates (Fig. 6, A and B). For instance, the peak values of PWL after CBD and DH-CBD (50 mg/kg i.p.) were 8.1 ± 0.5 and 10.7 ± 0.6 s in the WT mice, whereas the peak values of PWL after CBD and DH-CBD were 4.3 ± 0.7 and 5.9 ± 0.8 s in α3−/− mice. The values between the WT and α3−/− mice are significantly different (P < 0.001). Both CBD- and DH-CBD–induced analgesic effects remained unchanged in either CB1 knockout mice (Fig. 6, C and D) or CB2 knockout mice as compared with their WT littermates (Fig. 6, E and F).

Correlation analysis: cannabinoid potentiation of IGly is correlated with cannabinoid-induced analgesic potency

Fig. S1 lists the names and chemical structures of 11 synthetic cannabinoids structurally similar to CBD. We explored the structural and functional activity of these cannabinoids by measuring the following functional indexes: the binding affinity of the cannabinoids bound for both CB1 receptors and CB2 receptors (in purified brain membrane) and CB2 receptors (in transformed Escherichia coli bacteria cells), the potentiation of IGly induced by 1 µM cannabinoids and the EC50 values of cannabinoid potentiation of IGly in Hek 293 cells expressing the α3 GlyRs, and cannabinoid-induced analgesic effect in CFA-induced inflammatory pain in mice. Although these cannabinoids share a high degree of structural similarity, they differed significantly in their binding affinity for CB1/CB2 receptors, their potencies in potentiating IGly, and their inhibition of pain hypersensitivity in mice (Fig. S1). Except for inducing an analgesic effect, some of these cannabinoids, such as THC and HU210, substantially reduced body temperature, locomotor activity, and balance and coordination skills in mice (Fig. 7). There was a strong correlation between the cannabinoid-induced potentiation of IGly and cannabinoid-induced analgesic effect in chronic inflammatory pain in mice (Fig. 7 A, 1 µM cannabinoid-induced potentiation vs. i.p. 50 mg/kg cannabinoid-induced percentage changes of PWL, P < 0.001). There was also a strong correlation between the EC50 values of cannabinoid potentiation of IGly and cannabinoid-induced percentage changes of PWL (r2 = 0.662, P < 0.05). In contrast, the magnitudes of cannabinoid-induced analgesia in chronic pain were not significantly correlated with the binding affinity of cannabinoids.
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For either CB1 or CB2 receptors (Fig. 7, C and D, P > 0.05). Neither cannabinoid-induced potentiation of GlyRs (Fig. 7, D–F) nor cannabinoid-induced analgesia (Fig. 7, G–I) was significantly correlated with cannabinoid-induced psychoactive effects such as hypothermia, hypolocomotion, and incoordination (P > 0.05).

NMR analysis: a direct interaction between CBD and the S296-containing domain of the α3 GlyR

Our recent study using NMR analysis has revealed chemical shift of the S296 residue in the TM3 of purified α1 GlyR-TM proteins by THC titration (Xiong et al., 2011). However, there are two unsolved issues. First, it is unclear if cannabinoids can interact with the α3 GlyR, as the previous experiment was conducted with the α1 GlyR-TM domains. There are 12 residues different within the four TM domains between the α1 and α3 subunits. Second, changes in chemical shift, although indicative of cannabinoid–receptor interactions, are not always predictive of functional effects.

Figure 5. Antagonism of DD-CBD to DH-CBD–induced potentiation of Igly and analgesia in inflammatory pain.

(A) Structures of DH-CBD and DD-CBD. Trace records of Igly in HEK 293 cells expressing the α3 GlyRs without and with co-application of DD-CBD and DH-CBD. (B) DD-CBD inhibition of DH-CBD–induced potentiation of Igly in HEK 293 cells expressing the α3 GlyRs (n = 6). (C) The concentration–response curves of DH-CBD–induced potentiation of Igly without and with 1 μM DD-CBD (n = 5–7). (D) The effect of i.t. and i.p. application of DD-CBD on PWL to thermal stimulation after CFA paw injection in mice (n = 7–10). (E) Dose–dependent inhibition of i.p. DH-CBD–induced analgesic effect by i.p. application of DD-CBD in post-CFA mice (n = 7–10). (F) Dose–dependent inhibition of i.p. DH-CBD–induced analgesic effect by i.t. application of DD-CBD in post-CFA mice (n = 8–10). *, P < 0.05; ***, P < 0.001, as compared with vehicle injection. Data are representative of two independent experiments and expressed as mean ± SEM.

Figure 6. A decrease in CBD and DH-CBD–induced analgesia in the α3+/– mice but not in the CB1+/– and CB2+/– mice.

(A and B) The analgesic effect of 50 mg/kg CBD i.p. (A) or 50 mg/kg DH-CBD i.p. (B) on PWL to thermal stimulation after CFA injection in the α3+/– mice (n = 9–10). ***, P < 0.001, as compared with WT mice. (C and D) The analgesic effect of 50 mg/kg CBD i.p. (C) or 50 mg/kg DH-CBD i.p. (D) on PWL to thermal stimulation after CFA injection in the CB1+/– mice (n = 6). (E and F) The analgesic effect of 50 mg/kg CBD i.p. (E) or 50 mg/kg DH-CBD i.p. (F) on PWL to thermal stimulation after CFA injection in the CB2+/– mice (n = 6). Data are representative of two independent experiments and expressed as mean ± SEM.
interaction, provide only limited information about direct binding at the site involving S296. To address these issues, we first converted the α1 subunit to the α3 subunit by mutating all 12 residues to match the α3 sequence. Using the high-resolution NMR structure of α1 GlyR TM domains solved in the lyso-1-palmitoylphosphotidylglycerol (LPPG) lipids as a template, we generated a homology model of α3 GlyR-TM structure (Fig. 8 A). We next measured the interaction between CBD and α3 GlyR TM domains by chemical shift titration using two-dimensional (2D) 15N heteronuclear single quantum coherence (HSQC) spectroscopy and by 3D 15N-edited nuclear Overhauser effect spectroscopy (NOESY) as well as 2D homonuclear 1H–1H NOESY. HSQC spectroscopy revealed that S296 in the TM3 domain was distinctively sensitive to CBD titration. Representative contour plots of the S296 HSQC resonance at different CBD concentrations are depicted in Fig. 8 B. With increasing CBD concentrations, the S296 resonance exhibited CBD-dependent changes both in chemical shift and in intensity. It is worth noting that at an intermediate CBD concentration (105 µM), S296 resonance became two distinct peaks, suggesting two coexisting states of binding on a relatively slow exchange time scale or two different protein conformations at S296. As CBD concentration continued to increase, the binding or conformational state corresponding to the downfield resonance (Fig. 8 B, left peak) became dominant.

To determine whether or not CBD can directly interact with α3 GlyR-TM, we examined the intermolecular NOESY cross peaks between CBD and the protein. Fig. 8 C compares the contour plots of NOESY cross peaks between the aromatic protons of CBD (~6.2–6.4 ppm) and other proton resonances in the 1–4 ppm range in the absence and presence of α3 GlyR-TM, respectively. In the absence of the protein, intramolecular cross peaks of CBD in LPPG can be detected at ~1.3, ~1.6, and ~2.5 ppm (Fig. 8 C). In the presence of α3 GlyR-TM, an additional cross peak was observed at ~3.9 ppm. This resonance was assigned to Hβ of S296. The presence of an intermolecular cross peak is a strong indication that CBD interacts with the TM domain of α3 GlyR subunit specifically at a site involving S296. The transition from the free to the CBD-bound state seems to induce a conformational change in the protein at S296, as can be clearly seen from the gradual disappearance of the free S296 resonance and appearance of the bound.
resonance, with an intermediate coexistence of both peaks (Fig. 8 B). NMR titration and NOESY experiments suggest a direct interaction of CBD with residue S296. Based on this idea, we performed several parallel molecular dynamics simulations of 20 ns each with different random seeds to determine the molecular nature of the interaction between CBD and the binding site involving S296. Fig. 8 D depicts a typical frame from simulation trajectories showing details of the binding pocket for CBD. DD-CBD inhibited DH-CBD–induced potentiation of IGly in an apparent competitive manner, as revealed by our electrophysiological experiments. In this regard, we next compared the docking free energies between CBD and DD-CBD at the S296 site and found that binding affinities of CBD and DD-CBD are within the same order of magnitude with similar docking free energies (unpublished data). This finding favors the idea that DD-CBD inhibition of CBD potentiation of IGly is through a competitive mechanism by acting near or at the same site involving S296. Consistent with our NMR and MD simulation that CBD interacts with α3 GlyR at S296, substitution of S296 with an alanine in the α3 GlyR significantly reduced the magnitude of CBD potentiation by nearly 70% (Fig. 8, E and F).

Besides S296, residue T397 in the loop leading to the fourth transmembrane domain (TM4) of α3 GlyR, was also affected by the CBD titration (unpublished data). T397 is evidently in an intrinsically dynamic region of the protein, showing two conformations with an exchange rate slower than the NMR time scale (and hence two distinct HSQC resonances for T397) even in the absence of CBD. Binding of CBD at S296 also altered the motional characteristics of T397, resulting in the disappearance of one of the conformations at T397. We could not determine the functional role of the residue T397 in CBD-induced potentiation because the T397A mutation resulted in low expressions of the α3 GlyRs in HEK 293 cells.

**Kinetic analysis: DH-CBD accelerates GlyR activation rate and slows deactivation rate**

The observations mentioned in the previous section suggest that CBD directly interacts with S296-containing domain in the α3 GlyR protein. To further explore mechanistic detail of the interaction between cannabinoids and GlyRs, we examined the effect of DH-CBD on GlyR kinetics using fast drug perfusion in HEK-293 cells expressing α3 GlyRs. DH-CBD at 3 µM accelerated the initial slope of the activation phase of the current activated by 1 mM glycine (Fig. 9 A). The 10–90% activation times were 11.1 ± 0.8 and 5.8 ± 0.8 ms in the absence and presence of 3 µM DH-CBD, respectively. These values were significantly different (P < 0.05). The S296A mutation and DD-CBD pretreatment completely abolished DH-CBD–induced acceleration of receptor activation time (11.3 ± 0.7 vs. 11.4 ± 0.4 ms for S296A and 10.5 ± 1.3 vs. 10.8 ± 1.3 ms for DD-CBD, P = 0.4–0.6). DH-CBD significantly slowed the receptor deactivation time constant from 10.9 ± 0.5 to 41.3 ± 4.8 ms immediately after a 5-ms application of 1 mM glycine (Fig. 9 B, P < 0.001). The S296A mutation and DD-CBD prevented DH-CBD–induced acceleration of receptor deactivation time (11 ± 1.7 vs. 14.2 ± 3.7 ms for S296 and 11.7 ± 0.8 vs. 16.6 ± 3.3 ms for DD-CBD, P > 0.05). In contrast, DH-CBD did not significantly alter receptor desensitization time (3.4 ± 0.2 vs. 3.8 ± 0.4 s, P > 0.05). These observations suggest that DH-CBD may increase agonist...
not yet available. The current study has provided several lines of evidence to suggest that CBD and DH-CBD suppress persistent inflammatory and neuropathic pain by targeting the α3 GlyRs in rodents. Consistent with in vitro observation that DH-CBD was more efficacious than CBD in potentiating IGly, DH-CBD was more potent than CBD in reducing chronic pain. DH-CBD also attenuated i.t. PGE2-induced persistent pain hypersensitivity in mice. Several lines of evidence suggest that cannabinoid-induced analgesia is mediated through the α3 GlyR-dependent pathway. First, both CBD and DH-CBD-induced analgesic effects were significantly reduced in mice lacking the α3 GlyRs but not in mice lacking the CB1 and CB2 receptors. Second, DD-CBD inhibited DH-CBD-induced potentiation of the α3 GlyRs and analgesic effect in chronic pain. Third, structural and functional analysis reveals that the magnitude of the cannabinoid-induced analgesic effect was correlated with cannabinoid potentiation of the α3 GlyRs but not with the cannabinoid binding affinity for CB1 and CB2 receptors. Because of substantially reduced CB1 binding affinity, DH-CBD, even at high concentrations (50 mg/kg i.p.), did not produce the psychoactive effects commonly associated with cannabinoid activation of CB1 receptors. Collectively, glycinergic cannabinoids represent a new class of therapeutic agents that selectively relieve pathological pain by targeting the α3 GlyRs.

The data from NMR titration and NOESY experiments strongly indicates the direct interaction of CBD with residue S296. The change in NMR signal intensity upon CBD titration suggests that the protein motion at S296 is sensitive to CBD binding. The molecular model of the α3 GlyR TM domains reveals that S296 is located near the intracellular end of the TM3 helix, with its side chain facing the lipids. Direct interaction of CBD with α3 GlyR-TM protein was confirmed by the intermolecular NOESY cross peaks between CBD and the protein. There is a transition from the free to the CBD-bound state as indicated by the observation that a free S296 resonance and a bound resonance appeared sequentially with an intermediate coexistence of both peaks. This finding also favors a protein conformational change at S296 in the presence of CBD.

Our molecular dynamics simulations suggest that CBD-α3 GlyR binding interactions involve the S296 residue of the α3 GlyR TM domain on the principal side, and the lipid molecules on the complementary side. The molecular docking analyses reveal that the binding free energies of the potentiator CBD and the inhibitor DD-CBD at this protein-lipid interfacial site are very similar, suggesting that the binding affinities are within the same order of magnitude. This finding favors the idea that the DD-CBD inhibition of CBD potentiation of IGly is through a competitive mechanism by acting near or at the same site involving S296. It should be pointed out that both DH-CBD and DD-CBD are modulators of GlyRs. Unlike orthosteric ligands, these modulators bind to allosteric sites. The molecular nature of competitiveness of these modulators remains unknown. Although the data from the in vitro study together with the result of molecular dynamics...
simulations suggest that DD-CBD may compete for the same site with CBD, our experimental data alone are not sufficient to conclude that DD-CBD acts as a competitive antagonist of CBD. Future studies should be performed to examine the effect of DD-CBD on the purified α1 or α3 GlyR–TM proteins using NMR analysis.

The kinetic analysis suggests that DH-CBD increases the agonist binding affinity of GlyRs. DH-CBD accelerated receptor activation rate and, on the other side, slowed receptor deactivation rate. Although receptor activation time represents agonist binding and/or channel gating, receptor deactivation time reflects the kinetics of agonist unbinding/channel closing or combination of two. The DH-CBD–induced changes in GlyR kinetics appear relevant to DH-CBD–induced potentiation of I_{gly} because both S296A and DD-CBD, which inhibited DH-CBD potentiation of I_{gly}, abolished DH-CBD alteration of receptor gating kinetics. DH-CBD shifted in a parallel manner the glycine-concentration response curve to the left, favoring a hypothesis that DH-CBD allosterically increases the agonist binding affinity of GlyRs. However, this notion should be made with caution, as slow deactivation time of GlyRs could reflect a slow channel closing, unbinding rate, or both in the presence of DH-CBD. In this scenario, slow deactivation time could be a result of slow wash time of DH-CBD because of its hydrophobic nature. One can also argue that the deactivation rate could be contaminated with desensitization rate. However, it is unlikely to be the case in our study because DH-CBD did not significantly affect GlyR desensitization rate while slowing deactivation.

Several preclinical persistent and chronic pain models were tested in this study. Intraplantar CFA injection has been widely used as an inflammatory pain model. Both systemic and i.t. injection of DH-CBD significantly reduced mechanical pain hypersensitivity induced by CFA. PGE2 is one of the major proinflammatory substances that promote nociceptive processing in the spinal cord and peripheral tissues upon various noxious stimuli such as CFA (Vanegas and Schäible, 2001; Harvey et al., 2004, 2009; Zeilhofer et al., 2012). Consistent with the observations in CFA-induced inflammatory pain models, DH-CBD also produced an analgesic effect in i.t. injection of PGE2-induced nociception in mice. More importantly, i.t. application of DH-CBD exerted potent inhibition of chronic neuropathic pain in rats. Neuropathic pain is a substantial health issue because currently available therapies are far from satisfactory. Although neuropathic pain and inflammatory pain differ in pathogenesis, molecular mechanisms, and treatments, these two types of persistent pain may be modulated by similar synaptic mechanism at the spinal level (Zeilhofer et al., 2012). The data presented in our study suggest that the α3 GlyR contributes to the mechanisms that modulate both types of pain. Yet, we cannot exclude the potential involvement of other subtypes of GlyRs in the pain modulation. In addition to reducing chronic pain, DH-CBD can also attenuate acute pain. DH-CBD increased the time latency in the tail flick reflex in mice (Xiong et al., 2011). This analgesic effect induced by DH-CBD was abolished in mice depleted with the α3 GlyRs but remained intact in mice depleted with the CB1 receptors. Consistent with this idea, i.t. application of DH-CBD at higher doses also significantly increased the contralateral PWL from baseline in both inflammatory and neuropathic pain in rats.

i.t. application of DH-CBD seems the most efficacious way to suppress mechanical and thermal pain hypersensitivity in both inflammatory and neuropathic pain conditions. This idea is consistent with the distinct distribution pattern of the α3 GlyRs in lamina II of the spinal dorsal horn (Harvey et al., 2004). Moreover, the α3 GlyRs are either absent or less expressed in primary sensory neurons such as dorsal root ganglion neurons (Lynch, 2004). Oral administration of cannabinoids is not an ideal route for drug delivery because primary cannabionoids are largely metabolized by the liver (Huestis and Pertwee, 2005). It is worth mentioning that one of the common practices to deliver medicinal cannabis to humans is via sublingual spray, which bypasses the liver and delivers drugs directly into the blood stream (Nurmikko et al., 2007).

Collectively, we propose that i.t. injection of cannabinoids should be the most efficacious route to treat patients with chronic neuropathic pain.

Among 11 cannabinoid analogues evaluated in this study, DH-CBD has emerged as an ideal glycnergic cannabinoid that can be used to treat chronic pain without causing adverse effects. Unlike some of the analogues that not only showed relatively high efficacy in potentiating I_{gly} but also demonstrated relatively high affinity to bind to CB1 receptors, DH-CBD displayed a low affinity for CB1 receptors and at the same time is one of the most efficacious positive modulators of GlyRs. It has been shown consistently in our correlation analysis that most psychoactive effects induced by cannabinoids are associated with CB1 receptor binding affinity but not cannabinoid-induced potentiation of GlyRs. Conversely, the cannabinoid-induced analgesic effect in chronic pain is correlated with cannabinoid potentiation of GlyRs but not with cannabinoid binding affinity to CB1 receptors. These principles may apply to future studies in developing a new generation of glycnergic cannabinoids in the treatment of chronic pain. In addition to lacking a psychoactive side effect, glycnergic cannabinoids are unlikely to develop drug tachyphylaxis or tolerance, one of the major barriers for long-term pain management with currently available clinical agents. Repeated application of DH–CBD either i.p. or i.t. exhibited similar analgesic potency in both inflammatory and neuropathic pain. This finding is not unexpected because glycnergic cannabinoids act on the GlyRs as allosteric modulators instead of agonists or antagonists.

Collectively, we have provided evidence to suggest that glycnergic cannabinoids are ideal therapeutic agents in the treatment of inflammatory and neuropathic pain. They can effectively attenuate pathological pain without significantly causing major psychoactive side effect and analgesic tolerance. The mechanistic details of drug–receptor interaction could help to develop novel agents for the treatment of painful conditions and other diseases involving GlyR impairment.
Inflammatory pain model in mice. Inflammation was induced with intraplantar injection of 10 µl CFA (diluted 1:4 with saline; Sigma-Aldrich) to the left hind paw. The PWL to noxious heat was measured using a system described previously (Chaplan et al., 1994). The von Frey filaments were applied for 4–6 s to the test area between the footpads on the plantar surface of the hind paw. If a positive response occurred (e.g., abrupt paw withdrawal, licking, and shaking), the next smaller von Frey hair was used; if a negative response was observed, the next higher force was used. The test was continued until: (1) the responses to five stimuli were assessed after the first crossing of the withdrawal threshold or (2) the upper/lower end of the von Frey hair set was reached before a positive/negative response had been obtained. The PWL was determined according to the formula provided by Dixon (1980).

Measurement of thermal pain hypersensitivity in mice and rats. Thermal pain hypersensitivity was determined by measuring PWL to radiant heat stimuli (Hargreaves et al., 1988) with a plantar stimulus analogues meter (model 390; IITC). Animals were placed under individual plastic boxes on a heated glass floor (30°C) and allowed to habituate for at least 30 min before testing. Both hind paws were tested three times, with >2 min between trials. A cut-off time of 20 s was used to avoid sensitization and damage to the skin. The mean PWL of the three trials was used for data analysis.

Measurement of body temperature. Mouse body temperature was measured by inserting a thermometer (Thermalent TH-5; Physitemp) 2 cm into the rectum until a stable reading was obtained. Ambient room temperature was 22°C.

Locomotor activity. Mice were i.p. injected with the vehicle or cannabinoids. After 15 min, the spontaneous locomotor activity of mice was measured in a standard home cage in a 12-station photobeam activity system (Opto-M3 Activity Meter; Columbus Instruments) where the animals were placed individually 30 min after injection of drugs. Using infrared beams, activity was monitored in the horizontal directions. The total number of ambulatory beam breaks was recorded for 30 min and stored every 10 s.

Rotarod test. A computer-interfaced rotarod accelerating from 4–40 rotations per min over 300 s was used (ENV-575M; Med Associates). The shaft diameter is 3.2 cm. The mice were trained three trials per day with a 20-min interval for three consecutive days. Each trial ended when the mouse fell off the rotarod or after 300 s had elapsed. The time that each mouse maintained its balance on the rotating rod was measured as latency to fall.

HEK 293 cell transfection and recording. HEK 293 cells were cultured as described previously (Xiong et al., 2008). The plasmid cDNAs of the WT and mutant GlyR subunits and EP2R were transfected using the SuperFect Transfection kit (QIAGEN). The currents were recorded 1–2 d after transfection. HEK 293 cells, but not neurons, were lifted and continuously superfused with a solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1.2 mM MgCl2, 5 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH; ~340 mM ounseal with sucrose). Membrane currents were recorded in the whole-cell configuration using an amplifier (Axopatch 200B; Axon) at 20–22°C. Cells were held at ~60 mV unless otherwise indicated. Data were acquired using pClamp 9.2 software (Molecular Devices). Data were filtered at 1 KHz and digitized at 2 KHz. Bath solutions were applied through three barrel square glass tubing (Warner Instrument) with a tip diameter of ~200 µm. Drugs were applied through a Warner fast-step stepper-motor driven system. The solution exchange time constants were ~4 ms for an open pipette tip and 4–12 ms for whole-cell recording.
was confirmed by double-stranded DNA sequencing using a CEQ 8000 Genetic Analysis System (Beckman Coulter).

**Spinal cord slice preparation and electrophysiological recordings.** Lumbar spinal cord slices at the L5-L6 level were prepared from adult rats as we described previously (Pan and Pan, 2004; Zhou et al., 2008). We removed the lumbar spinal cord through laminectomy during isoflurane-induced anesthesia. We sliced the spinal cord (400 μm) using a vibratome and continually superfused the slices with artificial cerebrospinal fluid containing (in mM) 110.7 NaCl, 3.6 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 1.2 NaH₂PO₄, 11.0 glucose, and 25.0 NaHCO₃ (bubbled with 95% O₂/5% CO₂). Neurons in the lamina II of the spinal cord were visualized using a fixed-stage microscope (BX50WI; Olympus) with differential interference contrast/infrared illumination. We obtained all whole-cell patch-clamp recordings at 34°C using glass pipettes filled with a solution containing (in mM) 110 Cs₂SO₄, 5 TEA, 2.0 MgCl₂, 0.5 CaCl₂, 5.0 Hepes, 5.0 EGTA, 5.0 ATP-Mg, 0.5 Na-GTP, and 10 lidocaine N-ethyl bromide, adjusted to pH 7.2–7.4 with 1 M CsOH (290–300 mOsm). GlyR-mediated currents were recorded at the holding potential of 0 mV and elicited by puff application of 30 µM glycine directly to the recorded neuron using a positive pressure system (4 psi for 15 ms; Tooyee Company). The input resistance was continuously monitored, and the recording was abandoned if it changed >15%. Data acquisition and analysis of postsynaptic currents were done as described previously (Pan and Pan, 2004; Zhou et al., 2008).

**[3H]-CP55940 binding of CB1 and CB2 receptors.** Mouse brain tissues (CB1) and Rosetta(DE3) pLysS competent E. coli cells (transfected with human CB2 receptor cDNA) were collected and homogenized using a polytron homogenizer (Brinkman) at 500 rpm for 30 s in ice-cold 50 mM Tris-HCl, 1 mM EDTA, and 3 mM MgCl₂, pH 7.4. The homogenate was centrifuged at 48,000 g for 20 min at 4°C. The membrane pellet was suspended and incubated with [3H]-CP55940 (PerkinElmer) and various concentrations of cannabinoids in phosphate buffered saline (PBS) containing 0.2% (wt/vol) BSA. The filters were washed four times with 4 ml cold PBS containing 0.1% (wt/vol) BSA. The filters were punched into scintillation vials containing 3 ml liquid scintillation cocktail. The samples were counted in a scintillation counter at 50% efficiency. Assays were performed in triplicate, and each experiment was repeat at least three times.

**NMR spectroscopy.** Interaction between CBD and the transmembrane (TM) domain of the human GlyR α₃ subunit (α₃ GlyR-TM) was investigated by NMR spectroscopy. The full-length α₃ GlyR TM domains have a sequence of MLEQRGLGYYL LQYIPSLL LVIWSWSFWI NLDAAPARVA LGITTVTLTT TSQSGSRASL PKSVYKAIID IWLVACLLEFV FSALLEYAV NVFVRQHKEG GGFDRAKID IDTSIRACP LFALIFNFIV WIVYKILRKE DEFEHHHHHH. The DNA plasmid for protein expression was prepared by mutating all 12 amino acid residues in the α₁ GlyR-TM plasmid to match those in the α₃ sequence (Ma et al., 2008; Canlas et al., 2008; Xiong et al., 2011). The large intracellular loop between TM3 and TM4 domains was replaced with a glycine linker (GGGG). The same expression and purification protocols used for α₁ GlyR-TM (Ma et al., 2008; Canlas et al., 2008; Xiong et al., 2011) were followed. The NMR samples typically contained 100 µM α₃ GlyR-TM solubilized in ~15 mM of LPPG in 10 mM sodium phosphate buffer, pH 5.8. 2D ¹H HSQC spectroscopy and 3D ¹H-¹H NOESY, as well as 2D ¹H-¹H NOESY, were recorded on Avance 600 and 800 MHz spectrometers (Bruker) at 313 K, with a complex time-domain size of 1,024 × 128, 1,024 × 32 × 88, and 1,024 × 370, respectively. NOE mixing time was 120 ms. NMR chemical shift titration experiments were performed at CBD concentrations of 0, 5, 105, and 546 µM. The NMRPipe (Delaglio et al., 1995) and Topspin programs were used for NMR data processing, and the Sparky program (SPARKY 3; T.D. Goddard and D.G. Kneller, University of California, San Francisco, San Francisco, CA) was used for analyzing the spectra and for preparing the figures.

**Molecular dynamics simulations.** The structure of α₃ GlyR-TM was generated from the NMR structure of α₁ GlyR-TM using MODELLER 9v8 (Eswar et al., 2007) and manually inserted into a fully hydrated ternary lipid mixture as detailed previously (Cheng et al., 2007). The force-field parameters for CBD were generated using CHARMM general force field (Vannomneslaeghe et al., 2010) with high penalties further optimized using Gaussian 09 (Gaussian, Inc.) at the MP2/6-31G(d) level (Møller and Plesset, 1934) and tested using CHARMM c36b1 (Liu et al., 2004; Sadalino and Tang, 2004; Brooks et al., 2009). All simulations were performed using NAMD 2.7b1 (Phillips et al., 2005). Guided by the experimental NMR data, the initial CBD location near S296 was determined by docking, followed by production molecular dynamics simulations in replica lasting 20 ns each. The final simulation frames were used to analyze the binding site for CBD in α₃ GlyR-TM. The binding site was used in AutoDock 4.0 (Morris et al., 1998) to evaluate interaction between DD-CBD and α₃ GlyR-TM. For both CBD and DD-CBD, docking results were compiled from separate 300 runs using the Lamarckian genetic algorithm implemented in AutoDock 4.0. In each case, a population size of 300, a maximum of 27,000 generations, and a maximum of 15 million energy evaluations were used.

**Whole-cell kinetic measurements.** Receptor activation rate for current induced by 1 mM glycine was estimated by measuring the slope of the initial inward component of current between 10 and 90% of the maximal current (10–90% rise time). Receptor desensitization was induced by 1 mM glycine for 10 s in cells voltage-clamped at ~60 mV. The deactivation time was recorded for 30 s immediately after 10 ms application of 1 mM glycine. The time constants of deactivation and desensitization were determined by fitting with mono-exponential functions using the Levenberg-Marquardt algorithm in Clampfit 9.2 (Molecular Devices).

**Drugs.** Most of the chemicals including glycine were from Sigma-Aldrich. Solutions were prepared on the day of experiment. THC was obtained from the National Institute on Drug Abuse. HU210 and CP55940 were obtained from Tocris Bioscience. Agonists and other chemical agents were diluted either directly in the bath solution or dissolved in ethanol before further dilution. The final ethanol concentration was <8 mM, which did not significantly affect Iₙa, DD-CBD was originally dissolved in DMSO in a stock solution and further diluted in working solution (external buffer). The maximal concentration of DMSO in bath solution was <0.1%. This concentration of DMSO alone did not affect Iₙa. In behavioral tests, all drugs were dissolved in drug/emulphor/saline solution with a ratio of 1:1:18. Emulphor was obtained from North American Chemical. The vehicles used in our experiments did not affect latency responses when administered alone.

**Data analysis.** Statistical analysis of concentration-response data were performed with the use of the nonlinear curve-fitting program Prism (Graph-Pad Software). Data were fit using the Hill equation I/Iₙa = Bottom + (Top – Bottom)/(1 + 10^(LogEC₅₀ – Log(Agonist))/Hi×HillSlope), where I is the current amplitude activated by a given concentration of agonist, I/Iₙa is the maximum response of the cell, and EC₅₀ is the concentration eliciting a half-maximal response.

A one-way repeated measures ANOVA was used to compare the data between time points in each group. Data from different drug groups were compared using a two-way mixed model ANOVA. Tukey's honestly significant difference post-hoc test was used to compare specific data points in ANOVA. STATISTICA 6.0 software (StatSoft, Inc.) was used for analysis, and data are expressed as mean ± SEM. P < 0.05 was considered significant.

**Online supplemental material.** Figure S1 lists the names and chemical structures of 11 synthetic cannabinoids structurally similar to CBD. Figure S2 shows the procedures of chemical synthesis of cannabinoid analogues. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20120242/DC1.
See the text for the actual content of the page.


Chemical synthesis. DH-CBD, DD-CBD, 1-desoxy-THC, and K071 were synthesized after the procedure as described previously (Xiong et al., 2011).

Synthesis of CBD analogues. Proton nuclear magnetic resonance (1H NMR, 500 MHz) and carbon nuclear magnetic resonance (13C NMR, 500 MHz) spectra were recorded on a Bruker-500 instrument in CDCl3 (unless otherwise noted) with the values given in ppm (TMS as internal standard) and J (Hz) assignments of 1H resonance coupling. The high resolution electrospray ionization (ESI) mass spectra were obtained on a Waters LCT Premier time-of-flight (TOF) mass spectrometer. Thin-layer chromatography (TLC) was performed on 0.25 mm Analtech GHLF silica gel. Flash column chromatography was performed with Bodman silica gel LC 60 A. Elemental analyses were performed by Atlantic Microlabs, Inc.

**p-Mentha-1,8-dien-3-yl 3-pentylphenyl ether (K065, 3)**. A solution of (+)-trans-p-Mentha-1,8-dien-(2,8)-ol (945 mg, 6.21 mmol), m-pentyl phenol (926 mg, 5.64 mmol), and N,N-Dimethylformamid-dinopentylacetal (1.69 g, 7.34 mmol) in 33 ml anhydrous methylene chloride was stirred for 3 d at room temperature (20°C). The mixture was condensed and the crude product was purified by column chromatography on silica using Hexanes/Benzene (10:1) as a mobile phase to give the less polar product 2 as a colorless oil (710 mg, 43% based on 950 mg, 5.64 mmol), and 2-(3-methyl-6-(prop-1-en-2-yl)cyclohex-2-enyl)-5-chlorophenol (K271, 7) 

**p-Mentha-1,8-dien-3-yl 3-pentylphenyl ether (K065, 3)**. A solution of (+)-trans-p-Mentha-1,8-dien-(2,8)-ol (2550 mg, 3.61 mmol), m-(2-hydroxy)ethyl phenol (452 mg, 3.27 mmol), and N,N-Dimethylformamid-dinopentylacetal (972 mg, 4.20 mmol) in 20 ml anhydrous methylene chloride was stirred for 3 d at room temperature (20°C). The mixture was condensed and the crude product was purified by column chromatography on silica using hexanes/ethyl acetate (6:4) as a mobile phase to give product as a colorless oil (98 mg, 11% based on 550 mg, 3.61 mmol), and 2-(3-methyl-6-(prop-1-en-2-yl)cyclohex-2-enyl)-5-chlorophenol (K271, 7)

**p-Mentha-1,8-dien-3-yl 3-pentylphenyl ether (K065, 3)**. A solution of (+)-trans-p-Mentha-1,8-dien-(2,8)-ol (550 mg, 3.61 mmol), m-(2-hydroxy)ethyl phenol (420 mg, 3.27 mmol), and N,N-Dimethylformamid-dinopentylacetal (972 mg, 4.20 mmol) in anhydrous methylene chloride (15 ml) was formed with Bodman silica gel LC 60 A. Elemental analyses were performed by Atlantic Microlabs, Inc.
<table>
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<th>Chemical name</th>
<th>Chemical structure</th>
<th>$\text{CB}_1 K_i$ values</th>
<th>$\text{CB}_2 K_i$ values</th>
<th>Potentiation (1 $\mu$M)</th>
<th>$\text{EC}_{50}$ of potentiation (µM)</th>
<th>Changes of PWL</th>
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<td></td>
<td></td>
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<td>$\mu$M</td>
<td>%</td>
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Figure S1. Structural and functional relationships of 11 cannabinoids.
Figure S2. Schematic illustration of synthesis of CBD analogies. Reagents and conditions: (a) DMF-dineopentyl acetal, DCM, at room temperature for 3 d.