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## The membrane proximal region of the cannabinoid receptor CB<sub>1</sub>, N-terminus can allosterically modulate ligand affinity

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### Abstract

The human cannabinoid receptor, CB<sub>1</sub>, a G protein-coupled receptor (GPCR), contains a relatively long (~110 a.a.) amino terminus, whose function is still not defined. Here we explore a potential role for the CB<sub>1</sub> N-terminus in modulating ligand binding to the receptor. We find that although most of the CB<sub>1</sub> N-terminus is not necessary for ligand binding, previous studies have found that mutations introduced into a conserved membrane proximal region (MPR) do impair the receptors ability to bind ligand. Moreover, within the highly conserved MPR (~ residues 90–110) lie two cysteine residues that are invariant in all CB<sub>1</sub> receptors. We find these two cysteines (C98 and C107) form a disulfide in heterologously expressed human CB<sub>1</sub>, and this C<sub>98</sub>-C<sub>107</sub> disulfide is much more accessible to reducing agents than the previously known disulfide in extracellular loop 2 (EL2). Interestingly, the presence of the C<sub>98</sub>-C<sub>107</sub> disulfide modulates ligand binding to the receptor in a way that can be quantitatively analyzed by an allosteric model. The C<sub>98</sub>-C<sub>107</sub> disulfide also alters the effects of allosteric ligands for CB<sub>1</sub>, Org 27569 and PSNCBAM-1. Together, these results provide new insights into how the N-terminal MPR and EL2 act together to influence the high-affinity orthosteric ligand binding site in CB<sub>1</sub>, and suggest the CB<sub>1</sub> N-terminal MPR may be an area through which allosteric modulators can act.

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The cannabinoid receptor, CB<sub>1</sub>, is a G protein-coupled receptor (GPCR) found in high concentrations in the central nervous system (1). CB<sub>1</sub> has been shown to mediate neurotransmitter release in pre-synaptic terminals (2–4), by coupling with G<sub>i</sub> or G<sub>o</sub> proteins, which then inhibit adenylyl cyclase (5, 6), N- and P/Q-type calcium channels (7), and activate A-type inwardly rectifying potassium channels (8). The resulting modulation in amplitude and frequency of neurotransmission thus induced by CB<sub>1</sub> activation is thought to be one of the causes for the psychotropic effects known to accompany cannabis use.

From a biochemical and structural perspective, one intriguing question about CB<sub>1</sub> has been the purpose and role of its relatively long (~110 amino acid) N-terminus (Figure 1). The N-terminus has been shown to be involved in receptor biosynthesis and targeting, but its role in ligand binding and receptor activation is still not well defined.

One would not expect the entire N-terminus would be required for ligand binding, since the hydrophobic cannabinoid ligands will partition into the membrane and thus can only interact

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### ASSOCIATED CONTENT

**Supporting Information.** G protein activity of Δ103 truncation mutant, immunoblot analysis of CB<sub>1</sub> receptor mutants used in membrane radioligand binding assays, the effect of DTT on individual Cys to Ala mutants (C<sub>98A</sub>CB<sub>1</sub><sup>WT</sup> and C<sub>107ACB<sub>1</sub>WT</sub>), and the effect of other reducing agents on CB<sub>1</sub> mutants are given in the Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

with at most part of the extracellular N-terminus. Indeed, most of the N-terminus can in fact be deleted without abolishing ligand binding or G protein activation (9–11).

However, some of the N-terminal region closest to the membrane, the so-called membrane proximal region (MPR) is apparently required for ligand binding. Kendall and colleagues have shown that dipeptide insertions into the MPR affects binding of the agonist CP 55940 (10), and we have previously observed that a full deletion of the CB<sub>1</sub> N-terminus (up to residue 113) abolishes ligand binding, but retaining the MPR does not (Figure 2 and S1).

Interestingly, the CB<sub>1</sub> MPR is highly conserved among different species (see Figure 1), and contains two highly conserved cysteine residues (C98 and C107 in human CB<sub>1</sub>), which are invariant across CB<sub>1</sub> N-termini from mammals, birds, fish, and amphibians (Figure 1B). Although previous studies (including our own) have found that these cysteine residues can be mutated to alanine or serine without abolishing agonist and antagonist binding or G protein activation (12, 13), no further investigations into whether these residues form a disulfide have been reported, nor what potential role such a disulfide might play, nor if these residues alter the effects of allosteric CB<sub>1</sub> ligands.

Thus, in the present work, we set out to investigate if a potential disulfide in the CB<sub>1</sub> N-terminal MPR might affect or modulate ligand binding to CB<sub>1</sub>. Our goal was to first determine if a C<sub>97</sub>-C<sub>107</sub> disulfide is in fact present in CB<sub>1</sub> receptors, and if so, if this disulfide could be used as a tool to assess if it (and the MPR to which it is attached) plays a heretofore unappreciated roles in forming and stabilizing the orthosteric binding pocket for CB<sub>1</sub>, thereby acting as built in allosteric modulators of the receptor.

## Experimental Procedures

### Buffers

The definitions for buffers are: PBSSC [0.137 M NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>]; Hypotonic Buffer [5 mM Tris-HCl, 2 mM EDTA, PIC, pH 7.5]; TME [20 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, pH 7.4]; Rat A [320 mM sucrose, 2 mM Tris-EDTA, & 5 mM of MgCl<sub>2</sub>]; and RatB: [50 mM Tris-HCl, pH 7, 2 mM 2 mM Tris-EDTA & 5 mM MgCl<sub>2</sub>].

### Expression and membrane preparations of shCB<sub>1</sub> genes in COS-1 cells

The nomenclature used in the text for the various CB<sub>1</sub> mutants are as follows. Every mutant is referred to as CB<sub>1</sub>. The “wild type” CB<sub>1</sub> receptor refers to a mutant synthetic human CB<sub>1</sub> “wild type” receptor which contains all native 13 cysteine residues and a 1D4 antibody epitope on the C-terminus, and this is referred to as CB<sub>1</sub><sup>WT</sup>. Proteins containing deletions or mutations are denoted as a subscript to the left of CB<sub>1</sub>, and the cysteine “background” used for the mutant is denoted with a superscript on the right of CB<sub>1</sub>. Thus, for example, C<sub>98A</sub>CB<sub>1</sub><sup>WT</sup> indicates that a C98A mutation was introduced into a “wild-type” CB<sub>1</sub> cysteine background. The various mutant DNA were expressed in COS-1 cells by transient transfection and membranes prepared as previously described (13).

### Labeling and SDS-PAGE gel shift studies of CB<sub>1</sub> mutants to assess the presence of a disulfide bond

Purification, labeling, and SDS-PAGE analysis of CB<sub>1</sub> mutants were performed as described previously (11). In brief, CB<sub>1</sub> constructs were purified using a one-step immunoaffinity approach. While bound to the column samples were either subjected to a 20 fold molar excess of bimane (or not) prior to elution and SDS-PAGE analysis in the absence or

presence of reducing agent DTT. At least two separate gels were loaded to confirm the initially observed gel-shift.

### Membrane preparations of rat cannabinoid receptors

Rat cortices were purchased from Pel Freeze Biologicals (Rogers, AR) and membrane preparations were performed as previously described (14). Six grams of cortical tissue were homogenized in 45 ml of Rat A buffer and pelleted via centrifugation ( $1600 \times g$  for 10 min), washed twice as above, and the combined supernatant fractions were then centrifuged at  $39,000 \times g$  for 15 min. The pellet was resuspended in 90 mls of Rat B buffer, incubated at  $37^\circ C$  for 10 min, and subsequently centrifuged at  $11,000 \times g$  for 15 min whereupon the pellet was again resuspended in Rat B buffer, and incubated at  $30^\circ C$  for 40 min. Final centrifugation occurred at  $11,000 \times g$  for 15 min, pellets were homogenized to suspension in TME, aliquoted, snap frozen and stored at  $-80^\circ C$  until use. Protein concentration was determined using the modified DC protein assay kit (Bio-Rad).

### DTT treatment of CB<sub>1</sub> containing membranes

Membrane preparations containing receptor protein were diluted to 4.4 mg/ml of total protein. Importantly, samples were passed through a 24 gauge needle 5 times and allowed to sit on ice for 1 hour prior to treatments. Treatments consisted of diluting membrane preparations to 2.2 mg/ml in various concentrations of DTT (0–300 mM) and allowing the treated samples to nutate at room temperature for 20 min. Treated membranes were then immediately used for equilibrium binding studies.

### Radioactive Ligand binding studies

The ligand binding experiments were carried out as previously described (11, 13). Data was globally fit and error estimates for the parameters were derived from least square fits. All radioactive binding experiments were performed at least twice in duplicate, unless otherwise indicated. Additionally, an allosteric two-site model (equation 1), described previously (15), was used to fit our data:

$$FractionBound = \frac{[A] \left( 1 + \frac{\alpha[B]}{K_{B2}} \right)}{[A] \left( 1 + \frac{\alpha[B]}{K_{B2}} \right) + K_A \left( 1 + \frac{[B]}{K_{B1}} + \frac{[B]}{K_{B2}} \left( 1 + \frac{\beta[B]}{K_{B1}} \right) \right)} \quad (1)$$

where R denotes the receptor; A denotes the orthosteric ligand and B denotes the allosteric ligand. The  $K_A$ ,  $K_{B1}$  and  $K_{B2}$  are the dissociation constants where the subscript B1 and B2 represent the two sites that the allosteric ligand can interact with, orthosteric site and allosteric site, respectively. The cooperativity factors,  $\alpha$  and  $\beta$ , denote the allosteric interaction between A and B or between the two molecules of B. Cooperativity values greater than 1 denote positive cooperativity (increased affinity) whereas values less than one denote negative cooperativity (decreased affinity), and values equal to one are neutral.

## Results

### Biochemical Evidence of N-terminal disulfide in CB<sub>1</sub>

The only previously confirmed disulfide bond in CB<sub>1</sub> is in extracellular loop 2 (EL2), between C257 and C264<sup>1</sup>. For example, as shown in Figure 3A, a purified CB<sub>1</sub> mutant containing only the two cysteine residues at 257 and 264 (termed C<sub>98A,C107A</sub>CB<sub>1</sub><sup>PUR</sup>) is

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<sup>1</sup>Interestingly, this disulfide appears to take the place of the canonical disulfide bridge connecting EL2 to transmembrane helix 3 present in most Class A GPCRs.

unreactive to thiol-reactive fluorophores, unless first treated with reducing agents (see also Ref (11)) providing biochemical evidence that these two cysteines in extracellular loop two (EL2) are in a disulfide bond consistent with previous mutagenesis studies (12, 13).

We became interested in the possibility that C98 and C107 in the CB<sub>1</sub> N-terminus also form a disulfide based on observations made during experiments aimed at identifying a non-reactive CB<sub>1</sub> “background” mutant for fluorescence studies (11). We noticed that a purified CB<sub>1</sub> mutant containing the N-terminal cysteines, C98 and C107, in addition to the two EL2 cysteines C257 and C264 (called CB<sub>1</sub><sup>PUR</sup>) was also non-reactive to fluorescent labeling (Figure 3B), as was our non-reactive background C<sub>98A,C107A</sub>CB<sub>1</sub><sup>PUR</sup>.

Like C<sub>98A,C107A</sub>CB<sub>1</sub><sup>PUR</sup>, CB<sub>1</sub><sup>PUR</sup> also became susceptible to labeling with the thiol-reactive fluorescent probe bimane if treated with DTT prior to labeling suggesting that the C98 and C107 also form a reducible disulfide bond.

Upon closer examination, we also noticed a shift in the coomassie stain of CB<sub>1</sub><sup>PUR</sup> under non-reducing SDS-PAGE conditions (Figure 3A). This was confirmed by comparing the mobility of C<sub>98A,C107A</sub>CB<sub>1</sub><sup>PUR</sup> versus CB<sub>1</sub><sup>PUR</sup> on SDS-PAGE, both with and without reducing agent (Figure 3B). Reduction by DTT causes the purified CB<sub>1</sub><sup>PUR</sup> receptor to run more slowly, comparable to its C<sub>98A,C107A</sub>CB<sub>1</sub><sup>PUR</sup> counterpart (Figure 3B), providing further evidence that an N-terminal disulfide exists that imparts structure and/or stability to the CB<sub>1</sub> receptor.

### Pharmacological evidence that N-terminal cysteines are in a disulfide bond

We reasoned that any effect of an N-terminal disulfide bond on ligand binding must be more subtle than previously appreciated, since almost all of the N-terminus can be deleted while ligand binding ability is retained. Based on previous work by Karnik et al. (16), we decided to explore this phenomenon by systematically monitoring binding of either agonist or antagonist as a function of DTT, using transiently expressed receptors in COS cells (Figure 4).

These results show that DTT causes a decrease in agonist binding, with a surprising concomitant increase in antagonist binding to CB<sub>1</sub><sup>WT</sup> (Figure 4A). We next tested for this effect in a CB<sub>1</sub> mutant in which two cysteines were mutated to alanine (C98A and C107A) in an CB<sub>1</sub><sup>WT</sup> background (referred to as C<sub>98A,C107A</sub>CB<sub>1</sub><sup>WT</sup>). As seen in Figure 4B, the C<sub>98A,C107A</sub>CB<sub>1</sub><sup>WT</sup> mutant does not show the DTT dependent increase and decrease in antagonist and agonist binding, only a decrease in both at very high DTT concentrations. Furthermore, the individual Cys to Ala mutations (C<sub>98A</sub>CB<sub>1</sub><sup>WT</sup> and C<sub>107A</sub>CB<sub>1</sub><sup>WT</sup>) also behave like the double Cys to Ala mutant in that they do not show the DTT dependent increase and decrease in antagonist binding at the lower DTT concentrations (Fig. S5). Thus, we conclude the DTT effect described above is due to reduction of the C<sub>98</sub>-C<sub>107</sub> disulfide bond in the N-terminus.

We reasoned that reduction of the disulfide bond between C257 and C264 in EL2 explains why the higher DTT concentrations abolish binding of both agonist and antagonist, as this disulfide is known to be required for functional CB<sub>1</sub> ligand binding (12, 13). We confirmed this by testing the effect of DTT on our previously characterized C<sub>98A,C107A</sub>CB<sub>1</sub><sup>MC</sup> mutant (13) and found it behaves like C<sub>98A,C107A</sub>CB<sub>1</sub><sup>WT</sup> (Figure 4D). These results are consistent with the interpretation that the C<sub>257</sub>-C<sub>264</sub> disulfide bond in EL2 confers stability to the CB<sub>1</sub> receptor orthosteric site.

The necessity for higher concentrations of DTT also indicates C<sub>257</sub>-C<sub>264</sub> disulfide in EL2 is in less accessible and/or is more readily reversible than the C<sub>98</sub>-C<sub>107</sub> disulfide in the N-terminus. Other reducing agents (TCEP and GSH) showed a similar behavior, requiring

higher concentrations to reduce the EL2 disulfide (Figure S2), again supporting the presumably inaccessible location of the C<sub>98</sub>-C<sub>107</sub> disulfide bond. Furthermore, when the N-terminal cysteine pair is re-introduced into C<sub>98A,C107A</sub> CB<sub>1</sub><sup>MC</sup> (a mutant we call CB<sub>1</sub><sup>MC</sup>) the DTT dependent change in ligand binding is restored (Figure 4C).

Importantly, the DTT effect is also observed in experiments carried out on rat cerebral cortex membranes (CB<sup>RAT</sup>, Figure 4E), which contain native cannabinoid receptors. This important control confirms that the DTT effect on ligand binding is not just an artifact caused by the use of a transient expression system (COS cells) to express the mutant human CB<sub>1</sub> receptors.

Finally, we tested the possible functional effect of the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide bond using a CB<sub>1</sub>-G protein fusion system. These assays measured the ability of a CB<sub>1</sub> agonist to stimulate radioactive GTP $\gamma$ S<sup>[35]</sup> binding to a CB<sub>1</sub><sup>WT</sup> receptor with a G<sub>ai</sub> subunit fused to its C-terminal tail (13). The results, shown in Fig. 4F, indicate that addition of DTT under conditions that should selectively reduce the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide, but not the EL2 C<sub>257</sub>-C<sub>264</sub> disulfide, causes a small but reproducible shift in the EC<sub>50</sub> value of agonist required for G-protein activation (the conditions were the same as the maximum inflection point on Fig. 4A, 10 mM DTT treatment for 20 min prior to the assay).

### Analysis suggests the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide allosterically regulates the CB<sub>1</sub> orthosteric binding site

The behavior seen in Figure 4 is highly indicative of an allosteric effect, where reducing the allosteric N-terminal disulfide imparts positive cooperativity to the orthosteric (antagonist) ligand binding site, and negative cooperativity to agonist binding. We tested this using an allosteric two-site model, and find excellent agreement of the fits to our experimental data ( $R^2 = 0.93 \pm 0.02$ ). The model, represented in a cartoon in Figure 6A and schematically in Scheme 1, presumes that the DTT dependent enhancement/decrease of binding at the N-terminal disulfide is the “other site” (i.e., allosteric), and that EL2 disulfide is part of the orthosteric site, as it is known that the EL2 disulfide is required for orthosteric ligand binding (12, 13). Note that in this two-site model, DTT is not binding to these sites in a traditional sense, but rather, is modifying/interacting with these two sites in a manner that can be interpreted (for the case of this model) as ‘binding.’

Although not perfect, this two-site model provides a unique way to conceptualize and quantify our data, and the fits indicate both positive or negative cooperativity ( $\alpha$ ) is imparted on the orthosteric ligand by modulation of the ‘allosteric effect’ disulfide in the N-terminus (Table 2). Moreover, the mean dissociation constant indicates a high “affinity” of DTT towards for N-terminal cysteines. In contrast, the EL2 disulfide shows a lower “affinity” for DTT (Table 2), consistent with a model where the N-terminal cysteine residues more accessible than the EL2 disulfide.

It is important to note that the dissociation values derived from our fits likely depend on the experimental conditions (time, temperature, concentration, etc.), and we did not explore the effect of these possible variables. Also, it might formally be possible to fit our data with a dimer/oligomer model, but since we find no evidence that significant amounts of our CB<sub>1</sub> mutants form dimers through a disulfide by SDS-PAGE analysis (see Figure S3) we have not done so. The implications of conformational coupling between the N-terminal disulfide and the orthosteric site are discussed in greater detail below.

## The N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide bond affects the actions of CB<sub>1</sub> allosteric ligands Org 27569 and PSNCBAM-1

The fact that the N-terminal MPR can act to allosterically modulate ligand binding to CB<sub>1</sub> (described above) is reminiscent of the way some small molecule allosteric ligands can affect GPCRs. Several such allosteric ligands exist for CB<sub>1</sub>, but their ability to modulate receptor binding and function has not been tested in relation to the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide bond in CB<sub>1</sub> we describe here. Thus, we tested how a known CB<sub>1</sub> specific allosteric ligand Org 27569 (11, 17) is affected by the presence or absence of the N-terminal disulfide. We find the positive cooperativity of Org 27569 ( $\alpha = 4.9 \pm 0.4$ ) for agonist binding is significantly enhanced (Figure 5) when the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide is removed by either mutation or incubation of the sample with 10 mM DTT (combined mean value  $\alpha = 7.2 \pm 0.6$  for the two conditions [ $t(8) = -3.3$ ,  $p < 0.02$ ]). Interestingly, the effects of the allosteric antagonist, Org 27569 are additive with the allosteric effects of the N-terminal disulfide. For instance, the sum of cooperativity factors (4.9 ± 0.4 for ‘wt CB<sub>1</sub>’ (Figure 5) and 2.3 ± 0.5 for antagonist binding (Figure 4, Table 2) is equal to the higher cooperativity value observed for Org 27569 when the N-terminal disulfide is absent (7.2 ± 0.6 vs. 7.2 ± 0.6). We also tested the effect of DTT reduction on another allosteric ligand for CB<sub>1</sub>, PSNCBAM-1. As shown in Fig. 5C, addition of DTT at a concentration shown to reduce the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide bond resulted in an increase in agonist binding.

## Discussion

Our data indicate the membrane proximal region (MPR) of the CB<sub>1</sub> N-terminus can affect the ligand binding properties of the receptor in a previously unappreciated way, and yield insights into where known allosteric ligands may exert their action on CB<sub>1</sub>. Specifically, we find direct evidence for an intramolecular disulfide in the CB<sub>1</sub> N-terminal MPR (Figure 3B), and find that reduction of this disulfide results in altered ligand binding in an unexpected way when ligand binding is monitored as a function of increasing DTT (Figure 4).

At lower DTT concentrations, reducing the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide can impart either positive or negative cooperativity ( $\alpha$ ) on the orthosteric ligand binding site, depending on whether the ligand is an antagonist or agonist. High DTT concentrations caused a clear attenuation of ligand binding, presumably due to reduction of the EL2 disulfide (Figure 4). These results are consistent with previous mutation studies that suggest a disulfide between C257/C264 in EL2 is critical for the stability of the orthosteric ligand binding site (12, 13). Below, we discuss the implications resulting from analyzing this data using an allosteric two-site model.

### The effect of DTT on ligand binding can be described using an allosteric model

Our findings suggest that the CB<sub>1</sub> N-terminal cysteines allosterically regulates the binding characteristic for the orthosteric site on CB<sub>1</sub>, since reducing the N-terminal disulfide with DTT changes the affinity for agonist and antagonists in a reciprocal manner. This reciprocity is illustrated by the cooperativity factors – for instance, the inverse of the mean positive cooperative value for antagonist binding ( $\alpha_{SR}^{-1} = 0.50 \pm 0.09$ ) is close to the mean negative cooperative for agonist binding ( $\alpha_{CP} = 0.69 \pm 0.08$ ). It is also reasonable to assume the converse effects may exist, where agonist binding is enhanced by the presence of an N-terminal disulfide, and antagonist specific binding is reduced by the presence of an N-terminal disulfide.

### The N-terminal disulfide in CB<sub>1</sub> is more solvent accessible than the EL2 disulfide

Based on the K<sub>B1</sub> and K<sub>B2</sub> values determined from the model (Table 2), we find the EL2 disulfide (K<sub>B1</sub>) has an approximately 20-fold higher dissociation constant than the N-

terminal disulfide ( $K_{B2}$ ). Our data suggest that the N-terminal disulfide is much more accessible to reducing agents than the EL2 disulfide, consistent with our results using other reducing agents (see also Figure S2)

The fact that high DTT concentrations are required to reduce the EL2 disulfide is reminiscent of rhodopsin, where the highly conserved disulfide between EL2 and TM3 is completely buried and inaccessible to reducing agents (in the absence of denaturants) (18).

### Possible structural roles of an N-terminal disulfide in CB<sub>1</sub>

What insights do these data and analysis provide about the role of the CB<sub>1</sub> N-terminus? One insight from our data is that the CB<sub>1</sub> N-terminal MPR couples with the orthosteric ligand binding site. Based on this observation, we propose that this part of the N-terminus forms a ‘lid’ over the orthosteric binding site (as depicted in our model in Figure 6B). This idea is intriguing since almost all known GPCR structures to date show solvent accessible binding pockets, except for rhodopsin and sphingosine 1 phosphate receptor (S1P1), two GPCRs that, like CB<sub>1</sub>, bind hydrophobic ligands. Rhodopsin and S1P1 both show significant occlusion of the orthosteric binding pocket and limited solvent accessibility presumably due to the hydrophobic nature of their endogenous ligands. Since cannabinoid ligands are also hydrophobic, it is reasonable to speculate that CB<sub>1</sub> also possesses an occluded binding pocket.

In summary, our results suggest the highly-conserved N-terminal MPR of CB<sub>1</sub> may play several roles. One role is to interact with and allosterically regulate the orthosteric binding site of CB<sub>1</sub>, leading to a subtle change in conformational and functional states of the receptor. This may explain the highly conserved nature of this region. Another role for the CB<sub>1</sub> N-terminus, stabilized by the C<sub>98</sub>-C<sub>107</sub> disulfide, could also be to act as a domain over the binding pocket in CB<sub>1</sub>. Thus, we propose there is domain coupling at least between the CB<sub>1</sub> N-terminal MPR and the ligand binding pocket of CB<sub>1</sub>, where reduction of the exposed N-terminal disulfides can perturb the N-terminal domain structure and thus the entire conformational landscape of the extracellular domain.

Interestingly, the N-terminus of rhodopsin plays a similar role for that receptor. It forms a stable domain, and when constrained to the receptor by engineered disulfides, produces a receptor with enhanced thermostability (19). Other GPCRs have also shown a role of extracellular disulfides, including some chemokine receptors (20, 21) and the angiotensin II receptor (16). Interestingly, metabotropic glutamate and calcium sensing receptors have been reported to have intermolecular disulfides between receptors (22, 23), but we have not found evidence for this in CB<sub>1</sub> (Figure S3).

### Possible role for the CB<sub>1</sub> N-terminal MPR in binding allosteric ligands and/or enabling them to exert their influence

As with several other GPCRs, CB<sub>1</sub> has been shown to undergo regulation by allosteric exogenous ligands, several of which have been described (17, 24–29). Our results in Figure 5 indicate interplay between the agonist CP 55940, the allosteric ligands Org 27569 (and PSNCBAM-1), and the CB<sub>1</sub> MPR and N-terminal disulfide. One possible mechanism through which this occurs could be that allosteric ligands bind near this region and affect the orthosteric ligand binding/dissociation pathway, thereby preventing orthosteric ligand dissociation. Consistent with this, the allosteric compounds described by Price et al. have been shown to slow the dissociation of CP 55940 from the orthosteric binding site (17). Recent computational studies on the muscarinic acetylcholine receptor 3 suggest an orthosteric ligand with slower dissociation rates toward M3 can bind to a second site along the binding pathway (30). Thus, it is tempting to speculate that small-molecule allosteric

CB<sub>1</sub> allosteric ligands may work in a similar fashion. However, given the lipophilic nature of cannabinoid ligands, this site would presumably be in the egress pathway that connects the orthosteric binding site to the membrane, and not directly to extracellular parts of the N-terminus.

Interestingly, the lipid binding GPCR S1P1 structure hints at a lipid access channel between TM1 and TM7 (31). Reggio and coworkers have proposed a similar channel may exist between helices in cannabinoid receptors (32–34). If such a channel exists in CB<sub>1</sub>, it may represent an area where the N-terminal MPR of CB<sub>1</sub> induces its allosteric effect. In support of this model, we find that the absence of the N-terminal disulfide in the MPR enhances the cooperativity between the allosteric ligand Org 27569 and the agonist CP 55940 (Figure 5). However, since the cooperativity for these interactions appears to be additive, it is likely that these two allosteric events occur through somewhat different mechanisms.

### Possible biological roles for CB<sub>1</sub> N-terminal disulfide

Finally, although we have no direct evidence for this yet, it is tempting to speculate that the N-terminal disulfide in CB<sub>1</sub> could act as a redox sensor. Redox-dependent structural switches have been observed in other proteins, such as OxyR, INAD, and angiotensin II (35). A redox sensor in CB<sub>1</sub> could be neuroprotective, since traumatic brain injury results in the release of endocannabinoids in addition to reactive oxygen intermediates (36, 37). Taken together, this redox-switch could potentially enhance CB<sub>1</sub> receptor ligand occupancy and, in part, modulate the neuroprotective properties of CB<sub>1</sub> activation. Altering the effect would likely be subtle (as suggested by our functional data in Figure 5F), but it could change the ‘set point’ at which the receptor responds to endogenous signals.

We also note that the antioxidant tripeptide glutathione (GSH) can be released from neurons in a depolarization-dependent fashion (38) and GSH has been shown to play a role in modulation of excitatory neurotransmission (39) similar to endocannabinoids (40). Thus, GSH release may be a way to locally regulate presynaptic CB<sub>1</sub> receptor ‘set points.’ Moreover, impaired GSH homeostasis or increase in reactive oxygen species is associated with diseases (41) that coincide with CB<sub>1</sub> receptor-associated diseases, including Parkinson’s and Alzheimer’s (1). The importance of the potential disulfide bond-mediated redox sensitivity in normal and diseased states has implications in the development of antioxidant-based therapeutic approaches and warrants further exploration.

In summary, we find clear evidence for the presence of an extracellular disulfide bond between C98 and C107 in the N-terminus of CB<sub>1</sub>, evidence that the MPR of the CB<sub>1</sub> N-terminus acts to allosterically modulate agonist and antagonist binding, and evidence that this disulfide can affect the behavior of known CB<sub>1</sub> allosteric ligands. Together these data suggest the mechanism of action for some CB<sub>1</sub> allosteric ligands may lie in the MPR of the CB<sub>1</sub> N-terminus. Future studies are underway to test this theory and further investigate the mechanism of this allosteric effect.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## ABBREVIATIONS

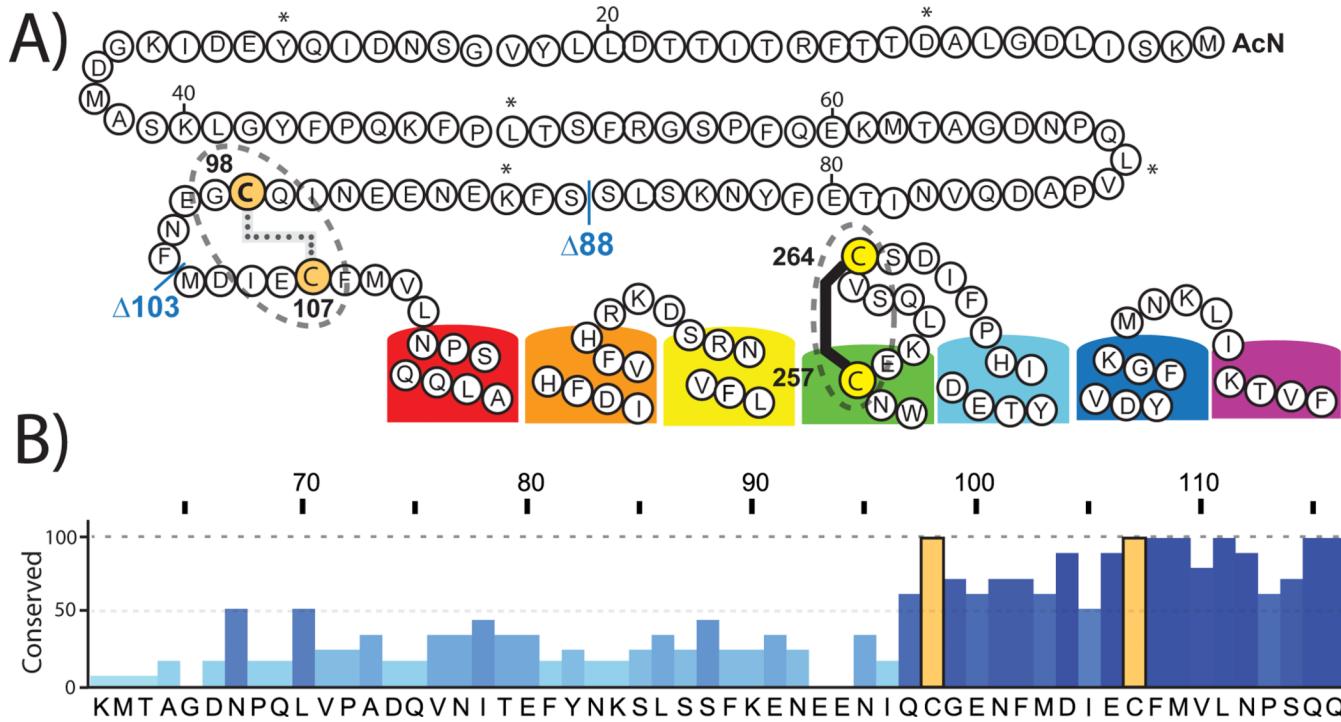
<b>GPCR</b>	G protein-coupled receptor
<b>CB<sub>1</sub></b>	cannabinoid type-1 receptor
<b>CP55940</b>	(-)-cis-3-[2-hydroxy-4-(1,1-dimethyl- heptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol
<b>SR141716A</b>	5-(4-chloro-phenyl)-1-(2,4-dichloro-phenyl)-4- methyl-1H-pyrazole-3-carboxylic acid piperidin-1-ylamide hydrochloride
<b>PDT-bimane</b>	2,3,6-trimethyl-5-[(2-pyridinyldithio)methyl]-1H,7H-pyrazolo[1,2-a]pyrazole-1,7-dione
<b>shCB1</b>	synthetic human CB1 receptor
<b>DTT</b>	Dithiothreitol
<b>CB<sub>1</sub><sup>WT</sup></b>	synthetic human “ <u>wild type</u> ” CB <sub>1</sub> receptor with all 13 cysteines retained and a 1D4 antibody epitope on the C-terminus
<b>Δ103CB<sub>1</sub><sup>WT</sup></b>	CB <sub>1</sub> <sup>WT</sup> with N-terminal truncation at site 103
<b>C98A,C107A CB<sub>1</sub><sup>WT</sup></b>	CB <sub>1</sub> <sup>WT</sup> receptor containing C98A and C107A mutations
<b>CB<sub>1</sub><sup>MC</sup></b>	“ <u>Minimal Cysteine</u> ” CB <sub>1</sub> background receptor has only N-terminal disulfide (C <sub>98</sub> -C <sub>107</sub> ), and EL2 disulfide (C <sub>257</sub> -C <sub>264</sub> )
<b>C98A,C107A CB<sub>1</sub><sup>MC</sup></b>	CB <sub>1</sub> <sup>MC</sup> receptor without C <sub>98</sub> -C <sub>107</sub> N-term disulfide (C98, C107 mutated to alanine)
<b>CB<sub>1</sub><sup>PUR</sup></b>	CB <sub>1</sub> <sup>MC</sup> receptor with N- and C- terminal truncations (Δ88 and Δ417, respectively)
<b>C98A,C107A CB<sub>1</sub><sup>PUR</sup></b>	CB <sub>1</sub> <sup>PUR</sup> receptor lacking the C <sub>98</sub> -C <sub>107</sub> N-term disulfide (C98, C107 mutated to alanine), a CB <sub>1</sub> mutant used for <u>purification</u>
<b>CB<sup>RAT</sup></b>	wild-type CB receptors present in membranes prepared from <u>rat</u> brains
<b>TCEP</b>	(tris(2-carboxyethyl)phosphine
<b>EL2</b>	extracellular loop 2
<b>TM</b>	transmembrane helix
<b>PIC</b>	protease inhibitor cocktail

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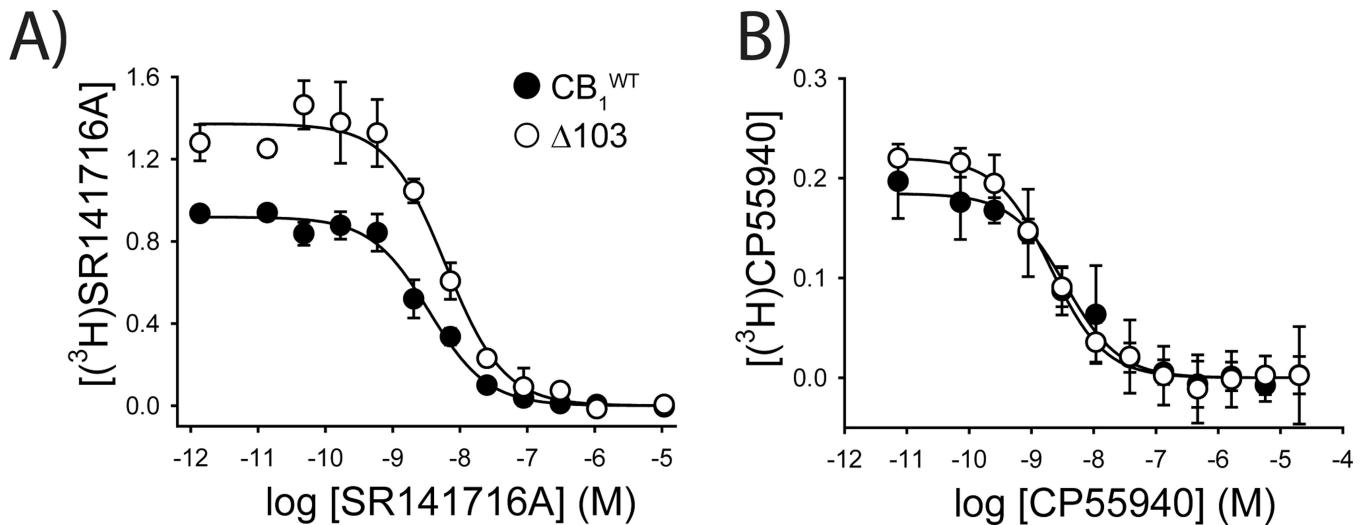
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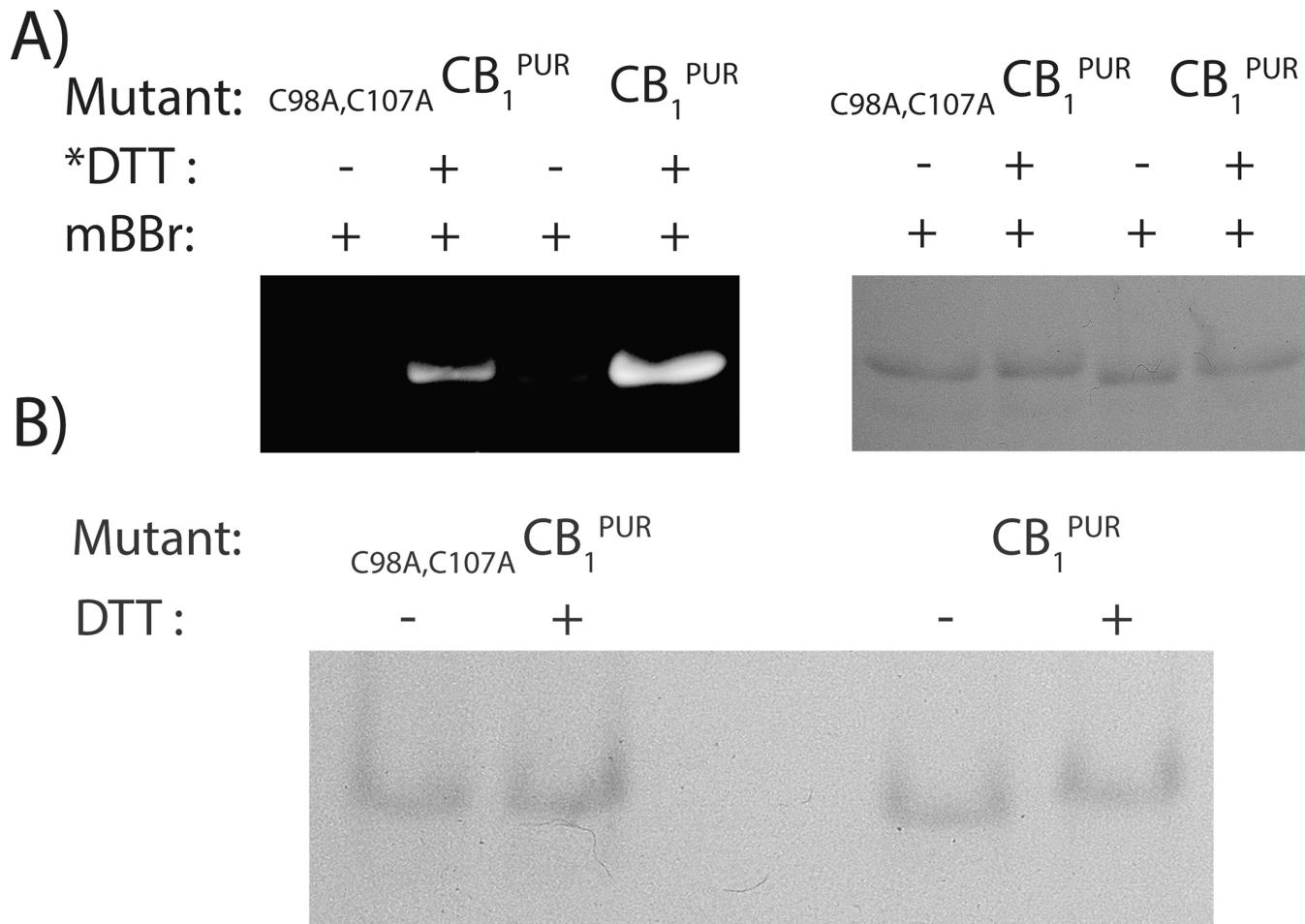
**Figure 1.**

The long N-terminus of CB<sub>1</sub> has a highly conserved membrane proximal region (MPR) containing two conserved cysteine residues. (A) Two-dimensional model of human cannabinoid (CB<sub>1</sub>) receptor showing the extracellular region as well as the sites of cysteines and deletions studied in the present work. Disulfide pairs C<sub>98</sub>-C<sub>107</sub> and C<sub>257</sub>-C<sub>264</sub> are depicted as filled yellow or orange circles. The sites for truncation mutants Δ88 and Δ103 are also indicated. (B) The CB<sub>1</sub> N-terminal MPR is highly conserved, as indicated by alignment conservation plot of various sequences taken from a broad selection of species (sequences extracted from GPCR.org). The multiple sequence alignment conservation was based on the AMAS program (42). Cysteines (human C<sub>98</sub> and C<sub>107</sub>) are colored in orange.

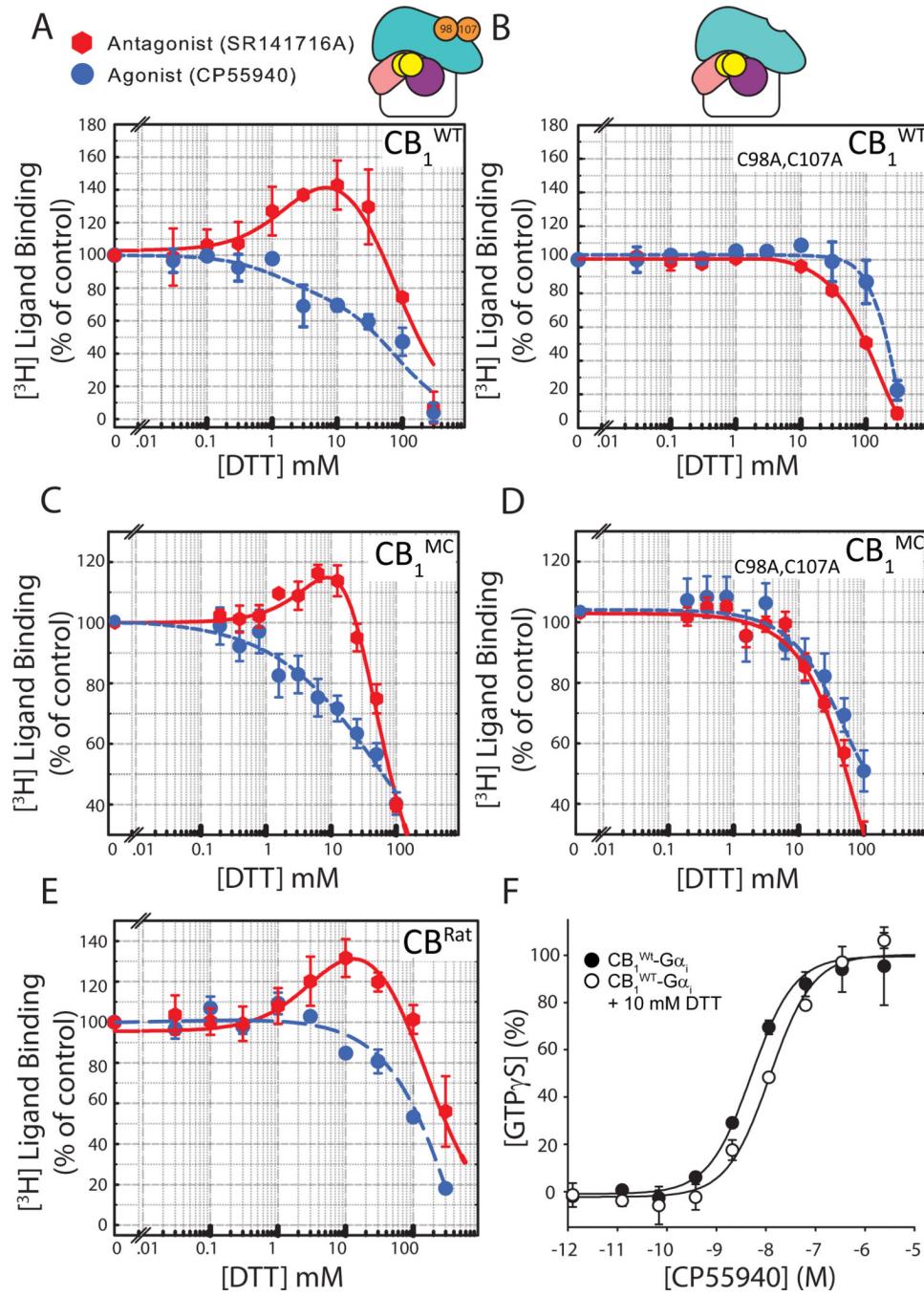


**Figure 2.**

Most of the  $\text{CB}_1$  N-terminus can be deleted without abolishing ligand binding. Competitive inhibition binding studies comparing  $\text{CB}_1^{\text{WT}}$  (●) and  $\Delta 103\text{CB}_1^{\text{WT}}$  (○) to binding tritiated (A) antagonist SR141716, and (B) agonist CP 55940. Binding was carried out using a Brandel 24-well filtration apparatus, and the data fit with a one-site binding model. Data represent one binding experiment performed in duplicate. See Experimental Procedures for more details.

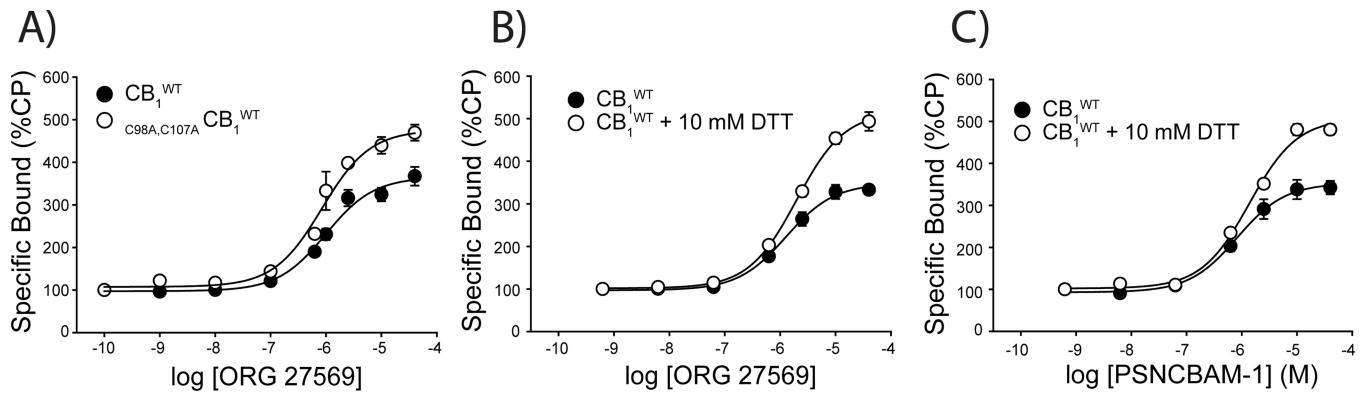
**Figure 3.**

Biochemical evidence for a disulfide between C98 and C107 in the CB<sub>1</sub> N-terminus is indicated by their lack of reactivity towards thiol-specific probes, and their presence causing a faster running species on a non-reduced SDS-PAGE. (A) The purified, minimal-cysteine construct mutants C98A,C107A CB<sub>1</sub><sup>PUR</sup> (containing C257 and C264) and CB<sub>1</sub><sup>PUR</sup> (containing C98,C107, C257 and C264) do not react to thiol-reactive fluorophore bimane as indicated by the lack of detectable in-gel bimane fluorescence in the absence of DTT (left), even though equivalent amounts of protein are present (right, coomassie staining of the same gel). However, reducing both samples with DTT (+DTT) prior to bimane labeling, results in label incorporation. (B) Evidence for a disulfide is also observed when the mobility for the purified CB<sub>1</sub> receptor C98A,C107A CB<sub>1</sub><sup>PUR</sup> and CB<sub>1</sub><sup>PUR</sup> are compared in the presence or absence of DTT. In non-reducing conditions, CB<sub>1</sub><sup>PUR</sup> runs faster than C98A,C107A CB<sub>1</sub><sup>PUR</sup>, but this behavior is abolished upon DTT treatment.

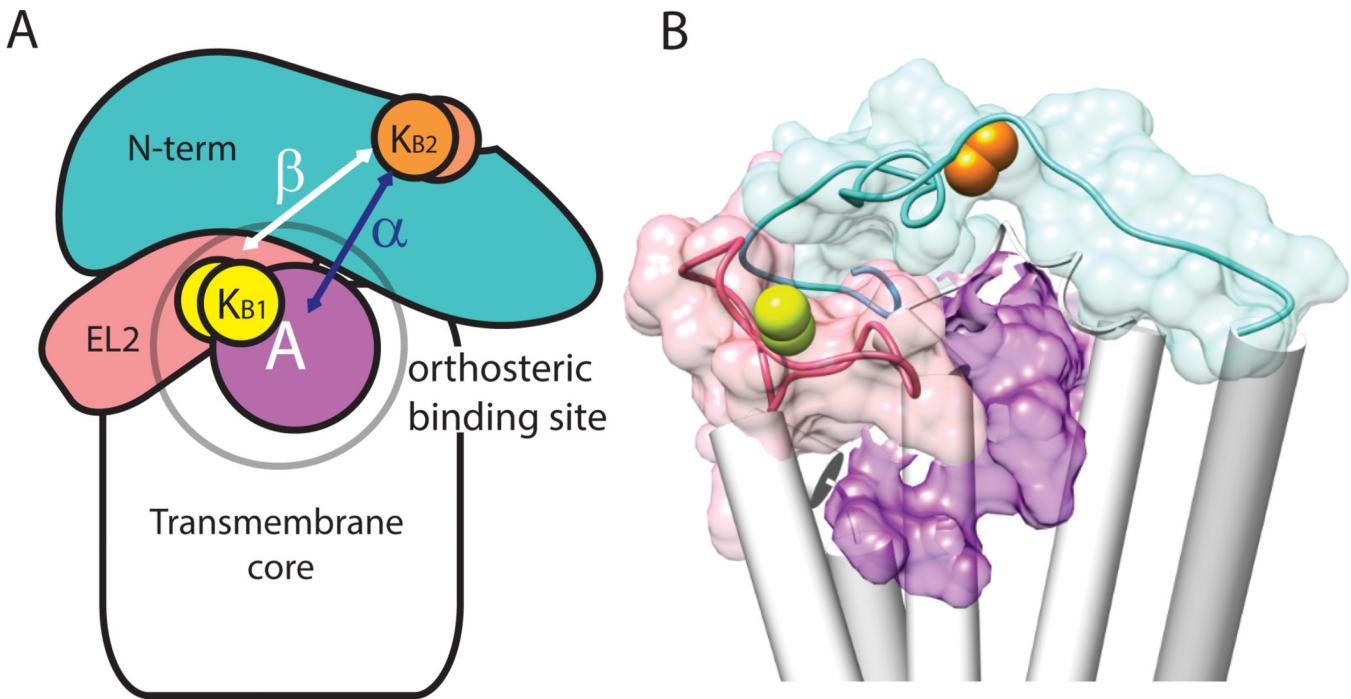
**Figure 4.**

Increasing concentrations of the reducing agent DTT causes different allosteric modulation of agonist (CP55940, blue circles) and antagonist (SR14171A, red hexagons) ligand binding to  $\text{CB}_1$ . (A) Effect of DTT on ligand binding to “wild-type” ( $\text{CB}_1^{\text{WT}}$ ) receptors. (B) Effect of DTT on ligand binding to a receptor lacking N-terminal cysteines ( $\text{C98A,C107A CB}_1^{\text{WT}}$ ). (C) Binding to  $\text{CB}_1$  mutant containing only C98, C107, C257, and C264 ( $\text{CB}_1^{\text{MC}}$ ). (D) Binding to  $\text{CB}_1$  mutant containing only C257 and C264 ( $\text{C98A,C107A CB}_1^{\text{MC}}$ ). (E) Binding to wild-type cannabinoid receptors present in membranes prepared from rat cortices ( $\text{CB}^{\text{RAT}}$ ). (F) Agonist-stimulated  $\text{GTP}\gamma\text{S}$  binding to  $\text{CB}_1^{\text{WT}}-\text{G}\alpha_i$  in the (●) absence ( $\text{EC}_{50} = 5.1 \pm 1 \text{ nM}$ ) or (○) presence of a 10 mM DTT pretreatment ( $\text{EC}_{50} = 12 \pm 2 \text{ nM}$ ). Experiments were

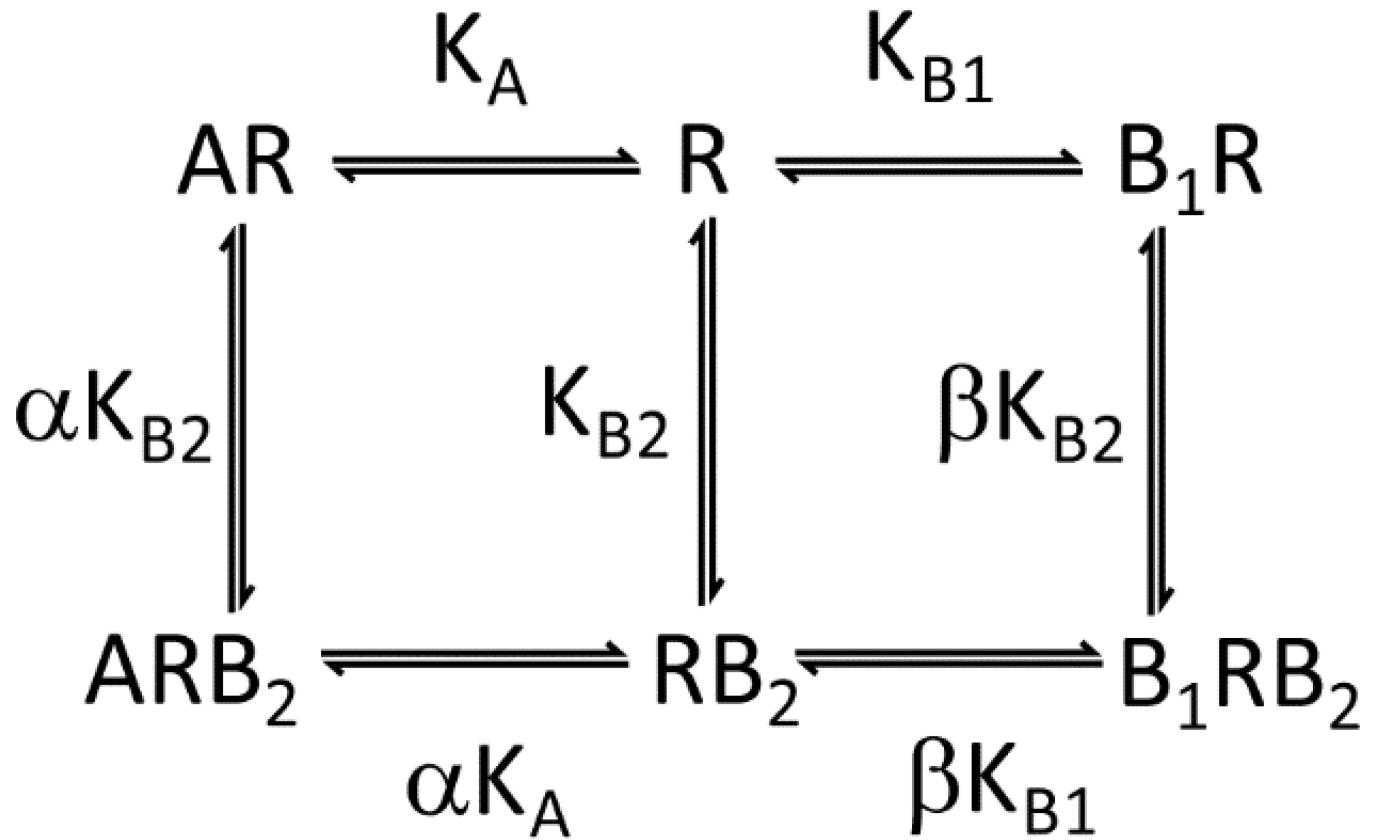
performed at least twice in duplicate and data are presented as the mean  $\pm$  S.E.M. Data is normalized to specific fraction bound for respective radioligands determined in the absence or presence of saturating concentration of respective cold ligand. For more details see Experimental Procedures.

**Figure 5.**

The CB<sub>1</sub> N-terminal disulfide alters the cooperativity of the allosteric ligand Org 27569 and PSCNBAM-1. Equilibrium binding of agonist [<sup>3</sup>H]CP55940 were determined in the presence of various concentrations of allosteric modulator. (A) The effect of Org 27569 on agonist binding of CB<sub>1</sub><sup>WT</sup> (●) compared with a CB<sub>1</sub> mutant lacking the N-terminal disulfide (A, C98A,C107A CB<sub>1</sub><sup>WT</sup>, ○). (B) Comparison of the effect of Org 27569 on CB<sub>1</sub><sup>WT</sup> treated with 10 mM DTT for 20 min prior to the assay (B, 10 mM DTT ○). (C) Comparison of the effect of PSCNBAM-1 on CB<sub>1</sub><sup>WT</sup> treated with 10 mM DTT for 20 min prior to the assay (C, 10 mM DTT ○). The data show that removing the CB<sub>1</sub> N-terminal disulfide enhances the effects of the allosteric ligands Org 27569 and PSCNBAM-1.. When the allosteric ternary complex is fit to the above data, the resulting allosteric cooperativity factors with respect to Org 27569 are  $4.9 \pm 0.4$  for CB<sub>1</sub><sup>WT</sup> and  $7.8 \pm 0.5$  or  $6.8 \pm 0.3$  for C98A,C107A CB<sub>1</sub><sup>WT</sup> or CB<sub>1</sub><sup>WT</sup> treated with 10 mM DTT, respectively. With respect to PSCNBAM-1, the cooperativity factors are  $4.2 \pm 0.2$  for CB<sub>1</sub><sup>WT</sup> and  $5.9 \pm 0.3$  for CB<sub>1</sub><sup>WT</sup> treated with 10 mM DTT. Each radioactive binding shown above is representative of at least two independent experiments performed in triplicate and is reported as the mean  $\pm$  S.E.M. The cooperativity factors were determined by fitting the combined respective data sets, and errors were determined from least squares fitting. See Experimental Procedures for more details.

**Figure 6.**

Proposed model showing how interaction with the CB<sub>1</sub> N-terminus effects orthosteric ligand binding pocket. (A) Cartoon model depicting the allosteric two-site model for regulation of CB<sub>1</sub> by the N-terminus. The model shows the orthosteric ligand, A (purple circle), and the orthosteric binding pocket (gray circle), which also includes the critical C<sub>257</sub>-C<sub>264</sub> disulfide in EL2 (pink). The N-terminal region that allosterically modulates binding is also shown (turquoise), as well as the N-terminal C<sub>98</sub>-C<sub>107</sub> disulfide (orange circles). The cooperativity factors governing interactions between K<sub>B2</sub> and the orthosteric site ( $\alpha$ ) or between the two disulfides ( $\beta$ ) are represented by blue and white arrows, respectively. (B) A homology model of CB<sub>1</sub> developed using coordinates from the structure of GPCR S1P1 (Protein Data Bank (PDB): 3V2Y). The model shows the three domains that are conformationally coupled in the model: (1) The N-terminal domain (turquoise, the C<sub>98</sub>-C<sub>107</sub> disulfide in orange), (2) Loop EL2 (pink, the C<sub>257</sub>-C<sub>264</sub> disulfide in yellow) and (3) the orthosteric ligand binding cavity (purple). Transmembrane helices 6 and 7 have been omitted for clarity.



**Scheme 1.**  
Allosteric two site model.

**Table 1**  
Nomenclature Used for CB<sub>1</sub> Mutants

Mutant <sup>1</sup>	Description/Comment	Cys residues (total number)	N-terminal disulfide (C <sub>98</sub> -C <sub>107</sub> )?
CB <sub>1</sub> <sup>WT</sup>	"Wild Type" CB <sub>1</sub> background receptor.	All 13 native Cys	+
C <sub>98A,C107A</sub> CB <sub>1</sub> <sup>WT</sup>	CB <sub>1</sub> <sup>WT</sup> without C <sub>98</sub> -C <sub>107</sub> N-term disulfide (C98, C107 mutated to alanine).	11 cysteines	-
CB <sub>1</sub> <sup>MC</sup>	"Minimal Cysteine" CB <sub>1</sub> background receptor. Has only N-term disulfide (C <sub>98</sub> -C <sub>107</sub> ), and EL2 disulfide (C <sub>257</sub> -C <sub>264</sub> ).	4 Cys: (C <sub>98</sub> , C <sub>107</sub> ) (C <sub>257</sub> , C <sub>264</sub> )	+
C <sub>98A,C107A</sub> CB <sub>1</sub> <sup>MC</sup>	CB <sub>1</sub> <sup>MC</sup> without C <sub>98</sub> -C <sub>107</sub> N-term disulfide (C98, C107 mutated to alanine).	2 Cys: (C <sub>257</sub> , C <sub>264</sub> )	-
CB <sub>1</sub> <sup>PUR</sup>	CB <sub>1</sub> <sup>MC</sup> with N- and C-terminal truncations (Δ88 and Δ417, respectively).	4 Cys: (C <sub>98</sub> , C <sub>107</sub> ) (C <sub>257</sub> , C <sub>264</sub> )	+
C <sub>98A,C107A</sub> CB <sub>1</sub> <sup>PUR</sup>	CB <sub>1</sub> <sup>PUR</sup> , without C <sub>98</sub> -C <sub>107</sub> N-term disulfide (C98, C107 mutated to alanine). CB <sub>1</sub> mutant used for purification.	2 Cys: (C <sub>257</sub> , C <sub>264</sub> )	-
CB <sup>RAT</sup>	Wild-type CB receptors present in membranes prepared from rat brains.		N.D.

<sup>1</sup> All CB<sub>1</sub> mutants contain 1D4 antibody epitope (last nine amino acids of rhodopsin) on C-terminus.

**Table 2**

## Mean Fit parameters

$K_{B1}^{C257-C264}$	$95 \pm 20$ mM	$\alpha_{SR141716A}$	$2.3 \pm 0.5$
$K_{B2}^{C98-C107}$	$4.4 \pm 1.3$ mM	$\alpha_{CP55940}$	$0.69 \pm 0.08$

An allosteric two site model (Eq 1) was used to fit the data in Figure 4. Mean parameter values  $\pm$  S.E.M. are listed above where,  $K_{B1}^{C257-C264}$  and  $K_{B2}^{C98-C107}$  are the dissociation constants for the orthosteric and allosteric effector disulfide respectively. The  $\alpha$  value is the cooperativity factor between A and B for CP55940 or SR141716A (denoted as subscript). The mean coefficient of determination for all fits is  $0.95 \pm 0.01$ . For more details see Supplemental Table 1 and Experimental procedures.